I. STUDIES ON THE SYNTHESIS, RESOLUTION, AND PROPERTIES OF SOME FLUORINATED AMINO ACIDS

II. STUDIES ON BLOOD GROUP A-SPECIFIC SUBSTANCE

III. SYSTEMATIC QUALITATIVE TESTS FOR CERTAIN ACIDIC ELEMENTS IN ORGANIC COMPOUNDS

IV. ISOLATION OF COMPOUNDS TOXIC TO TOMATO PLANTS FROM THAMNOSMA MONTANA

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ABSTRACT

I. Syntheses are described of the following fluorinated amino acids: ortho-, meta-, and para-fluorophenylalanine, 2-fluoro-4-methoxy-phenylalanine and 2-fluorotyrosine.

The apparent dissociation constants of the above mentioned phenylalanine derivatives have been determined by electrometric titration. The ultra-violet absorption spectra of the mono-fluorophenylalanines and fluorotyrosines have been obtained.

A study has been made of the factors influencing the asymmetric specificity of the papain catalyzed synthesis of amides.

The attempted preparation of fluorocyclohexanol is described.

II. Studies on blood group A-specific substance have been made. These include a study of methods of isolation, a study of the electrophoretic properties of blood group A-specific substance, and a colorimetric method for the estimation of the activity of substances inhibiting the isoagglutination of blood group A-cells. Also the appearance of sheep cell lysins and human A-cell agglutinins in rabbits immunized with a partially purified blood group A-specific substance from hog gastric mucin has been observed.

III. Qualitative tests for certain inorganic elements (and carbon) in organic compounds are described.

IV. A survey of desert shrubs for materials toxic to tomato seedlings grown in nutrient culture has been made. From Thamnosma montana three crystalline compounds toxic to tomato seedlings have been isolated.

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II. Studies on Blood Group A-Specific Substance

# STUDIES ON THE SYNTHESIS, RESOLUTION AND PROPERTIES OF SOME FLUORINATED AMINO ACIDS

## Introduction

In recent years there has been an increasing interest in the preparation of various analogs of naturally occurring compounds to determine the effect that different substituents may have on the physical and physiological properties of these compounds. The introduction of a halogen atom into the ring of the aromatic amino acids has been one such extensively studied modification. Of these halogen containing amino acids, the fluorine containing amino acids have been particularly interesting. In these laboratories and in others DL-3-fluorotyrosine has been synthesized and resolved (1,2). The isomeric nuclear substituted fluorophenylalanines were first synthesized by Schiemann and Roselius (3); m-fluorophenylalanine was also synthesized by Mead (4). DL-3,5-difluorotyrosine; DL-3-fluoro-5-iodotyrosine, DL-2-fluorothyronine, DL-3-fluorothyronine, DL-3'-fluorothyronine; DL-3',5'-difluorothyronine, DL-3'-fluoro-5'-iodo-3,5-diiodothyronine, DL-3'-fluoro-3,5-diiodothyronine and DL-3,5-diiodo-3',5'-difluorothyronine have all been synthesized (4,5,6,7,8).

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* A discussion of reasons for the interest in thyronine analogs will be found in the thesis by Benson (9).

In section I-A of this thesis are described the preparations of o-,m-, and p-fluorophenylalanine by two methods which had not previously been applied to the syntheses of these compounds. Also described in this section are the preparations of two new fluorine containing amino acids: 2-fluoro-4-methoxy-phenylalanine and 2-fluoro-tyrosine. This latter compound may prove to be interesting as a competitive inhibitor to tyrosine in enzyme systems which use tyrosine as a substrate. The DL-fluorophenylalanines have been found by Mitchell and Niemann to be very effective competitive inhibitors of the metabolism of phenylalanine as determined by tests with a wild type strain of Neurospora crassa; a mole ratio of o-fluorophenylalanine/phenylalanine of 1.1 produces a 50% inhibition of growth (10). 3-fluorotyrosine is also a very good inhibitor. As might be expected, there is considerable difference in the action and activity of L-3-fluorotyrosine and D-3-fluorotyrosine. For this reason it was thought to be desirable to resolve the fluorophenylalanines which were prepared. Complete asymmetric enzymatic specificity was not observed in the first resolution attempted, i.e. in the resolution of N-carbobenzoxy-DL-o-fluorophenylalanine (11,12). Therefore, before attempting the enzymatic resolution of the other fluorophenylalanines experiments were carried out with various

acyl derivatives of DL-phenylalanine to determine the effect that the acylating group might have on the specificity of the enzymatic action of papain. The results of these investigations and the resolution of the N-acetyl derivatives of the isomeric DL-fluorophenylalanines are reported in section I-B of this thesis.

The absorption spectra and apparent dissociation constants of the three isomeric DL-chlorophenylalanines have been recently reported by Nevenzel, Shelberg and Niemann (13,14). Section I-C of this thesis is an extension of their work to the fluorinated amino acids whose preparation is described herein.

The fluorine containing aliphatic amino acids previously have not been studied as extensively as have the fluorinated aromatic amino acids. It would seem extremely likely that they also would have pronounced action as inhibitors in the metabolism of their naturally occurring analogs. Section I-D of this thesis deals with preliminary attempts to prepare $\gamma$-fluorolysine. While a total synthesis was not achieved, syntheses of 4-chlorocyclohexanol and 1,4-dichlorocyclohexane are described. It is possible that the first named compound may be useful in synthesizing chloro- and/or fluorolysine.

# A. SYNTHESES OF FLUORINE CONTAINING AROMATIC AMINO ACIDS

## Discussion

The isomeric fluorophenylalanines have been previously prepared by the condensation of fluorobenzaldehyde with hippuric acid (Erlenmeyer azlactone synthesis (3,4)). The aldehydes were prepared by chromyl chloride oxidation of fluorotoluene or by hydrolysis of fluorobenzal chloride which had been obtained by chlorination of fluorotoluene. The approximate overall yield from the toluidine to the amino acid was 2.3, 7.0, and 5.0% for the o-, m-, and p-fluorophenylalanine, respectively (the yields were 3.1, 10.3, and 9.5% from the corresponding fluorotoluene (3,15).

The feasibility of preparation of the aldehydes by a method described by McFadyen and Stevens (16) was investigated for two reasons. First, it was a different synthetic approach to the preparation of fluorobenzaldehyde; and, second, it would be a further test of the generality of the McFadyen-Stevens reaction.

The synthetic steps and the yields obtained in the preparation of the isomeric fluorophenylalanines were as indicated in Table I.

The overall yields obtained by the synthetic routes shown in Table I were comparable to those obtained by the

Table I

Synthetic Steps Used and Yields Obtained in Preparation of Fluorophenylalanines

| $R\text{-}C_6H_4\text{-}R'$ | | Yield | | |
|---|---|---|---|---|
| R | R' | ortho | meta | para |
| $NO_2$ | COOH | | | |
| $NO_2$ | $COOC_2H_5$ | | 85% | |
| $NH_2$ | $COOC_2H_5$ | | 96% | |
| $NH_2$ | COOH | 70% | | 89% |
| $NH_2$ | $COOC_2H_5$ | 83% | 64% | 79% |
| $N_2BF_4$ | $COOC_2H_5$ | 52% | 67% | 64% |
| F | $COOC_2H_5$ | 89% | 97% | 95% |
| F | $CONHNH_2$ | 93% | 98% | 89% |
| F | $CONHNHS(=O)(=O)C_6H_5$ | 50% (dist.) | 50% (dist.) | 42% (dist.) |
| F | CHO | | | |
| F |  | 36% (recrystallized) | 33% (recrystallized) | 32% (recrystallized). |
| F | $CH_2CH(NH_2)COOH$ | 80% | 84% | 81% |
| Yield from starting material | | 3.6% | 4.6% | 4.1% |
| Yield from fluorinated starting material | | 12% | 13% | 9.2% |

procedure of Schiemann and Roselius. The fluorine atom appeared to exert no influence on the McFadyen-Stevens reaction and yields comparable to those which they obtained for other compounds were obtained.

In no case was a sharp melting oxazalone obtained in the condensation of the fluorobenzaldehyde with hippuric acid in the presence of acetic anhydride. Definite evidence was obtained in the case of the condensation of p-fluorobenzaldehyde and hippuric acid that 2-methyl-4-(p-fluorobenzal)-oxazalone-5 was present in the resulting reaction mixture as well as the expected 2-phenyl-4-(p-fluorobenzal)-oxazalone-5. Thus, it appears that trans-acylation has occurred in the course of the reaction. Undoubtedly it also occurred in the condensation of the ortho- and meta-fluorobenzaldehydes; in fact, the absorption spectrum obtained for a low melting m-fluorobenzal-oxazalone fraction strongly suggests the presence of 2-methyl-4-(m-fluorobenzal)-oxazalone-5 (see experimental). It is reported in (17) that "the question of acyl interchange has been examined. There has been no report of transacylation when hippuric acid was used, nor is this surprising, for the N-benzoyl group is not readily displaced by another". It was reported that when phenaceturic acid or caproyl glycine was refluxed with acetic anhydride, benzaldehyde, and sodium acetate, transacylation occurred, and the resulting product was largely or in part 2-methyl-4-benzal-oxazalone-5. The preparation of oxazalones of

fluorobenzaldehyde and the oxazalone of benzaldehyde should be repeated under identical conditions; if trans-acylation occurs only with the fluorobenzaldehydes, an interesting research problem would be the study of the effect of substituents in the aromatic ring on trans-acylation in the Erlenmeyer condensation.

The second method of synthesis of fluorophenylalanines used was the condensation of fluorobenzyl chloride with the sodium salt of either acetamido-malonic ester (Method IIB) or ethyl acetamido-cyano acetate (Method IIA) (18,19,20). The fluorobenzyl chloride was prepared by vapor phase, ultraviolet light catalyzed, chlorination of fluorotoluene and yields of purified product in excess of 80% were readily obtained (21). The condensation of fluorobenzyl chloride with either acetamide-malonic ester or ethyl acetamido-cyano acetate gave 60 to 90% yields of the condensation product. However, in the case of the condensation of m-fluorobenzyl chloride and ethyl acetamido-cyano acetate no crystalline product could be obtained, only a highly colored oil; for this reason condensation with acetamido-malonic ester is more desirable. The condensation product can be hydrolyzed either directly to the amino acid or it can be hydrolyzed to the N-acetyl derivatives which are the desired starting materials for enzymatic resolutions with papain. Overall yields of the DL-fluorophenylalanines from

the fluorotoluenes of 25 to 45% can be readily obtained by the foregoing method. This method is therefore the most practical one yet described for the syntheses of the fluorophenylalanines.

As has been previously observed for o-chlorophenylalanine (13), o-fluorophenylalanine is very difficult to recrystallize from aqueous solutions as a well defined crystalline product. This may be due in part to its relatively high solubility in water. It was observed that the fluorophenylalanines can be recrystallized from 6 F HCl as very nicely crystalline products which are stable to drying in vacuo over solid NaOH. Thus, o-fluorophenylalanine is obtained as the amino acid hydrochloride monohydrate, while the meta- and para- fluorophenylalanines are obtained as the amino acid hydrochloride.

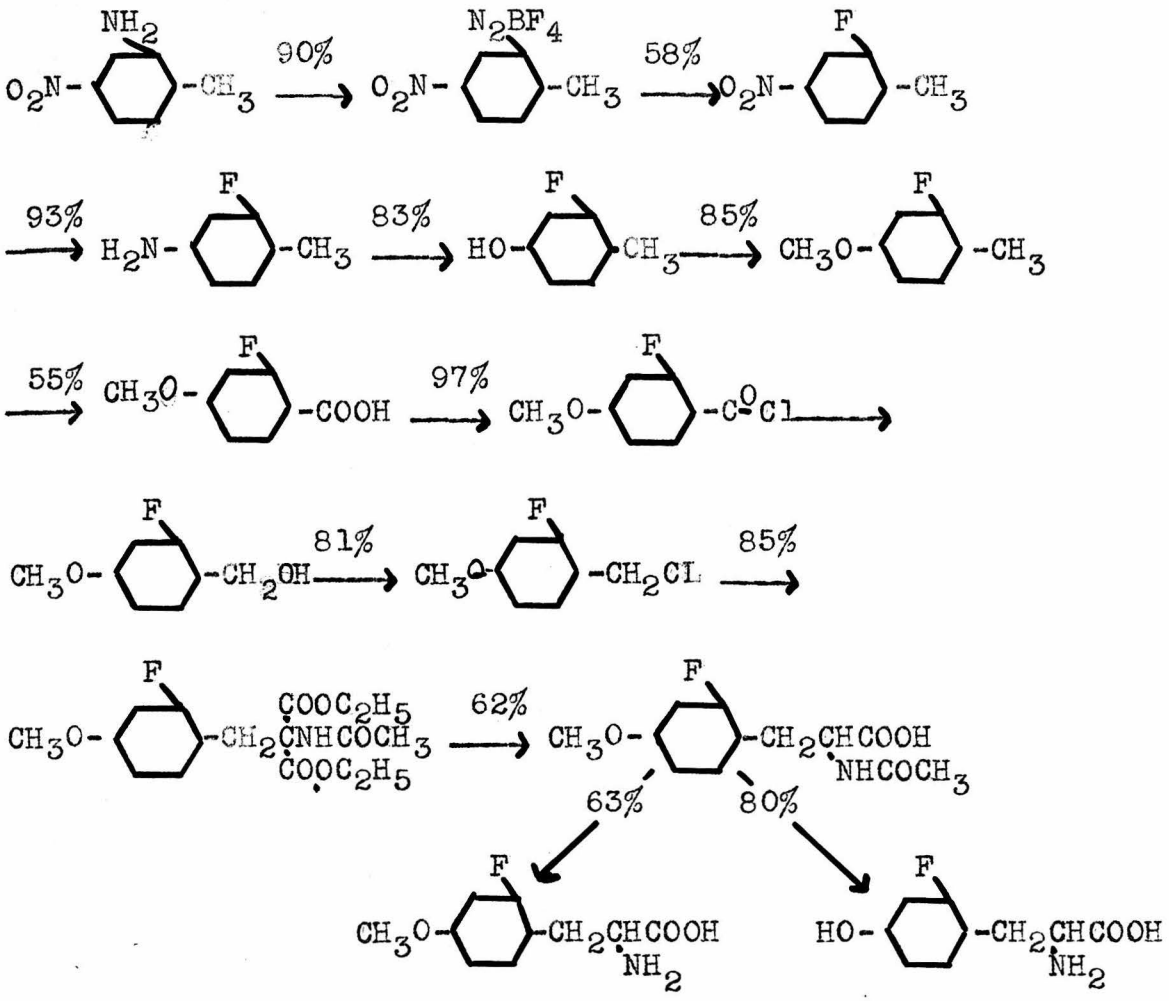
The method of synthesis of 2-fluorotyrosine (and 2-fluoro-4-methoxy-phenylalanine) was as indicated in Table II.

All reactions gave yields of greater than 50%. It is likely that the yield in the permanganate oxidation of 2-fluoro-4-methoxytoluene to the corresponding benzoic acid could be increased, particularly by recovering the unoxidized material. Direct hydrolysis $\alpha,\beta$-(2-fluoro-4-methoxy-benzyl)-acetamidomalonic ester to 2-fluorotyrosine would probably increase the overall yield significantly. The conversion of 2-methyl-5-nitrobenzenediazonium fluoroborate to 2-fluoro-4-nitrotoluene had to be carried out with great caution, as pointed out in the experimental section, due to the violence and rapidity

with which it may decompose. However, satisfactory yields were obtained in this decomposition.

Table II

Synthetic Steps and Yields Obtained in the Preparation of 2-Fluorotyrosine and 2-Fluoro-4-methoxy-phenylalanine




| | 2-Fluoro-4-methoxy phenylalanine | 2-Fluorotyrosine |
|---|---|---|
| Overall Yield | 5.0% | 6.3% |

Several attempts were made to oxidize 2-fluoro-4-methoxy-toluene to the corresponding aldehyde. In one instance the oxidizing agent used was chromic anhydride in acetic anhydride-sulfuric acid; this oxidation did not proceed smoothly and no product was obtained. The second oxidation attempted was with chromyl chloride in chloroform (actually tried on p-cresyl methyl ether), and although the odor of aldehyde was detected, difficulties in isolating the product made this method seem impractical. It is likely that the oxidation to the aldehyde could be carried out with greater ease before reduction of the nitro group and its subsequent conversion to an -OMe group.

Several trial reductions with lithium aluminum hydride of anisoyl chloride to the corresponding alcohol followed by distillation produced only tars. It is possible that a trace of acid present in the ether solution caused polymerization. However, when the alcohol obtained by the reduction of 2-fluoro-4-methoxy-benzoyl chloride was not distilled but, instead was converted directly to 2-fluoro-4-methoxy-benzyl chloride, the latter product was obtained in an overall yield of 81% from the acid chloride.

Only a small portion of the N-acetyl-2-fluoro-4-methoxy-phenylalanine has been converted to the free amino acids; the remainder has been left as the N-acetyl derivative in case it is desired to resolve it enzymatically.

## Experimental*

### Preparation of o-Fluorophenylalanine (Method I)

Ethyl o-aminobenzoate was prepared by refluxing 289 grams (2.1 moles) of o-aminobenzoic acid (EK White Label) with 2 liters of absolute ethanol (99.75% absolute as determined by the "Nujol" method (22)) and 170 grams of anhydrous hydrogen chloride for 36 hours (23). The ethanolic hydrogen chloride was distilled from the reaction product under reduced pressure. The ester hydrochloride was dissolved in one liter of water; the solution was made slightly basic with sodium carbonate and extracted with ether. After drying the combined ether extracts, the ether was removed from the ester, and the ester was distilled under reduced pressure. Yield: 255 grams (73%) of ethyl o-aminobenzoate, a colorless liquid, b. p. 143-147°/19 mm. (uncorr.). Lit. b. p. 145-147°/15 mm.

A second preparation starting with 421 grams (3.07 moles) of o-aminobenzoic acid yielded 337 grams (67%) of ethyl o-aminobenzoate, b. p. 136°/11 mm.

o-Carbethoxybenzenediazonium fluoroborate (23,24).- To one liter of 5 F HCl, 337 grams (2.04 moles) of ethyl o-aminobenzoate were added with stirring over a 5 minute period; a moderately-coarse crystal meal was thus obtained. The mixture was cooled to 0°C, and 160 grams of sodium nitrite

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* Unless otherwise indicated, microanalyses were performed by the late Dr. G. Oppenheimer and Mr. G. A. Swinehart.

in 300 ml. of water were added to the stirred mixture over a 45 minute period; the temperature was not allowed to rise above 7°C. The slight excess of nitrous acid present was decomposed with urea, 220 ml. of 12 F HCl were added, followed by the portionwise addition of a solution of 285 grams of sodium fluoroborate in 400 ml. of cold water. After approximately three-quarters of the sodium fluoroborate solution had been added, so much precipitated product was present that mechanical stirring became ineffective, and the flask had to be swirled by hand after the addition of each additional portion of the sodium fluoroborate solution. After all the sodium fluoroborate had been added, the mixture was allowed to stand in an ice bath for approximately 20 minutes; then the precipitate was removed by filtration through a large Buchner funnel, and dried as thoroughly as possible by suction. The precipitate was washed successively with two 400 ml. portions of cold water, two 400 ml. portions of cold methyl alcohol, and two 200 ml. portions of ether. The product was dried to constant weight in vacuo over concentrated sulfuric acid. Yield: 440 grams (82%) of o-carbethoxybenzenediazonium fluoroborate, a white solid, decomp. point 105-107° (uncorr.). Lit. decomp. point 106° (24).

Other preparations with 165 grams (1 mole) and 225 grams (1.35 moles) of ethyl o-aminobenzoate yielded 223 grams

(84%) and 258 grams (73%), respectively, of o-carbethoxybenzenediazonium fluoroborate, decomp. point, 105-108°, mostly 107-108°. A sample has been stable at room temperature for about 2 years.

Ethyl o-Fluorobenzoate (23,24).- 474 grams (1.86 moles) of dry o-carbethoxybenzenediazonium fluoroborate were decomposed in three portions. The decompositions were carried out in the following manner: approximately 150 grams of o-carbethoxybenzenediazonium fluoroborate were placed in a 1 liter RBST flask. The flask was connected with ST bends to a suction flask cooled in an ice bath, and then to a water aspirator. The diazonium fluoroborate was gently heated near the top edges with a Bunsen burner until decomposition had started. Further heat was applied as necessary to maintain the decomposition at such a rate that the pressure in the system varied from 100-150 mm. Near the end of the decomposition, some foaming tended to occur; this ceased when the reaction was completed. Some solid material sublimed during the decomposition. On cooling the reaction mixture partially solidified. Each decomposition required approximately 40 minutes. The material which sublimed fumed vigorously on exposure to laboratory air. It was combined in ether with the small amount of liquid which had been carried over into the suction flask, and

then redistilled to yield 25 grams of a liquid, b. p. 57-58°/18 mm.; this material was not readily soluble in cold water, 5% HCl, or 5% NaOH, but was soluble in concentrated sulfuric acid and less soluble in 85% phosphoric acid. A portion was refluxed with sodium hydroxide-methanol and the neutralization equivalent determined, it was found to be approximately 68.5-69.5. Theory for o-fluorobenzoyl fluoride is 71. It is possible that the "sublimate" contained the free acid and the acid fluoride. However, further work was not done to characterize this product.

The product in the decomposition flask was distilled and the main fraction of ethyl o-fluorobenzoate, b. p. 105-109°/20 mm. (uncorr.) weighed 160 grams. A solid residue weighing approximately 105 grams remained. This residue was dissolved in dilute sodium hydroxide, extracted with ether, the ether extracts washed until neutral and dried. After stripping off the ether, approximately 23 grams additional ester was obtained, b. p. 108-112°/20 mm. (uncorr.). The above fractions of ethyl o-fluorobenzoate were combined and approximately 15 grams of low boiling material were distilled off. The main fraction was dissolved in ether, washed with dilute sodium hydroxide (to remove any phenolic or acidic materials present), the ether solution was dried over Drierite, and the ester

fractionated. 153 grams (51%) of ethyl o-fluorobenzoate were obtained, b. p. 103-104°/19 mm. (uncorr.); $\eta_D^{25} = 1.489_7$. Lit. b. p. 102°/12 mm. (24).

A second preparation of 436 grams (1.65 moles) of o-carbethoxybenzenediazonium fluoroborate was decomposed in 2 portions, the product after the decomposition appeared to be mostly liquid. As in the previous decomposition, some solid material sublimed during the decomposition. The decomposition product was rinsed into a distilling flask with dry ether and fractionated. Approximately 5 grams of low boiling material were obtained. The main fraction of ethyl o-florobenzoate, b. p. 98-100°/12.5 mm. weighed 165 grams. It was dissolved in ether, extracted with dilute sodium hydroxide, the ether solution dried over Drierite, and redistilled through a 20 cm. Vigreux column. Yield: 147 grams (53%), b. p. 101-102°/12.5 mm. (uncorr.), $\eta_D^{25} = 1.490_5$.

<u>o-Fluorobenzhydrazide</u> (<u>25,26</u>).- 91 grams (1.55 moles) of hydrazine hydrate (Lemke, 85%) were placed in a one liter RBST flask fitted with a stirrer, reflux condenser and a dropping funnel. The solution was warmed to 75° on a water bath, and 147 grams (0.87 moles) of ethyl o-fluorobenzoate were added to the stirred hydrazine hydrate solution over a 2 hour period. The first portions of ester had to be added slowly, since the ester dissolved only slowly in the hydrazine hydrate. The mixture was warmed to 85-90° for

approximately 2 hours, and then placed in the cold room overnight. The precipitate was filtered off on a Buchner funnel and washed with three 25 ml. portions of cold 95% alcohol, and two 30 ml. portions of cold ether. It was dried *in vacuo* over sulfuric acid. Yield, 76 grams (57%) of o-fluorobenzhydrazide, colorless needles, m. p. 74.5-76.5°. By concentration of the filtrate and washings from above, 45.3 grams (33%) additional o-fluorobenzhydrazide were obtained, m. p. 74.0-76.5°; thus the total yield was 121 grams (90%). The o-fluorobenzhydrazide obtained was not purified further.

A second preparation using 87.3 grams (0.52 mole) of ethyl o-fluorobenzoate yielded a total of 71.4 grams (89%) of ethyl o-fluorobenzhydrazide. A third preparation using 42.2 grams (0.25 mole) of ethyl o-fluorobenzoate gave 31.8 grams (84%) of product.

Preliminary experiments indicated that benzene might be a satisfactory solvent for the recrystallization of o-fluorobenzhydrazide; therefore, a portion of the above product was recrystallized from benzene; some material was not readily soluble in the benzene. The insoluble material was found to melt at approximately 230-240° (decomp.), but was not further identified. The o-fluorobenzhydrazide was subsequently recrystallized from a cyclohexane-benzene mixture

and obtained as long needles, m. p. 72.0-73.0° (uncorr.); Lit. m. p. 70° (25).

Anal. Calcd. for $C_7H_7ON_2F$ (154.1): C, 54.54; H, 4.58; N, 18.18. Found: C, 54.68; H, 4.84; N, 18.14.

o-Fluorobenzhydrazide is soluble in alcohol and in water, slightly soluble in benzene and chloroform, less soluble in cyclohexane and ligroin.

sym-o-Fluorobenzoylbenzenesulfonhydrazide.- 116 grams (0.75 mole) of o-fluorobenzhydrazide were dissolved in 200 ml. of pyridine (Barrett's refined, redistilled) and cooled in an ice bath (16). To the stirred solution 134 grams (0.76 mole) of benzenesulfonyl chloride (EK White Label) were added at such a rate that the temperature was maintained at 20-30°. The addition required approximately 45 minutes. The reaction mixture was allowed to stand overnight at room temperature, and then slowly poured into a stirred mixture of 200 ml. of 12 F HCl and 500 grams of ice. The precipitate was removed by filtration and washed thoroughly with eight 200 ml. portions of water and finally with four 50 ml. portions of cold 95% ethanol.

Yield: 218 grams (98%) of sym-o-fluorobenzoylbenzenesulfonhydrazide, m. p. 165-170°, mostly 169-170° (corr.).

For analysis 2.0 grams of the above product was recrystallized from 50 ml. of benzene and then from 10 ml. of

absolute ethanol; obtained 0.9 grams pure sym-o-fluorobenzoylbenzenesulfonhydrazide as colorless prisms, m. p. 172-173.5 (corr.).

Anal. Calcd. for $C_{13}H_{11}O_3N_2SF$ (294.3): C, 53.05; H, 3.77; N, 9.52.

Found: C, 53.15; H, 3.74; N, 9.41 (Elek). C, 53.21; H, 3.99; N, 10.42.

A second preparation using 60 grams (0.39 mole) of o-fluorobenzhydrazide, 100 ml. of redistilled pyridine and 76 grams (0.43 mole) of benzenesulfonyl chloride yielded 100 grams (87%) of sym-o-fluorobenzoylbenzenesulfonhydrazide, m. p. 172-173° (corr.) after two recrystallizations from approximately 1 liter of absolute ethanol. For analysis 5.0 grams was recrystallized again from 60 ml. of absolute ethanol, obtained 4.5 grams of long colorless hexagonal prisms, m. p. 172-173° (corr.).

Anal. Calcd. for $C_{13}H_{11}O_3N_2SF$ (294.3): C, 53.05; H, 3.77; N, 9.52.

Found: C, 53.05; H, 4.13; N, 10.23(?).

o-Fluorobenzaldehyde and 2-Phenyl-4-(o-fluorobenzal)-oxazalone-5 (3,15,16).- In a 2 liter erlenmeyer flask fitted with a reflux condenser, 24.3 grams (0.082 moles) of sym-o-fluorobenzoylbenzenesulfonhydrazide were heated in 120 ml. of ethylene glycol (E. K. White Label or Mefford, pure) to 155-160° (the o-fluorobenzoylsulfonhydrazide dissolved).

To the hot solution 24.5 grams of anhydrous sodium carbonate were added in one portion, and the mixture was heated at 155-160° for 2 1/2-3 minutes, then 250 ml. of hot water were added through the reflux condenser and the mixture was cooled to room temperature in an ice bath. Subsequently it was extracted with 1 liter of ether (peroxide free) in 5 portions. The ether extracts were washed with water and dried over calcium chloride. The ether was distilled off and finally the pressure was reduced to 60 mm. and the bath temperature increased to 80°. The residue containing the crude aldehyde weighed 6.35 grams (62% yield maximum) and was used without further purification to prepare the oxazalone.

6.35 grams (0.05 mole) of crude o-fluorobenzaldehyde, 4.25 grams of freshly fused powdered sodium acetate, 9.2 grams (0.05 mole) of hippuric acid (EK White Label), and 15.7 ml. of acetic anhydride (B and A Reagent) were intimately mixed and heated on a boiling water bath for 20 minutes (27,28). The oxazalone started to precipitate after 5-10 minutes of heating. After standing overnight at room temperature, 35 ml. of water were added and the mixture was stirred for approximately 1/2 hour. The precipitate became somewhat oily. After filtration, the crude oxazalone was again washed with 35 ml. of cold water and then with 150 ml. of hot water in

small portions. The oxazalone was subsequently triturated with 40 ml. of 95% ethanol, whereupon it became nicely crystalline, and a large amount of colored impurities were removed. After further washings with successive small portions of cold ethanol, 6.4 grams (47%) of 2-phenyl-4-(o-fluorobenzal)-oxazalone-5 were obtained, softens 115-154°, m. p. 154-164° (corr.). The overall yield from o-fluorobenzoylbenzenesulfonhydrazide was 29%.

The preparation of o-fluorobenzaldehyde was repeated using 35.0 grams (0.12 mole) and 36.3 grams (0.12 mole) of o-fluorobenzoylbenzenesulfonhydrazide; yields of crude aldehyde of 8.0 grams (54%) and 12.0 grams (78%) were obtained. The crude aldehyde yielded 8.2 grams (48%) (overall yield: 26%) and 14.1 grams (54%) (overall yield: 43%), respectively, of 2-phenyl-4-(o-fluorobenzal)-oxazalone-5, m. p. 158-165° (corr.).

Another larger preparation of o-fluorobenzaldehyde was carried out using 211 grams of sym-o-fluorobenzoylbenzenesulfonbenzhydrazide. The decomposition was carried out in 4 portions. The aldehyde was extracted from the ethylene glycol-water solution from each decomposition with 250 ml. of ether (peroxide free) and then the aqueous-ethylene glycol solution was extracted for 24 hours with ether in a continuous liquid-liquid extractor. After drying over calcium

chloride, the ether was removed through a 20 cm. Vigreux column, and the aldehyde was fractionated under reduced pressure. 45.1 grams (50%) of o-fluorobenzaldehyde was obtained, b. p. 90-91°/46 mm, $\eta_D^{25} = 1.518_0$; Lit. b. p. 80.5°/36 mm., 175°/760 mm.; $\eta_D^{15} = 1.5121$.

37.4 grams (0.30 mole) of distilled o-fluorobenzaldehyde were refluxed for 70 minutes with 24 grams of freshly fused sodium acetate, 54.5 grams (0.30 mole) of hippuric acid (EK White Label) and 95 ml. of acetic anhydride (Baker C. P.). After cooling to room temperature, the solid reaction product was mostly yellow with small areas of orange-brown impurities. Upon stirring with 150 ml. of distilled water, most of the product was crystalline and very little oily material was present. The precipitate was removed by filtration, washed several times with 100 ml. portions of cold water, then with 100 ml. portions of hot water. Subsequent washing with cold ethanol removed most of the orange color and a light yellow product remained. After drying, 35.4 grams (44%) (22% overall yield from the fluorobenzoylbenzenesulfonhydrazide) of 2-phenyl-4-(o-fluorobenzal)-oxazalone-5 were obtained; m. p. 155-163° (corr.) with softening from 95-155° (corr.)

38.1 grams of 2-phenyl-4(o-fluorobenzal)-oxazalone-5 were dissolved in 2 1/2 liters of refluxing absolute ethanol, filtered hot, and allowed to cool slowly to room temperature. Long needlelike yellow crystals formed, and after several

hours, the precipitate was removed by filtration and washed with four 100 ml. portions of cold absolute ethanol. Yield: 29.0 grams, m. p. 167-169° (corr.). For analysis a sample was recrystallized again from ethanol, m. p. 167.0-168.0° (corr.).

Anal. Calcd. for $C_{16}H_{10}O_2NF$ (267.2): C, 71.90; H, 3.77; N, 5.24. Found: C, 72.17; H, 3.93; N, 5.28. Schiemann and Roselius (3) obtained the oxazalone from o-fluorobenzaldehyde in 35% yield after recrystallization from acetic acid. They reported a m. p. of 165.5-166.5°.

The absorption spectrum of the analyzed sample dissolved in chloroform was compared with that of a sample purified by chromatography on silicic acid-celite using benzene as a developer, and subsequently elutioning and recrystallizing from alcohol. The spectra were found to be the same. The spectra of the purified 2-phenyl-4-(o-fluorobenzal)-oxazalone-5 and the crude oxazalone are shown in Figure I.

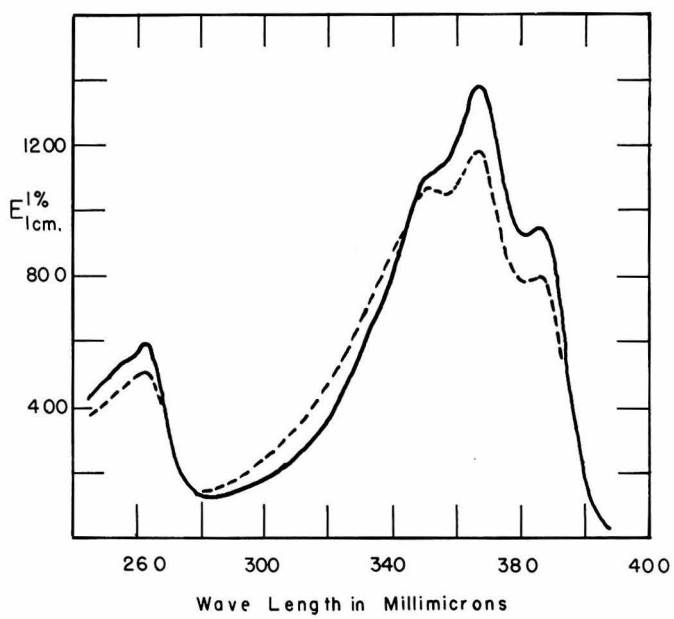


Figure 1. Ultra-Violet Absorption Spectra of pure 2-Phenyl-4-(o-fluorobenzal)-oxazalone-5, m. p. 167-169° _____, and of crude oxazalone, m. p. 155-163° ---------- (in chloroform).

$\varepsilon_{max}$ and $\varepsilon_{min}$ for the pure compound are given in Table I.

Table I

$\varepsilon_{max}$ and $\varepsilon_{min}$ for 2-Phenyl-4-(o-fluorobenzal)-oxazalone-5 in chloroform

| $\lambda$ (mμ) | | $\varepsilon$ |
|---|---|---|
| 386.5 | (max) | 25,400 |
| 382 | (min) | 24,800 |
| 366.5 | (max) | 37,000 |
| 285 | (min) | 3,300 |
| 263 | (max) | 16,000 |

o-Fluorophenylalanine (3).- 28 grams (0.1 mole) of 2-phenyl-4-(o-fluorobenzal)-oxazalone-5 were refluxed with 21 grams of red phosphorus, 130 ml. of acetic anhydride (Baker C. P.) and 130 ml. of hydriodic acid (sp. gr. 1.70) for 11 hours (28). The red phosphorus was removed by filtration, and the filtrate was evaporated under reduced pressure to a thick syrup. Water was added and the solution was reconcentrated under reduced pressure. The syrup was dissolved in 150 ml. of water and the aqueous solution was extracted with several portions of ether, then neutralized to pH 5.4 with ammonium hydroxide and allowed to stand several days at room temperature. A gel-like precipitate was obtained. It was removed by filtration and washed with water and alcohol. A second precipitate was obtained by evaporation of the filtrate and

washings. The two fractions were combined and recrystallized from approximately 100 ml. of water. 4.7 grams (25%) of o-fluorophenylalanine were obtained, decomp. point 244-249° (corr.).

Anal. calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 58.96; H, 6.0 (?); N, 7.6. By concentration of the filtrate and washings from the recrystallization 3.6 grams (20%) additional o-fluorophenylalanine was obtained after recrystallization, decomp. point 245-249° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.05; H, 5.6; N, 7.46 (corrected for ash).

Thus a total yield of 8.3 grams (45%) of o-fluorophenylalanine was obtained. Schiemann and Roselius (3) reported a yield of 33% for the preparation of o-fluorophenylalanine from the oxazalone, and a m. p. of 258.5-259 (with decomp.).

Another preparation of o-fluorophenylalanine from 30.6 grams (0.11 mole) of 2-phenyl-4-(o-fluorobenzal)-oxazalone-5 yielded 16.8 grams (80%) of crude o-fluorophenylalanine, decomp. point 235-241° (corr.). This product was recrystallized by dissolving in 300 ml. of boiling water, filtering hot, and adding 600 ml. of ethanol. Recrystallization repeated. 13.5 grams (64%) of o-fluorophenylalanine

was obtained, decomp. point 244-248° (corr.).
Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.11; H, 5.48; N, 7.60 (Elek). This sample was used to determine the ionization constants and absorption spectrum reported in section I-C of this thesis.

It is to be noted that o-fluorophenylalanine was found to be very difficult to recrystallize satisfactorily from water or water-ethanol solutions; it tended to form a gel from which it was very difficult to remove the mother liquor and which was very difficult to wash properly. It has been found, however, that o-fluorophenylalanine can be recrystallized nicely from 6 F HCl, long prisms being obtained; the product on drying in vacuo over NaOH is o-fluorophenylalanine hydrochloride monohydrate.

The overall yield of o-fluorophenylalanine from o-aminobenzoic acid was approximately 3.6%.

Preparation of m-Fluorophenylalanine (Method I)

Ethyl m-nitrobenzoate was prepared by refluxing 456 grams (2.7 moles) of m-nitrobenzoic acid (EK White Label) with 3 liters of absolute ethanol (99.8% absolute) and 225 grams of anhydrous hydrogen chloride for approximately 2 days. The ethanolic-hydrogen chloride was removed by distillation at reduced pressure, and the remaining ester was dissolved

in ether, extracted with dilute sodium carbonate and then water until neutral. The ether-ester solution was dried over Drierite, the ether distilled, and the product fractionated at reduced pressure through a 20 cm. Vigreux column. 453 grams (85%) of ethyl m-nitrobenzoate were obtained, b. p. 128-130°/2 mm; 138-139°/4 1/2 mm.; m. p. 39.5-41.2°. Lit. b. p. 296-298°/760; m. p. 41° (47°).

Ethyl m-aminobenzoate was prepared by the catalytic reduction of ethyl m-nitrobenzoate with platinum oxide catalyst (31,32). A stock solution of 446 grams of ethyl nitrobenzoate was prepared in 95% ethanol, total volume 2 liters. A 133 ml. aliquot (29.7 grams, 0.15 mole) of the stock solution was used for each reduction. The platinum catalyst was prepared from recovered platinum as described in (32). In the first reduction approximately 300 mg. of catalyst were used; subsequent reductions were performed by the addition of 25-35 mg. of fresh catalyst to the reaction vessel (which also contained the used catalyst). In this manner the reduction rate was controlled and prevented from becoming either too slow or too rapid. Each reduction required approximately 1/2 hour; however, it was usually continued about 1/2 hour after the reduction was thought to be complete. In the apparatus used, about 38-40 lbs. of hydrogen were consumed for each reduction.

The combined, filtered ethyl alcohol-ethyl m-aminobenzoate solution was concentrated under reduced pressure and the remaining ester was fractionated through a Vigreux column. Yield: 363 grams (96%) of ethyl m-aminobenzoate, b. p. 137-138°/3 mm. Lit. 294°/760 mm.

m-Carbethoxybenzenediazonium fluoroborate was prepared as described above for the ortho isomer (23). However, the product was not washed with methanol or ether. From 354 grams (2.1 moles) of ethyl m-aminobenzoate, 360 grams (64%) of m-carbethoxybenzenediazonium fluoroborate were obtained as a light peach colored solid, decomp. point 75-76° (uncorr.).

Ethyl m-fluorobenzoate was prepared as described for the ortho isomer by the decomposition in 2 portions of 355 grams of m-carbethoxybenzenediazonium fluoroborate (23). The crude products in the decomposition flask and in the suction trap flask were rinsed into a distilling flask with ether and fractionated under reduced pressure. Approximately 13 grams of a low boiling forerun were obtained, b. p. 73°/48 mm. It is presumably comparable to the low boiling fraction obtained from the ortho isomer, and was not further characterized. The main fraction of ethyl m-fluorobenzoate boiled at 97-99°/16 mm. and weighed 153 grams (67%). Approximately 60 grams of high boiling material in the

distilling flask were discarded. The crude ester was dissolved in ether and washed with dilute acid and dilute base, the ether solution dried over Drierite, and the product refractionated through a 20 cm. Vigreux column, all fractions collected had a b. p. of 103°/21 mm, and $\eta_D^{25}$ = 1.4831, yield: 146 grams.

m-Fluorobenzhydrazide was prepared by the reaction of 137 grams (0.82 mole) of ethyl m-fluorobenzoate with 88 grams (1.51 moles) of hydrazine hydrate (Lemke-85%) as described above (26). However, the product precipitated in the reaction mixture, so that it was necessary to add approximately 25 ml. of ethanol to the mixture when approximately 1/2 of the ester had been added. The reaction mixture was placed in the cold room overnight and the product was then filtered and washed with three 25 ml. portions of cold ethanol and two 25 ml. portions of ether. The m-fluorobenzhydrazide crystallized as long prisms, and weighed 117 grams (93%) after drying over sulfuric acid in vacuo; m. p. 138.0-139.5 (corr.). From the filtrate and washings 5.6 grams (4%) additional m-fluorobenzhydrazide was obtained by concentration, m. p. 137-139° (corr.). The total yield was therefore 97%.

For analysis 2 grams of the main fraction were recrystallized from ethanol, thus obtaining 1.6 grams, m. p. 138.0°-139.5° (corr.).

Anal. Calcd. for $C_7H_7ON_2F$ (154.1): C, 54.54; H, 4.58; N, 18.18. Found: C, 54.72; H, 4.72; N, 18.27.

sym-m-Fluorobenzoylbenzenesulfonhydrazide was prepared as described above by the reaction of 118 grams (0.76 mole) of m-fluorobenzhydrazide dissolved in 600 ml. of pyridine (redistilled) with 135 grams (0.77 mole) of benzenesulfonyl chloride (EK White Label) (16). After standing overnight at room temperature, the reaction product was poured slowly into a stirred mixture of 600 ml. of 12 F HCl and 1500 grams of ice. The mixture was placed in the cold room for several hours, then the product was removed by filtration and washed with six 250 ml. portions of water, and finally with two 50 ml. portions of cold 95% ethanol. 221 grams (98%) of sym-m-fluorobenzoylbenzenesulfonhydrazide, m. p. 181-182° (corr.) were obtained. For analysis 2 grams of the above product were recrystallized from 40 ml. of benzene; it precipitated as long colorless needles, m. p. 182-183° (corr.). Anal. Calcd. for $C_{13}H_{11}O_3N_2SF$ (294.3): C, 53.05; H, 3.77; N, 9.52. Found: C, 53.09; H, 3.76; N, 10.13. N, 9.39 (Elek).

m-Fluorobenzaldehyde was prepared by the decomposition of 215 grams of sym-m-fluorobenzoylbenzenesulfonhydrazide in 4 portions as described above (16). The aldehyde was extracted from each aqueous-ethylene glycol solution with one 250 ml. portion of ether (peroxide free), and then the aqueous-glycol solutions were combined and extracted in a continuous liquid-liquid extractor for 24 hours. After drying, the ether was

distilled from the extracts and the m-fluorobenzaldehyde was fractionated through a 20 cm. Vigreux column. 45.6 grams (50%) of m-fluorobenzaldehyde were obtained, b. p. 98-99°/50 mm.; $\eta_D^{25} = 1.515_7$; Lit. b. p. 76°/26 mm., 173°/760 mm.; $\eta_D^{24} = 1.5159_4$ (15,29).

2-Phenyl-4-(m-fluorobenzal)-oxazalone-5 was prepared by gently refluxing for 60 minutes 45.2 grams (0.36 mole) of m-fluorobenzaldehyde, 30 grams of freshly fused sodium acetate, 66 grams (0.37 mole) of hippuric acid (EK White Label) and 113 ml. of acetic anhydride (Baker C. P.)(27,28). After standing overnight at room temperature, the yellow-orange solid was well washed with four 100 ml. portions of cold water, and then with four 100 ml. portions of hot water. Subsequently the product was washed with ten 15 ml. portions of cold 95% ethanol. 59 grams (61%) of crude 2-phenyl-4-(m-fluorobenzal)-oxazalone-5 were obtained, m. p. 109-139° (uncorr.). Small scale experiments indicated that the crude oxazalone could be satisfactorily recrystallized from absolute ethanol, therefore 57 grams of the crude oxazalone were dissolved in 2 liters of hot absolute ethanol (required approximately 20 minutes), filtered and allowed to cool to room temperature. The precipitate of long light yellow needles was removed by filtration and washed with six 50 ml. portions of absolute ethanol. Obtained 30.1

grams (33%) of 2-phenyl-4-(m-fluorobenzal)-oxazalone-5, m. p. 158.5-159.5° (corr.). Recrystallization did not change the melting point.

Anal. Calcd. for $C_{16}H_{10}O_2NF$ (267.2): C, 71.90; H, 3.77; N, 5.24. Found: C, 71.94; H, 3.93; N, 5.18 (Elek). Schiemann and Roselius obtained the oxalzalone in a 70% yield after recrystallization from acetic acid, m. p. 156-156.5 (3).

The filtrate and washings from the above recrystallization were concentrated and several additional fractions were obtained, all with wide melting ranges; for example the second fraction weighed 10.8 grams and had a melting range of 108-154° (uncorr.), mostly less than 120°. Fraction 4 weighed 2.0 grams and had a m. p. of 108-118°, its absorption spectrum was taken (see below).

The absorption spectra of the crude product, the recrystallized azlactone, and F-4 were taken in chloroform and are shown in Figure II.

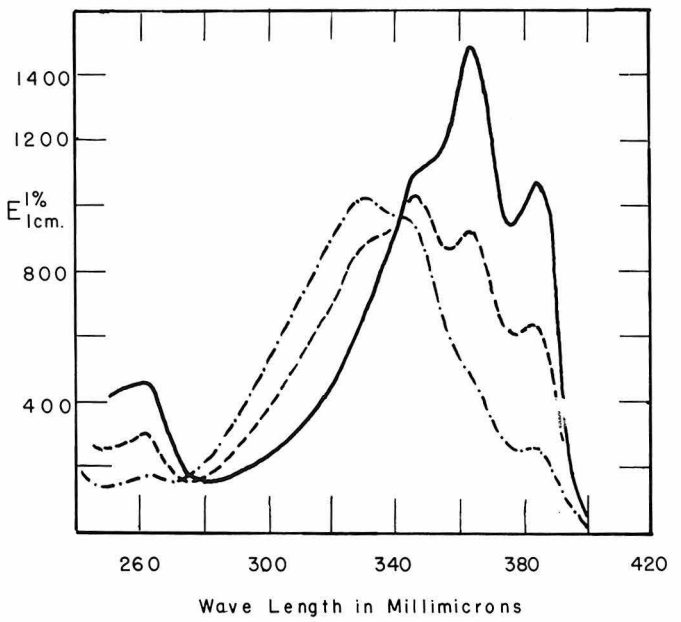


Figure 2. Ultra-Violet Absorption Spectra of pure 2-Phenyl-4-(m-fluorobenzal)-oxazalone-5, m. p. 158.5-159.5° ————————, crude oxazalone, m. p. 109-139° – – – – – –, and of F-4, m. p. 108-118° .-.-.-.-.-.-.- (in chloroform).

$\varepsilon_{max}$ and $\varepsilon_{min}$ are given for the pure oxazalone are given in Table II.

Table II

$\varepsilon_{max}$ and $\varepsilon_{min}$ for 2-Phenyl-4-(m-fluorobenzal)-oxazalone)-5 in Chloroform

| | $\lambda$(mμ) | | $\varepsilon$ |
|---|---|---|---|
| Recrystallized once from absolute ethanol; m. p. 158.5-159.5° (corr.) softens 157.5° | 383 | (max) | 27,200 |
| | 378.5 | (min) | 26,000 |
| | 363.5 | (max) | 38,100 |
| | 282 | (min) | 3,900 |
| | 262 | (max) | 12,200 |

m-Fluorophenylalanine (3,4) was prepared by refluxing 29 grams of recrystallized 2-phenyl-4-(m-fluorobenzal)-oxazalone-5 for 16 hours with hydriodic acid, acetic anhydride, and red phosporus (28). After removing the red phosphorus by filtration, the filtrate was evaporated to dryness under reduced pressure, and the solid remaining was dissolved in 150 ml. of water, extracted well with ether, and filtered. It was then made basic with ammonium hydroxide and the excess ammonium expelled by boiling (the amino acid started to crystallize as needles from the boiling solution), and the solution was placed in the cold room overnight. The product was removed by filtration, washed with water and dried. 13.1 grams (64%) of m-fluorophenylalanine was

obtained, decomp. point 240-242° (corr.). From the filtrate and washing 4.0 grams (20%) additional product was obtained by concentration, decomp. point 223-229° (corr.). The main fraction was again recrystallized by dissolving in 250 ml. of water, filtering, and adding 500 ml. of ethanol. Long, fine needles were obtained. After filtering, washing, and drying, the product weighed 10.1 grams, decomp. point 240-242° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.07; H, 5.73; N, 7.78. Schiemann and Roselius (3) reported a 50% yield of m-fluorophenylalanine from the oxazalone, m. p. 262-263° (decomp.).

A portion of the main fraction above was used to determine the absorption spectrum and ionizations constants of m-fluorophenylalanine as reported in section I-C of this thesis.

The overall yield of m-fluorophenylalanine from m-nitrobenzoic acid was approximately 4.6%.

Preparation of p-Fluorophenylalanine (Method I)

Ethyl p-aminobenzoate was prepared by refluxing 290 grams (2.1 moles) of p-aminobenzoic acid (Paragon Testing Lab.) with 2 liters of absolute ethanol (99.75% absolute) and 153 grams of dry hydrogen chloride for 48 hours (23). The

ethanol was removed by distillation under reduced pressure. The ester-hydrochloride was dissolved in 1.5 liters of water and a saturated solution of sodium carbonate was carefully added. The ester precipitated and was filtered and washed well with water. Subsequently it was dissolved in 2 liters of warm ethanol and precipitated by the addition of approximately 6 liters of water. The precipitate was removed by filtration and again reprecipitated from ethanol. After drying, obtained 278 grams (80%) of ethyl p-aminobenzoate, m. p. 89.5-90.5° (uncorr.), lit. m. p. 91-92°.

A second preparation using 403 grams (2.9 moles) of p-aminobenzoic acid (EK White Label), 3 liters of absolute ethanol, and 225 grams of anhydrous hydrogen chloride yielded 431 grams (89%) of ethyl p-aminobenzoate (this product was not recrystallized), m. p. 88-90° (uncorr.).

p-Carbethoxybenzenediazonium fluoroborate was prepared as described above using 431 grams (2.6 moles) of ethyl p-aminobenzoate, 1100 ml. of 5 F HCl, 200 grams of sodium nitrite, 275 ml. additional 12 F HCl, and 350 grams of sodium fluoroborate in 500 ml. of water (23). The precipitated diazonium fluoroborate was removed by filtration and washed with three 350 ml. portions of cold water, three 400 ml. portions of cold methyl alcohol, and two 400 ml. portions of ether. The product was light tan, and after drying

in vacuo over concentrated sulfuric acid, it weighed 556 grams (81%); decomp. point 97-98° (uncorr.). Lit. decomp. point 93-94° (23,24).

A second preparation using 275 grams (1.6 moles) of ethyl p-aminobenzoate yielded 352 grams (77%) of dried p-carbethoxybenzenediazonium fluoroborate. Only slight decomposition has occurred in a sample of the above compound which has been stored for 2 years in a screw-cap bottle. Ethyl p-fluorobenzoate was prepared by the decomposition as described above of 341 grams of p-carbethoxybenzenediazonium fluoroborate (in one portion) (23). The decomposition required approximately 1 hour; no white solid appeared to sublime, but approximately 50 ml. of liquid were distilled or sprayed over into the trap. The material in the decomposition flask solidified on cooling to room temperature. The product in the trap flask was rinsed into a distilling flask with ether and distilled. Approximately 14 grams of material, b. p. 68-85°/27 mm. were obtained, and 35 grams of material b. p. 85-105°/27 mm. The low boiling material was again refractionated and yielded 9.8 grams of a product, b. p. 58-62°/34 mm. The higher boiling fraction was added to the main reaction product and distilled under reduced pressure, 140 grams, (62%), b. p. 112-114°/35 mm. were obtained. The main fraction was dissolved in ether and washed with dilute sodium hydroxide, the ether solution was dried

and redistilled to yield 136 grams (61%) of ethyl p-fluorobenzoate, b. p. 113-114°/35 mm., m. p. 26-27°. Lit. b. p. 105-106°/25 mm. (23, 24).

A second decomposition in two portions of 550 grams of p-carbethoxybenzenediazonium fluoroborate was carried out. The decomposition products (including the liquid in the trap flask) were combined with ether in a distilling flask and fractionated through a Vigreux column, 32.0 grams of a product b. p. 66°/29 mm. and 246 grams of ethyl fluorobenzoate, b. p. 104-105°/19 mm. were obtained. The ester was dissolved in ether, and extracted with dilute base, ether solution dried, and refractionated to yield 232 grams (66%) of ethyl p-fluorobenzoate, b. p. 89-90°/12 mm.; m. p. 26-27°. The low boiling fractions were not further characterized.

p-Fluorobenzhydrazide was prepared as described for the ortho isomer using 64 grams (1.09 moles) of hydrazine hydrate (Lemke-85%) and 110 grams (0.66 mole) of ethyl p-fluorobenzoate (26). A large amount of product precipitated out of the hot mixture. After allowing it to crystallize at 4°, the product was removed by suction filtration and washed with five 20 ml. portions of cold 95% ethanol, and four 20 ml. portions of ether. After drying, the product weighed 93 grams (92%), m. p. 147-158°. A second crop of 5 grams was obtained by

concentration of the mother liquors. It was found that p-fluorobenzhydrazide could be recrystallized from acetone, chloroform, or ethyl acetate; the latter was chosen as most satisfactory. 94 grams of crude p-fluorobenzyhydrazide were recrystallized by dissolving in 1800 ml. of refluxing ethyl acetate, filtering hot to remove a small amount of insoluble material, and allowing to cool to room temperature. The finely crystalline slightly yellow precipitate was removed by filtration and washed with ethyl acetate and ether; yield: 64 grams, m. p. 161.5-163.0° (corr.). Anal. Calcd. for $C_7H_7ON_2F$ (154.1): C, 54.54; H, 4.58; N, 18.18. Found: C, 54.57; H, 4.66; N, 18.22 (Elek). A second fraction of 7 grams was obtained by placing filtrate and washings in the cold room; m. p. 159-162° (corr.).

A second preparation of p-fluorobenzhydrazide was performed using 138 grams (2.3 moles) of hydrazine hydrate (85%) and 231 grams (1.38 moles) of ethyl p-fluorobenzoate. 215 grams (98%) of p-fluorobenzhydrazide, m. p. 153-162° (corr.) were obtained. It was used without further purification.

sym-p-fluorobenzoylbenzenesulfonbenzhydrazide was prepared by the method described above using 60 grams (0.39 mole) of p-fluorobenzhydrazide, 600 ml. of pyridine (Barrett's refined, redistilled), and 75 grams (0.42 mole) of benzenesulfonyl chloride (EK White Label). After the

reaction mixture had stood overnight at room temperature, approximately 400 ml. of pyridine were distilled off under reduced pressure, and the mixture was poured with stirring onto a mixture of 250 ml. of 12 F HCl and 300 grams of ice. After standing, the precipitate was filtered and washed well with six 100 ml. portions of water. The light tan product was dried in vacuo, yield: 102 grams (88%), m. p. 170-178° (corr.). The product was dissolved in 500 ml. of hot absolute ethanol, treated with Norite to decolorize, and recrystallized. After concentration of the ethanol solution, 76 grams of nearly white crystals were obtained, m. p. 179-180° (corr.). It is to be noted that sym-p-fluorobenzoylbenzenesulfonbenzhydrazide is much more soluble in ethanol than the ortho and meta-compounds and it was therefore difficult to obtain it in a colorless crystalline form. For analysis a sample was recrystallized from benzene and from alcohol, m. p. 179.0°-180.5° (corr.).

Anal. Calcd. for $C_{13}H_{11}O_3N_2SF$ (294.3): N, 9.52.
Found: N, 9.85 (Elek).

A second preparation was made using 195 grams (1.26 moles) of p-fluorobenzhydrazide, 2.0 liters of pyridine (redistilled), and 224 grams of benzenesulfonyl chloride (EK White Label). The pyridine was not removed from this mixture, instead the mixture was poured directly onto a

mixture of 2 liters of 12 F HCl and 5 kg. of ice, washed with water and finally with several small portions of cold ethanol (the ethanol removed a large amount of color as well as some product which was later recovered). Yield: 285 grams (76%), m. p. 175-179°. Since the yield was less than expected, the acid-pyridinium hydrochloride solution was concentrated under reduced pressure and 49 grams (13%) additional product was obtained, m. p. 177-179° (corr.). Total yield: 89%. The sym-p-fluorobenzoylbenzenesulfonhydrazide from this preparation was used without further purification.

p-Fluorobenzaldehyde and 2-Phenyl-4-(p-fluorobenzal)-oxazalone-5.- Three decompositions by the method described above of sym-p-fluorobenzoylbenzenesulfonhydrazide were made using a total of 76 grams (0.26 mole) of the sulfonhydrazide made in the first described preparation above (16). The yield of crude aldehyde obtained (not distilled) varied from 51-67% of theory. It was converted to the oxazalone by the previously described method (3,27,28), and the product (total yield 21.5 grams) was obtained in 46-53% yield (overall yield 27-29%), m. p. 129-170° (corr.). Attempts to obtain a sharp melting compound by recrystallization from ethanol were not successful.

Another preparation of p-fluorobenzaldehyde was made using six 50 gram portions (1.0 mole) of sym-

p-fluorobenzoylbenzenesulfonhydrazide. The decomposition mixture from each decomposition was first extracted once with 250 ml. of ether (peroxide free) and then combined and extracted in a continuous liquid-liquid extractor for 24 hours. After drying, the ether extracts were distilled and fractionated through a 20 cm. Vigreux column. Yield: 54 grams (42%) of p-fluorobenzaldehyde, b. p. 97-99°/48 mm; $\eta_D^{25} = 1.518_0$. Lit. b. p. 104.5°/74 mm., 173°/760 mm. $\eta_D^{19} = 1.52001$ (15,29).

2-Phenyl-4-(p-fluorobenzal)-oxazalone-5 was prepared by heating 53.7 grams (0.43 mole) of distilled p-fluorobenzaldehyde, 35 grams of sodium acetate, 78.5 grams (0.44 mole) of hippuric acid (EK White Label) and 135 ml. of acetic anhydride (Baker C. P.), for 1 1/4 hours (the mixture darkened considerably on heating). After cooling a nicely crystalline yellow precipitate formed; it was allowed to stand overnight at room temperature, then 200 ml. of water were slowly added to the reaction mixture with stirring (only a small amount of oily material formed). After filtering, the precipitate was washed well with several additional 100 ml. portions of cold water, and then with three 100 ml. portions of hot water. The oxazalone was subsequently allowed to stand overnight at 4° with 60 ml. of ethanol and than washed with eight 20 ml. portions of cold ethanol. After drying there was obtained 69 grams (60%) of crude 2-phenyl-4-

(p-fluorobenzal)-oxazalone-5, m. p. 133-165° (corr.). 65 grams of the crude oxazalonewere dissolved in 4 liters of hot absolute ethanol(required approximately 1 hour to dissolve), filtered, and allowed to cool slowly to room temperature. A yellow, very nicely crystalline product (needles) formed; it was removed by filtration, washed well with five 100 ml. portions of absolute ethanol and dried. Recovered 34.5 grams (32% from aldehyde) of pure 2-phenyl-4-(p-fluorobenzal)-oxazalone-5, m. p. 184-185.5 (corr.). Anal. Calcd. for $C_{16}H_{10}O_2NF$ (267.2): C, 71.90; H, 3.77; N, 5.24. Found: C, 71.92; H, 3.83; N, 5.22 (Elek). Schiemann and Roselius (3) obtained the oxazalone from the aldehyde in 75% yield, m. p. 181-182°. From the combined filtrate and washings 25 grams additional product was recovered in several fractions, all had wide melting ranges, for example fraction 3 weighed 8.3 grams and had a m. p. of 133-148° (corr.).

Chromatographic separations of portions of F-3 on a silicic acid-celite column using benzene as a solvent and as a developer yielded approximately equal amounts of compounds melting at 153-154° (corr.) (yellow needles) and 184-185° (corr.) (2-phenyl-4-(p-fluorobenzal)-oxazalone-5). The lower melting compound moved less rapidly on the column than did the higher melting compound. Both compounds were visible only as very light yellow zones on the column, and it was

difficult to ascertain their exact position.

A fractionation of 21 grams of the wide melting fractions obtained in the recrystallization above by 2 recrystallizations from 75 ml. of benzene yielded 8.8 grams of a product (F-B-1) (yellow needles) m. p. 153-154.5° (corr.), believed to be 2-methyl-4-(p-fluorobenzal)-oxazalone-5. Anal. Calcd. for: $C_{11}H_8O_2NF$ (205.2): C, 64.50; H, 3.93; N, 6.84. Found: C, 64.61; H, 3.96; N, 6.74 (Elek).

It was originally thought that the two forms of oxazalone which had been obtained were cis-trans isomers; therefore an attempt was made to transform the low melting form into the high melting form (33) by dissolving in pyridine, and pouring onto iced-dilute hydrochloric acid. The m. p. of the product obtained by this procedure had not changed from that of the original material. Therefore it seemed unlikely that the compounds were isomers; instead the possibility that trans-acylation had occurred was investigated and the following experiments were performed:

α-Acetamido-p-fluoro-cinnamic acid ethyl ester and α-Acetamido-p-fluoro-cinnamic acid were prepared by the method of Carter (33). 200 mg. of the low melting azlactone (m. p. 153-154.5°) were dissolved in 1 ml. of benzene, 0.1 ml. of 1 F sodium ethoxide was added and the mixture

was allowed to stand for about 5 minutes; 0.2 ml. of 1 F HCl was added, and the mixture was shaken vigorously for 3 minutes. No precipitate had formed, therefore 1 ml. of 60-70° ligroin was added, a precipitate formed rapidly. It was removed by filtration, washed with ligroin and then water; dried. Yield: 200 mg. (80%) of p-fluoro-α-acetamido cinnamic acid ethyl ester, m. p. 116-117° (corr.).

Anal. Calcd. for $C_{13}H_{15}O_3NF$ (252.3): C, 62.00; H, 6.00; N, 5.71. Found: C, 62.24; H, 5.87; N, 5.43 (Elek).

154 mg. of the ester were dissolved in 4 ml. of ethanol, 3 ml. of 0.5 F NaOH were added and the mixture was allowed to stand at room temperature for approximately 2 1/2 hours, 5 ml. of water were added and then the solution was extracted with ether. The aqueous solution was filtered, warmed to 50°, and 3 ml. of 1 F HCl added. After standing overnight at 4°, very nicely crystalline white needles formed m. p. 214-216 (corr.) (decomp.); yield 102 mg. (75%) of α-acetamido-p-fluoro-cinnamic acid.

Anal. Calcd. for $C_{11}H_{10}O_3NF$ (223.2): C, 59.19;

H, 4.52; N, 6.28. Found: C, 59.03; H, 4.88; N, 6.18.

α-Benzamido-p-fluoro-cinnamic acid ethyl ester and α-Benzamido-p-fluoro-cinnamic acid (3) were prepared in the same manner as described above (33). From 200 mg. of 2-phenyl-4-(p-fluorobenzal)-oxazalone-5 (m. p. 184-185.5°) 211 mg. (90%) of α-benzamido-p-fluoro-cinnamic acid ethyl ester, m. p. 122-123° (corr.) were obtained.

Anal. Calcd. for $C_{18}H_{16}O_3NF$ (313.3): C, 69.00; H, 5.14; N, 4.47. Found: C, 69.31; H, 5.13; N, 4.43.

178 mg. of the ester were converted to the acid by the method described above and 130 mg. (77%) of α-benzamido-p-fluorocinnamic acid were obtained as white needles, m. p. 224-226° (corr.) (decomp.). (3) gives 224-225° (decomp.) as the melting point for α-benzamido-p-fluoro-cinnamic acid.

Anal. Calcd. for $C_{16}H_{12}O_3NF$ (285.3): C, 67.37; H, 4.24; N, 4.91. Found: C, 67.27; H, 4.22; N, 4.95 (Elek).

Therefore it appears very probable that the oxazalone with a m. p. 153-154.5° is 2-methyl-4-(p-fluorobenzal)-oxazalone-5 and that trans acylation has occurred. In order

to make the identification more positive, 2-methyl-4-(p-fluorobenzal)-oxazalone-5 should be synthesized by the usual methods. It is also probable that trans-acylation occurred in the preparation of the ortho and meta isomers as described in this thesis. In the former case the product is probably considerably lower melting and more soluble and was removed in the washing procedure employed. This would explain the lower yield of oxazalone obtained with this isomer. It is very likely, however, that 2-methyl-4-(m-fluorobenzal)-oxazalone-5 can be isolated by fractional crystallization or by chromatographic methods from the meta-isomer. The existence of trans-acylation in the oxazalone formation should be further investigated. When it occurs, the yields of amino acid could be increased by using the crude azlactone.

The absorption spectra in chloroform of the crude oxazalone (m. p. 133-165°), of 2-phenyl-4-(p-fluorobenzal)-oxazalone-5, 2-methyl-4-(p-fluorobenzal)-oxazalone-5, and 2-methyl-4-benzal-oxazalone-5* were taken and are shown in Figure III. $\epsilon_{max}$ and $\epsilon_{min}$ are given in Table III.

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* Prepared by Mr. J. L. O'Brien.

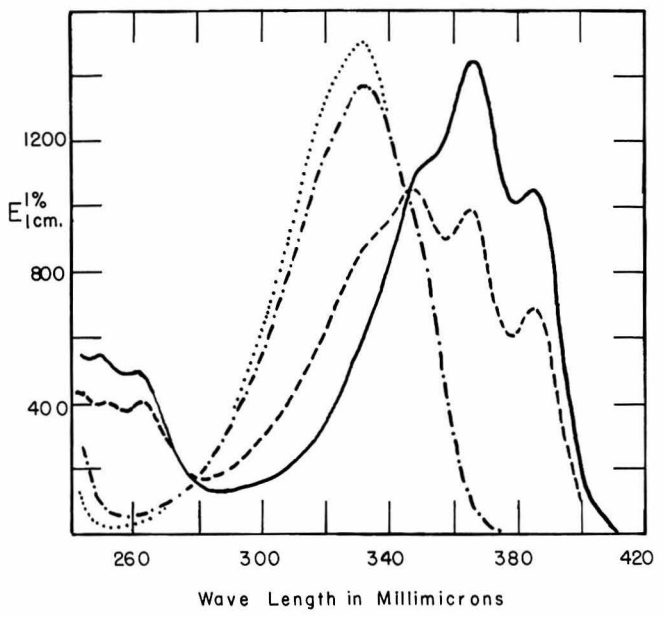


Figure 3. Ultra-Violet Absorption Spectra of pure 2-Phenyl-4-(p-fluorobenzal)-oxazalone-5, m. p. 184-185.5° _____, 2-methyl-4-(p-fluorobenzal)-oxazalone-5, m. p. 153-154.5 -.-.-.-.-.-., crude oxazalone, m. p. 133-165°- - - - - , and of 2-methyl-4-benzal-oxalone-5..... (in chloroform).

Table III

$\varepsilon_{max}$ and $\varepsilon_{min}$ for 2-Phenyl-4-(p-fluorobenzal)-oxazalone-5, 2-Methyl-4-(p-fluorobenzal)-oxazalone-5 and 2-Methyl-4-benzal-oxazalone-5

in Chloroform

| | $\lambda_{m\mu}$ | | $\varepsilon$ |
|---|---|---|---|
| 2-Phenyl-4-(p-fluorobenzal)-oxazalone-5 Recrystallized from Ethanol. m. p. 184-185.5° (corr.). | 385 | (max) | 28,000 |
| | 380 | (min) | 26,800 |
| | 366.5 | (max) | 38,600 |
| | 286 | (min) | 3,530 |
| | 262 | (max) | 12,700 |
| | 258 | (min) | 9,750 |
| | 252 | (max) | 11,000 |
| | 248 | (min) | 10,800 |
| 2-Methyl-4-(p-fluorobenzal)-oxazalone-5 Recrystallized from Ethanol and from Benzene. m. p. 153-154.5° (corr.). | 331.5 | (max) | 28,000 |
| | 257 | (min) | 1,230 |
| 2-Methyl-4-benzal-oxazalone-5 m. p. 154-155° (corr.). | 332 | (max) | 28,000 |
| | 254 | (min) | 525 |

p-Fluorophenylalanine (3) was prepared by reductively hydrolyzing with hydriodic acid, acetic anhydride, and red phosphorus 13.9 grams (0.05 mole) of the crude oxazalone obtained from the first preparation described above (28). After extraction with ether and recrystallization from aqueous ammonia, 3.5 grams (40%) of p-fluorophenylalanine were obtained as nicely crystalline plates, decomp. point 256-258° (corr.). Amino N, Calc.: 7.6, Found: 7.65 (Cunningham).

A second preparation was made using 31.2 grams (0.12 mole) of recrystallized 2-phenyl-4-(p-fluorobenzal)-oxazalone-5. It was reductively hydrolyzed with hydriodic acid, acetic anhydride and red phosphorus for 24 hours. After removal of the red phosphorus, the mixture was evaporated to dryness under reduced pressure, dissolved in 150 ml. of water and extracted well with ether, filtered, and then made basic with ammonium hydroxide. The excess ammonium hydroxide was expelled by boiling, and a crystalline precipitate (plates) formed in the hot solution. After several days in the cold room, the product was filtered, washed with water, alcohol, and then ether. Obtained 15.9 grams (74%) of p-fluorophenylalanine, decomp. point 252-258° (corr.). From the filtrate and washing 1.6 grams (7%) of additional p-fluorophenylalanine was isolated, decomp. point 256-258° (corr.). Total yield: 81%.

The main fraction was recrystallized by dissolving in 500 ml. of boiling water, filtering and adding 2 volumes of ethanol. Very nicely crystalline plates were obtained which weighed, after washing and drying, 13.3 grams, decomp. point 259-261° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.29; H, 5.67; N, 7.57.

This sample was used to determine the ionization constants and absorption spectrum reported in section I-C of this thesis. Schiemann and Roselius (3) reported a 22% yield of p-fluorophenylalanine, m. p. 263.5-264° (with decomp.) from the oxazalone.

The overall yield of p-fluorophenylalanine from p-aminobenzoic acid was approximately 4.1%.

Preparation of o-Fluorophenylalanine (Method II)*

o-Fluorobenzyl chloride was prepared by the vapor phase, ultra-violet light catalyzed chlorination of o-fluorotoluene (EK White Label) (the boiler temperature was allowed to rise to 174°)(uncorr.) (21). The chlorination of 40 grams (0.36 mole) of o-fluorotoluene required approximately 2 hours, and after fractionation under reduced pressure 42.6 grams (82%) of o-fluorobenzyl chloride, b. p. 86-88.5°/40 mm., $\eta_D^{25}$ = 1.5122 were obtained. Lit. b. p. 83°/32 mm., 67.5-68°/16 mm.; $\eta_D^{24}$ = 1.51538 (25,34).

α,α'-(o-Fluorobenzyl)-acetamido-malonic ester.- 7.0 grams (0.30 mole) of sodium were dissolved in 475 ml. of absolute ethanol (99.8% absolute); 66.3 grams (0.31 mole) of acetamido-malonic ester (Winthrop Chem.) and 38.5 grams (0.27 mole) of o-fluorobenzyl chloride were added and the mixture was refluxed for 4 hours (18,19). It was then filtered hot to remove sodium chloride, and 2 volumes of water were added. The product was allowed to crystallize overnight at 4°; then it was filtered and recrystallized from 30% ethanol; obtained 78.8 grams (89%) of α,α'-(o-fluorobenzyl) acetamido-malonic ester, m. p. 107-108° (corr.).

Anal. Calcd. for $C_{16}H_{20}O_5NF$ (325.3): C, 59.07; H, 6.20; N, 4.30. Found: C, 59.03; H, 6.19; N, 4.37.

* The preparation of N-acetyl-o-fluorophenylalanine from fluorotoluene was done by Mr. Wildon Fickett, and is included in this thesis for the sake of completeness.

N-Acetyl-o-fluorophenylalanine was prepared by refluxing 74.8 grams (0.23 mole) of $\alpha,\alpha'$-(o-fluorobenzyl)-acetamido-malonic ester with 600 ml. of 2.5 F NaOH for 4 hours, cooling slightly, and adding 470 ml. of 3 F HCl and refluxing for an additional hour (18,19). The solution was filtered hot, and the product was allowed to crystallize upon cooling. 35.8 grams of crude N-acetyl-o-fluorophenylalaninewere obtained. m. p. 142.5-145°. It was recrystallized from 30% alcohol and 32.4 grams (63%) of pure N-acetyl-o-fluorophenylalanine were recovered, m. p. 147-149° (corr.).

Anal. Calcd. for $C_{11}H_{12}O_3NF$ (225.2): C, 58.65; H, 5.37; N, 6.22. Found: C, 58.46; H, 5.51; N, 6.35.

o-Fluorophenylalanine (3) was prepared by refluxing 10.0 grams (0.044 mole) of N-acetyl-o-fluorophenylalanine with 80 ml. of 6 F HCl for 14 hours. The hydrolysate was placed in the cold room overnight, and the precipitate was removed by filtration. A portion of the precipitate was recrystallized from 6 F HCl; obtained 1.54 grams (17%) of o-fluorophenylalanine hydrochloride monohydrate after drying in vacuo over NaOH, decomp. point 243-250° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF \cdot HCl \cdot H_2O$ (237.7): C, 45.48; H, 5.51; N, 5.89. Found: C, 45.45; H, 5.48; N, 5.84 (Elek). Neut. Eq. Found: 236.5, 239.0.

The remainder of the o-fluorophenylalanine was isolated by crystallization from aqueous ammonia; obtained 5.1 grams

(63%) (total o-fluorophenylalanine isolated, 84%) decomp. point 245-249° (corr.). It was recrystallized by dissolving in 150 ml. of hot water and adding 300 ml. of ethanol. Obtained 3.2 grams (F-2-A), decomp. point 245-250° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 60.20 (?); H, 5.39; N, 7.79 (Elek).

The filtrate was evaporated to 25 ml. and 50 ml. of ethanol were added; obtained a second fraction (F-2-B) of 1.4 grams, decomp. point 255-258° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.64. Found: C, 59.08; H, 5.45; N, 7.62 (Elek).

o-Fluorophenylalanine is very difficult to obtain from water or water-ethanol mixtures in any form but a gel. However, from 6 F HCl, a very nicely crystalline product is obtained (long prisms), and it is recommended that o-fluorophenylalanine be recrystallized from this solvent whenever possible. It is obtained as the amino acid hydrochloride monohydrate, stable to drying in vacuo over NaOH.

The overall yield of o-fluorophenylalanine from o-fluorotoluene by the above method was approximately 37%.

Preparation of m-Fluorophenylalanine (Method IIA)

m-Fluorobenzyl chloride* was prepared by the vapor phase,

* m-Fluorobenzyl chloride and p-fluorobenzyl chloride were prepared in the junior year Organic Chemistry Laboratory by Messrs. Arcand, Jennings, Picciotto, and Viglierchio. The author wishes to express his appreciation for their efforts and also for the cooperation of Prof. H. J. Lucas.

ultra-violet light catalyzed chlorination of m-fluorotoluene (EK White Label) (21). The boiler temperature was allowed to increase to 175°. From 209 grams (1.9 moles) of m-fluorotoluene 226 grams (82%) of m-fluorobenzyl chloride, b. p. 178-182°/745 mm. were obtained. The product was refractionated through a Vigreux column, and 213 grams of m-fluorobenzyl chloride were obtained, b. p. 84°/29 mm., $\eta_D^{25}$ = 1.5100. Lit. b. p. 67-68°/15 mm., 73°/23 mm., $\eta_D^{17.5}$ = 1.51412 (15,34). Ethyl-(m-fluorobenzyl)-acetamido-cyanoacetate, N-Acetyl-m-fluorophenylalanine and m-fluoro-phenylalanine (3).- 23.0 grams (1.0 mole) of sodium were dissolved in 1 liter of absolute ethanol (99.8%), 176 grams of ethyl acetamido-cyanoacetate (1.05 moles) (Winthrop Chem.) and 145 grams (1.0 mole) of redistilled m-fluorobenzyl chloride were added and the mixture was refluxed for 11 hours (20). The precipitated sodium chloride was removed by filtration and the filtrate was allowed to cool. It was placed in the cold room for several days but no precipitate was obtained. The addition of water to a small portion of the solution produced only an oil. The ethanol was removed by distillation under reduced pressure and the remaining oil was refluxed for 11 hours with 1 liter of 4 F NaOH, and then 350 ml. of 12 F HCl were added and refluxing was continued for 4 hours. 200 ml. of 6 F NaOH were added, and the

solution was filtered to remove a small amount of insoluble material, and then made slowly acid with approximately 150 ml. of 12 F HCl. After standing overnight in the cold room, the colored precipitate was removed by filtration, washed with water and dried. Yield: 72 grams (32%) of N-acetyl-m-fluorophenylalanine, m. p. 147-152° (corr.).

21 grams of N-acetyl-m-fluorophenylalanine were hydrolyzed with 200 ml. of 6 F HCl for 24 hours. The hydrolysate was placed in the cold room overnight and the amino acid hydrochloride was removed by filtration, dissolved in hot water, made basic with ammonium hydroxide, and the excess ammonia expelled by boiling. The solution was filtered, and 100 ml. of ethanol added to the filtrate. A crystalline precipitate (needles) formed rapidly. After standing overnight at 4°, the precipitate was filtered, washed with water, ethanol, and then ether. It was recrystallized by dissolving in 300 ml. of hot water, and adding 300 ml. of ethanol. Long needles were obtained; yield after filtering, washing, and drying was 14.2 grams (84%) of m-fluorophenylalanine, decomp. point 248-251° (corr.).
Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.25; H, 5.42; N, 7.71 (Elek).

The ionization constants and spectrum of m-fluorophenylalanine were determined on this fraction.

m-Fluorophenylalanine can be recrystallized easily from 6 F HCl and the resulting product dried <u>in vacuo</u> over NaOH to

yield the amino acid hydrochloride. 1.0 gram of the above amino acid was recrystallized from 50 ml. of 6 F HCl; obtained 1.05 grams of m-fluorophenylalanine hydrochloride.
Anal. Calcd. for $C_9H_{10}O_2NF.HCl$ (219.6): C, 49.20; H, 5.05; N, 6.38. Found: C, 49.32; H, 5.07; N, 6.28 (Elek). Neut. Equiv. Found: 220.0, 221.1.

A second preparation of N-acetyl-m-fluorophenylalanine was made by the above method using 125 ml. of absolute ethanol (99.8% absolute), 2.5 grams (0.11 mole) of sodium, 18.7 grams (0.11 mole) of ethyl acetamido-cyano acetate (Winthrop Chem.) and 15.2 grams (0.105 mole) of m-fluorobenzyl chloride. The mixture was refluxed for 3 hours, filtered hot to remove the sodium chloride, evaporated under reduced pressure, and the oil hydrolyzed with 150 ml. of 2.5 F NaOH for 5 1/2 hours. 35 ml. of 12 F HCl were added and the mixture was refluxed for 2 hours. It was then filtered to remove a large amount of colored oil, and the filtrate was allowed to stand overnight at room temperature. The crystalline, nearly white precipitate was filtered, washed with water and a small amount of ethanol and dried. Yield: 2.8 grams, m. p. 150-153.5° (corr.). The filtrate and washings from above were refluxed with the oil for a short time, filtered, and the filtrate placed in the cold room. The precipitate which formed was filtered and washed with water. Yield: 6.6 grams, m. p. 151-155° (corr.). Total

yield: 9.4 grams (40%). It is possible that the yield could have been increased by using ethanol freshly distilled from sodium, and by slightly less vigorous hydrolysis conditions (see below). The overall yield from m-fluorotoluene was 33%, and the yield of m-fluorophenylalanine would have been approximately 28% (est.).

Preparation of m-Fluorophenyalanine (Method IIB)

$\alpha,\alpha'$-(m-Fluorobenzyl)-acetamido-malonic ester was prepared in the same manner as described above for the ortho isomer by refluxing 300 ml. of absolute ethanol (99.8%), 4.7 grams (0.2 mole) of sodium, 46.0 grams (0.21 mole) of acetamido-malonic ester (Winthrop Chem.) and 28.9 grams (0.20 mole) of m-fluorobenzyl chloride for 5 hours (18,19). The precipitated sodium chloride was removed from the hot mixture by filtration and 2 volumes of warm water slowly added to the filtrate. Very nicely crystalline needles of prisms formed rapidly. After cooling the product was filtered, washed with cold 30% ethanol, then water, and dried. Yield of $\alpha,\alpha'$-(m-fluorobenzyl)-acetamido-malonic ester was 44.2 grams (68%), m. p. 117-121° (corr.).

A second preparation was made using 200 ml. of absolute ethanol (99.8% absolute), 2.4 grams (0.1 mole) of sodium, 23.0 grams (0.11 mole) of acetamido-malonic ester (Winthrop Chem.) and 14.7 grams (0.1 mole) of m-fluorobenzyl chloride.

After filtering hot, the ethanol filtrate was allowed to stand overnight in the cold room, and the precipitate of long prisms obtained was filtered, washed with a small quantity of cold alcohol and water. Dried. Yield: 15.0 grams (46%), m. p. 118-121. From the filtrate and washings an additional 10 grams, m. p. 118-121° of product was obtained by concentration to approximately 50 ml. Total yield; 76%. For analysis a small portion of the first fraction was recrystallized from 50% ethanol, m. p. 119-121° (corr.).
Anal. Calcd. for $C_{16}H_{20}O_5NF$ (325.3): C, 59.07; H, 6.20; N, 4.30. Found: C, 59.12; H, 6.15; N, 4.28 (Elek).

N-acetyl-m-fluorophenylalanine.- 63.2 grams of α,α'-(m-fluorobenzyl)-acetamido-malonic ester were refluxed 4 hours with 500 ml. of 2 F NaOH, cooled slightly, 80 ml. of 12 F HCl slowly added, and the mixture refluxed an additional 2 hours (18,19). It was filtered hot (pH 3) and 25 ml. of 12 F HCl were slowly added. A nicely crystalline precipitate of N-acetyl-m-fluorophenylalanine formed (long prisms) which was filtered, washed with water and dried. Yield: 40.1 grams (92%) m. p. 154-156.5° (corr.). For analysis 5.0 grams of the above product were recrystallized from water. Recovered 4.6 grams, m. p. 154-156° (corr.).
Anal. Calcd. for $C_{11}H_{12}O_3NF$ (225.2): C, 58.65; H, 5.37; N, 6.22. Found: C, 58.71; H, 5.31; N, 6.26 (Elek).

The overall yield of N-acetyl-m-fluorophenylalanine from m-fluorotoluene was 55%, and assuming a yield of 80% in the

conversion of the acetyl compound to the free amino acid, a yield of approximately 45% m-fluorophenylalanine would be obtained by this method.

Preparation of p-Fluorophenylalanine (Method IIA)

p-Fluorobenzyl chloride was prepared by the vapor phase, ultra-violet light catalyzed chlorination of p-fluorotoluene (EK White Label) (21). The boiler temperature was allowed to rise to 175°. From 207 grams (1.9 moles) of p-fluorotoluene 212 grams (83%) of p-fluorobenzyl chloride, b. p. 175-178°/745 mm. (uncorr.) were obtained. The product was refractionated at reduced pressure through a 20 cm. Vigreux column; obtained 205 grams; b. p. 86-87°/30 mm.; $\eta_D^{25}$ = 1.5103. Lit. b. p. 76°/20 mm. (34).

Ethyl-(p-fluorobenzyl)-acetamido-cyanoacetate was prepared by the method described above using 23 grams (1.0 mole) of sodium, 1 liter of absolute ethanol (99.8% absolute), 174 grams (1.0 mole) of ethyl acetamido-cyanoacetate (Winthrop Chem.) and 145 grams (1.0 mole) of p-fluorobenzyl chloride (20). The mixture was refluxed for 3 hours, filtered hot to remove the sodium chloride (the sodium chloride was washed with 200 ml. of hot ethanol and the washings were added to main filtrate), and the filtrate placed in the cold room overnight. A crystalline precipitate was obtained,

filtered off, washed with three 50 ml. portions of cold ethanol, and dried. Yield: 164 grams (57%) of ethyl-(p-fluorobenzyl)-acetamido-cyanoacetate, m. p. 164-166° (corr.). To the filtrate and washings were added 1 1/2 liters of water, and 37 grams additional product was obtained, m. p. 159-164° (corr.); total yield:70%. The second fraction was recrystallized from ethanol and 31 grams of productwere obtained, m. p. 164-166° (corr.).

For analysis 6.0 grams of the main fraction were recrystallized from 50 ml. of 95% ethanol. Recovered 4.5 grams of ethyl-(p-fluorobenzyl)-acetamido-cyanoacetate, m. p. 165-166° (corr.).

Anal. Calcd. for $C_{14}H_{15}O_3N_2F$ (278.3): C, 60.41; H, 5.43; N, 10.05. Found: C, 60.42; H, 5.47; N, 10.00 (Elek).

N-Acetyl-p-fluorophenylalanine.- 78 grams (0.27 mole) of ethyl-(p-fluorobenzyl)-acetamido-cyanoacetate were hydrolyzed for 5 hours in 1 liter of 1 F NaOH, then cooled slightly and 90 ml. of 12 F HCl slowly added; the mixture was refluxed two hours more, filtered hot to remove a small amount of insoluble material (pH 4) and 10 ml. of 12 F HCl were slowly added (20). A crystalline precipitate formed on cooling. After standing overnight at 4°, the precipitate was filtered, washed with water and dried. Yield: 38.0 grams (60%), m. p. 150-151.5° (corr.).

For analysis 5.0 grams of the second preparation were recrystallized from 75 ml. of water. Recovered 4.6 grams, m. p. 150.5-152.0° (corr.).

Anal. Calcd. for $C_{11}H_{12}O_3NF$ (225.2): C, 58.65; H, 5.37; N, 6.22. Found: C, 58.74; H, 5.45; N, 6.30 (Elek).

p-Fluorophenylalanine (3).- 10.0 grams of N-acetyl-p-fluorophenylalanine (0.044 mole) were hydrolyzed for 12 hours with 75 ml. of 6 F HCl. The hydrolysate was placed in the cold room overnight and then the precipitated amino acid hydrochloride was removed by filtration. A portion of this product was recrystallized from 20 ml. of 6 F HCl; obtained 1.16 grams (12%) of p-fluorophenylalanine hydrochloride after drying in vacuo over NaOH, decomp. point 241-252° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF \cdot HCl$ (219.6): C, 49.20; H, 5.05; N, 6.38. Found: C, 49.40; H, 5.00; N, 6.31 (Elek). Neut. Equiv. 220.0; 219.6. The remainder of the product was recrystallized from approximately 75 ml. of aqueous ammonia, and then again by dissolving in 100 ml. of water and adding 200 ml. of ethanol. Crystalline plates were obtained. Yield: 4.7 grams (58%) (total yield: 72%), decomp. point 265-267° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.04; H, 5.48; N, 7.72 (Elek).

A portion of this sample was used for the determination of absorption spectrum and ionization constants reported in section I-C of this thesis.

The overall yield of p-fluorophenylalanine from p-fluorotoluene was 27%.

Preparation of p-Fluorophenylalanine (Method IIB)

α,α'-(p-fluorobenzyl)-acetamido-malonic ester.- 4.7 grams (0.20 mole) of sodium were dissolved in 300 ml. of absolute ethanol (99.8% absolute); then 46.0 grams (0.21 mole) of ethyl acetamido-malonate (Winthrop Chem.) and 29.0 grams (0.20 mole) of p-fluorobenzyl chloride were added and the mixture was refluxed for 4 hours (18,19). It was then filtered hot to remove sodium chloride and to the hot filtrate 600 ml. of water were slowly added. The solution was placed in the cold room overnight and the crystalline precipitate was filtered and washed with water; dried. Obtained 33.5 grams (51%) of α,α'-(p-fluorobenzyl)-acetamido-malonic ester, m. p. 130-138° (corr.). After recrystallization from ethanol 28.6 grams were recovered, m. p. 145-147.5° (corr.). For analysis, a small portion was recrystallized again from ethanol, m. p. 145-147.0° (corr.). Anal. Calcd. for $C_{16}H_{20}O_5NF$ (325.3): C, 59.07; H, 6.20; N, 4.30. Found: C, 59.14; H, 6.28; N, 4.29 (Elek).

A second preparation was carried out using 200 ml. of absolute ethanol (99.8% absolute), 2.4 grams (0.10 mole) of sodium, 23.0 grams (0.11 mole) of acetamido-malonic ester, and 14.5 grams (0.10 mole) of p-fluorobenzyl chloride. After refluxing for 9 hours, the mixture was filtered and placed in the cold room overnight. The precipitate was filtered and washed with cold alcohol. Obtained 14.5 grams (45%) of product, m. p. 145-147° (corr.).

A third preparation was made using 200 ml. of ethanol freshly distilled from sodium, 2.3 grams (0.1 mole) of sodium, 23 grams (0.11 mole) of acetamido-malonic ester, and 14.8 grams (0.10 mole) of p-fluorobenzyl chloride. It was refluxed for 3 hours, filtered and placed at 4° overnight. The precipitate was filtered and washed with cold ethanol. Obtained 25.8 grams (76%) of $\alpha,\alpha$-(p-fluorobenzyl)-acetamido-malonic ester, m. p. 143-147° (corr.). From the filtrate an additional 2.5 grams (7%) of product, m. p. 143-146° (corr.) was isolated by concentration to 50 ml. and addition of an equal volume of water. Thus the total yield was 83%.

N-Acetyl-p-fluorophenylalanine.- 65.8 grams (0.20 mole) of $\alpha,\alpha$-(p-fluorobenzyl)-acetamido-malonic ester were hydrolyzed for 4 hours in 1 liter of 1.2 F NaOH, then cooled slightly and 100 ml. of 12 F HCl added and the mixture was

refluxed for 2 hours, filtered (pH 3) (18,19). 20 ml. of 12 F HCl were slowly added and the solution was allowed to cool slowly. After storage overnight at 4°, the precipitate was filtered and washed with water; dried. Obtained 40.1 grams (88%) of N-acetyl-p-fluorophenylalanine, m. p. 150-153° (corr.).

The overall best yield of N-acetyl-p-fluorophenylalanine by this method was 60%. Assuming a 75% yield in the preparation of p-fluorophenylalanine from N-acetyl-p-fluorophenylalanine, the amino acid can be prepared in 45% yield from fluorotoluene by this procedure.

## Preparation of 2-Fluoro-4-methoxy-phenylalanine and 2-Fluorotyrosine

2-Methyl-5-nitrobenzenediazonium fluoroborate (35).- 100 grams (0.66 mole) of 4-nitro-2-aminotoluene (EK White Label) were added to 300 ml. of 5 F HCl, cooled to 0° C, and 45 grams of sodium nitrite in 90 ml. of water were added over a 1/2 hour period (24). Urea was added to react with the excess sodium nitrite, and the solution was then filtered to remove a small amount of highly colored insoluble material. 70 ml. of 12 F HCl were added, followed by 90 grams of sodium fluoroborate dissolved in 120 ml. of water. After standing at 0° for 1/2 hour, the precipitated diazonium fluoroborate was filtered, washed with cold water, methanol, then ether; dried *in vacuo* over sulfuric acid. Yield: 152 grams (92%) of 2-methyl-5-nitrobenzenediazonium fluoroborate, decomp. point 129-130° (uncorr.).

A second preparation was made using 300 grams (1.97 moles) of 4-nitro-2-aminotoluene (EK White Label), 900 ml. of 12 F HCl, 130 grams of sodium nitrite in 275 ml. of water; after diazotization was complete, 200 ml. of 12 F HCl and 270 grams of sodium fluoroborate in 350 ml. of water were added. The precipitate was filtered, washed with cold water, methanol, and ether; dried. Yield: 438 grams (89%), decomp. point 130-132° (uncorr.).

2-Fluoro-4-nitrotoluene (35).- Several procedures were tried in decomposing 2-methyl-5-nitrobenzenediazonium fluoroborate.

The first decomposition was carried out as suggested in (35). 150 grams of 2-methyl-5-nitrobenzenediazonium fluoroborate were placed in a 1 liter ST flask. 700 grams of dry sand (2-3 volumes) were mixed by shaking with the diazonium fluoroborate. The flask was connected by suitable joints to a condenser and 2 successive suction flasks cooled in ice, and finally to a water aspirator. The compound was decomposed by gentle heating at such a rate that the pressure in the system was maintained at 50-120 mm. The decomposition product was highly colored, and the sand present made it difficult to ascertain when decomposition was complete.

The sand was extracted with 500 ml. of ether (the material in the trap flasks was added to this extract), the ether extract was washed with water, then dried over Drierite, and finally distilled at 90-100°/10 mm. Obtained 30.5 grams, m. p. 33.5-35.0°. The sand was extracted a second and third time with 500 ml. portions of ether, and after washing, drying and distilling, a total of 20.4 grams additional product was obtained, m. p. 33-35°. The combined product was recrystallized from 50 ml. of ligroin. The 2-fluoro-4-nitrotoluene was obtained as large, light yellow crystals, m. p. 34-35°, total weight 49 grams (52%). Lit. b. p. 65-68°/2 mm., m. p. 34-35° (35).

A second decomposition was carried out in a manner similar to above, except no sand was used. The decomposition was rapid and only a small flame was needed to start decomposition. The residue in the flask was finally heated somewhat more strongly to complete decomposition. The residue was highly colored and a large amount was not readily soluble in ether. After the flask had been rinsed once with 75 ml. of ether, it was accidentally cracked and some material was undoubtedly lost. The ether extract was washed with water until neutral, then dried, and the product distilled under reduced pressure through a 20 cm. Vigreux column. Obtained 22 grams (35%); b. p. 96-97°/8 mm.

The third and most satisfactory method of decomposition was as follows: The decomposition apparatus was a 1 liter RBST flask attached to an upright 40 cm. air condenser and a 40 cm. downward water cooled condenser (assembled in the form of an inverted U). A 250 ml. suction flask was connected at the bottom of the downward condenser, and this in turn was connected to another 250 ml. suction flask with an inlet projecting 1/2 way into the water with which this latter flask was partially filled. The whole apparatus was assembled in the hood.

Approximately 50 gram portions were decomposed each time. The decomposition was carried out by gently heating the diazonium compound at a small local spot near the surface

with a burner or even a match, and as soon as the reaction had just started, heating was discontinued and the reaction was allowed to proceed spontaneously. After the reaction had stopped, the decomposition flask was gently heated until no more boron trifluoride and nitrogen were evolved.

The product in the decomposition flask, condenser and trap flasks was combined with ether, washed with water until neutral; the ether solution dried and distilled through a 20 cm. Vigreux column. In all a total of 338 grams of 2-methyl-4-nitrobenzenediazonium fluoroborate was decomposed in 6 decompositions by this latter method. 121 grams (58%) of 2-fluoro-4-nitrotoluene, b. p. 94-95°/8 mm., m. p. 33.5-34.8°, were obtained.

It should be emphasized that the decomposition always had to be carried out with caution and on a moderately small scale as it is more vigorous than the other decompositions of this type which the author has carried out. However, the yield is satisfactory.

2-Fluoro-4-aminotoluene (35).- 139 grams (0.92 mole) of 2-fluoro-4-nitrotoluene were dissolved in sufficient absolute ethanol to make a total volume of 1/2 liter. 100 ml. portions (0.18 mole) of this stock solution were used for each reduction (31). The compound was reduced using the Parr hydrogenation apparatus. Approximately 80 mg. of platinum oxide catalyst (32) were

used for the first reduction, and about 25 mg. additional catalyst were added for each subsequent reduction. 47-48 lbs. of hydrogen were required for each reduction. The filtered ethanol solution was dried and the product was fractionated through a Vigreux column, obtaining 104 grams (93%) of 2-fluoro-4-aminotoluene, a light yellow liquid, b. p. 97°/15 mm.; lit. b. p. 200-205° (35).

2-Fluoro-4-hydroxy-toluene.- 22.2 grams (0.18 mole) of 2-fluoro-4-aminotoluene were added to a stirred solution of 250 ml. of 2 F HCl. A fine white crystal meal formed. It was cooled to 0°, and 12.5 grams of sodium nitrite in 30 ml. of water was added over a 15 minute period. Urea was added to react with excess nitrous acid. The ice cold mixture was slowly added through a separatory funnel to 200 ml. of boiling water and the resulting phenol was steam distilled as it formed (36). The two phase distillate was extracted with three 75 ml. portions of ether; the ether extracts were dried and fractionated. Obtained 17.6 grams (79%) of 2-fluoro-4-hydroxy-toluene, b. p. 102-102.5°/21 mm. as a light yellow solid.

A second preparation used 77 grams (0.61 mole) of 2-fluoro-4-aminotoluene, 125 ml. of conc. sulfuric acid, 1 liter of water, and 43 grams of sodium nitrite in 100 ml. of water. After the excess nitrous acid had been discharged

with urea, the diazonium compound was slowly added to 700 ml. of boiling water and the product steam distilled. The distillate was extracted with ether and the product fractionated; yield: 67 grams (86%) of 2-fluoro-4-hydroxy-toluene, b. p. 88-89°/10 mm.

2-Fluoro-4-methoxy-toluene.- 85 grams (0.67 mole) of 2-fluoro-4-hydroxy-toluene were dissolved in 300 ml. of 2.2 F NaOH, cooled to 10°, and through a separatory funnel, 86 grams (0.68 mole) of dimethyl sulfate (B and A Reagent) were added to the stirred solution at such a rate that the temperature remained between 10-15° (37). The mixture was then warmed to 50° for 2 hours, then allowed to stand at room temperature for several days. Finally it was heated to 80-90° for 2 hours. The oil phase was separated from the cooled solution, and then the aqueous solution was extracted with ether. These ether extracts were combined with the main oil phase and dried over Drierite. The product was fractionated; obtained 80 grams (85%) of 2-fluror-4-methoxy-toluene, b. p. 81-82.5°/24 mm.

Attempted preparation of 2-Fluoro-4-methoxy-benzaldehyde.-

Several attempts were made to prepare 2-fluoro-4-methoxy-benzaldehyde, none of which proved to be satisfactory. They are described briefly here.

Method I (38). 19.9 grams (0.14 mole) of 2-fluoro-4-methoxy-toluene were dissolved in 225 ml. of glacial acetic

acid and 220 ml. of acetic anhydride, cooled to 10° and 34 ml. of conc. sulfuric acid added. After cooling to 5°, 40 grams of chromium trioxide (tech. flakes) were added over a 15 minute period. No increase in temperature was evident, After 15 minutes more, the temperature began to increase rapidly, finally to 40-45° and flashes of light were observed in the flask. The solution was poured onto iced water, but no precipitated aldehyde diacetate was obtained.

A second oxidation was attempted using 10.8 grams (0.8 mole) of 2-fluoro-4-methoxy-toluene dissolved in 100 ml. of acetic acid, 100 ml. of acetic anhydride and 18 ml. of conc. sufluric acid. 20 grams of chromium trioxide dissolved in 150 ml. of glacial acetic acid were added over a 2 hour period, and the temperature was maintained below 8°, usually at 3-4°. The solution was poured onto 1 liter of iced water, but only a very small precipitate was obtained, and it was concluded that this oxidation was also unsuccessful.

Method II was oxidation with chromyl chloride (39). p-Cresyl methyl ether (anisole) prepared from the corresponding phenol with dimethyl sulfate (37) was used for a trial oxidation. Chromyl chloride was prepared from chromium trioxide, conc. HCl, conc. sulfuric acid according to a method described in (40,41).

For the oxidation, 40 grams of chromyl chloride were dissolved in 50 ml. of chloroform, cooled to 5°, and 15 grams of p-cresyl methyl ether dissolved in 30 ml. of chloroform were slowly added to the stirred mixture over a 2 hour period; the temperature was maintained below 10°. A large amount of brown precipitate formed upon the addition of the p-cresyl methyl ether; presumably this was the $RCH(OCrCl_2OH)_2$ complex. The mixture was allowed to stand for several weeks at room temperature, then water and a slight excess of sodium bisulfite were added (the latter to reduce chromium to the trivalent state and to decompose the addition compound), and the mixture was steam distilled. However it foamed so much that steam distillation was not successful. The odor of aldehyde was present, and it is possible that this method could be used. However, since the presence of negative groups has been reported to make compounds difficult to oxidize by this method (29), it was decided that the method described below would be more practical.

2-Fluoro-4-methoxy-benzoic acid was prepared by the oxidation of 2-fluoro-4-methoxy toluene with potassium permanganate (42). In a 2 liter flask equipped with a reflux condenser and a stirrer were placed 29.0 grams (0.20 mole) of 2-fluoro-4-methoxy-toluene, 77.5 grams of potassium permanganate (C. P.) and 1 liter of water. The mixture was stirred and

gently refluxed for 3 hours. There was still some unoxidized material, so 10 grams of additional potassium permanganate were added, and the unreacted product was steam distilled with about 500 ml. of water (5-10 grams unreacted). The remaining solution was filtered to remove manganese dioxide, the manganese dioxide cake was washed with three 50 ml. portions of hot water, and the filtrate was slowly acidified with 30 ml. of 12 F HCl. A white crystalline precipitate formed. After storage in the cold room overnight, the precipitate was filtered and dried. Yield: 16.9 grams (48%) of 2-fluoro-4-methoxy-benzoic acid, m. p. 190-193° (corr.).

A second oxidation of 24 grams of 2-fluoro-4-methoxy-toluene resulted in a yield of 16.2 grams (55%) of 2-fluoro-4-methoxy-benzoic acid, m. p. 190-193° (corr.). For analysis a portion of this product was recrystallized from toluene, m. p. 192-193.5° (corr.).

Anal. Calcd. for $C_8H_7O_3F$ (170.1): C, 56.47; H, 4.15. Found: C, 56.71; H, 4.24.

In larger preparations, it would be profitable to recover the unoxidized material with a resultant increase in yield on the basis of compound actually oxidized. Comparable yields were obtained in trial oxidations of p-cresyl methyl ether. This is indicative that the fluorine atom does not affect the oxidation with permanganate of 2-fluoro-4-methoxy-toluene.

2-Fluoro-4-methoxy benzoyl chloride was prepared by refluxing 29.8 grams (0.175 mole) of 2-fluoro-4-methoxy-benzoic acid for 10 hours with 40 ml. of redistilled thionyl chloride (43). The thionyl chloride was distilled off (until boiler temperature was 140°), and the product was fractionated through a Vigreux column. Obtained 32.0 grams (97%) of 2-fluoro-4-methoxy-benzoyl chloride, b. p. 120-122°/3 mm.; m. p. 37-40°; most of it solidified at 38.5°.

2-Fluoro-4-methoxy-benzyl alcohol and 2-Fluoro-4-methoxy-benzyl chloride.- 200 ml. of 0.5 F lithium aluminum hydride in absolute ether were placed in a 1 liter RBST flask equipped with a sealed stirrer, dropping funnel, and a reflux condenser (protected from $CO_2$ and $H_2O$ by Ascarite-Drierite tubes) (30). 30.8 grams (0.16 mole) of 2-fluoro-4-methoxy-benzoyl chloride dissolved in 300 ml. of absolute ether were added to the stirred solution over a 1/2 hour period. After standing a short time, 10 ml. of water were slowly added, and then the mixture was poured into iced dilute sulfuric acid and extracted with ether. The ether extracts were washed with water until neutral and then dried. The ether was distilled off until the boiler temperature was 60°; the weight of the crude product was 30 grams. It was used without further purification*.

* Trial experiments reducing p-anisoyl chloride to the alcohol indicated that the alcohol polymerized readily on attempted distillation (perhaps due to a trace of acid being present), so the product was used without further purification in the above preparation.

2-Fluoro-4-methoxy-benzyl chloride was prepared by adding the above prepared crude alcohol slowly to 25 ml. of redistilled thionyl chloride*, allowing the mixture to stand overnight, and then warming at 90° for 1 hour (44). The thionyl chloride was distilled at reduced pressure (boiler temperature kept below 120°), and then the product was distilled at 7 mm. Obtained 23 grams (81% from acid chloride) of 2-fluoro-4-methoxy-benzyl chloride, b. p. 110-112°/7 mm.; sp. gr. approx. 1.22.

α,α -(2-Fluoro-4-methoxy-benzyl)-acetamido-malonic ester.- 3.1 grams (0.13 mole) of sodium were dissolved in 250 ml. of ethanol (99.8% absolute) 30.0 grams (0.14 mole) of acetamido-malonic ester (Winthrop Chem.) and 21.8 grams (0.125 mole) of 2-fluoro-4-methoxy-benzyl chloride were added and the mixture was refluxed for 4 hours; filtered hot to remove sodium chloride (18,19). The precipitate was washed with two 50 ml. portions of hot alcohol, and 600 ml. of water were added to the filtrate. A crystalline precipitate formed on cooling. The precipitate was removed by filtration, washed with 25 ml. of cold 30% ethanol, and three 25 ml. portions of water.

* A more satisfactory yield (77%) was obtained with anisyl alcohol by the above method, than by adding anisyl alcohol in pyridine to thionyl chloride (25%) or by adding thionyl chloride to anisyl alcohol (56%) (44).

Yield: 36.8 grams (85%) of α,α-(2-fluoro-4-methoxy-benzyl)-acetamido-malonic ester, m. p. 112-120, mostly 118-120°. For analysis a portion was recrystallized from absolute ethanol, m. p. 120-121.5 (corr.).

Anal. Calcd. for $C_{17}H_{22}O_6NF$ (355.3): C, 57.45; H, 6.24; N, 3.94. Found: C, 57.55; H, 6.31; N, 3.89 (Elek).

N-acetyl-2-fluoro-4-methoxy-phenylalanine.- 35.5 grams (0.10 mole) of α,α-(2-fluoro-4-methoxy-benzyl)-acetamido-malonic ester were refluxed for 6 hours with 175 ml. of 2.5 F NaOH, and than 95 ml. of 5 F HCl were added and the mixture was refluxed for 2 hours (24,25). The mixture was allowed to crystallize overnight at 4°; obtained 15.8 grams (62%) of N-acetyl-2-fluoro-4-methoxy-phenylalanine, m. p. 162-170° (corr.). This product was recrystallized from a small volume of alcohol; recovered 13.7 grams, m. p. 169.5-171.5 (corr.).

Anal. Calcd. for $C_{12}H_{14}O_4NF$ (255.2): C, 56.46; H, 5.53; N, 5.48. Found: C, 56.51; H, 5.45; N, 5.55 (Elek).

2-Fluoro-4-methoxy-phenylalanine was prepared by refluxing 1.0 grams (0.004 mole) of N-acetyl-2-fluoro-4-methoxy-phenylalanine for 7 hours with 40 ml. of 3 F HCl. After the HCl had been removed by distillation at reduced pressure, the product was dissolved in 20 ml. of water, made basic with ammonium hydroxide, and the excess ammonia expelled by boiling. The solution was placed in the cold room;

after 5 hours only a small amount of turbidity had formed which was removed by filtration through a hardened filter paper. 15 ml. of ethanol were added to the filtrate and a crystalline precipitate formed (long needles). It was filtered, washed with several small portions of water and ethanol. Yield: 530 mg. (63%) of 2-fluoro-4-methoxy-phenylalanine, decomp. point 218-226° (corr.). It was recrystallized from 40 ml. of 50% ethanol; recovered 425 mg. decomp. point 217-221° (corr.).

Anal. Calcd. for $C_{10}H_{12}O_3NF$ (213.2): C, 56.33; H, 5.68; N, 6.57. Found: C, 56.35; H, 5.79; N, 6.64 (Elek).

The absorption spectrum and ionization constants for this compound were determined and are reported in section I-C of this thesis.

2-Fluorotyrosine was prepared by refluxing 1.4 grams (0.0055 mole) of N-acetyl-2-fluoro-4-methoxy-phenylalanine with 20 ml. of HBr (Reagent grade) for 7 hours. After removal of the hydrobromic acid by distillation at reduced pressure, the oil remaining was dissolved in 30 ml. of water, made basic with ammonium hydroxide, and the excess ammonia was expelled by boiling. After storage in the cold room, a precipitate of long needles was obtained, filtered and washed with water and alcohol. Yield: 870 mg (80%) of 2-fluorotyrosine, decomp. point 280-285° (corr.). This

product was dissolved in approximately 75 ml. of boiling water, boiled with a small amount of Norite to remove color, filtered and allowed to crystallize. Recovered 635 mg. of 2-fluorotyrosine, decomp. point 280-285° (corr.). Anal. Calcd. for $C_9H_{10}O_3NF$ (199.2): C, 54.27; H, 5.06; N, 7.04. Found: C, 54.16; H, 5.02; N, 7.07 (Elek).

The overall yields of 2-fluoro-4-methoxy-phenylalanine and 2-fluorotyrosine from 2-amino-4-nitrotoluene were 5.0% and 6.3%, respectively. It is probable that the yield could be increased at several steps in this procedure. 10 grams of N-acetyl-2-fluoro-4-methoxy-phenylalanine have not been hydrolyzed in case it is desired to resolve it enzymatically into the D and L isomers.

## Summary

The three nuclear substituted mono-fluorophenylalanines have been prepared by two different methods. By vapor phase chlorination of fluorotoluene, condensation of the fluorobenzyl chloride with acetamido-malonic ester, and hydrolysis to the amino acid, the fluorophenylalanines can be easily prepared in yields of 40-50%.

Trans-acylation has been found to occur in the condensations of fluorobenzaldehydes and hippuric acid in the presence of acetic anhydride and sodium acetate.

2-Fluoro-4-methoxy-phenylalanine and 2-fluorotyrosine have been prepared.

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## B. A STUDY OF THE FACTORS INFLUENCING ASYMMETRIC SPECIFICITY IN THE PAPAIN CATALYZED SYNTHESIS OF AMIDES

### Discussion

An N-acylated amino acid can be enzymatically resolved by papain through the asymmetric synthesis of the anilide or phenylhydrazide of the acylated L-amino acid; these derivatives crystallize from the reaction mixture and can subsequently be converted to the free amino acid (1). The acylated D-amino acid can be recovered unchanged from the reaction mixture and converted by usual methods to the free amino acid, or if desired into peptides. The reaction involved is as follows:

$$1\ \ R\underset{\substack{|\\ NH\\ |\\ CO\\ |\\ R'}}{CHCO_2}^- + \emptyset NH_2 \xrightarrow[\text{CYSTEINE}\ pH\ 4.6]{\text{PAPAIN}} 1/2\ R\underset{\substack{|\\ NH\\ |\\ CO\\ |\\ R'}}{CHCONH\emptyset} + 1/2\ R\underset{\substack{|\\ NH\\ |\\ CO\\ |\\ R'}}{CHCO_2}^- + 1/2\ OH^-$$

ACYLATED DL-AMINO ACID

ACYLATED L-AMINO ACID ANILIDE

ACYLATED D-AMINO ACID

Because of the versatility possible in subsequent reactions when a carboalkoxy residue is used as the acylating agent, this type of blocking group has been used by others (1,2,3,4) in the resolution of alanine, leucine, lysine, glutamic acid and $\alpha$-amino adipic acid. Apparently a high degree of specificity was obtained in these resolutions.

We first tried to resolve N-carbobenzoxy-DL-o-fluorophenylalanine by this procedure and noted that non-asymmetric synthesis of N-carbobenzoxy-o-fluorophenylalanylphenylhydrazide occurred when the acylated amino acid was incubated with activated papain, L-cysteine, and the theoretical amount of phenylhydrazine, i.e. 1/2 mole of phenylhydrazine/mole of amino acid.

At the same time that the enzymatic synthesis of the N-carbobenzoxy-o-fluorophenylalanylphenylhydrazide was being carried out and with another portion of the same enzyme preparation, an enzymatic synthesis of N-carbobenzoxy-L-alanylphenylhydrazide from N-carbobenzoxy-DL-alanine was specific; at least the product obtained was sharp melting and had the same rotation as the product obtained by Dr. Brown in these laboratories and from which pure L-alanine has been obtained. This would seem to rule out any possible objection that the particular enzyme preparation which we had was "not normal". Therefore the cause for the non-asymmetric synthesis must either have been the acylating group or the presence of the fluorine atom in the aromatic ring. Accordingly, before attempting further resolutions of the fluorophenylalanines it was deemed advisable to study the effect that the acylating group might have in the enzymatic resolution of acyl phenylalanines.

The investigation of asymmetric specificity was carried out with the following N-substituted DL-phenylalanines: N-carbobenzoxy, N-carboethoxy, N-carbomethoxy, N-acetyl, and N-benzoyl. All but N-carbomethoxy-DL-phenylalanine were crystalline compounds.

As described more completely in the experimental section, the enzymatic syntheses were performed by incubating the substrate of the acylated DL-amino acid with activated papain, L-cysteine, and redistilled phenylhydrazine - 1/2 mole of the latter/mole of amino acid- in a sodium acetate-acetic acid buffer of pH 4.6 and at a temperature of 40°. The formal strength of the buffer was varied with the initial concentration of substrate, being so chosen that little change in pH would be expected as the reaction proceeded, in practice, the pH change was less than 0.1 unit. It was observed that less protein denaturation seemed to occur in buffers of greater formal strength and it is recommended that in the future a buffer at least 0.5 F in sodium acetate and acetic acid be used. The papain was activated by precipitating 4 times with methanol from an $H_2S$ saturated, aqueous solution. After several days of incubation, usually 3 or 4, the precipitated acyl amino acid phenylhydrazide was removed, additional phenylhydrazine was added, the pH readjusted to 4.6, and incubation continued for several days,

whereupon the precipitate was again removed. Addition of base and incubation was usually repeated a third time.

Tables I and II summarize the results obtained.

Table I

RESOLUTION OF ACYL PHENYLALANINES*

| ACYL AMINO ACID | CONC. SUBSTRATE MOLES/L. | CONC. PAPAIN GMS./L. | | F-1 | F-2 | F-3 | F-4 |
|---|---|---|---|---|---|---|---|
| N-CARBOMETHOXY-DL-PHENYLALANINE | 0.05 | 4.4 | YLD | 60% | 25% | 15% | |
| | | | % L | 85% | 85% | 55% | |
| N-CARBOETHOXY-DL-PHENYLALANINE | 0.05 | 2.0 | YLD | 96% | 38% | 32% | 17%** |
| | | | % L | 82% | 58% | 7% | 0% |
| N-CARBOBENZOXY-DL-PHENYLALANINE | 0.012 | 2.5 | YLD | 99% | 63% | 25% | |
| | | | % L | 78% | 38% | 7% | |
| N-BENZOYL-DL-PHENYLALANINE | 0.01 | 2.5 | YLD | 79% | 22% | 0% | |
| | | | % L | 96% | 95% | | |
| N-ACETYL-DL-PHENYLALANINE | 0.25 | 3.0 | YLD | 61% | 20% | 2% | |
| | | | % L | 98% | 99% | 97% | |

*Yields are calculated on the assumption that only the L-isomer can form a phenylhydrazide. The percentage of the fraction which is L is calculated from the rotation of the crude acyl phenylalanylphenylhydrazide which is obtained from the reaction mixture and the rotation of the final pure isomeric product; the remainder of the fraction is D. The approximate composition was also checked by the yield of L or D and DL-acyl phenylalanylphenylhydrazide obtained after fractional recrystallization.

**Obtained by isolating as a crude oil (by ether extraction) the N-carboethoxy-phenylalanine remaining after the removal of F-3 and incubating with a fresh portion of enzyme and phenylhydrazine.

Table II

RESOLUTION OF ACETYL-DL-PHENYLALANINE

| CONC. ACETYL-DL-PHENYLALANINE MOLES/L. | CONC. PAPAIN GMS./L. | | F-1 | F-2 | F-3 |
|---|---|---|---|---|---|
| 0.25 (Resolution I) | 3.0 | YLD | 61% | 20% | 2% |
| | | % L | 98% | 99% | 97% |
| 0.25 (Resolution II) | 25 | YLD | 62% | 0% | |
| | | % L | 100% | | |
| 0.25* (Resolution III) | 3.0 | YLD | 99% | 10% | |
| | | % L | 100% | 99% | |
| 0.25* (Resolution IV) | 4.4 | YLD | 84% | 0% | |
| | | % L | 100% | --- | |

These experiments indicate that the asymmetric enzymatic specificity is not complete in the case of the carbomethoxy-phenylalanine, carboethoxyphenylalanine, and carbobenzoxy-phenylalanine. Qualitatively the rate of formation of the phenylhydrazide was slower in the case of N-carbomethoxy phenylalanine than was the case with the other two carbo-alkoxy derivatives studied. It is also to be noted that no fraction was obtained with a preponderance of the D-form over the L-form in the case of N-carbomethoxy-phenylalanine. This may prove to be significant in understanding the specificity

* A 1-FOLD EXCESS OF PHENYLHYDRAZINE WAS USED INITIALLY.

of papain and may be an indication of the effect the side chain size may have on enzymatic rates and specificity. Based on the assumption that the reaction rate is 1st order with respect to the acylated amino acid concentration, it would appear that the rate of formation of the D-carboethoxy or carbobenzoxy-phenylalanylphenylhydrazides is approximately 1/8 to 1/10 of the rate of formation of the corresponding L-derivative. Lack of complete data at the present time prevents a more detailed analysis of rate as a function of concentration and nature of substrate, base, and enzyme. It would be desirable to carry out the enzymatic synthesis of N-carbobenzoxy- or N-carboethoxy-D and -L-phenylalanyl-phenylhydrazides in the presence of the activated enzyme, L-cysteine, phenylhydrazine and the pure N-carbobenzoxy- or carboethoxy-D-phenylalanine and the pure N-carbobenzoxy- or carboethoxy-L-phenylalanine in order to determine relative rates and to investigate the possibility that enzymatic synthesis might not occur in the presence of the D-isomer alone.

As can be seen from Table I a high degree of asymmetric specificity was obtained with N-benzoyl-DL-phenylalanine and N-acetyl-DL-phenylalanine. On the basis of optical rotation of the crude product, a minimum of 95% N-benzoyl-L-phenylalanylphenylhydrazide and 98% N-acetyl-L-phenylal-anylphenylhydrazide was obtained, even when an excess of

phenylhydrazine was used and incubation was continued over a fortnight. From the filtrate from the resolution mixture, the N-benzoyl-D-phenylalanine and N-acetyl-D-phenylalanine were recovered in good yield.

Since there was a large variation in the enzyme substrate ratio, particularly in that used with N-acetyl-DL-phenylalanine as compared with N-carbobenzoxy-DL-phenylalanine, it might be supposed that this ratio was important in determining specificity, and that in those cases where the amount of substrate was large as compared to the amount of enzyme that a high degree of specificity was obtained only because the enzyme was "saturated" with the L-isomer, not because it was inherently completely specific for the L-isomer. That this is not a valid argument is indicated by the following observations:

1). As shown in Table II an 8 fold increase in enzyme-substrate ratio in the case of N-acetyl-DL-phenylalanine caused no decrease in optical purity of the resulting phenylhydrazide. A lower yield also was obtained with a large excess of enzyme, this may be an indication that the substrate-enzyme complex may have a small dissociation constant. However, this experiment should be repeated with a wide range of substrate-enzyme concentrations and perhaps with a variation in base concentration.

2). The enzyme-substrate ratio is large and comparable in the resolution of N-benzoyl-DL-phenylalanine and N-carbobenzoxy-DL-phenylalanine, yet in the former case a high order of specificity was observed, in the latter, a low order of specificity.

3). A similar degree of specificity was observed in the case of N-carbobenzoxy and N-carboethoxy-DL-phenylalanine, yet there is approximately a 4-fold difference in enzyme-substrate ratio.

The ortho-, meta, and para-fluorophenylalanines have been enzymatically resolved through the use of the N-acetyl derivatives and complete specificity was observed in all cases, even though a 1-fold excess of phenylhydrazine was used initially. In all cases the N-acetyl-L-fluorophenylalanylphenylhydrazide was obtained in approximately 90% yield and essentially optically pure. This is further evidence that the fluorine atom is not responsible for the non-asymmetric synthesis originally observed in the case of N-carbobenzoxy-DL-o-fluorophenylalanine, but that the acylating group and the side chain of the amino acid play an important role in determining the specificity of the action of papain. There is no evidence whether the base used plays an important part in determining specificity, but it seems unlikely to the author that it would.

The N-acetyl derivatives of phenylalanine and fluorophenylalanine are more desirable to use than the N-benzoyl derivatives because of their much greater solubility at pH 4.6. This decreases greatly the volume in which the resolution can be performed and thus the amount of enzyme which must be activated and the amount of L-cysteine which must be used are smaller.

It is interesting to note that N-carbobenzoxy-L-phenylalanylphenylhydrazide is higher melting and more insoluble (in toluene) than the corresponding DL-compound, while the opposite was the case for N-carboethoxy- and N-carbomethoxy-phenylalanylphenylhydrazide (Table III). While N-acetyl-DL-phenylalanine and the N-acetyl-DL-fluorophenylalanines all melt in the range of 150-155°, there is a marked difference in the melting point and solubility of the corresponding N-acetyl-D- derivatives, with a marked decrease in m. p. as the substituent is shifted around the nucleus from the ortho to the para position. The low melting point of N-acetyl-D-p-fluorophenylalanine made it difficult to isolate since it did not crystallize out of the resolution mixture upon acidification. If the resolution were to be repeated, the addition of a seed crystal would probably cause crystallization of the N-acetyl-D-p-fluorophenylalanine from the acidified resolution solution and a higher yield of this isomer would be obtained. The optical rotations of the

Table III

Some Physical Properties of Phenylalanine Derivatives

| Parent Compound | | Ring Substituent: none | ortho F | meta F | para F |
|---|---|---|---|---|---|
| DL-Phenylalanine | d.p. | | 245-250° | 248-251° | 265-267° |
| Lit. | d.p. | 271-273° | 259° | 263° | 264° |
| L-Phenylalanine | d.p. | 283° (Lit.) | 226-232° | 239-243° | 250-255° |
| | $[\alpha]_D^{25}(H_2O)$ | -35.0° (Lit.) | -15° | -23° | -23° |
| D-Phenylalanine | d.p. | 283° (Lit.) | 224-228° | 230-234° | 227-232° |
| | $[\alpha]_D^{25}(H_2O)$ | +35.0° (Lit.) | +15° | +22° | +24° |
| N-Acetyl-DL-phenylalanine | m.p. | 152-154° | 147-149° | 154-156° | 149-151° |
| N-Acetyl-D-phenylalanine | m.p. | 171-172° | 168-170° | 158-160° | 142-143° |
| Lit. | m. p. | 172° | | | |
| $[\alpha]_D^{25}$(ethanol) | | -46.0° | -28.6° | -40.4° | -38.6° |
| Lit. $[\alpha]_D^{25}$(ethanol) | | -51° | | | |
| N-Benzoyl-DL-phenylalanine | m.p. | 184-187.5° | | | |
| Lit. | m.p. | 187-188° | | | |
| N-Benzoyl-D-phenylalanine | m.p. | 139.5-140.5° | | | |
| Lit. | m.p. | 145-146° | | | |
| $[\alpha]_D^{25}$(0.4F NaOH) | | -18.0° | | | |
| Lit. $[\alpha]_D^{25}$(1 F NaOH) | | -17.1° | | | |
| N-Carbomethoxy-DL-phenylalanine | m.p. | syrup | | | |
| | Lit. | syrup | | | |
| N-Carboethoxy-DL-phenylalanine | m.p. | 73-75.5° | | | |
| N-Carbobenzoxy-DL-phenylalanine | m.p. | 103-104° | 108-109° | | |
| | Lit. | 103° | | | |
| N-Carbobenzoxy-D-phenylalanine | m.p. | | 103-105° | | |
| | $[\alpha]_D^{25}$(acetone) | | +15.7° | | |
| N-Acetyl-L-phenylalanyl-phenylhydrazide | m.p. | 207-208° | 215-216.5° | 209-210° | 229-232° |
| | Lit. | 205° | | | |
| $[\alpha]_D^{25}$(pyridine) | | -34.6° | -29.2° | -30.5° | -36.2° |
| Lit. $[\alpha]_D^{23}$(pyridine) | | -33.5° | | | |

Table III

Some Physical Properties of Phenyalanine Derivatives (Cont.)

| Parent Compound | | Ring Substituent: none | Ring Substituent: ortho F |
|---|---|---|---|
| N-Benzoyl-L-phenylalanyl-phenylhydrazide | m.p.<br>$[\alpha]_D^{25}$(pyr.) | 214.5-216.5°<br>-61.7° | |
| N-Carbomethoxy-DL-phenylalanyl-phenylhydrazide | m.p. | 181-182° | |
| N-Carbomethoxy-L-phenylalanyl-phenylhydrazide | m.p.<br>$[\alpha]_D^{25}$(pyr.) | 170-174°<br>-23.8° | |
| N-Carboethoxy--DL-phenylalanyl-phenylhydrazide | m.p. | 171-172.5° | |
| N-Carboethoxy--L-phenylalanyl-phenylhydrazide | m.p.<br>$[\alpha]_D^{25}$(pyr.) | 156.5-159.5°<br>-22.2° | |
| N-Carboethoxy--D-phenylalanyl-phenylhydrazide | m.p.<br>$[\alpha]_D^{25}$(pyr.) | 156-159°<br>+23.4° | |
| N-Carbobenzoxy-DL-phenylalanyl-phenylhydrazide | m.p. | 160-161.5° | 153.5-155.5° |
| N-Carbobenzoxy-L-phenylalanyl-phenylhydrazide | m.p.<br>$[\alpha]_D^{25}$(pyr.)<br>$[\alpha]_D^{25}$($CHCl_3$)<br>$[\alpha]_D^{25}$(acetone) | 176-179°<br>-24.1°<br>-29.2° | 171-172°<br><br>-31.0° |
| N-Carbobenzoxy-D-phenylalanyl-phenylhydrazide | m.p.<br>$[\alpha]_D^{25}$(pyr.) | 178-179°<br>+24.4° | |

fluorophenylalanines also showed a dependence upon the position of the fluorine atom.

Any explanation of the lack of specificity which has been observed in terms of the acylating group must account for the high degree of specificity which has been observed in the syntheses of several carboalkoxy-aliphatic-amino acid phenylhydrazides, i.e. alanine, and also for the fact that mere overall length of the acylating group apparently is not of prime importance, as illustrated by the roughly comparable size of carbobenzoxy and benzoyl groups, both relatively large, and acetyl and carbomethoxy groups, both relatively small. Further experiments should be done to determine what other carboalkoxy-amino acids are resolved non-asymmetrically and what effect other acylating groups such as $-C(=O)-NH\phi$, $-C(=S)-OC_2H_5$, $-C(=S)-O\phi$, $-C(=S)-NH\phi$, and $-O_2S\phi$ may have on the specificity of the enzyme action.

## Experimental*

### Preparation of Acylated Amino Acids; Substrates for Enzymatic Syntheses.-

N-Carbobenzoxy-DL-o-fluorophenyalanine.- 17.4 grams (0.094 mole) of DL-o-fluorophenylalanine (Prepared by Method I, section I-A) were dissolved in 100 ml. of 1 F NaOH, 17.8 grams of carbobenzoxy chloride (0.10 mole) (5) and 100 ml. of 1 F NaOH were added dropwise simultaneously to the cold stirred amino acid solution. After warming to room temperature the aqueous solution was extracted with ether to remove any unreacted carbobenzoxy chloride, and then acidified with hydrochloric acid until just turbid. Upon standing a large amount of product crystallized, and additional HCl was added and the mixture was allowed to stand in the cold room overnight. The precipitate was filtered, washed with water and dried. Yield 26.2 grams (90%) of N-carbobenzoxy-DL-o-fluorophenylalanine, m. p. 106.5-108.5 (corr.). For analysis a portion of the above product was recrystallized from toluene, m. p. 108.5-109.5 (corr.).

Anal. Calcd. for $C_{17}H_{16}O_4NF$ (317.3): C, 64.34; N, 5.08; N, 4.42. Found: C, 63.97; H, 5.09; N, 4.50.

N-Carbobenzoxy-DL-alanine was prepared by Dr. David Brown (4) by the above procedure from DL-alanine (Lemke) and carbobenzoxy chloride (6).

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* See footnote page 11.

N-Carbobenzoxy-DL-phenylalanine was prepared by Mr. Robert MacAllister from DL-phenyalanine (Winthrop Chem.) and carbobenzoxy chloride by the above method. It was recrystallized from toluene before use. m. p. 103-104° (corr.). Lit. m. p. 103° (6).

N-Acetyl-DL-phenylalanine was prepared by the reaction of 33.4 grams (0.2 mole) of DL-phenylalanine (Winthrop Chem.) dissolved in 100 ml. of 4 F NaOH with 70 grams of acetic anhydride (B and A Reagent). After acidification and storage at 4° overnight, 37 grams (88%) of N-acetyl-DL-phenylalanine were obtained, m. p. 151-154° (corr.). Recrystallization from water raised the m. p. to 152-154° (corr.); lit. m. p. 151-152° (7).

N-Acetyl-DL-o-fluorophenylalanine was that prepared by Mr. Wildon Fickett (Method II, this thesis, section I-A).

N-Acetyl-DL-m-fluorophenylalanine was prepared by the acetylation (by the method described above for phenylalanine) of DL-m-fluorophenylalanine prepared by Method I of this thesis (section I-A). From 7.0 grams (0.038 mole) of DL-m-fluorophenylalanine, there were obtained 7.0 grams (81%) of N-acetyl-m-fluorophenylalanine, m. p. 151-155° (corr.). A portion was recrystallized from water for analysis, m. p. 154.5-156° (corr.).

Calcd. for $C_{11}H_{12}O_3NF$ (225.2): N, 6.22. Found: N, 6.40.

N-Acetyl-DL-p-fluorophenylalanine was prepared by the acetylation as described above of 9.25 grams (0.05 mole) of DL-p-fluorophenylalanine (prepared by Method I, section I-A). There were obtained 9.9 grams (86%) of N-acetyl-DL-p-fluorophenylalanine, m. p. 148.5-151 (corr.). Several other small preparations gave yields of 88-90%. The yield could be increased to 95% by extraction with ether of the acidified filtrate after the main precipitate had been removed. A portion was recrystallized from water for analysis, m. p. 149-151° (corr.).

Anal. Calcd. for $C_{11}H_{12}O_3NF$ (225.2): N, 6.22. Found: N, 6.62.

N-Benzoyl-DL-phenylalanine was prepared by simultaneous dropwise addition of 30 grams (0.21 mole) of benzoyl chloride (EK White Label) and 35 ml. of 6 F NaOH to a vigorously stirred solution of 33 grams (0.20 mole) of DL-phenylalanine (Winthrop Chem.) in 100 ml. of cold 2 F NaOH. After standing for several hours, the solution was acidified, and after storage overnight at 4°, the product was filtered and washed. After recrystallization from water obtained 49 grams (91%) of N-benzoyl-DL-phenylalanine, m. p. 184-187.5° (corr.). Lit. m. p. 187-188°.

N-Carbomethoxy-DL-phenylalanine was prepared by the simultaneous dropwise addition of 50 grams (0.5 mole) of carbomethoxy chloride (methyl chloroformate) (EK White Label) and 50 ml. of 6 F NaOH to a cold, vigorously stirred solution

of 33 grams (0.2 mole) of DL-phenylalanine (Winthrop Chem.) in 135 ml. of 1.5 F NaOH. On careful acidification with HCl an oil was obtained. Attempts to crystallize a portion of the oil from toluene, ethanol, or water were unsuccessful. Therefore the main portion was dissolved in ethanol, and the ethanol allowed to evaporate slowly at room temperature. The product did not crystallize after long standing at room temperature, but remained as a viscous syrup. The yield was 41.7 grams (97%). The crude syrup was used in the resolution to be described. Previously (8) carbomethoxy-DL-phenylalanine has been obtained only as a syrup.

N-Carboethoxy-DL-phenylalanine was prepared by the reaction by the method described above of 27 grams (0.25 mole) of carboethoxy chloride (ethyl chloroformate) (prepared by the addition of the calculated amount of ethanol to liquid phosgene and distilling the product; the fraction of b. p. 90-94° was used) and 35 ml. of 6 F NaOH with 33 grams (0.2 mole) of DL-phenylalanine (Winthrop Chem.) dissolved in 85 ml. of 2.7 F NaOH. On acidification, a solid was first obtained, but the addition of more acid produced an oil. The oil was "recrystallized" by the addition of ligroin to a benzene solution, and finally by fractional crystallization from an aqueous solution by the slow addition of HCl. The solid fraction obtained had a m. p. of 73-75.5° and weighed 26.5 grams (56%).

Anal. Calcd. for $C_{12}H_{15}O_4N$ (237.2): C, 60.75; H, 6.37; N, 5.90. Found: C, 60.87; H, 6.22; N, 6.15.

Preparation of Active Papain.- Several preparations of active papain were made for resolution experiments starting with 50 or 100 grams of crude papain. A preparation on a 50 gram scale is more convenient and requires less time (2 days); therefore one such preparation is described briefly below. The procedure used was approximately that described in (1,4). Every effort was made to keep the enzyme solution cold during all operations. No investigation has been made of the factors influencing papain activity, indeed it may be that fewer activation steps are necessary (12). However, due to the nature of the experiments, it was decided that a "standard" activation procedure should be used.

50 grams of "Hygrade Papain" (Wallenstein Lab.) were ground to a fine powder in a cold mortar, suspended in 200 ml. of cold water, and stirred in the cold room for about 4 hours. The solution was filtered by suction through Whatman No. 1 filter paper to remove the insoluble material, and the precipitate was washed with 50 ml. of cold water (precipitate discarded). The filtrate was a clear yellow solution. It was cooled in an ice bath and saturated with

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* All centrifugation was done in the cold room using the large centrifuge. The 250 ml. centrifuge bottles were capped with rubber diaphrams.

$H_2S$ for 3 hours, centrifuged at 2000 RPM for 20 minutes and the precipitate discarded. Sufficient methanol was added to the turbid supernatant to bring the methanol concentration to 70% (v/v). The methanol was added slowly to the stirred solution cooled in a salt-ice bath over a 15 minute period; stirring was continued for 45 minutes after all the methanol had been added. The white precipitate was centrifuged out at 2000 RPM for 20 minutes; the supernatant was decanted and discarded. The precipitate was dissolved in 200 ml. of cold $H_2S$ saturated water, saturated with $H_2S$ and stored overnight in the cold room. The following day the solution was resaturated with $H_2S$ and centrifuged. A clear yellow supernatant was obtained. Sufficient methanol was slowly added to the supernatant to bring the methanol concentration to 70%; after one hour the precipitate was centrifuged out, dissolved in 200 ml. of water previously saturated with $H_2S$ and the solution again saturated with $H_2S$ for 2 hours. Upon centrifugation, only a small amount of precipitate was obtained. The enzyme was again reprecipitated from the supernatant with methanol, centrifuged, dissolved in $H_2S$ saturated water, saturated with $H_2S$ for 2 hours, and the small precipitate centrifuged out. The papain was reprecipitated a fourth time with methanol. The volume of the suspension placed in each centrifuge tube was measured, so that a known aliquot of the precipitated enzyme could be dissolved in water and lyophilized to determine the actual concentration of papain used in the

resolutions (the yield was usually 25-30%). The remainder of the enzyme was dissolved in a sodium acetate-acetic acid buffer, pH 4.6, and used for the enzymatic resolutions. A clear, slightly greyish solution was obtained when the enzyme was dissolved in the buffer.

## Enzymatic Syntheses

N-Acetyl-DL-phenylalanine Resolution I.- 10.0 grams (0.048 mole) of N-acetyl-DL-phenylalanine were dissolved in 7.1 ml. of 3.9 F NaOH and 150 ml. of 0.5 F sodium acetate-0.5 F acetic acid buffer (pH 4.6). 2.77 grams (0.025 mole) of redistilled phenylhydrazine, 0.80 grams of L-cysteine hydrochloride (Van Camp Lab.) and 0.62 grams of activated papain were added; the solution was diluted to 200 ml. and incubated in a stoppered 200 ml. flask at 40°. No turbidity was present at 2 hours, but a large amount of precipitate had formed within 9 hours. After 85 hours the precipitate was filtered, washed with water and dried. Yield: 4.36 grams (61%) of N-acetyl-L-phenylalanylphenylhydrazide, (I-F-1), m. p. 201-205° (corr.), $[\alpha]_D^{25} = -33.2°$ (C,6; pyridine).

2.73 grams of phenylhydrazine were added to the filtrate; the pH readjusted to 4.6, and the solution was incubated at 40° for 96 hours. The precipitate was filtered, washed with water and dried. Yield, 1.47 grams (I-F-2) (20%), m. p.

204-206° (corr.), $[\alpha]_D^{25}$ = -33.8° (C,7; pyridine).

The filtrate from I-F-2 was incubated at 40° for 16 days. The precipitate obtained weighed 140 mg., m. p. 202-206° (corr.), $[\alpha]_D^{25}$ = -32.6° (C,7; pyridine). The total yield of crude N-acetyl-L-phenylalanylphenylhydrazide was 83%.

The filtrate from the above product was acidified with 20 ml. of HCl and extracted in a continuous liquid-liquid extractor with ether for 24 hours. The ether extract was poured into an evaporating dish and allowed to evaporate to a thick syrup. The product did not crystallize, but upon the addition of 30 ml. of water it soon crystallized. The precipitate was filtered, washed with water and dried. Yield of N-acetyl-D-phenylalanine, 3.4 grams (68%), m. p. 165-167° (corr.), $[\alpha]_D^{25}$ = -32.4° (C,8; pyridine). Lit. m. p. 172° (9).

I-F-1 (4.3 grams) was recrystallized by dissolving in 700 ml. of hot toluene (dissolved slowly), filtering hot to remove a small amount of insoluble protein, and allowing to cool slowly. The precipitate formed as a gel, and was filtered while the solution was still warm. The precipitate was washed with three 35 ml. portions of warm toluene. Obtained 3.97 grams of product, m. p. 206.5-208° (corr.), $[\alpha]_D^{25}$ = -34.4°; -34.7° (C,8; pyridine). Lit. m. p. 205°, $[\alpha]_D^{23}$ = -33.5 (C,4.5; pyridine). (1). The filtrate from above was evaporated and 0.17 gram additional product was obtained, m. p. 200-205° (corr.), $[\alpha]_D^{25}$ = -33.2° (C,8; pyridine).

N-Acetyl-DL-phenylalanine Resolution II.- This experiment was performed to determine the effect on specificity that a large amount of enzyme and cysteine might have. 10.0 grams (0.048 mole) of N-acetyl-DL-phenylalanine were dissolved in 16.0 ml. of 3.9 F NaOH and 100 ml. of 0.5 F sodium acetate -0.5 F acetic acid buffer. 2.7 grams (0.025 mole) of redistilled phenylhydrazine, 6.4 grams of L-cysteine hydrochloride (Van Camp Lab.) and 5.0 grams of activated papain were added. The solution was diluted to 200 ml. with buffer and incubated in a stoppered 200 ml. flask at 40°. A large amount of precipitate had formed within 2 hours. After 87 hours the precipitated N-acetyl-L-phenylalanylphenylhydrazide was filtered, washed with water and dried. Yield: 4.51 grams (63%) of N-acetyl-L-phenylalanylphenylhydrazide (II-F-1), m. p. 202-205° (corr.), $[\alpha]_D^{25} = -34.8°$ (C,6; pyridine).

To the filtrate from II-F-1 were added 2.7 grams of phenylhydrazine, the pH was readjusted to 4.6, and incubation was continued for 20 days. No appreciable additional precipitate was obtained.

The solution was acidified with HCl and extracted with ether in a continuous liquid-liquid extractor. Some material was lost, so recovery was not quantitative. The N-acetyl-D-phenylalanine obtained from this resolution was combined with that obtained from Resolution III (see below).

II-F-1 (4.5 grams) was dissolved in 60 ml. of boiling ethanol, filtered and allowed to cool to room temperature. The precipitate was filtered and washed with several small portions of ethanol. Recovered 2.8 grams, m. p. 207-208° (corr.), $[\alpha]_D^{25} = -34.6°$ (C,8; pyridine).

Anal. Calcd. for $C_{17}H_{19}O_2N_3$ (297.3): C, 68.66; H, 6.44; N, 14.14. Found: C, 68.41; H, 6.54; N, 13.92.

The ethanol filtrate and washings from the recrystallization of II-F-1 were evaporated to 25 ml. and 50 ml. of water were added. The precipitate was filtered, washed, and dried. Yield: 1.14 grams, m. p. 206-207°, $[\alpha]_D^{25} = -33.9°$ (C,7; pyridine).

N-Acetyl-DL-phenylalanine Resolution III.- This experiment was performed to determine the effect of an initial one-fold excess of phenylhydrazine on the specificity of resolution and upon the yield. 5.0 grams (0.024 mole) of N-acetyl-DL-phenylalanine were dissolved in 0.66 ml. of 3.9 F NaOH and 75 ml. of 0.5 F sodium acetate-0.5F acetic acid buffer. 2.9 grams (0.027 mole) of redistilled phenylhydrazine, 0.40 grams of L-cysteine hydrochloride (Van Camp Lab.) and 0.31 grams of activated papain were added and the solution was diluted to 100 ml. with buffer. Incubation was at 40°. The solution was not turbid at two hours, but at 9 hours a large amount of precipitate had formed. After 88 hours of incubation,

the precipitate was filtered, washed with water, and dried. Yield: 3.57 grams (III-F-1) (99%), m. p. 206-208° (corr.),

To the filtrate from III-F-1 were added 1.37 grams of phenylhydrazine and incubation was continued for 4 days. A small additional precipitate was obtained, III-F-2, weight 400 mg. (11%), m. p. 206-208°, $[\alpha]_D^{25} = -33.6°$ (C,7; pyridine).

The filtrate from III-F-2 was incubated for 17 additional days, but no further precipitate was obtained. It was then acidified with HCl and extracted with ether in a liquid-liquid extractor. The ether phase was evaporated and the product combined with that from Resolution II. Total yield: 3.4 grams, m. p. 145-163°, $[\alpha]_D^{25} = -26°$ (C,8; pyridine). This fraction was recrystallized from a small volume of ethanol to yield 2.2 grams of N-acetyl-D-phenylalanine, m. p. 168-170° (corr.), $[\alpha]_D^{25} = -32.6°$ (C,8; pyridine). This fraction was then combined with the N-acetyl-D-phenylalanine from Resolution I and recrystallized once again from a small volume of ethanol. Obtained 4.3 grams, m. p. 167-168.5° (corr.) $[\alpha]_D^{25} = -33.1°$ (C,8; pyridine). Lit. m. p. 172° (95).

III-F-1 was recrystallized from ethanol; recovered 2.7 grams of N-acetyl-L-phenylalanylphenylhydrazide, m. p. 204-207°, $[\alpha]_D^{25} = -34.7°$ (C,8; pyridine).

N-Acetyl-DL-phenylalanine Resolution IV.- This was a repetition of Resolution III. 27.1 grams (0.13 mole) of N-acetyl-DL-phenylalanine were dissolved in 3.3 ml. of 3.9 F NaOH and 300 ml. of 0.5 F sodium acetate -0.5 F acetic acid buffer. 2.0 grams of L-cysteine hydrochloride (Van Camp Lab.), 14.1 grams (0.13 mole) of redistilled phenylhydrazine and 2.2 grams of activated papain were added. The solution was diluted to 500 ml. with buffer and incubated at 40° in a stoppered 500 ml. flask. The precipitate was removed after 85 hours incubation, washed with water, and dried. Yield: (F-1), 16.4 grams (84%), m. p. 202-205° (corr.), $[\alpha]_D^{25}$ = -35° (C,7; pyridine). 1.0 grams of L-cysteine hydrochloride and 2.7 grams of redistilled phenylhydrazine were added to the filtrate from F-1 and incubation continued for 50 hours at 40°. Essentially no further precipitate was obtained. The solution was filtered to remove a small amount of insoluble material and the filtrate was acidified with 12 F HCl and allowed to stand 2 hours at 0°. The precipitated N-acetyl-D-phenylalanine was filtered off and washed with water. Yield (F-2) was 7.8 grams (58%), m. p. 164-167° (corr.) $[\alpha]_D^{25}$ = -31°. The filtrate was extracted with ether in a liquid-liquid extractor; the ether extract was evaporated and residue triturated with water. Obtained 5.4 grams (40%) of N-acetyl-D-phenylalanine, $[\alpha]_D^{25}$ = -30.5° (C,7; pyridine). The fractions of N-acetyl-D-phenylalanine were recrystallized from a small volume of alcohol and then from water to yield

pure N-acetyl-D-phenylalanine, m. p. 171-172° (corr.); $[\alpha]_D^{25}$ = -32.9° (C,9; pyridine); $[\alpha]_D^{25}$ = -46.0° (C,8; ethanol).

Anal. Calcd. for $C_{11}H_{13}O_3N$ (207.2): C, 63.75; H, 6.32; N, 6.76. Found: C, 63.89; H, 6.39; N, 6.57.

Lit. $[\alpha]_D^{26^\circ}$ = -51° (ethanol) (9). Value reported for N-acetyl-L-phenylalanine $[\alpha]_D^{25}$ = + 47.6° (ethanol) (10).

F-1 (16.3 grams) was recrystallized from ethanol; recovered 14.9 grams of N-acetyl-L-phenylalanylphenylhydrazide in the main fraction, m. p. 207-208° (corr.), $[\alpha]_D^{25}$ = -34.6° (C,9; pyridine), and a second fraction of 650 mg., m. p. 204-207° (corr.), $[\alpha]_D^{25}$ = -33.8° (C,8; pyridine).

N-Benzoyl-DL-phenylalanine Resolution.- 10.5 grams (0.039 mole) of N-benzoyl-DL-phenylalanine were dissolved in 27.4 ml. of 3.9 F NaOH and 3.5 liters of 0.1 F sodium acetate -0.1 F acetic acid buffer (pH 4.6). 2.2 grams (0.020 mole) of redistilled phenylhydrazine, 14.0 grams of L-cysteine hydrochloride (Van Camp Lab.) and 9.7 grams of activated papain were added and the solution was diluted to 3.8 liters, stoppered, and incubated at 40°. The solution was turbid within 1 hour, and a fine precipitate had formed within 8 hours. The solution was clear at the end of two days incubation. After 88 hours incubation, the precipitated N-benzoyl-L-phenylalanylphenylhydrazide was filtered, washed

with water and dried. Yield: 5.55 grams (F-1) (79%) m. p. 199-209° with softening 194-199°, $[\alpha]_D^{25}$ =-56.6° (C,6; pyridine). 2.2 grams of phenylhydrazine were added to the filtrate from F-1 and incubation was continued for 90 hours. The precipitate was filtered, washed with water and dried. Obtained 1.55 grams (F-2) (22%), m. p. 203-209°, $[\alpha]_D^{25}$ = -54.9° (C,8; pyridine).

The filtrate from F-2 was acidified with HCl and extracted in a continuous liquid-liquid extractor for 24 hours, the ether extract was allowed to evaporate in an evaporating dish, and the product was triturated with water and filtered. After washing and drying it weighed 5.0 grams (F-3) (95%), m. p. 132-138° (corr.) (too colored to obtain a rotation).

F-1 (5.5 grams) was dissolved in 175 ml. of hot ethanol, filtered hot to remove insoluble protein, and allowed to cool and stand at room temperature overnight. A nicely crystalline precipitate (needles) was obtained. Yield: 3.37 grams (F-1-A), m. p. 214.5-216.5° (corr.) $[\alpha]_D^{25}$ = -61.7° (C,7; pyridine).

Anal. Calcd. for $C_{22}H_{21}O_2N_3$ (359.4): C, 73.51; H, 5.89; N, 11.70. Found: C, 73.68; H, 5.97; N, 11.77.

The filtrate from F-1-A was concentrated and 1.27 grams additional product was obtained, m. p. 165-195°, $[\alpha]_D^{25}$ = -49.2° (pyridine). An additional recrystallization of this latter product from ethanol yielded 1.00 grams of N-acetyl-L-phenylalanylphenylhydrazide, m. p. 215-217° (corr.), $[\alpha]_D^{25}$ = -61.9° (C,8; pyridine).

F-2 was recrystallized from ethanol and the product melted at 215-217° (corr.), $[\alpha]_D^{25}$ = -61.3° (C,7; pyridine).

Although no pure N-benzoyl-DL-phenylalanylphenylhydrazide was isolated, it seems probable from the rotation of F-1 and F-2 that a small amount (about 5%) was formed.

N-Benzoyl-D-phenylalanine (F-3) was dissolved in 50 ml. of ethanol, allowed to stand in the cold room for several weeks; no precipitate was obtained. 25 ml. of water were added, and after standing for a short time a small amount (500 mg.) of highly colored solid precipitated out; discarded. The solution was heated to boiling and 400 ml. of water were added; then it was boiled with Norite to remove color and after filtering it was allowed to cool slowly; a nicely crystalline white precipitate formed. It was filtered and washed with water, Yield: 3.3 grams, m. p. 137-139° (corr.) $[\alpha]_D^{25}$ = +22.6 (C,8; pyridine). It was recrystallized several additional times from dilute acid solutions, m. p. 139.5-140.5° (corr.), $[\alpha]_D^{25}$ = +23.8° (C,8; pyridine) $[\alpha]_D^{25}$ = -18.0° (C,8; 0.39 F NaOH).

Anal. Calcd. for $C_{16}H_{15}O_3N$ (269.3): C, 71.35; H, 5.62; N, 5.21. Found: C, 71.56; H, 5.85; N, 5.04.

Lit. m. p. 145-146°, $[\alpha]_D^{20}$ = -17.1° (C,7; 1 F NaOH) (11).

N-Carbobenzoxy-DL-phenylalanine Resolution.- 14.0 grams (0.047 mole) of N-carbobenzoxy-DL-phenylalanine were dissolved in 28.7 ml. of 3.9 F NaOH and 3.5 liters of 0.1 F sodium acetate- 0.1 F

acetic acid buffer (pH 4.6) and 2.66 grams (0.024 mole) of redistilled phenylhydrazine, 14.0 grams of L-crysteine hydrochloride (Van Camp Lab.) and 9.7 grams of activated papain were added. The solution was diluted to 3.8 liters and incubated in a stoppered gallon container at 40°. It was turbid within 10-15 minutes, and clear after about 1 1/2 days. After 88 hours incubation the precipitated N-carbobenzoxy-phenylalanylphenylhydrazide was filtered, washed with water and dried. Yield: 9.04 grams (F-1) (99%)*, m. p. 156-168° (corr.) $[\alpha]_D^{25} = -14.0°$ (C,6; pyridine). To the filtrate from F-1 were added 2.76 grams of phenylhydrazine, the pH was readjusted to 4.6 and incubation was continued for 4 days. The solution became turbid within several hours, and a relatively large amount of precipitate had formed in several days. The precipitated N-carbobenzoxy-phenylalanylphenylhydrazide was filtered, washed and dried; yield: 5.78 grams (F-2) (63%), m. p. 156-168°, mostly 156-160° (corr.), $[\alpha]_D^{25} = +6.0°$ (C,8; pyridine). To the filtrate from F-2 was added 1 gram of phenylhydrazine, and incubation was continued for 5 days. The precipitate (F-3) after washing and drying, weighed 2.27 grams (25%), m. p. 173-177°, with softening 155-173° (corr.), $[\alpha]_D^{25} = +20.9°$ (C,7; pyridine). Thus the total yield of N-carbobenzoxy-phenylalanylphenylhydrazide was 187% of theory.

*Yields are calculated on the assumption that only the L-phenylhydrazide can be formed.

F-1 (9.0 grams) was dissolved in 150 ml. of boiling toluene, filtered hot to remove about 400 mg. of insoluble protein, and allowed to cool slowly to about 60°, when it was filtered. The precipitate (F-1-A) contained 5.63 grams, m. p. 168-172° (corr.), $[\alpha]_D^{25} = -19.2°$ (C,8; pyridine). Upon cooling the filtrate there was obtained 2.00 grams (F-1-B), m. p. 158-160° (corr.) $[\alpha]_D^{25} = -1.5°$ (C,7; pyridine). F-1-A was again fractionally recrystallized from 250 ml. of toluene and there were obtained 4.44 grams of N-carbobenzoxy-L-phenylalanylphenylhydrazide (F-1-A-1), m. p. 178-179° (corr.), $[\alpha]_D^{25} = -24.1°$ (C,8; pyridine) from the first precipitate and 1.2 grams (F-1-A-2) from the filtrate with a m. p. of 158-160° (corr.), $[\alpha]_D^{25} = -0.4°$ (C,7; pyridine). F-1-A-1 was again fractionally recrystallized from toluene; all fractions obtained had a m. p. of 177-179° (corr.), $[\alpha]_D^{25} = -24.6°$ (C,8; pyridine), $[\alpha]_D^{25} = -29.2°$ (C, 2.5; chloroform).

Anal. Calcd. for $C_{23}H_{23}O_3N_3$ (389.4): C, 70.93; H, 5.95; N, 10.80. Found: C, 71.07; H, 6.21; N, 10.41.

F-1-B and F-1-A-2 were combined and recrystallized from toluene; recovered 3.0 grams, of nearly pure N-carbobenzoxy-DL-phenylalanylphenylhydrazide, m. p. 160-161.5° (corr.), $[\alpha]_D^{25} = -1.2°$ (C,8; pyridine).

Anal. Calcd. for $C_{23}H_{23}O_3N_3$ (389.4): C, 70.93; H, 5.95; N, 10.80. Found: C, 70.73; H, 5.96; N, 10.74.

Thus 4.4 grams of N-carbobenzoxy-L-phenylalanylphenylhydrazide and 3.0 grams of N-carbobenzoxy-DL-phenylalanylphenylhydrazide were recovered from F-1.

By repeatedly fractionally recrystallizing F-2 and F-3 from toluene in a similar manner to F-1 there were obtained 3.3 grams of N-carbobenzoxy-D-phenylalanylphenylhydrazide, m. p. 178-179° (corr.), $[\alpha]_D^{25} = +24.4°$ (C,7; pyridine); Anal. Calcd. for $C_{23}H_{23}O_3N_3$ (389.4): C, 70.93; H, 5.95; N, 10.80. Found: C, 71.07; H, 5.99; N, 10.86: and 3.5 grams of N-carbobenzoxy-DL-phenylalanylphenylhydrazide, m. p. 159.5-161° (corr.), $[\alpha]_D^{25} = 0.0°$ (C,9; pyridine).

N-Carboethoxy-DL-phenylalanine Resolution I.- 11.9 grams (0.05 mole) of N-carboethoxy-DL-phenylalanine were dissolved in 6.4 ml. of 3.9 F NaOH and 800 ml. of 0.1 F sodium acetate -0.1 F acetic acid buffer (pH 4.6). 2.8 grams (0.026 mole) of redistilled phenylhydrazine, 4.0 grams of L-cysteine hydrochloride (Van Camp Lab.) and 2.0 grams of activated papain were added. The solution was diluted to 1 liter, and incubated in a stoppered 1 liter flask at 40° for 104 hours. The precipitate (I-F-1) weighed after filtering, washing and drying 7.44 grams (91%), m. p. 151-164° (corr.) $[\alpha]_D^{25} = -16.0°$ (C,6; pyridine).

The filtrate from I-F-1 was incubated with 2.7 grams additional phenylhydrazine for 72 hours. The precipitated phenylhydrazide which had formed was filtered, washed with

water, and dried. This fraction (I-F-2) weighed 3.1 grams (38%), m. p. 167-171°, with softening from 153 to 167° (corr.), $[\alpha]_D^{25} = -3.7°$ (C, 9; pyridine).

The pH of the filtrate from I-F-2 was readjusted to 4.6 and incubation was continued for 16 days. The precipitate (I-F-3) was removed, it weighed 2.46 grams (30%), m. p. 155-170°, mostly 155-158° (corr.), $[\alpha]_D^{25} = +19.2°$ (C,7; pyridine).

The filtrate from I-F-3 was combined with a similar filtrate from a second resolution (see below), acidified with 30 ml. of 12 F HCl, and extracted in a continuous liquid-liquid extractor for 48 hours. The ether extract was evaporated; an oily residue was obtained which could not be crystallized. It was dissolved in 200 ml. of hot 0.1 F sodium acetate-0.1 F acetic acid buffer, filtered to remove a small amount of insoluble material and cooled, 1.2 grams of L-cysteine hydrochloride and 4 grams of phenylhydrazine were added, and the solution was adjusted to pH 4.6. 1.0 gram of activated papain was added and the solution was incubated for 56 hours. A precipitate formed rapidly. The precipitate (I + II-F-4) was filtered, washed and dried. It weighed 2.65 grams (17%), m. p. 154-160°; $[\alpha]_D^{25} = +23° \pm 2°$ (C,6; pyridine).

The filtrate from I + II-F-4 was incubated for one week, obtained 1.46 grams additional product (9%), m. p. 152-158°

(corr.) (too colored to obtain a rotation). It was recrystallized from toluene, obtained 1.15 grams of N-carboethoxy-D-phenylalanylphenylhydrazide, m. p. 156-160.5° (corr.) $[\alpha]_D^{25} = +23.4$ (C,8; pyridine).

N-Carboethoxy-DL-phenylalanine Resolution II.- 10.2 grams (0.043 mole) of N-carboethoxy-DL-phenylalanine were incubated with 2.47 grams (0.023 mole) of redistilled phenylhydrazine, 4.0 grams of L-cysteine hydrochloride, and 2.0 grams of activated papain as described for Resolution I. The first fraction (II-F-1) of N-carboethoxy-phenylalanyl-phenylhydrazide obtained after 96 hours incubation weighed 6.55 grams (93%), m. p. 151-165° (corr.), $[\alpha]_D^{25} = -14.1°$ (C,6; pyridine). The second fraction (II-F-2) obtained after incubation for 72 additional hours with 2.5 grams added phenylhydrazine weighed 2.69 grams (38%), m. p. 168-173°, with softening 154-168°, $[\alpha]_D^{25} = -3.7°$ (C,9; pyridine). A third fraction (II-F-3) weighing 2.37 grams (30%), m. p. 155-158° (corr.), $[\alpha]_D^{25} = +19.3°$ (C,8; pyridine) was obtained after 16 days additional incubation. The filtrate from II-F-3 was combined with that from I-F-3 and was treated as described above.

By repeated fractional crystallization of I-F-1, I-F-2, II-F-1 and II-F-2 from toluene, there were obtained as the more insoluble component 7.5 grams of N-carboethoxy-DL-phenylalanylphenylhydrazide, m. p. 171-172.5° (corr.);

$[\alpha]_D^{25}$ = -0.4° (C,8; pyridine);

Anal. Calcd. for $C_{18}H_{21}O_3N_3$ (327.4): C, 66.03; H, 6.47; N, 12.84. Found: C, 66.19; H, 6.67; N, 12.67; and 7.5 grams of N-carboethoxy-L-phenylalanylphenylhydrazide, m. p. 156.5-159.5° (corr.); $[\alpha]_D^{25}$ = +22.2° (C,8; pyridine) as the more soluble component.

Anal. Calcd. for $C_{18}H_{21}O_3N_3$: N, 12.84. Found: N, 12.57.

I-F-3 and II-F-3 were fractionally recrystallized to yield 3.3 grams of N-carboethoxy-D-phenylalanylphenylhydrazide, m. p. 156-159° (corr.); $[\alpha]_D^{25}$ = +21.5° (C,8; pyridine).

Anal. Calcd. for $C_{18}H_{21}O_3N_3$ (327.4): N, 12.84. Found: 12.83.

N-Carbomethoxy-DL-phenylalanine Resolution.- 10.7 grams (0.048 mole) of N-carbomethoxy-DL-phenylalanine were dissolved in 12.7 ml. of 3.9 F NaOH and 800 ml. of 0.2 F sodium acetate-0.2 F acetic acid buffer (pH 4.6). 2.6 grams (0.024 mole) of redistilled phenylhydrazine, 4.0 grams of L-cysteine hydrochloride (Van Camp Lab.) and 4.4 grams of activated papain were added, the solution diluted to 1 liter with buffer, and incubated in a stoppered 1 liter flask at 40°. The precipitate of N-carbomethoxy-phenylalanyl-phenylhydrazide formed slowly. It was removed by filtration after 96 hours incubation. Yield: (F-1), 4.6 grams, (61%), m. p. 166-177°, mostly 166-169° (corr.); $[\alpha]_D^{25}$ = -17.3° (C,8; pyridine). To the filtrate from F-1 there were added

3.0 grams of cysteine hydrochloride and 3.6 grams of phenylhydrazine; the pH was readjusted to 4.6 and incubation was continued for 45 hours. The precipitate (F-2) weighed 1.74 grams (23%) m. p. 167-169° (corr.); $[\alpha]_D^{25} = -17.3°$ (C,8; pyridine).

The filtrate from F-2 was incubated for 7 days and the precipitated phenylhydrazide (F-3) obtained weighed 1.14 grams, (15%), m. p. 179-182°, with softening from 170-179° (corr.); $[\alpha]_D^{25} = -2.6°$ (C,8; pyridine).

The filtrate from F-3 was acidified with HCl and extracted with ether, but only a non-crystallizable oil could be obtained from the ether extract; it was discarded.

F-1 and F-2 were combined and repeatedly fractionally recrystallized from toluene; obtained 3.7 grams of N-carbomethoxy-L-phenylalanylphenylhydrazide, m. p. 170-174° (corr.); $[\alpha]_D^{25} = -23.8°$ (C,8; pyridine).

Anal. Calcd. for $C_{17}H_{19}O_3N_3$(313.3): C, 65.15; H, 6.11; N, 13.42. Found: C, 65.16; H, 6.11; N, 13.30.

From F-3 and the more insoluble fractions obtained from the recrystallization of F-1 and F-2 there were obtained 2.0 grams of N-carbomethoxy-DL-phenylalanylphenylhydrazide, m. p. 181-182.5° (corr.); $[\alpha]_D^{25} = -0.2°$ (C,8; pyridine).

Anal. Calcd. for $C_{17}H_{19}O_3N_3$ (313.3): C, 65.15; H, 6.11; N, 13.42. Found: C, 64.87; H, 6.04; N, 13.66.

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## THE ENZYMATIC SYNTHESIS OF N-CARBOBENZOXY-D AND L-*o*-FLUOROPHENYLALANYLPHENYLHYDRAZIDES

*Sir:*

Previous studies on the resolution of acylated DL-amino acids by the asymmetric enzymatic synthesis of the anilide or phenylhydrazide of the acylated L-amino acid[1] have given no indication that appreciable quantities of the anilide or phenylhydrazide of the acylated D-amino acid may also be formed. We wish to report a case where substantial quantities of the D-phenylhydrazide have been synthesized despite the fact that the amount of amine present was insufficient to permit quantitative conversion of both the D- and L-acids.

25.0 g. (0.079 mole) of N-carbobenzoxy-DL-*o*-fluorophenylalanine was incubated with 20 g. of activated papain, 36.0 g. of L-cysteine hydrochloride, and 4.3 g. (0.040 mole) of redistilled phenylhydrazine at 40° for five days. The precipitated N-carbobenzoxy-*o*-fluorophenylalanylphenylhydrazide was recovered and recrystallized from toluene to give 11.0 g. of N-carbobenzoxy-*o*-fluorophenylalanylphenylhydrazide (I); m. p. 152–160°; 5.0 g. of additional papain, 12.0 g. of cysteine hydrochloride and 1.00 g. of phenylhydrazine was added to the filtrate from (I), the solution was incubated for five days at 40°, and the precipitate recrystallized from toluene to give 3.0 g. of N-carbobenzoxy-DL-*o*-fluorophenylalanylphenylhydrazide (II); m. p. 153.5–155.7° (cor.); $[\alpha]^{25}D$ 0.0° (3% in acetone). (I) was fractionally recrystallized from toluene to give 4.0 g. of N-carbobenzoxy-L-*o*-fluorophenylalanylphenyl-hydrazide (III); m. p. 171.0–172.0° (cor.); $[\alpha]^{25}D$ −31.0° (3% in acetone). *Anal.* Calcd. for $C_{23}H_{22}O_3N_3F$: C, 67.8; H, 5.4; N, 10.3. Found: C, 67.9; H, 5.7; N, 10.3; and 4.0 g. of (II); m. p. 155.5–156.5° (cor.); $[\alpha]^{25}D$ 0.0° (3% in acetone). *Anal.* Calcd. for $C_{23}H_{22}O_3N_3F$: C, 67.8; H, 5.4; N, 10.3. Found: C, 67.9; H, 5.6; N, 10.3. The filtrate from (II) was concentrated under reduced pressure, acidified, and the oily solid recrystallized from toluene to give 5.6 g. of an approximately equimolar mixture of N-carbobenzoxy-D-*o*-fluorophenylalanine and N-carbobenzoxy-DL-*o*-fluorophenylalanine. Fractional recrystallization from toluene gave 1.0 g. of N-carbobenzoxy-D-*o*-fluorophenylalanine (IV); m. p. 103–105° (cor.); $[\alpha]^{25}D$ +15.7° (5% in acetone). *Anal.* Calcd. for $C_{17}H_{16}O_4NF$: C, 64.3; H, 5.1; N, 4.4. Found: C, 64.4; H, 5.1; N, 4.2; and 1.9 g. of N-carbobenzoxy-DL-*o*-fluorophenylalanine (V); m. p. 108.5–110.0° (cor.); $[\alpha]^{25}D$ 0.2° (5% in acetone). *Anal.* Calcd. for $C_{17}H_{16}O_4NF$: C, 64.3; H, 5.1; N, 4.4. Found: C, 64.5; H, 5.3; N, 4.5.

A simultaneous enzymatic resolution of N-carbobenzoxy-DL-alanine using an aliquot of the same enzyme preparation gave N-carbobenzoxy-L-alanylphenylhydrazide in 75% yield after one recrystallization; m. p. 154.5–155.5° (cor.); $[\alpha]^{25}D$ −27.2° (5% in acetone).

Other experiments not reported here indicate that the behavior noted with *o*-fluorophenylalanine is not unique and it is clear that further study on the effect of the nature of the side chain, of the base, and of the acyl group on the course of the enzymatic synthesis is required. Such investigations are now in progress.

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(1) M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 707 (1937).

N-Acetyl-DL-o-fluorophenylalanine Resolution.- 25.5 grams (0.11 mole) of N-acetyl-DL-o-fluorophenylalanine were dissolved in 350 ml. of 0.5 F sodium acetate-0.5 F acetic acid buffer (pH 4.6) and 3.3 ml. of 3.9 F NaOH. 12.3 grams (0.11 mole) of redistilled phenylhydrazine, 2.0 grams of L-cysteine hydrochloride (Van Camp Lab.) and 2.2 grams of activated papain were added; the solution was diluted to 500 ml. and incubated in a 500 ml. stoppered flask at 40°. A large amount of precipitate had formed within 9 hours. The solution was incubated for 110 hours, then the precipitate (F-1) was removed by filtration. After washing and drying F-1 weighed 15.5 grams (87%), m. p. 210-213° (corr.) (too colored to obtain a rotation). The filtrate from F-1 was incubated with 1.0 gram additional L-cysteine hydrochloride and 2.5 grams phenylhydrazine for 6 days. 0.95 gram (5%) additional N-acetyl-o-fluorophenyalanylphenylhydrazide was obtained, m.p. 210-213° (corr.) $[\alpha]_D^{25} = -29.2°$ (C,5; pyridine).

The filtrate from F-2 was acidified with 50 ml. of 12 F HCl and allowed to stand in the cold room overnight. The precipitate was filtered off; obtained 10.4 grams (82%) of N-acetyl-D-o-fluorophenylalanine (F-3), m. p. 166-170° (corr.); $[\alpha]_D^{25} = -15.9°$ (C,9; pyridine).

F-1 and F-2 were combined and recrystallized from 400 ml. of ethanol. Obtained 14.3 grams of N-acetyl-L-o-fluorophenylalanylphenylhydrazide, m. p. 215-216.5° (corr.); $[\alpha]_D^{25}$ = -29.6° (C,7; pyridine).

Anal. Calcd. for $C_{17}H_{18}O_2N_3F$ (315.3): C, 64.75; H, 5.75; N, 13.33. Found: C, 64.66; H, 6.08; N, 13.59.

The small additional fraction obtained from the filtrate from the recrystallization of F-1 and F-2 had the same m. p. and rotation.

The N-acetyl-D-o-fluorophenylalanine (F-3) was recrystallized from a small volume of alcohol and then from 75 ml. of water. A nicely crystalline precipitate was obtained, F-3-1,6.3 grams, m. p. 168-170° (corr.) $[\alpha]_D^{25}$ = -28.6° (C,8; ethanol) $[\alpha]_D^{25}$ = -16.4° (C,8; pyridine).

Anal. Calcd. for $C_{11}H_{12}O_3NF$ (225.2): C, 58.65; H, 5.37; N, 6.22. Found: C, 58.72; H, 5.69; N, 5.95.

N-Acetyl-DL-m-fluorophenylalanine Resolution.- 9.54 grams (0.042 mole) of N-acetyl-DL-m-fluorophenylalanine were dissolved in 1.3 ml. of 3.9 F NaOH and 125 ml. of 0.5 F sodium acetate-0.5 F acetic acid buffer (pH 4.6) 0.8 gram of L-cysteine hydrochloride (Van Camp Lab.), 0.9 gram of activated papain, and 4.7 grams (0.044 mole) of redistilled phenylhydrazine were added. The solution was diluted to 200 ml. and incubated in a stoppered 200 ml. flask at 40°.

A large amount of precipitate formed within 9 hours; the solution was incubated for 116 hours and then the precipitate of N-acetyl-m-fluorophenylalanylphenylhydrazide (F-1) was removed by filtration. It weighed, after washing and drying, 5.7 grams (85%), m. p. 206-209° (corr.); $[\alpha]_D^{25}$ = -30.1° (C,8; pyridine). The filtrate from F-1 was incubated with 0.4 gram additional cysteine hydrochloride and 1.4 grams of phenylhydrazine for 48 hours. The precipitate (F-2) weighed 160 mg., m. p. 206-209° (corr.); $[\alpha]_D^{25}$ = -29.6° (C,8; pyridine).

The filtrate from F-2 was acidified with 20 ml. of 12 F HCl and allowed to stand overnight at 4°. The precipitated N-acetyl-D-m-fluorophenylalanine (F-3) was filtered; yield: 2.90 grams (61%), m. p. 156.5-158.5° (corr.); $[\alpha]_D^{25}$ = -29.0° (C,8; pyridine). F-1 and F-2 were combined and recrystallized from 50 ml. of ethanol. The first fraction obtained (F-1-1) weighed 4.33 grams, m. p. 209-211° (corr.); $[\alpha]_D^{25}$ = -30.6° (C,8; pyridine); the second fraction (F-1-2) weighed 970 mg. m. p. 198-208° (corr.); $[\alpha]_D^{25}$ = -30.5° (C,8; pyridine). F-1-2 was recrystallized again and the m. p. was raised to 209-211° (corr.). Both fractions were then combined and recrystallized once more from ethanol, m. p. 209-210° (corr.); $[\alpha]_D^{25}$ = -30.9° (C,9; pyridine).

Anal. Calcd. for $C_{17}H_{18}O_2N_3F$ (315.3): C, 64.75; H, 5.75; N, 13.33. Found: C, 64.52; H, 5.74; N, 13.33.

The N-acetyl-D-m-fluorophenylalanine was recrystallized from a small volume of ethanol, m. p. 158.5-160.0 (corr.); $[\alpha]_D^{25}$ = -29.0° (C,7; pyridine); $[\alpha]_D^{25}$ = -39.9° (C,7; ethanol) and then from 50 ml. of water. Obtained a

nicely crystalline product, yield: 1.9 grams, m. p. 159-160° (corr.); $[\alpha]_D^{25} = -40.4°$ (C,8; ethanol). Anal. Calcd. for $C_{11}H_{12}O_3NF$ (225.2): C, 58.65; H, 5.37; N, 6.22. Found: C, 58.37; H, 5.63; N, 5.91.

N-Acetyl-DL-p-fluorophenylalanine Resolution.- 14.2 grams (0.063 mole) of N-acetyl-DL-p-fluorophenylalanine were dissolved in 2.0 ml. of 3.9 F NaOH and 150 ml. of 0.5 F sodium acetate-0.5 F acetic acid buffer (pH 4.6). 1.2 grams of L-cysteine hydrochloride (Van Camp Lab.), 6.8 grams (0.063 mole) of redistilled phenylhydrazine and 1.4 grams of activated papain were added. The solution was diluted to 300 ml. with buffer and incubated at 40° in a stoppered 300 ml. flask. A large amount of precipitate formed within 9 hours. After 88 hours incubation the precipitated N-acetyl-L-p-fluorophenylalanylphenylhydrazide (F-1) was filtered. After washing and drying it weighed 8.7 grams (87%), m. p. 223-230° (corr.); $[\alpha]_D^{25} = -37.0°$ (C,8; pyridine). To the filtrate from F-1 were added 0.6 gram of cysteine hydrochloride and 1.9 grams of phenylhydrazine and incubation was continued for 4 days. The precipitate (F-2) obtained weighed 0.62 grams, (6%), m. p. 229-232° (corr.); $[\alpha]_D^{25} = -36.2°$ (C,8; pyridine).

The filtrate from F-2 was slowly acidified with 30 ml. of 12 F HCl and placed in the cold room overnight. Only a small protein precipitate formed and it was discarded. The

acid filtrate was then extracted for 30 hours in a continuous liquid-liquid extractor with ether. The ether extract was allowed to evaporate but only an oily residue was obtained. It was dissolved in dilute sodium hydroxide, extracted with ether, and then slowly acidified. When approximately neutral a small amount of oily material precipitated, the solution was decanted from this oil and the oil was discarded (after attempts to crystallize it failed). The solution was made acid and additional oil came down. It was placed in the cold room for 1 week and the oil solidified; in addition large colorless crystals formed. The colorless crystals separated were/ mechanically from the oil, yield: 1.8 grams. The crystals were crystallized several times from water, obtained 0.72 gram of N-acetyl-D-p-fluorophenylalanine, m. p. 142-143° (corr.); $[\alpha]_D^{25} = -38.6^\circ$ (C,8; ethanol).

Anal. Calcd. for $C_{11}H_{12}O_3NF$ (225.2): C, 58.65; H, 5.37; N, 6.22. Found: C, 58.86; H, 5.41; N, 6.39.

It is probable that if another resolution of N-acetyl-DL-p-fluorophenylalanine were to be carried out, the addition of a seed crystal of N-acetyl-D-p-fluorophenylalanine to the acid solution after removal of the N-acetyl-L-p-fluorophenylalanylphenylhydrazide would cause the precipitation of the N-acetyl-D-p-fluorophenylalanine present and that better recovery could thus be obtained.

F-1 and F-2 were combined and recrystallized from 100 ml. of ethanol. The first fraction obtained weighed 7.6 grams, m. p. 233-235° (corr.); $[\alpha]_D^{25} = -36.4°$ (C,9; pyridine);

Anal. Calcd. for $C_{17}H_{18}O_2N_3F$ (315.3): C, 64.7; H, 5.75; N, 13.33. Found: C, 64.58; H, 5.89; N, 13.06.

The second fraction weighed 0.89 grams, m. p. 224-230° $[\alpha]_D^{25} = -36.2°$ (C,8; pyridine). A recrystallization of this fraction raised the m. p. to 232-234° (corr.).

Preparation of D and L-fluorophenylalanines.

L-(-)-o-Fluorophenylalanine.- 13.0 grams of N-acetyl-L-o-fluorophenylalanylphenylhydrazide were hydrolyzed for 40 hours in 100 ml. of hot 6 F HCl. The hydrochloric acid was removed under reduced pressure, the residue was dissolved in 50 ml. of water and made basic with 15 ml. of conc. ammonium hydroxide. The solution was then extracted with five 20 ml. portions of peroxide free ether. The solution was filtered, then evaporated by boiling over an open flame to 30 ml. (excess ammonia was expelled). The solution was placed in the cold room overnight; a precipitate formed but very little water could be removed from it by filtration. It was stirred with 20 ml. of ethanol and filtered. This removed a great deal of color that was present. The

precipitate was warmed with 30 ml. of ethanol, filtered; repeated. The product was nearly white and appeared to be needles. It weighed 5.9 grams (78%), m. p. 214-220° (decomp.). This fraction was recrystallized again from water-ethanol. Obtained 4.6 grams, decomp. point, 226-232°.

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 57.19; H, 5.73; N, 7.58 (Elek).

It was then recrystallized from 200 ml. of 50% ethanol, and then recrystallized from 75 ml. of 6 F HCl. From this latter solvent a very nicely crystalline product was obtained (long prisms), which was dried over NaOH *in vacuo*. Recovered 3.4 grams, decomp. point 226-231° (corr.).

Anal. Calcd. for $C_9H_{10}O_2NF \cdot HCl \cdot H_2O$ (237.7): C, 45.48; H, 5.51; N, 5.89. Found: C, 45.63; H, 5.57; N, 5.82 (Elek); Neut. Equiv. 237.5.

$[\alpha]_D^{25} = -15°$ (calc. on basis of free amino acid; (*C*,2; 0.1 F NaCl, pH 5.5). From the filtrates from the aqueous-ethanol recrystallizations there was recovered 0.93 gram additional product as the hydrochloride hydrate after recrystallization from 6 F HCl, decomp. point 225-228° (corr.);

$[\alpha]_D^{25} = -15°$ (calc. on basis of free amino acid; *C*,2; 0.1 F NaCl, pH 5.4).

*D*-(±)-*o*-Fluorophenylalanine.- 5.7 grams of N-acetyl-D-*o*-fluorophenylalanine were hydrolyzed by refluxing with 100 ml. of 6 F HCl for 17 hours. After the HCl had been removed

under reduced pressure, the residue was dissolved in 30 ml. of distilled water, made slightly alkaline with ammonium hydroxide and filtered to remove a small amount of insoluble material. It was boiled to expel excess ammonia, then allowed to cool to room temperature. The precipitate which formed was gelatinous and it was very difficult to remove any water upon filtering. The precipitate (containing approximately 10 ml. of water) was warmed with 10 ml. additional water, allowed to cool and filtered. Repeated twice more. Finally it was washed with alcohol which tended to make the precipitate more crystalline by removing the water. Yield: 2.4 grams, decomp. point 221-226° (corr.); $[\alpha]_D^{25} = +15°$ (C,2; water). From the filtrate and washings there was obtained 1.75 grams additional product, decomp. point 223-231° (corr.); $[\alpha]_D^{25} = +15°$ (C,2; water). Total yield: 4.1 grams (89%).

The first fraction obtained was recrystallized from 40 ml. of water; recovered 1.7 grams, decomp. point 231-234° (corr.); $[\alpha]_D^{25} = +15°$ (C,2; water).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.0; H, 5.50; N, 7.65. Found: C, 57.41; H, 5.76; N, 7.51. It was then recrystallized from 120 ml. of 70% ethanol and finally from 50 ml. of 6 F HCl. From this latter solvent a nicely crystalline precipitate was obtained as long prisms. Recovered 1.9 grams of D- (+)-o-fluorophenylalanine hydrochloride monohydrate, decomp. point 224-228° (corr.);

Anal. Calcd. for $C_9H_{10}O_2NF \cdot HCl \cdot H_2O$ (237.7): C, 45.48; H, 5.51; N, 5.89. Found: C, 45.61; H, 5.59; N, 5.84 (Elek). Neut. Equiv. 243. From the filtrates from the aqueous-ethanol recrystallizations there was recovered after recrystallization from 6 F HCl 2.3 grams additional D-(+)-o-fluorophenylalanine hydrochloride hydrate, decomp. point 224-228° (corr.); Neut. Equiv. 238.

It is recommended that the D and L-o-fluorophenylalanine be recrystallized from HCl whenever possible.

L-(-)-m-Fluorophenylalanine.- 3.4 grams of N-acetyl-L-m-fluorophenylalanylphenylhydrazide were refluxed for 40 hours with 30 ml. of 6 F HCl. The hydrochloric acid was removed at reduced pressure; the residue was dissolved in 50 ml. of water and made basic with 10 ml. of conc. ammonium hydroxide. It was then extracted with five 20 ml. portions of peroxide free ether and filtered to remove a small amount of insoluble material. The excess ammonia was expelled by boiling and the solution was evaporated to 30 ml. and again filtered hot. Upon cooling, a nicely crystalline precipitate formed (needles). It was filtered and washed with water and then ethanol. Yield: 1.3 grams (67%), of L-(-)-m-fluorophenylalanine, decomp. point 235-237° (corr.); $[\alpha]_D^{25} = -23°$ (C,2; water). It was recrystallized from 30 ml. of 50% ethanol and then from 30 ml. of water. Obtained 0.83 grams, decomp. point 239-243° (corr.) $[\alpha]_D^{25} = -24°$ (C,2; water).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.04; H, 5.47; N, 7.68 (Elek).

D-(+)-m-Fluorophenylalanine.- 1.5 grams of N-acetyl-D-m-fluorophenylalanine were hydrolyzed for 15 hours with 50 ml. of 6 F HCl. The hydrochloric acid was removed under reduced pressure. The residue was dissolved in 35 ml. of water, filtered, made slightly basic, boiled to expel the excess ammonia and again filtered hot. Upon slowly cooling to room temperature a nicely crystalline precipitate of fine needles formed. After standing overnight it was filtered, washed with water and dried. Yield: 0.58 grams (48%) of D-(+)-m-fluorophenylalanine, decomp. point 228-231° (corr.); $[\alpha]_D^{25} = +22$ (C,2; water).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.06; H, 5.49; N, 7.70 (Elek).

From the filtrate there was obtained by the addition of ethanol 280 mg. additional D-(+)-m-fluorophenylalanine (23%), decomp. point 230-234° (corr.).

L-(-)-p-Fluorophenylalanine.- 7.5 grams of N-acetyl-L-p-fluorophenylalanylphenylhydrazide were refluxed with 80 ml. of 6 F HCl for 42 hours. The hydrochloric acid was removed under reduced pressure, the residue was dissolved in 70 ml. of water and 15 ml. of conc. ammonium hydroxide. It was extracted with six 20 ml. portions of peroxide free ether,

and then the aqueous solution was evaporated over an open flame to 35 ml. A nicely crystalline precipitate of flat plates formed. It weighed, after filtration and washing with water and ethanol, 2.7 grams (63%), decomp. point 246-248° (corr.). It was recrystallized from 100 ml. of 50% ethanol, recovered 2.04 grams of L-(-)-p-fluorophenylalanine, decomp. point 250-255° (corr.); $[\alpha]_D^{25} = -23°$ (C,2; water).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 59.09; H, 5.45; N, 7.61.

D-(+)-p-Fluorophenylalanine.- 600 mg. of N-acetyl-D-p-fluorophenylalanine were hydrolyzed for 12 hours with 25 ml. of 6 F HCl. The hydrochloric acid was removed under reduced pressure and the residue was dissolved in 10 ml. of water, made slightly basic with ammonium hydroxide, filtered, and evaporated to 5 ml. to expel the excess ammonia. 5 ml. of ethanol were added, and upon cooling long needles slowly precipitated. The precipitate was filtered and washed with aqueous ethanol and then ethanol. Yield: 300 mg. (61%) of D-(+)-p-fluorophenylalanine, decomp. point 227-232° (corr.); $[\alpha]_D^{25} = +24°$ (C,2; water).

Anal. Calcd. for $C_9H_{10}O_2NF$ (183.2): C, 59.01; H, 5.50; N, 7.65. Found: C, 58.87; H, 5.57; N, 7.74 (Elek).

## Summary

A study has been made of the factors influencing asymmetric specificity in the papain catalyzed synthesis of amides. It has been found that the syntheses of N-carbomethoxy-, N-carboethoxy-, and N-carbobenzoxy-phenylalanylphenylhydrazides are partially non-asymmetric, while the syntheses of N-acetyl-, and N-benzoylphenylalanyl-phenylhydrazides are greater than 95% asymmetric.

The N-acetyl-, ortho-, meta-, and para-fluorophenylalanines have been resolved enzymatically with papain.

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## C. THE APPARENT ACID DISSOCIATION CONSTANTS AND THE ULTRA-VIOLET ABSORPTION SPECTRA OF THE ISOMERIC FLUOROPHENYLALANINES AND THE ULTRA-VIOLET ABSORPTION SPECTRA OF FLUOROTYROSINES

### Discussion and Results

The ultra-violet absorption spectra and apparent acid dissociation constants of phenylalanine and the chlorophenylalanines have recently been reported by Nevenzel, Shelberg, and Niemann (1,2). In this section are reported the results of an extension of this work to the mono-fluorophenylalanines and to the mono-fluorotyrosines.

The methods used were essentially the same as those used by Nevenzel and thoroughly discussed in (1,2); therefore no formal discussion of them will be given here*.

Table I summarizes the results of the investigation to determine the apparent acid dissociation constants of the

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* In the work reported in this thesis, $pK'_{COOH}$ was calculated from about 14 values in the pH range 2.0-3.75, and $pK^{+}_{NH_3}$ from an equal number of values in the pH range 7.0-9.5. The blank correction in the acid range was calculated and an experimentally determined linear "correction factor" was applied ranging from -1% at pH 2.70 to -8% at pH 2,00. With this "correction factor", consistent values were obtained at all points. The "blank correction" in the alkaline range was determined experimentally- in the range 7-9.5 it was negligible and was, at most, 0.01 ml. at pH 9.8. The amino acid solutions had a known concentration of about 0.02 F; they were prepared in 0.1 F NaCl ($CO_2$ free). The NaOH used was 0.1692 F; the HCl was 0.1936 F.

fluorophenylalanines. Included in the table for comparison are the results obtained by Nevenzel for phenylalanine and the chlorophenylalanines.

Table I

The Apparent Dissociation Constant of Phenylalanine and Some Halogenated Derivatives

| Compound | Final Temp. (°C) | $pK'^{**}_{COOH}$ | No. Values | Final Temp. (°C) | $pK'^{**}_{NH_3^+}$ | No. Values |
|---|---|---|---|---|---|---|
| Phenylalanine | | 2.16± 0.05 | 5 | | 9.12±0.02 | 8 |
| | 23.1 | 2.16± 0.01 | 16 | 24.3 | 9.12±0.01 | 14 |
| o-Fluorophenylalanine* | 23.5 | 2.12 ± 0.01 | 14 | 24.2 | 9.01±0.01 | 13 |
| | 23.1 | 2.11 ± 0.01 | 13 | 24.5 | 9.01±0.01 | 13 |
| m-Fluorophenylalanine | 24.0 | 2.10 ± 0.01 | 13 | 24.5 | 8.97±0.01 | 15 |
| | 24.0 | 2.10 ± 0.01 | 15 | 24.9 | 8.98±0.01 | 16 |
| p-Fluorophenylalnine | 24.9 | 2.12 ± 0.01 | 15 | 24.9 | 9.04±0.01 | 11 |
| | 24.3 | 2.13 ± 0.01 | 13 | 24.5 | 9.06±0.01 | 14 |
| 2-Fluoro-4-methoxyphenylalanine | 23.2 | 2.18 ± 0.01 | 15 | 24.5 | 9.03±0.01 | 11 |
| o-Chlorophenylalanine | | 2.23 ± 0.04 | 45 | | 8.94±0.03 | 40 |
| m-Chlorophenylalanine | | 2.17 ± 0.04 | 24 | | 8.91±0.01 | 25 |
| p-Chlorophenylalanine | | 2.08 ± 0.06 | 52 | | 8.96±0.03 | 40 |

* The fluorophenylalanine giving the upper value of each pair was prepared by Method I and that giving the lower value by Method II (see section I-A).

**The deviation shown is the average absolute deviation.

The data in Table I indicates that whereas the substitution of a chlorine atom for a hydrogen atom in the aromatic ring increased the acid strength of the ammonium group of phenylalanine by about 0.2pK units (about 50%), the substitution of a fluorine atom for a hydrogen atom decreased the pK value by about 0.1-0.15 units (about 25-40% increase in acidity). The differences in acid strengths of the -COOH groups are probably within the limit of experimental error, but for the fluorophenylalanines they are in the expected direction (see below).

The smaller effect of fluorine substitution as compared with chlorine substitution is what would be predicted by the modern theories of organic chemistry (see (3) for a discussion). It has been found for a number of compounds in the aromatic series (for example the benzoic acids) that meta- and para-substituted fluorobenzoic acids are slightly weaker than the corresponding chlorobenzoic acids. The effect of nuclear substitution is diminished as the acidic group is removed from the ring. Thus, in phenylpropionic acid, the effect of halogen substitution is small. The ammonium group is presumably more susceptible to the influence of substituents in the nucleus than the carboxyl group due to its closer proximity. The effect observed was slightly greater with meta substitution than with para substitution. This is as would be predicted for an ortho-para

directing substituent. A substituent situated in the ortho position to the side chain can exert its influence in several ways; accordingly it is difficult to predict the expected effect.

The apparent dissociation constants for 2-fluoro-4-methoxy-phenylalanine are shifted in the expected direction since a para-alkoxyl group always decreases the acid strength (3), and the dissociation constants found are slightly smaller than those observed for o-fluorophenylalanine.

The ultra-violet absorption spectra of the mono-fluorophenylalanines, the mono-fluorotyrosines, and 2-fluoro-4-methoxy-phenylalanine are shown in Figures I, II, and III. For comparative purposes the abosrption spectra of phenylalanine and L-tyrosine are also shown in Figures I and II. $\varepsilon_{max.}$ and $\varepsilon_{min.}$ of the above compounds are shown in Table II.

An inspection of the Figures or of the Table shows the most striking difference in the spectra to be the large increase in absorption of the fluorophenylalanines in the low intensity B (benzenoid) (4) absorption (260 mμ) region as compared to that of either phenylalanine or the chlorophenylalanines. This is in accordance with the absorption of fluorobenzene as compared to chlorobenzene and benzene which are in the ratio 1650/310/250 (4).

The bathochromic effect caused by the introduction of a fluorine atom in place of a hydrogen atom in the aromatic ring of phenylalanine is about 5 mμ. This is 5-6 mμ less than the effect observed when a chlorine atom is introduced. There appears to be a definite increase of 2.5 mμ in the bathochromic effect as the substituent is shifted around the ring from the ortho to the para position. There also appears to be a definite decrease in the intensity of the absorption as the fluorine atom is shifted around the nucleus from the ortho to the para position. The bathochromic shift has not been large enough to permit the observation of a definite maximum in the high intensity E (ethylenic) (4) absorption (200-220 mμ) region in the case of the fluorophenylalanines, whereas it was sufficiently large in the case of the chlorophenylalanines to permit such observation in the region 210-220 mμ.

The effect of fluorine substitution in tyrosine is not as striking as observed for the phenylalanines. Little increase in $\varepsilon_{max.}$ at 270-275 mμ was observed and the slight shift of $\varepsilon_{max.}$ was towards shorter wave lengths.

Table II

$\varepsilon_{max.}$ and $\varepsilon_{min.}$ for Several Nuclear Substituted Phenylalanines

| Compound | λ(mμ) | | ε |
|---|---|---|---|
| DL-Phenylalanine (in 0.1 F aqueous sodium chloride) | 208 | | 8050 |
| | 234 | (min.) | 40 |
| | 247 | | 131 |
| | 248 | | 132 |
| | 252 | (max.) | 168 |
| | 254 | (min.) | 158 |
| | 257.5 | (max.) | 202 |
| | 261 | (min.) | 145 |
| | 263 | (max.) | 155 |
| DL-o-Fluorophenylalanine (in 0.1 F aqueous sodium chloride) | 208 | | 7700 |
| | 229 | (min.) | 45 |
| | 261.5 | (max.) | 845 |
| | 265 | (min.) | 537 |
| | 267.5 | (max.) | 760 |
| DL-m-Fluorophenylalanine (in 0.1 F aqueous sodium chloride) | 208 | | 7400 |
| | 229 | (min.) | 40 |
| | 257 | | 589 |
| | 258 | | 590 |
| | 262 | (max.) | 820 |
| | 266 | (min.) | 500 |
| | 268 | (max.) | 745 |
| DL-p-Fluorophenylalanine (in 0.1 F aqueous sodium chloride) | 208 | | 6900 |
| | 230 | (min.) | 40 |
| | 264 | (max.) | 710 |
| | 267 | (min.) | 400 |
| | 270 | (max.) | 635 |
| DL-o-Chlorophenylalanine (in 0.1 F aqueous sodium chloride) | 213 | (max.) | 8300 |
| | 238 | (min.) | 43 |
| | 263 | | 199 |
| | 264 | | 200 |
| | 266 | (max.) | 203 |
| | 272 | (min.) | 142 |
| | 273 | (max.) | 144 |
| DL-m-Chlorophenylalanine (in 0.1 F aqueous sodium chloride) | 213 | (max.) | 8900 |
| | 237 | (min.) | 47 |
| | 260 | | 215 |
| | 262 | | 220 |
| | 267 | (max.) | 266 |
| | 272 | (min.) | 173 |
| | 274 | (max.) | 201 |

Table II
(cont.)

$\varepsilon_{max.}$ and $\varepsilon_{min.}$ for Several Nuclear Substituted Phenylalanines

| Compound | $\lambda$(mμ) | | $\varepsilon$ |
|---|---|---|---|
| DL-p-Chlorophenylalanine | 221 | (max.) | 11,200 |
| (in 0.2 F aqueous sodium | 240 | (min.) | 68 |
| chloride or water) | 260 | (max.) | 226 |
| | 262 | (min.) | 214 |
| | 267 | (max.) | 261 |
| | 273 | (min.) | 169 |
| | 275 | (max.) | 182 |
| L-Tyrosine | 223 | (max.) | 8600 |
| (in 0.1 F aqueous sodium | 245 | (min.) | 165 |
| chloride) | 275 | (max.) | 1400 |
| DL-2-Fluorotyrosine | 220.5 | (max.) | 8925 |
| (in 0.1 F aqueous sodium | 242 | (min.) | 185 |
| chloride) | 272 | (max.) | 1580 |
| DL-3-Fluorotyrosine | 221 | (max.) | 8250 |
| (in 0.1 F aqueous sodium | 243 | (min.) | 200 |
| chloride) | 272 | (max.) | 1520 |
| DL-2-Fluoro-4-methoxy- | 222 | (max.) | 11000 |
| phenylalanine | 243 | (min.) | 200 |
| (in 0.1 F aqueous sodium | 271 | (max.) | 1730 |
| chloride) | | | |

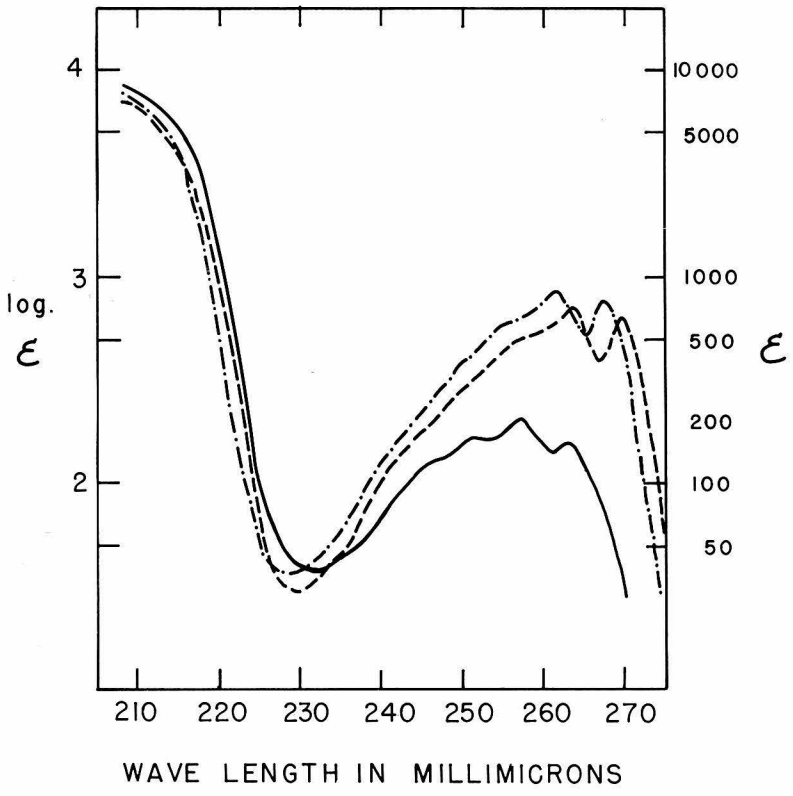


Figure 1. Ultra-Violet Absorption Spectra:
of Phenylalanine _____
o-Fluorophenylalanine .-.-.-.-.-.-.-.-
p-Fluorophenylalanine - - - - - - - -
in 0.1 F NaCl. The spectrum of m-Fluoro-
phenylalanine is not shown; it is nearly
identical with the spectrum of o-fluoro-
phenylalanine

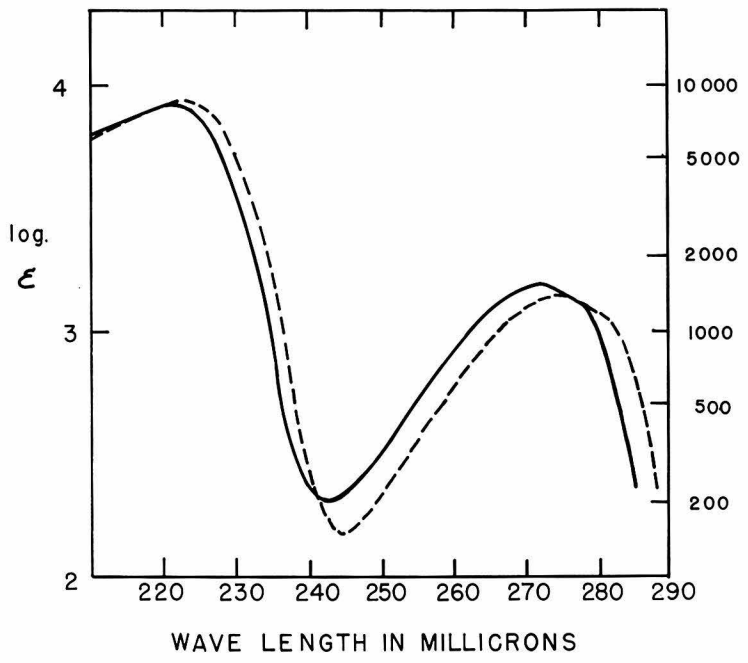


Figure 2. Ultra-Violet Absorption Spectra of 3-Fluorotyrosine _____ and L-tyrosine in ----------
0.1 F NaCl

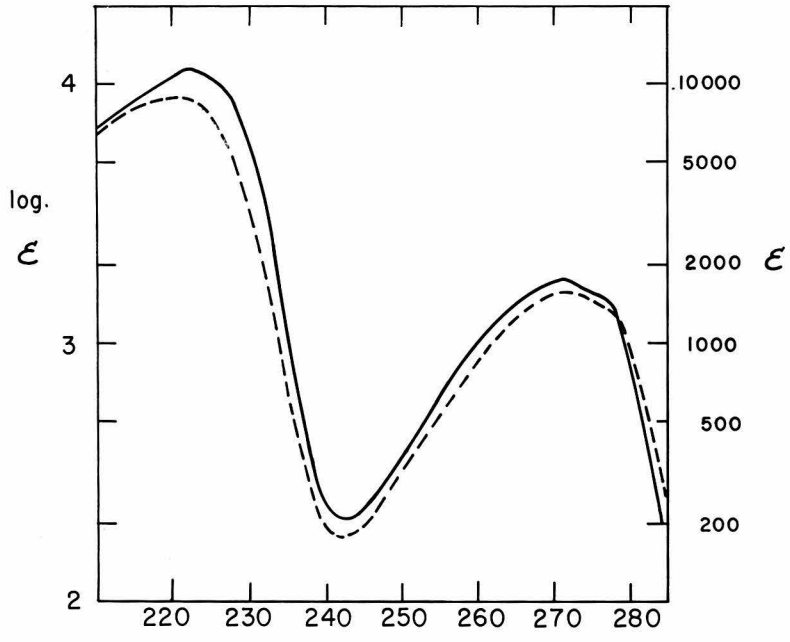


Figure 3. Ultra-Violet Absorption Spectra of 2-Fluoro-4-methoxy-phenylalanine_____ and 2-Fluorotyrosine - - - - - - in 0.1 F NaCl.

## Summary

The apparent acid dissociation constants of the monofluorophenylalanines have been measured. An expected slight increase in acid strength of the ammonium group of 0.1-0.15 pK units has been observed. The differences in acid strength of the COOH groups were probably within the limit of experimental error.

The ultra-violet absorption spectra of the fluorophenylalanines show bathochromic shifts of the maxima in the 260 mμ region of 5-7.5 mμ. A very large increase in the intensity of absorption in this region was observed.

The ultra-violet absorption spectra of 2-fluoro and 3-fluoro-tyrosine show only small differences from that of the parent compound.

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## D. ATTEMPTED SYNTHESIS OF 4-FLUOROCYCLOHEXANOL

### Discussion

It was proposed to synthesize some fluorinated amino acids in the aliphatic series. Fluorolysine was chosen for our first attempts since it was thought that fluorocyclohexanol might be available by reduction of fluorophenol. The fluorophenol can be readily obtained by preparation of the diazonium fluoroborate of anisidine and subsequent decomposition.

It should be possible to oxidize fluorocyclohexanol to fluorocyclohexanone and then convert the ketone to a fluorinated lysine by the same procedures that are used conventionally to prepare lysine from cyclohexanone (1,2,3). A consideration of the number of isomers possible in the rearrangement of the cyclohexanoneoxime made it seem desirable to prepare 4-fluorocyclohexanol which would eventually lead to $\gamma$-fluorolysine, even though this fluorinated derivative might be less interesting than one in which the fluorine atom was nearer to one of the end groups. However, if the projected procedure were practical for the synthesis of $\gamma$-fluorolysine, it would probably be possible to extend the method to other fluorinated derivatives of lysine, and perhaps even to other halogenated derivatives.

Accordingly we have prepared 4-fluorophenol by the usual methods as described in the experimental section. Several reductions of this compound were attempted. One method was catalytic hydrogenation at high pressure and temperatures ranging from 150-250° over a Raney nickel catalyst. Under the more vigorous conditions some hydrogen fluoride was evolved. One attempt under less vigorous conditions yielded some fluorocyclohexane or cyclohexane. An attempted reduction over platinum oxide catalyst at approximately 150° was more promising in that a significant amount of hydrogen was used, but on opening the bomb it was evident that a considerable amount of hydrogen fluoride had been evolved. A significant yield of fluorocyclohexane or more likely of cyclohexane was obtained. It seems probable however that high pressure reduction over a platinum catalyst in acetic acid as a solvent and at room temperature might produce the desired fluorocyclohexanol (4). This appears to the author to be the most promising method.

A second approach to the synthesis is from 4-chlorocyclohexanol. This compound is readily prepared as described in the experimental section from hydroquinone. Hydroquinone is reduced catalytically to 1,4-dihydroxycyclohexane which is subsequently dehydrated to yield 1,4-epoxycyclohexane. Treatment of this compound with concentrated hydrochloric acid at room temperatures produces 4-chlorocyclohexanol in

good yield. It is believed that the trans compound has been obtained as the main product. 4-chlorocyclohexanol is a very promising starting material for the preparation of $\gamma$-chlorolysine, but it is to be anticipated that it will be difficult to replace the chlorine atom by a fluorine atom. Also, some of the steps involved in the conversion of cyclohexanoneoxime to lysine involve rather drastic conditions and it is possible that dehydrohalogenation might occur. It is possible that 1,4-epoxycyclohexane can be converted to 4-bromocyclohexanol by treatment under the proper conditions with HBr. It would be expected that the replacement of a bromine atom by a fluorine atom would be more readily achieved than would the replacement of a chlorine atom by a fluorine atom. It is also possible that 4-bromocyclohexanol could be converted into a bromolysine if dehydrohalogenation were not encountered.

Treatment of 1,4-epoxycyclohexane with hot concentrated hydrochloric acid yields the 1,4-dichlorocylohexane in good yield. It is believed that the product is the cis-dichlorocyclohexane. It has been reported that the action of concentrated HBr on 1,4-epoxycyclohexane yields the trans-dibromide; thus this series of compounds appears to have some interesting chemistry connected with it.

Attempted Synthesis of 4-Fluorocyclohexanol.

4-Methoxy-benzenediazonium fluoroborate (5).- 493 grams (4.0 moles) of p-anisidine (EK White Label) in 1800 ml. of 5 F HCl were diazotized with 300 grams of sodium nitrite dissolved in 600 ml. of water. The excess nitrous acid was discharged with urea, 250 ml. of 12 F HCl added, and then 540 grams of sodium fluoroborate dissolved in 600 ml. of water were added. After 1/2 hour the precipitate was filtered, washed with cold water, methyl alcohol, and ether. After drying in vacuo over sulfuric acid it weighed 638 grams, decomp. point 139-140° (corr.). Lit. m. p. 100°, decomp. point 139° (5). From one-half of the filtrate and washings (remainder had been discarded) 41 grams additional product was obtained, decomp. point 139-141° (corr.). Total yield was 680 grams (76%) of 4-methoxy-benzenediazonium fluoroborate. Schiemann (5) reported a 85% yield.

4-Fluoroanisole (5).- 200 grams (0.90 mole) of dried 4-methoxy-benzenediazonium fluoroborate were placed in a 1 liter RBST flask connected by suitable joints to a condenser and two 250 ml. suction flasks connected in series, and finally to a water aspirator. Both suction flasks were immersed in an ice bath, and the second one was partially filled with water. The decomposition was carried out by carefully heating the diazonium compound at such a rate that the pressure in the system was maintained at 50-100 mm.

Most of the product distilled during the decomposition and was collected as a dark red liquid in the first flask. The product was combined in ether, washed with water until neutral, dried, and distilled. Obtained 98 grams (85%) of 4-fluoroanisole, b. p. 80.6°/52 mm., $\eta_D^{25}$ = 1.4852. Lit. b. p. 50.7°/13 mm., 57.2°/19 mm.; $\eta_D^{23}$ = 1.4862.

A second decomposition of 240 grams (1.1 moles) in a similar manner gave a yield of 108 grams (79%), b.p. 80-81°/52 mm.; $\eta_D^{25}$ = 1.4851. A third decomposition of 236 grams (1.1 (moles) yielded 100 grams (74%) of p-fluoroanisole, b. p. 80-81°/52 mm.; $\eta_D^{25}$ = 1.4852. Schiemann (5) reported a 67% yield on the decomposition.

p-Fluorophenol (5,1).- 272 grams of p-fluoroanisole were refluxed with 1 liter of benzene (distilled from $AlCl_3$) and 310 grams of anhydrous aluminum chloride (Merck Reagent, Anhydrous) for 40 hours (6,7). 700 ml. of benzene were distilled off (boiler temperature was 110°). 500 ml. of iced water were slowly added to the $AlCl_3$-phenol residue in the flask, then 200 ml. of 12 F HCl were added. The benzene-phenol phase was separated and the aqueous phase was extracted with ether and the ether extracts were combined with the benzene-phenol fraction. The product was distilled under reduced pressure. Yield: 220 grams (91%) of 4-fluorophenol, b. p. 85-86°/15 mm.; m. p. 46.5-47.5°. Lit. b. p. 74-75°/12 mm.; m. p. Form I, 26.5-27: Form II, 46.5°.

Attempted Reduction of 2-Fluorophenol by Catalytic Hydrogenation.-

Several attempts were made to reduce fluorophenol to fluorocyclohexanol, all apparently unsuccessful. They are briefly described here.

Trial 1.- 47 grams (0.42 mole) of p-fluorophenol were dissolved in sufficient absolute alcohol to make a total volume of 100 ml. and placed in the 450 ml. glass liner of the high pressure hydrogenation apparatus (9,10). 4.2 grams of Raney nickel catalyst(prepared as described in (8)) were added and the mixture was allowed to stand overnight under 1700 lbs. hydrogen pressure (no pressure drop was observed), then the mixture was shaken and heated at 150° for 3 hours (no hydrogen was consumed). The bomb was cooled and 3.5 grams additional catalyst was added, and hydrogenation attempted again at 1700 lbs. and 175-185° for 7 hours. Little or no pressure drop occurred. Nearly all of the phenol was recovered unchanged.

Trial 2.- The phenol was redistilled to eliminate any "catalyst poisons", and another reduction was attempted using 41 grams (0.36 mole) of p-fluorophenol and 3 grams of Raney nickel catalyst. The hydrogenation was for 8 hours at 2000 lbs. of hydrogen and 200-270°. Little or no hydrogen was consumed. After cooling the glass liner indicated that hydrogen fluoride had been evolved; the conditions were probably too vigorous. 27 grams of p-fluorophenol were recovered after distillation.

Trial 3.- Used 52 grams (0.46 mole) of p-fluorophenol recovered from Trial 1 and 2 and 3 grams of Raney nickel catalyst. Hydrogenation was carried out at 125-180° for 1/2 hour. 700-750 lbs. of hydrogen were consumed (3/4 mole; theory 1.4 moles). After cooling, 3 grams additional catalyst was added, and hydrogenation was continued at 150-185° and 2000 lbs. for 4 hours. Little or no additional hydrogen was used.

The product from the hydrogenation was dissolved in ether, extracted with 100 ml. of 6 F NaOH, the ether extract washed with water and the ether distilled off. 3.0 grams of material remained in the boiler with a boiling point greater than 68°. The odor was similar to ligroin or cyclohexane. Approximately 22 grams of fluorophenol were recovered unchanged.

Trial 4.- 56 grams (0.50 mole) of p-fluorophenol (recovered from previous hydrogenation attempts) were placed with 1.2 grams of platinum oxide catalyst (11) in the 450 ml. glass liner in the high pressure hydrogenation apparatus. It was heated at 130-175° and 1400 lbs. hydrogen pressure for 45 minutes; approximately 1000 lbs. of hydrogen were used. The bomb was refilled with hydrogen, 750 lbs. were used in the next 5 hours at 170°, and the bomb was refilled again with hydrogen, but only 150 lbs. additional hydrogen was taken up in 1 1/2 hours at 190-160°. The heat was turned off and the bomb was allowed to shake overnight, little additional hydrogen was used. On opening the bomb, a moderate amount

of solid material was present; this solid was insoluble in ether; it was probably silica from the action of hydrogen fluoride and water on the glass liner. Three liquid phases were present; the lower and top ones were colorless, the center one was light yellow. The phases were separated in a separatory funnel. The lower phase weighed 7.5 grams and all but 3 ml. was soluble in ether. The central phase weighed 20 grams. The upper phase weighed 18 grams and contained 17 grams of material b. p. 78-80°; thus it was probably either fluorocychlohexane or, more likely, it was cyclohexane. It was not further identified. The central phase was distilled through a column; obtained 12.6 grams, b. p. 87-89°/18 mm. (probably fluorophenol).

It is possible that conditions can be found by which it will be possible to reduce fluorophenol to fluorocyclohexanol; particularly using platinum oxide as the catalyst in glacial acetic acid at room temperature (4), or possibly with a Raney nickel catalyst prepared under hydrogen (12) and used at somewhat lower temperatures than those employed above.

Preparation of 4-Chlorocyclohexanol.

1,4-Dihydroxycyclohexane and 1,4-Epoxycyclohexane.- The procedure followed was essentially that described in (13).

Hydroquinone (B and A Reagent) was reduced in 80-100 grams portions dissolved in methanol using Raney nickel in the high pressure hydrogenation apparatus. Each reduction

was carried out at 150° with an initial hydrogen pressure of 1000-1500 lbs. The bomb had to be refilled several times with hydrogen during hydrogenation. The methanol solution from each hydrogenation was filtered, the methyl alcohol fractionated off until the boiler temperature was 240°. The boiler was cooled, 50 grams of activated alumina were added, and the mixture was heated (with a heating mantle). The 1,4-epoxycyclohexane and water were fractionated as formed through a 20 cm. Vigreux column. The boiler temperature remained at 240° until the reaction was nearly completed, when it increased rapidly. The last product was removed under slightly reduced pressure. The dehydration required 5 to 7 hours.

The two phase distillate was separated, and the lower phase was extracted several times with ether; the ether extracts were added to the upper phase, dried and refractionated. There was obtained from 403 grams (3.7 moles) of hydroquinone (4 reductions and dehydrations) a total of 180 grams (50%) of 1,4-epoxycyclohexane, b. p. 119-121°/746 mm., $\eta_D^{25} = 1.4467$, Anal. Calcd. for $C_6H_{10}O$ (98.1): C, 73.5; H, 10.3. Found: C, 73.5; H, 10.3 (Deutsch), and 33 grams of a fraction b. p. 121-150°. This latter fraction was redistilled to yield 20 grams of additional 1,4-epoxycyclohexane, b. p. 119-122°/746 mm. (total yield 55%). Lit. b. p. 120.1°/760 mm. calc. 119°/746 mm.; $\eta_D^{20} = 1.4477$ (13).

Another reduction and dehydration (3 portions) of 186 grams (1.7 moles) of hydroquinone yielded 83 grams of 1,4-epoxycyclohexane, b. p. 117-121°/748 mm. (50% yield).

It is reported in (13) that approximately 47-58% of the dihydroxycyclohexane resulting from hydrogenation of hydroquinone is trans and the remainder is cis. By the procedure which was used in (13) only a 35% yield of 1,4-epoxycyclohexane was obtained from the mixed isomers, while 28% yield was obtained from the pure cis-isomer, and 73% from the pure trans-isomer. The slightly modified procedure described above gives somewhat larger yields from the mixture then those obtained in (13).

4-Chlorocyclohexanol (14,15,16).- 26.7 grams (0.27 mole) of 1,4-epoxycyclohexane were dissolved in 100 ml. of 12 F HCl and allowed to stand for 5 days at room temperature*. 100 ml. of distilled water were added and the second dense phase was separated. The acid-aqueous phase was extracted with three 30 ml. portions of ether and the ether extracts were added to the oil phase. After washing with water to remove acid, the extracts were dried and the product distilled at reduced pressure. Yield, 28.5 grams (78%) of 4-chlorocyclohexanol. b.p. 134-135°/38 mm.; a solid at room temperature. Lit. b. p. 105°/12 mm. (14).

A second preparation was made in a similar manner and from 68.5 grams (0.70 mole) of 1,4-epoxycyclohexane, 70.6

* It was found that heating at 100° for 1 hour yielded mainly the 1,4-dichloride, b. p. 118-122°/70 mm. (14,15); liquid at 4°. Lit. b. p. 79-80°/13 mm.; m. p. trans, 102°; cis, liquid. Anal. Calcd. for $C_6H_{10}Cl_2$ (153.1): C, 47.08; H, 6.59; Cl, 46.3. Found: C, 46.59; H, 6.69 (Collins); Cl, 48.0.
Therefore the above compound is presumably the cis-dichloride. Oldberg, Pines and Ipatieff (13) report that the trans-dibromide is obtained by refluxing the epoxide with conc. HBr.

grams (75%) of product, b. p. 125-127°/32 mm., m. p. 47-75° were obtained. It had to be distilled rapidly to avoid crystallization in the condenser. Two recrystallizations of 14 grams of the above product from 75 ml.of ligroin yielded 8.2 grams, m. p. 81-83° of 4-chlorocyclhexanol.
Anal. Calcd. for $C_6H_{11}OCl$ (134.6): C, 53.53; H, 8.23; Cl, 26.35. Found: C, 53.73; H, 8.18; Cl, 26.86 (Peeler).
The above compound is probably the trans-form of 4-chlorocyclohexanol.

It is probable that 4-bromocyclohexanol can be prepared in an analogous fashion. However, it has been reported that 4-bromocyclohexanol cannot be distilled (15). From the bromo- and chloro-cyclohexanols it may be possible to prepare a γ-halogenated lysine.

## Summary

Possible methods of preparation of halogenated lysines are discussed. Several catalytic reductions of 4-fluorophenol with Raney nickel or platinum oxide were not successful.

The preparation of 4-chlorocyclohexanol (trans) and 1,4-dichlorocyclohexane from hydroquinone is described. It is possible that the former compound might be a useful intermediate in the preparation of γ-chlorolysine.

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## II. STUDIES ON BLOOD GROUP A-SPECIFIC SUBSTANCE

The blood group specific substances have been the subject of numerous investigations since Landsteiner in the early part of this century began to study the individual differences in animal bloods. The isolation of materials responsible for the immunological differences in erythrocytes has been a difficult problem. It has been observed that substances which give similar immunological reactions are available from other sources such as hog gastric mucosa and the fluid from some human ovarian cysts,

In this thesis are described studies which were made in collaboration with Drs. Holzman and Brown on the isolation and properties of blood group A-specific substance from natural sources. Included are observations which have been made on the appearance of sheep cell lysins and human A cell agglutinins in rabbits immunized with a partially purified blood group A-specific substance from hog gastric mucin, a colorimetric method for the estimation of the activity of substances inhibiting the isoagglutination of blood group A- cells, and the use of ion exchange resins in the isolation of blood group A-substance. Also described are some of the electrophoresis studies which were made in these laboratories with blood group A-substance and some observations made of the reaction of blood group A-substance with lead tetraacetate.

A large part of the work which is included herein is in the form of published papers since these contain a satisfactory presentation and discussion of the data. Additional information on these and other phases of the work is contained in the notebooks and theses of Dr. Holzman and Dr. Brown.

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A.

# A Study of the Isolation of Blood Group A-Specific Substance from Commercial Hog Gastric Mucin and Some Observations on the Separation of A-Substance from Other Natural Sources [1]

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## INTRODUCTION

The serological properties of blood group A-specific substances[2] isolated from a variety of sources (for bibliography, see (1)) are the most characteristic features of these preparations, and, consequently, immunological techniques have been relied upon to evaluate starting materials and preparative procedures. However, the data of previous investigators have permitted only limited comparisons to be made of the efficacy of different procedures for isolating A-substance (2, 3). This situation, which has been commented upon before (3), exists because of the variation in the methods used by different authors in carrying out the serological tests and the unpredictable degree to which

[1] This work was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the California Institute of Technology.

[1a] Contribution No. 1085.

[2] By "group A-specific substance" here, is meant any material effective in inhibiting isoagglutination of human type A erythrocytes by type B serum and also effective in inhibiting lysis of sheep erythrocytes by human A-cell immune rabbit sera. Evidence has recently been presented (23) that two different group-specific substances are obtainable from human urine of type A individuals, and that one of these materials is effective in inhibiting hemolysis of sheep erythrocytes by immune rabbit sera while the other is active in inhibiting isoagglutination of human A cells. We have no evidence for the existence of two such different group-specific substances. We have not examined urine, however, as a source of group-specific materials.

such tests are influenced by changes in experimental details. Hence, we believe that questions involving the usefulness of various natural products as starting materials for the preparation of A-substance and, particularly, of the most advantageous procedure for effecting its isolation can best be answered by the isolation of a large number of A-substance preparations and by the parallel testing of these preparations by several serological procedures. Accordingly, we have undertaken a comparative study of the preparative procedures described in the literature (for references, see Table I). Most of our work has been done with commercial hog gastric mucin, a material in which A-substance has long been known to be abundantly present. By these experiments we have been able to secure definite evidence bearing on the question of the similarity of A-substance preparations isolable in different ways from hog mucin, and it has been possible to determine which of the several preparative methods that have been proposed is the most satisfactory from the standpoint of yield and potency of product. It has also been possible to compare A-substance preparations obtained from other sources with those obtained from hog gastric mucin.

## Experimental

*Serological and Other Analytical Procedures.* Two serological tests have been used. The *inhibition of hemolysis test,* which will be discussed in detail shortly (8), makes use of a colorimetric determination of the extent of lysis of sheep erythrocytes by human A-cell immune rabbit sera in the presence of guinea pig serum as a source of complement. The precision of the hemolysis test is high, and the results obtained by it can be used to distinguish preparations which differ by only 10–20% in serological activity. Each of the fractions obtained has also been assessed by an *inhibition of isoagglutination test,* using the same lot of human type B serum throughout, and using pooled human type A erythrocytes. In this latter test preparations must differ in activity by approximately 100% to be distinguishable. A detailed description and discussion of the inhibition of isoagglutination test has been given (9). We have also measured the amount of color given by each fraction when treated, after alkaline digestion, with a *modified Ehrlich reagent* (*p*-dimethylaminobenzaldehyde) (9). The intensity of color obtained with this reagent after heating blood group A-specific substance with alkali has been found to be correlated with the isoagglutination in-

hibition titers of the specific substance preparations (9). Since the existence of the correlation is dependent in part upon differences in preparations detectable by means of the inhibition of isoagglutination test, only very little correlation is demonstrable for the relatively similar and potent preparations discussed below. However, it has been found that for these preparations there exists a marked correlation between the results of the color test and of the relatively precise inhibition of hemolysis test. For this reason the color produced with Ehrlich's reagent is interpreted as a characteristic property of blood group A-specific substance.

*The Separation of A-Substance from Hog Gastric Mucin.* The general isolation procedures employed were: 1, sodium sulfate fractionation from aqueous solution (2); 2, alcohol fractionation from aqueous solution (3); 3, alcohol fractionation from a 90% phenol solution (2); 4, electrodialysis followed by alcohol fractionation; 5, a combination of two or more of the foregoing procedures. Table I contains abbreviated descriptions of the method of isolation of each fraction that is considered in this communication.

*The Separation of A-Substance from Human Erythrocytes.* The alcohol extraction procedures of Hallauer (7) and of Kossjakow (10) have been followed, with large amounts of dried cells and with dried stromata obtained by centrifuging aqueous suspensions of cells from which hemoglobin had been removed by repeated washing. Material extracted by boiling absolute ethanol from dry stromata (7), fraction 45 (see Table I), as well as fractions arising in a similar way from whole erythrocytes or arising from either source by extraction with 95% ethanol in the cold, has nearly as much potency in inhibiting isoagglutination and in inhibiting hemolysis as any of the other fractions arising from the alcohol extraction procedure of Hallauer (7). This ethanol-soluble material is obtained as the major part of all material extracted from dry stromata (7% yield) and was discarded by Hallauer. Because of the insolubility of the material in water, suspensions of it were prepared for serological testing by dilution of an absolute alcohol solution with physiological salt solution. The activity of fraction 45 in inhibiting isoagglutination and in inhibiting hemolysis is about 500 times less than that of the highly active preparation, fraction 66 (see Table I), from hog gastric mucin. Several other materials (see Table I) have been obtained by alcohol extraction in 0.2–0.3% yield from lyophilized erythrocytes and these fractions have all had from 150 to 300 times less activity in the inhibition of hemolysis test and from 500 to 1,000 times less activity in the inhibition of isoagglutination test than a potent preparation from hog mucin. Digestion of stromata with crude pancreatin has given a fraction, 161, obtained in 10% yield, which has the same order of activity as fractions obtained by alcohol extraction from erythrocytes.

The extremely low yield and serological activity of group A-specific substance isolated from human erythrocytes is illustrated by the data in Table I (*cf.* also Bray, Henry and Stacey (11)). This situation may be due to the relatively minute concentration of active material in erythrocytes or to the possibility that the procedures we have used in extracting it are relatively ineffective. This latter possibility is sug-

gested by recent experiments of Calvin and coworkers (12) which are in agreement with earlier observations of Schiff and Adelsberger (13) and Landsteiner and van der Scheer (14). It is probable that our alcohol-soluble fraction 45 obtained as a major fraction from stromata is similar to these lipid-rich fractions obtained by other workers. The similarity in yield of preparations with comparable activity from stromata and from whole erythrocytes is evidence that a large part of the active material was retained in the course of lysing the erythrocytes and washing the insoluble residue. This is in contrast with what might be expected from the results reported by Calvin *et al.* (*loc. cit.*). Further evidence is given by the experiments of Belkin and Wiener (15) who found that the titer of A-substance in their stromata preparations was higher than that in the parent erythrocytes and that the ratio of these titers was inversely proportional to the yield of stromata obtained. Thus, the unsatisfactory yield of A-substance from human erythrocytes is most probably attributable not to the inefficacy of the isolation procedures but rather to the very small amount of active material present.

*The Separation of A-Substance from Human Ovarian Cyst Fluids.* According to the observations of Yosida (16) and the extensive investigations carried out by Morgan and Van Heyningen (17), King and Morgan (18), and Morgan and Waddell (19), the fluid from pseudomucinous ovarian cysts may contain a blood group-specific substance in water-soluble form which has similar serological specificity to that present in the stromata of the individual's erythrocytes. We have examined such cyst fluids, and from one containing A-specific substances (Fluid No. 162, Table I), have separated two preparations by alcohol fractionation. One of these, fraction 113, precipitated by 50% (v/v) ethanol, has about one-third the potency of the native cyst fluid in inhibiting hemolysis; the other, fraction 119, precipitated by 66% (v/v) ethanol, has about twice the potency. It may be significant that fraction 119 has as much antihemolytic activity as some of the less pure fractions, 109 and 115 (see Table I), from hog gastric mucin, and yet has 4–6 times less potency in inhibiting isoagglutination than these fractions. Morgan and Van Heyningen report (17) no such discrepancy in the activities of two materials isolated from cyst fluids by rejection of material soluble in 90% phenol.

*The Specific Substance in Commerical Hog Pepsin.* We have isolated from Wilson pepsin (1:10,000, soluble powder), in the small yield of 1–2%, fractions 22 and 26 which are extremely potent in inhibiting hemolysis, one of which, fraction 22 (see Table I), also has a high degree of activity in inhibiting isoagglutination. These materials are more active as antihemolytic factors than any other substance we have obtained directly from any source. Only fractions 47 and R.7 F.5A, obtained from hog gastric mucin by other than the usual mild procedures (see Table I and the discussion below), have activities nearly or equally as high. Fractions 22 and 26 do not exhibit the augmented equivalent N-acetylglucosamine content of the somewhat degraded preparations 47, 48, R.6 F.5A, and R.7 F.5A and give somewhat less color in the modified Ehrlich procedure (9) than our highly active material from mucin, fraction 66. In the case of fraction 22, obtained according to Landsteiner and Chase (6) by alcohol fractionation of an aqueous solution of pepsin previously heated at pH 6 and pH 3.5, there is no indication of extensive degradation, judged from its still relatively high inhibition of isoagglutination titer. This titer of fraction 26, however, is not

commensurate with its antihemolytic activity and, thus, the autolysis procedure of Landsteiner and Harte (3) must be regarded as bringing about some degradation of the specific substance. It appears, then, that the specific substance isolable from pepsin (fractions 22 and 26) differs from that obtained from hog gastric mucin (fraction 66), 1, in having a lower equivalent per cent N-acetylglucosamine, 2, in having a somewhat diminished inhibition of isoagglutination potency, and 3, in having enhanced activity in inhibiting hemolysis. The extent to which these properties are related is unknown. When the serological properties of the fractions from pepsin are considered together with their behavior in the modified Ehrlich procedure, it is apparent that these specific substances partially resemble the somewhat degraded preparations, 47 and R.7 F.5A, whose preparations are discussed below. A preparation of Parke, Davis and Co. pepsin (1:3,000, granulated) proved to be valueless as a source of A-substance, confirming the findings of Freudenberg *et al.* (20) that different preparations of pepsin vary widely in their content of group A-specific substance. The Fairchild Bros. and Foster, 1:15,000, pepsin used by Landsteiner and co-workers in their isolation studies (3, 6) would appear to have the highest concentration of A-substance of any commercial preparation.

## Discussion

The data in Table I indicate that, irrespective of the method of isolation used, no fraction was obtained from hog gastric mucin which had more than 2–4 times the activity in the inhibition of isoagglutination test or 2–3 times the activity in the inhibition of hemolysis test of a centrifuged (Sharples), undialyzed suspension of the original mucin (fractions 62 and 135, Table I). The inhibition of hemolysis potency of the most active fraction derived by application of any one isolation procedure alone was the same, within 20%, as the potency of one obtained by any other procedure (*cf.* fractions R.1 F.2A, R.2 F.2A, R.3 F.2, 87, R.5 F.3, 128, 143, 120, 31, 66, 69. Table I), which might be taken as evidence that the active material obtained is a fairly well-defined preparation, although not necessarily homogeneous. It is also apparent from the data of Table I that, within this group of similar fractions, significant differences occur with respect to the equivalent N-acetylglucosamine content [3] (9), and even with respect to the serological activity itself, indicating that preparations of A-substance

[3] Various values can be found for the equivalent N-acetylglucosamine content of an A-substance preparation depending upon the experimental conditions selected for the analysis of N-acetylglucosamine itself and for that of the specific substance preparations. However, the values determined for different A-substance preparations under standardized conditions are highly significant in a relative rather than an absolute way, since the existence of correlations with the serological tests can be demonstrated.

TABLE I

*Properties of Some Blood Group A-Specific Substance Preparations*

| Starting material | Procedure followed in isolation | Note containing description of fraction | Fraction number | Yield (from starting material) | Inhibition of hemolysis titer[a,bbb] | Inhibition of isoagglutination titer[b,bbb] | Color with modified Ehrlich reagent[c,bbb] |
|---|---|---|---|---|---|---|---|
| | | | | *Per cent* | | | |
| Hog gastric mucin granules. Wilson Lab. Item No. 443 | Sodium sulfate fractionation of a centrifuged aqueous suspension (2). | *d* | R.1F.2A | 10 | 0.075±0.005 | 45±15 | 11.7±0.3 |
| | | | R.2F.2A | 15 | 0.11 ±0.01 | 45±15 | 12.1±0.3 |
| | Electrodialysis of uncentrifuged aqueous suspension followed by sodium sulfate fractionation. | *e* | R.3F.2 | | 0.090±0.010 | 60±20 | 12.1±0.3 |
| | Aqueous suspension centrifuged once in Sharples at pH 4.8. Electrodialyzed. Fractionated with ethanol. | *f* | 62 | 75 | 0.23 ±0.01 | 40±10 | 7.1±0.4 |
| | | *g* | 111 | 33 | 0.19 ±0.01 | 75±30 | 8.4±0.3 |
| | | *h* | 112 | 26 | 0.24 ±0.00 | 50±15 | 7.5±0.3 |
| | | *i* | 87 | 11 | 0.093±0.005 | 95±30 | 11.8±0.3 |
| | Aqueous suspension fractionated with ethanol after preliminary heating at pH 4.2 (3). | *j* | R.4F.3 | 25 | 0.12 ±0.01 | 80±20 | 11.0±0.3 |
| | | | R.5F.3 | 29 | 0.11 ±0.01 | 85±20 | 11.0±0.2 |
| | Aqueous suspension fractionated with ethanol after centrifuging twice at pH 4.4 in Sharples. | *k* | 125 | 60 | 0.18 ±0.01 | 45±15 | 9.0±0.2 |
| | | *l* | 123 | 34 | 0.24 ±0.01 | 40±15 | 8.5±0.3 |
| | | *m* | 128 | 21 | 0.11 ±0.00 | 75±20 | 11.8±0.1 |
| | | *n* | 135 | 72 | 0.18 ±0.02 | 45±15 | 7.6±0.2 |
| | | *o* | 142 | 38 | 0.24 ±0.00 | 45±15 | 8.1±0.2 |
| | | *p* | 143 | 22 | 0.11 ±0.01 | 85±20 | 10.6±0.1 |

TABLE I—*Continued*

*Properties of Some Blood Group A-Specific Substance Preparations*

| Starting material | Procedure followed in isolation | Note containing description of fraction | Fraction number | Yield (from starting material) | Inhibition of hemolysis titer[a,bbb] | Inhibition of isoagglutination titer[b,bbb] | Color with modified Ehrlich reagent[c,bbb] |
|---|---|---|---|---|---|---|---|
| | | | | *Per cent* | | | |
| | Aqueous suspension fractionated with ethanol after centrifuging at pH 4.4 and then at pH 7.2 in Sharples. | *q* | 97 | 65 | 0.18 ±0.01 | 45±15 | 9.0±0.1 |
| | | | 124 | 51 | 0.17 ±0.01 | 40±15 | 9.3±0.2 |
| | | *r* | 109 | 30 | 0.25 ±0.01 | 40±10 | 11.0±0.2 |
| | | | 115 | 33 | 0.24 ±0.01 | 45±15 | 11.1±0.1 |
| | | *s* | 120 | 15 | 0.10 ±0.00 | 75±20 | 9.3±0.1 |
| | | | 121 | 17 | 0.10 ±0.00 | 80±20 | 9.6±0.2 |
| | Ditto | *t* | 127 | 53 | 0.17 ±0.00 | 45±15 | 10.6±0.1 |
| | | | 129 | 51 | 0.18 ±0.00 | 45±15 | 10.6 |
| | Ethyl alcohol fractionation of a centrifuged 90% phenol solution (2). | *u* | 39 | 49 | 0.20 ±0.04 | 40±15 | 11.2±0.2 |
| | | *v* | 52 | 32 | 0.13 ±0.01 | 50±15 | 11.4±0.1 |
| | | *w* | 31 | 13 | 0.11 ±0.00 | 55±10 | 11.4±0.1 |
| | | *x* | 36 | 10 | *v.* Note *uu* | 55±15[uu] | 9.5±0.5[uu] |
| | Ethyl alcohol fractionation of aqueous solution of fraction 52. | *y* | 66 | 4 | 0.080±0.010 | 110±30 | 12.7±0.1 |
| | | *z* | 68 | 5 | 0.11 ±0.00 | 90±30 | 12.7±0.1 |
| | Sodium sulfate fractionation of aqueous solution of fraction 52. | *aa* | 69 | 1 | 0.10 ±0.01 | 75±20 | 10.7±0.1 |
| | | *bb* | 71 | 22 | 0.12 ±0.01 | 80±20 | 12.7±0.1 |

TABLE I—*Continued*

*Properties of Some Blood Group A-Specific Substance Preparations*

| Starting material | Procedure followed in isolation | Note containing description of fraction | Fraction number | Yield (from starting material) | Inhibition of hemolysis titer[a.bbb] | Inhibition of isoagglutination titer[b.bbb] | Color with modified Ehrlich reagent[c.bbb] |
|---|---|---|---|---|---|---|---|
| | | | | *Per cent* | | | |
| Hog Gastric Mucin(4)[4,5] | Ethyl alcohol fractionation of 90% phenol solution (2,4).[4] | *cc* | Kabat 1A(4)<br>960-GM-2[4] | | 0.10 ±0.01<br>0.14 ±0.01 | 50±20<br>55±25 | 11.3±0.1<br>10.0±0.2 |
| | Tryptic digestion followed by alcohol fractionation[4,5]. | *dd* | 960-GM-1B[4]<br>960-GM-1C[5] | | 0.17 ±0.02<br>0.13 ±0.01 | 55±25<br>55±25 | 9.6±0.2<br>10.5±0.1 |
| Hog Stomach Linings[6] | Autolysis, followed by alcohol fractionation.[6] | *ee* | M-330[6] | | 0.14 ±0.01 | 55±25 | 10.7±0.1 |
| Hog gastric mucin granules. Wilson Lab. Item No. 443 | Separation of "neutral polysaccharide" by selective adsorption and precipitation (5). | *ff* | 47 | 2 | 0.065±0.010 | 30±10 | 13.3±0.5 |
| | Fractionation of part of the product by precipitation with sodium sulfate. | *gg* | 48 | 5 | 0.080±0.020 | 30±10 | 12.3±0.1 |

[4] We are indebted to Dr. E. Brand for samples of Kabat's preparation, 1A, and for two Sharp and Dohme preparations, 960-GM-1B and 960-GM-2, as well as for information concerning the methods of preparation of the latter two materials.

[5] We are indebted to Dr. R. H. Barnes, of Sharpe and Dohme, Inc., for a sample of preparation 960-GM-1C and for information concerning the method used in its preparation.

[6] We are indebted to Dr. J. A. Leighty, of the Lilly Research Laboratories, for a sample of preparation M-330 and for information concerning the method used in its preparation.

TABLE I—*Continued*

*Properties of Some Blood Group A-Specific Substance Preparations*

| Starting material | Procedure followed in isolation | Note containing description of fraction | Fraction number | Yield (from starting material) | Inhibition of hemolysis titer[a,bbb] | Inhibition of isoagglutination titer[b,bbb] | Color with modified Ehrlich reagent[c,bbb] |
|---|---|---|---|---|---|---|---|
| | | | | *Per cent* | | | |
| | Digestion of fraction R.4F.3 with papain-HCN. Subsequent fractionation with ethanol and with acetic acid and acetone (3). | *hh* | R.6F.5A | 31[vv] | 0.10 ±0.01 | 25±10 | 12.3±0.2 |
| | Heating fraction R.5F.3 with formamide. Subsequent fractionation with ethanol, acetic acid and acetone(3). | *ii* | R.7F.5A | 26[ww] | 0.050±0.005 | 10± 5 | 13.4±0.3 |
| Pepsin, soluble powder, 1:10000, Wilson Lab. | Alcohol fractionation of an aqueous solution previously heated at pH 6 and pH 3.5 (6). | *jj* | 22 | 1 | 0.050±0.005 | 70±15 | 11.6±0.3 |
| | Autolysis, followed by alcohol fractionation (3). | *kk* | 26 | 2 | 0.040±0.005 | 35±10 | 11.7±0.1 |
| Fluid from pseudomucinous ovarian cyst from patient of blood group A.[7] | Centrifugation, followed by alcohol fractionation. | *ll* | 162 | | 0.65 ±0.20 | 4.5±1.5 | 1.7±0.3 |
| | | *mm* | 113 | | 1.9 ±0.3 | 4± 3 | 0.3±0.2 |
| | | *nn* | 119 | | 0.27 ±0.03 | 7± 5 | 3.4±0.3 |

[7] We are indebted to Dr. Roy W. Hammack, of the Pathology Laboratories of the Hospital of the Good Samaritan, Los Angeles, Cal., for a sample of fluid aspirated from a pseudomucinous ovarian cyst (No. B-3731-45) removed from a patient of blood group A.

TABLE I—*Continued*

*Properties of Some Blood Group A-Specific Substance Preparations*

| Starting material | Procedure followed in isolation | Note containing description of fraction | Fraction number | Yield (from starting material) | Inhibition of hemolysis titer[a,bbb] | Inhibition of isoagglutination titer[b,bbb] | Color with modified Ehrlich reagent[c,bbb] |
|---|---|---|---|---|---|---|---|
| | | | | *Per cent* | | | |
| Lyophilized erythrocytes, pooled from a large number of donors of blood group A.[8] | Successive extractions with ethanol-water solutions of decreasing alcohol content, followed by concentration of extract and precipitation with acetone (7). Subsequent fractionation with ethanol. | *oo* | 1 | 0.3 | 16±2 | 0.1±0.04 | less than 0.3 |
| | | *pp* | 56 | 7[zz] | 13±2 | 0.9±0.5 | less than 0.3 |
| | | *qq* | 57 | 22[zz] | 140±15 | less than 0.03 | less than 0.3 |
| | | *rr* | 169 | 60[zz] | 45±5 | | less than 0.3 |
| Lyophilized stromata from pooled erythrocytes from donors of blood group A[vv]. | Extraction with boiling absolute ethanol (7). | *ss* | 45 | 7<br>0.2[zz] | 34±3 | 0.2±0.06[aaa] | less than 0.3 |
| | Digestion with crude pancreatin at pH 7.9 at 40°C. | *tt* | 161 | 10<br>0.3[zz] | 29±3 | | 0.5±0.3 |

[8] We are indebted to the Hyland Laboratories, Los Angeles, Cal., for a sample of lyophilized, pooled erythrocytes removed from human donors of blood group A. The erythrocytes were washed twice with physiological saline prior to lyophilization.

Notes. *a.* $\gamma$ of test substance present in system in which erythrocytes are 50% hemolyzed. *b.* $\mu$l. of serum completely inhibited/$\gamma$ of test substance. *c.* Expressed as equivalent *per cent* of N-acetylglucosamine in test substance. *d.* Insoluble in 30% sodium sulfate. Precipitate electrodialyzed. Material from supernatant. *e.* Insoluble when supernatant after electrodialysis made 30% in sodium sulfate. Dialyzed. *f.* Material from undialyzed centrifugate. *g.* Material from supernatant of electrodialyzed centrifugate. *h.* Material precipitated upon electrodialysis of centrifugate. *i.* Material soluble when supernatant after electrodialysis made 47% (v/v) in ethanol but insoluble when made 65% (v/v) in ethanol. Again electrodialyzed. Material from supernatant. *j.* Material soluble in 40% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Not dialyzed. *k.* Material from dialyzed centrifugate. *l.* Material insoluble in 40% (v/v) ethanol. Dialyzed. *m.* Material soluble in 40% (v/v) ethanol, but insoluble in 65% (v/v) ethanol. Dialyzed. *n.* Material from undialyzed centrifugate. *o.* Material insoluble in 40% (v/v) ethanol. Not dialyzed. *p.* Material soluble in 40% (v/v) ethanol, but insoluble in 65% (v/v) ethanol. Not dialyzed. *q.* Material from dialyzed centrifugate. *r.* Centrifugate. Material soluble in 30% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Upon reprecipitation, insoluble in 45% (v/v) ethanol. Dialyzed. *s.* Centrifugate. Material soluble in 30% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Upon reprecipitation, soluble in 45% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Dialyzed. *t.* Centrifugate. All of material insoluble in 65% (v/v) ethanol. Dialyzed. *u.* Material insoluble in 10% (v/v) ethanol. Not dialyzed. *v.* Material insoluble in 10% (v/v) ethanol. Dialyzed. *w.* Material from supernatant after electrodialysis of fraction 39. *x.* Material precipitated by electrodialysis of fraction 39. *y.* Material soluble in 47% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Electrodialyzed. Material from supernatant. *z.* As in *y*, except material precipitated upon electrodialysis. *aa.* Precipitated by 30% sodium sulfate. Electrodialyzed. Material from supernatant. *bb.* As in *aa*, except material precipitated upon electrodialysis. *cc.* Preparation made by Sharp and Dohme. *dd.* Preparation made by Sharp and Dohme. *ee.* Preparation made by Lilly Research Laboratories. *ff.* Material not precipitated by $Zn(OH)_2$ at pH 7.1 but precipitated by acetone-acetic acid. Material from supernatant after electrodialysis of final precipitate. *gg.* Precipitated by basic lead acetate, then by 75% (v/v) ethanol, then by 94% (v/v) acetic acid. Not subjected to precipitation by $Zn(OH)_2$. Instead, extracted by water. Material insoluble when extract made 30% in sodium sulfate. Electrodialyzed. *hh.* Insoluble in 65% (v/v) ethanol after digestion and dialysis. Then insoluble in 75% (v/v) ethanol containing HCl. Soluble in 90% (v/v) acetic acid, but insoluble upon addition of acetone. Dialyzed. *ii.* Insoluble in 66% (v/v) ethanol in formamide. Precipitated twice by 68% (v/v) ethanol from water and precipitated from 50% acetone-40% acetic acid. Precipitated by 80% (v/v) ethanol from HCl solution. Dialyzed. Precipitated by 90% acetone. Dialyzed. *jj.* Material insoluble in 65% (v/v) ethanol. Reprecipitated. Dialyzed. *kk.* Precipitated by 63% (v/v) ethanol twice, then by acetone-acetic acid, then by ethanol. *ll.* Total solids from the centrifuged fluid. *mm.* Material insoluble when fluid made 1% in sodium acetate and 50% (v/v) in ethanol. Precipitate dialyzed and the non-dialyzable suspension filtered. Filtrate lyophilized. *nn.* Material soluble when fluid made 50% (v/v) in ethanol but insoluble at 66% (v/v) ethanol. Precipitate

taken up in water, filtered through Seitz pad and reprecipitated by 66% (v/v) ethanol. Precipitate taken up in water, filtered, filtrate dialyzed. Solution filtered through Seitz pad. Heavily opalescent filtrate lyophilized. *oo.* Material insoluble in a boiling absolute ethanol extract but soluble in an approximately 50% ethanol extract. Extract concentrated at room temperature, filtered through a Seitz pad. Fraction 1 precipitated by 5 volumes of acetone from a solution 0.9% in NaCl. Not dialyzed. *pp.* Material (from fraction 1) which is insoluble in absolute ether and in absolute ethanol under reflux but which is soluble in water at room temperature. Fraction 56 precipitated when aqueous extract made 50% (v/v) in ethanol. Not dialyzed. *qq.* Like fraction 56 except material soluble when extract made 50% (v/v) in ethanol, but insoluble at 66% (v/v) ethanol. Not dialyzed. *rr.* Like fraction 57 except material soluble when extract made 66% (v/v) ethanol. Not dialyzed. *ss.* Material extracted by boiling absolute ethanol. Extract concentrated under reduced pressure at 35°C. and the residue lyophilized. *tt.* Material soluble and non-dialyzable after digestion for 42 hours and inactivation at 85°C. for approximately 30 minutes. *uu.* Analytical data in doubt because of slight solubility of the fraction in physiological salt solution. *vv.* Yield calculated from fraction R.4 F.3. *ww.* Yield calculated from fraction R.5 F.3. *xx.* Yield calculated from fraction 1. *yy.* Stromata obtained by centrifugation at 25,000 r.p.m. in the Sharples of aqueous suspensions of erythrocytes and retention of the precipitated material. Precipitate washed until essentially free of hemoglobin, centrifuging it at 5000 r.p.m. after each washing. *zz.* Yield calculated from lyophilized erythrocytes. *aaa.* Serological activity of a suspension prepared by dilution of an ethanol solution with physiological salt solution. *bbb.* The analytical data reported are the mean results of duplicate or triplicate analyses and are given together with the average deviation.

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(*e.g.*, fractions 66, 68, 87, R.3 F.2) which are more active than those obtainable by the use of the hitherto described procedures (2, 3) can be isolated by relatively complex, yet still mild, techniques. These more active, undegraded preparations have perhaps a 20% higher A-substance content than those (fractions R.5 F.3, 128, 143, 31) isolated by the less involved procedures, as indicated by the three criteria of activity discussed in the "Experimental" part above.

Kabat and co-workers have reported (21, 22) that the A-substances isolated from commercial hog gastric mucin by the 90% phenol extraction procedure of Morgan and King (2) and by subsequent purifications are about 60% pure on the basis of specifically precipitable hexosamine. Their report is based on the comparative results of a quantitative precipitin test (4) in which A-substance preparations derived only from serologically active hog gastric mucosae were found to participate to the extent of 100% of the hexosamine present. Our experiments indicate that obtaining substances more active than fractions 128 (alcohol fractionation out of water), R.2 F.2A (sodium

sulfate fractionation out of water), and 31 (phenol extraction with subsequent alcohol fractionation) is not unique to the use of hog gastric mucosae from "secretor" hogs. By the procedures outlined in Table I significant amounts of such more active materials can be obtained from Wilson hog gastric mucin also. We do not believe that our findings with respect to the possibility of isolating highly active fractions from commercial hog mucin are in any way at variance with the findings of Kabat and co-workers (21, 22) that some hog gastric mucosae contain A-substance while others do not, and that in the latter mucosae there is material which is superficially similar to A-substance, but which has no significant serological activity. Thus, although the commercial hog gastric mucin with which we and others have worked may contain such material, the fact that no detailed information is available about the structure of either material leaves entirely open the question of what techniques may suffice to separate them.

In much of our preparative work we have used the technique of electrodialysis and have observed certain phenomena which we believe are significant. When an A-substance preparation is electrodialyzed under a potential gradient of *ca.* 18 volts/cm., a solid separates in the cell. This precipitation seems to be associated with an increase in the hydrogen ion activity of the cell and, ultimately, a pH of 4 or less may be attained. Accumulation of the precipitated solid begins near the cellophane membrane separating the central compartment from the anode compartment. The material which is precipitated can be dispersed or dissolved by the addition of alkali. In addition to the A-substance which is present in the precipitate there is a considerable amount dissolved in the clear or only slightly opalescent portion of the solution which overlays the turbid phase. Where comparison has been made between the serological activity of a precipitate obtained by electrodialysis and that of the material dissolved in its supernatant, it has been found that the more soluble fraction is 20–25% more potent in inhibiting hemolysis than the less soluble fraction, in the range where both activities are low (*cf.* fractions 111 and 112) and also where both are high (*cf.* fractions 66 and 68 and fractions 69 and 71). A-substance preparations which give rise in part to precipitated material when they are electrodialyzed cannot be regarded as homogeneous.

The so-called "neutral polysaccharide" (fraction 47), obtained by the procedure of Meyer, Smyth and Palmer (5), in which the original

mucin is heated with 2% sodium carbonate for 15 minutes at 70°C. and from which an "acidic polysaccharide" is removed in acetic acid solution as a gelatin salt, has a very high potency in the inhibition of hemolysis test (see Table I), in accord with the findings of Landsteiner as reported by Meyer *et al.* (5). Its activity in this test is greater than that of any other fraction obtained by us from hog gastric mucin with the single exception of R.7 F.5A which was obtained by heating another A-substance preparation in formamide solution at 150°C. (3). The equivalent N-acetylglucosamine content of fraction 47 is also higher than that of any fraction except R.7 F.5A. We have found, however, that the potency of fraction 47 and the closely related fraction 48 in the inhibition of isoagglutination test is very low, and, thus, is in no way commensurate with the hemolysis inhibition activity. This indication of alteration in A-substance in the course of Meyer's procedure (5) has not previously been recognized, although the findings of Morgan and King (2) with respect to the lability of A-substance in alkaline solution strongly suggest that the initial alkaline treatment used by Meyer *et al.* might profoundly alter the serological properties of the product.

The relative serological activities of fractions R.6 F.5A and R.7 F.5A compared with those of the fractions from which they were derived (see Table I) confirm the findings of Landsteiner and Harte (3) and of Morgan and King (2) with respect to the effects of treating A-substance with papain-HCN or with formamide at 150°C. By both of these procedures the activity of the A-substance in inhibiting hemolysis is increased and in inhibiting isoagglutination is decreased.

We have found that the alcohol fractionation procedure of Landsteiner and Harte (3) with some modifications possesses advantages over other procedures for the preliminary concentration of A-substance from commercial (Wilson) hog gastric mucin for the purpose of further purification and investigation, conveniently providing an undegraded preparation which in yield and potency is equivalent or superior to those obtained by other procedures. The desirable modifications in the Landsteiner and Harte procedure are: a, omission of the initial heating at 100°C.; and b, substitution of a thorough centrifugation in the open bowl of the Sharples of the aqueous suspension of mucin at a pH of 4.5. Centrifugation as indicated serves to remove about 15% of the weight of the mucin granules taken. The fraction which is removed at 40%

(v/v) alcohol concentration is obtained in 35% yield and is 40–50% less active in inhibiting hemolysis than a centrifuged suspension of mucin. The most active fraction is obtained in 20% yield from mucin and has essentially twice the inhibition of hemolysis potency of crude mucin.

## Summary

A study has been made of the relative merits of all previously reported procedures and of some new variants of these in isolating blood group A-specific substance from Wilson hog gastric mucin. In this study it has been possible, for the first time, to make direct comparisons of the serological activities of the fractions arising from all of these procedures. No fraction had more than 2–4 times the activity in inhibiting isoagglutination or 2–3 times the activity in inhibiting hemolysis of a centrifuged, undialyzed suspension of the original mucin. By rather complex procedures materials were obtained which had about 20% more activity in inhibiting hemolysis than those derived by any one of the hitherto described techniques.

Observations are reported on the isolation of A-substance from human erythrocytes and ovarian cyst fluids, and from hog pepsin.

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# B. THE USE OF ION EXCHANGE RESINS IN THE ISOLATION OF BLOOD GROUP A-SPECIFIC SUBSTANCE FROM HOG GASTRIC MUCIN*

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Ion exchange resins (1) have been used in the investigation of A substance hydrolysates (2), but apparently no attempt has been made to determine whether they can be profitably used in the isolation or purification of undegraded A substance[1] from sources such as hog gastric mucin. Studies along these lines are reported in this communication.

## EXPERIMENTAL

*Ion Exchange Resins*—De-Acidite (The Permutit Company, New York), washed on a 40 mesh screen with distilled water, was treated several times with aqueous 4 per cent sodium carbonate and repeatedly washed with distilled water, by suspension and decantation, until the supernatant liquid was colorless and had a pH of 8.0 or less. The resin collected on a suction filter contained 70 to 80 per cent water. 1 gm. (dry weight) of the resin contributed less than 0.05 milliequivalent of base to 75 ml. of distilled water when the suspension was allowed to stand, with frequent shaking, for 2 days at 25°. Under the same conditions 1 gm. (dry weight) of the resin removed 95 per cent of the hydrochloric acid from 75 ml. of a 0.066 M solution and 85 per cent from 75 ml. of a 0.093 M solution.

Amberlite IR-4 (The Resinous Products and Chemical Company, Philadelphia) prepared as described above, contained approximately 35 per cent water, and 1 gm. (dry weight) of the resin removed more than 95 per cent of the hydrochloric acid present in 25 ml. of a 0.166 M solution.

Amberlite IR-100 (The Resinous Products and Chemical Company), treated with 1 per cent hydrochloric acid and washed free of chloride, gave a product containing approximately 35 per cent water.

*A Substance Preparations*—A 2 per cent suspension of hog gastric mucin granules (Wilson), adjusted to pH 4.4 to 4.5 with glacial acetic acid, was

* This work was supported in part by a grant from the United States Public Health Service.

† Contribution No. 1225.

[1] Defined as a substance effective in inhibiting isoagglutination of human blood group A cells by group B serum and also effective in inhibiting lysis of sheep erythrocytes by human blood group A cell immune rabbit sera.

centrifuged twice in the open bowl of a Sharples centrifuge at 20,000 R.P.M. The centrifugate was used either directly or after ethanol fractionation (3, 4).

*Procedure*

Sufficient exchange resin was added, unless otherwise indicated, to a 1 to 2 per cent aqueous solution of the A substance preparation to provide 4 to 5 gm. (wet weight) of freshly washed resin for each gm. of dissolved solid. The suspension was stirred for 2 to 3 hours at 5°, filtered, and the operation

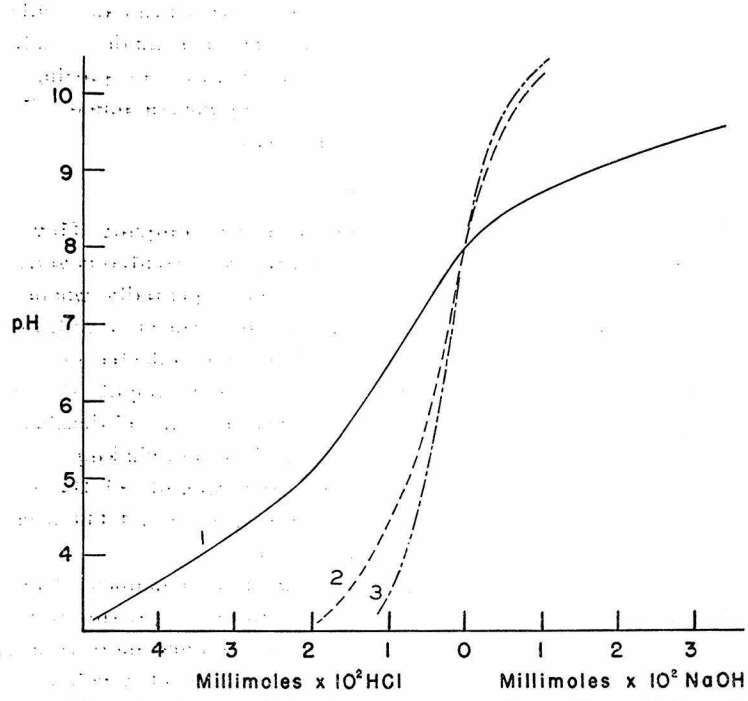


FIG. 1. Titration curves of A substance preparations. Curve 1, Fraction 136; Curve 2, Fraction 128; Curve 3, Fraction 141.

repeated. The resulting solution was either lyophilized or subjected to further treatment with a different exchange resin. Equivalent N-acetylglucosamine contents and inhibition of hemolysis titers were determined as described previously (4, 5). Titration curves were determined by adding sufficient hydrochloric acid to 50 mg. of solid in 5 ml. of water to decrease the pH to 3.0 or less and then titrating with 0.040 M sodium hydroxide. Since an inflection point was observed at pH 8, the data are plotted in Fig. 1 with reference to the number of millimoles of acid or base required to change the pH of the above solution from pH 8 to some other pH.

## DISCUSSION

Within limits the equivalent N-acetylglucosamine content (5) of an A substance preparation can be taken as an index of the A substance activity of the preparation, evaluated in terms of inhibition of isoagglutination or inhibition of hemolysis (4, 5). Preliminary experiments based upon the determination of the equivalent N-acetylglucosamine content of an A substance solution before and after treatment with a solid adsorbent previously equilibrated with water indicated that of a number of adsorbents tested the anion exchange resins De-Acidite and IR-4 and the cation exchange resin IR-100 were sufficiently promising to warrant further investigation.[2]

The results obtained by treating an aqueous solution of hog gastric mucin with the above ion exchange resins, either singly or in combination, are given in Table I. It is noteworthy that successive application of De-Acidite and IR-100 gave a preparation which was as active in the hemolysis test[3] as those obtained from mucin by ethanol fractionation (3, 4). The separation of a resin-treated mucin solution into a clear supernatant and a less active viscous precipitate is reminiscent of the behavior observed when A substance preparations obtained from mucin by ethanol fractionation are electrodialyzed (4).[4] Although resin treatment will give products[3] as active as those obtained by ethanol fractionation, there is no evidence that further treatment of the product with any of the above resins will lead to more active preparations such as those obtained by a combination of ethanol fractionation and electrodialysis.[4]

A substance preparations of varying purity, obtained from hog gastric mucin by ethanol fractionation or electrodialysis, were subjected to resin treatment and, in contrast to the experience with mucin, only in one instance was an increase in activity observed (Table II). In the isolation of A substance from hog gastric mucin, it has been noted that an increase in A substance activity is usually accompanied by an increase in the equivalent N-acetylglucosamine content of the preparation (4).[4] However, with A substance preparations containing more than 10 per cent equivalent N-acetylglucosamine, the equivalent N-acetylglucosamine content may not necessarily increase with A substance activity upon further purification and in some instances may actually decrease.[4] This behavior is not unexpected, since it has been pointed out that blood group-specific substances other than the A substance contain alkali-labile bonds involving N-acetylglucosamine

[2] It is possible that with a different test or under different conditions other adsorbents may have been selected.

[3] Comparable titers were also obtained with the inhibition of the isoagglutination test which would indicate that resin treatment does not cause the degradation of A substance.

[4] Holzman, G., and Niemann, C., unpublished experiments.

TABLE I

*Inhibition of Hemolysis Titer and Equivalent N-Acetylglucosamine Content of Hog Gastric Mucin Suspension after Successive Treatments with Several Exchange Resins*

| Description of fraction* | Fraction No. of product | Yield | Inhibition of hemolysis titer† | Equivalent N-acetyl-glucos-amine content‡ |
|---|---|---|---|---|
| Aqueous suspension of hog gastric mucin granules centrifuged twice at pH 4.4; centrifugate | 135 | 72% from mucin | 0.19 ± 0.02 | 7.6 |
| Fraction 135 treated twice with De-Acidite | 136 | 83% from No. 135 | 0.16 ± 0.01 | 8.9 |
| Fraction 136 treated twice with IR-100; clear supernatant and | 137 (ppt.) | 50% from No. 136 | 0.11 ± 0.01 | 10.8 |
| viscous ppt. obtained upon standing 24 hrs.; separated | 138 (supernatant) | 23% from No. 136 | 0.105 ± 0.005 | 11.6 |
| Fraction 137 treated twice with IR-4 | 139 | 100% from No. 137 | | 11.2 |
| Fraction 138 treated twice with IR-4 | 140 | 100% from No. 138 | | 11.7 |
| Fraction 136 treated twice with IR-100 and twice with IR-4 | 141 | 67% from No. 136 | 0.12 ± 0.01 | 11.2 |
| Fraction 135 treated alternately 4 times with De-Acidite and | 152 (supernatant) | 32% from mucin | 0.14 ± 0.02 | 10.5 |
| IR-100; allowed to stand 4 days; turbid supernatant and viscous ppt. obtained; separated | 154 (ppt.) | 20% from mucin | 0.19 ± 0.02 | 9.2 |
| Fraction 152 treated with De-Acidite and IR-100 | 153 | 88% from No. 152 | 0.12 ± 0.01 | 11.2 |

* 4 to 5 gm. (wet weight) of the exchange resin per gm. of material treated were used for each treatment, except in the preparation of Fractions 167 and 168, in which 8 gm. of De-Acidite per gm. were used for each treatment, and in the preparation of Fractions 152, 154, and 153, in which 0.5 gm. portions of De-Acidite and IR-100 per gm. were used for each treatment.

† Micrograms of test substance present in system in which sheep erythrocytes are 50 per cent hemolyzed by an anti-human A cell immune rabbit serum. The titers reported are the average of two or three determinations and are reported together with the average deviation of the several determinations.

‡ Expressed as equivalent per cent of N-acetylglucosamine in test substance (5). The analytical data reported are the mean results of duplicate or triplicate analyses. The absolute deviation was in no case larger than 0.3 per cent (equivalent N-acetylglucosamine).

(5, 6), and it is known that A- and O-specific substances can be separated, at least in part, by relatively simple fractionation procedures (7).[4]

TABLE II

*Inhibition of Hemolysis Titer and Equivalent N-Acetylglucosamine Content of Some Blood Group A-Specific Substance Preparations after Treatment with Exchange Resins*

| Description of fraction* | Fraction No. of product | Yield | Inhibition of hemolysis titer† | Equivalent N-acetyl-glucosamine content‡ |
|---|---|---|---|---|
| Aqueous suspension of hog gastric mucin granules centrifuged twice at pH 4.4; centrifugate (Fraction 135) fractionated with ethanol; material soluble in 40% (by volume) ethanol, insoluble in 65% (by volume) ethanol | 143 | 22% from mucin | 0.11 ± 0.01 | 10.6 |
| Fraction 143 treated twice with De-Acidite | 144 | 77% from No. 143 | 0.105 ± 0.015 | 12.0 |
| Fraction 144 treated twice with IR-100 | 145 | 100% from No. 144 | 0.105 ± 0.010 | 12.3 |
| Fraction 145 treated twice with IR-4 | 147 | 100% from No. 145 | 0.11 ± 0.01 | 12.1 |
| Aqueous suspension of hog gastric mucin granules; centrifugate fractionated with ethanol; material soluble in 30% (by volume) ethanol, insoluble in 65% (by volume) ethanol; upon reprecipitation, insoluble in 45% (by volume) ethanol; dialyzed, then electrodialyzed | 110 | | 0.20 ± 0.02 | 11.8 |
| Fraction 110 treated twice with De-Acidite | 167 | 74% from No. 110 | 0.21 ± 0.02 | 13.4 |
| Fraction 110 treated twice with IR-4 | 165 | 91% from No. 110 | 0.20 ± 0.02 | 11.8 |
| Same as Fraction 110, except upon reprecipitation, material soluble in 45% (by volume) ethanol, insoluble in 65% (by volume) ethanol; dialyzed, then electrodialyzed | 126 | | 0.088 ± 0.005 | 10.4 |
| Fraction 126 treated twice with De-Acidite | 168 | 60% from No. 126 | 0.070 ± 0.005 | 11.9 |
| Fraction 126 treated twice with IR-4 | 166 | 88% from No. 126 | 0.086 ± 0.005 | 10.5 |

See the corresponding foot-notes to Table I.

The effectiveness of De-Acidite and IR-100 in reducing the " buffering capacity" of mucin solutions and partially purified A substance preparations is clearly illustrated in Fig. 1. The anion exchange resin IR-4 was found to be markedly inferior to De-Acidite in this respect. Analysis of Fractions

135, 136, and 137 for total nitrogen, amino nitrogen, and amino acid nitrogen (Table III) revealed that De-Acidite was instrumental in removing acidic nitrogenous non-blood group-specific substances containing little or no amino nitrogen, whereas the substances removed by IR-100 contained substantial amounts of amino and amino acid nitrogen. At least part of the materials removed by De-Acidite are non-dialyzable.[5] It is interesting to note that in a centrifuged mucin solution approximately 20 per cent of the solids are not precipitated by 66 per cent ethanol, whereas approximately 30 per cent are removable by successive treatment with De-Acidite and IR-100.

It has been observed that many A substance preparations are contaminated by non-blood group-specific substances[6] exhibiting marked specific absorption in the 260 to 270 mμ region (8). An A substance preparation (Fraction 110) containing a substantial amount of the "260 mμ component" ($E^{1\%}_{1\,cm.}$ at 260 mμ = 13.2) was treated with De-Acidite and the resulting preparation (Fraction 167) was found to have a value of $E^{1\%}_{1\,cm.}$ at 260 mμ of 3.6. Treatment with IR-100 or IR-4 caused little or no decrease in extinction. The fact that De-Acidite was effective and IR-4 was relatively ineffective in removing the "260 mμ component" would indicate that the removal of this component by De-Acidite is not a simple anion exchange. An explanation of the mode of action of De-Acidite leading to the loss of the "260 mμ component," a gain in the equivalent N-acetylglucosamine content, and no significant change in A activity must await the accumulation of additional data.

TABLE III

*Nitrogen Content of A Substance Preparations*

| Fraction No. | Total N | Amino N | Amino acid N |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| 135 | 8.4 | 1.9 | 1.0 |
| 136 | 7.3 | 2.0 | 1.2 |
| 137 | 6.4 | 0.16 | 0.09 |

## SUMMARY

The treatment of hog gastric mucin with the two ion exchange resins De-Acidite and IR-100 has given apparently undegraded A substance preparations which are as active as those obtained from the same source by

[5] Bennett, E. L., unpublished data.

[6] While the "260 mμ component" has not been obtained free of A substance, all evidence (8) points to its non-blood group specific character.

ethanol fractionation. This enrichment in A substance is due to the partial removal by these two resins of some of the non-blood group-specific components normally present in hog gastric mucin.

The authors wish to express their indebtedness to Dr. D. H. Brown and Dr. G. Holzman for their assistance in this investigation.

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# C. THE APPEARANCE OF SHEEP CELL LYSINS AND HUMAN A CELL AGGLUTININS IN A RABBIT IMMUNIZED WITH A PARTIALLY PURIFIED BLOOD GROUP A-SPECIFIC SUBSTANCE FROM HOG GASTRIC MUCIN.

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*Contribution from The Gates And Crellin Laboratories of Chemistry, California Institute of Technology No. 1089*

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It has been reported that purified preparations of A-substance derived from human erythrocytes (1, 2), hog gastric mucin (3, 4), and human ovarian cyst fluids (5, 6) are without significant antigenicity when injected into rabbits. Morgan and co-workers have found (3, 5, 6) that the antigenicity of substances from the latter two sources can be made complete by combining them with the protein component of the specific O somatic antigen of *Bact. dysenteriae* (Shiga). Recently, A-substance from various body fluids has been converted to a complete antigen in another way (7), i.e., by introducing the p-nitrobenzyl- group, reducing it to p-aminobenzyl-, diazotizing, and coupling with, for example, ovalbumin. Morgan and King have suggested (4) that antigenicity may result when the polysaccharide-amino acid complex which possesses A specificity is associated through secondary valency bonds with an intact protein. In contrast with the observations mentioned above on the non-antigenicity of A-substance are the experiments of Hodyo who in 1939 reported (8) the production of group-specific precipitins by rabbits immunized with extracts of hog gastric mucosa. In 1944 Witebsky, Klendshoj, and McNeil (9) successfully used commercial preparations of A-substance for the immunization of human volunteers and in 1945, Kabat and Bezer (10) obtained human sera with high anti-A titer by immunizing individuals of groups O or B with a partially purified blood group A-substance derived from hog gastric mucin. In neither of the latter two cases was an added protein component present in the antigen used. Except for the work of Hodyo, where a crude mucosal extract elicited the formation of group specific precipitins, no clear-cut case of a significant positive response in rabbits receiving injections of A-substance has been reported. However, we have recently observed an instance of the appearance of human A cell agglutinins and of sheep cell lysins in the serum of a rabbit which had been subjected to a protracted course of immunization with 5 mg. doses, administered intravenously, of a partially purified blood group A specific substance (fraction 112A) derived from hog gastric mucin (see table 1).

The method of preparation of the antigen we have used is described in note 1, table 1. The relationship of the preparative method we have employed in this instance to those which have been employed by other workers in the field of the blood group specific substances (cf. Landsteiner and Chase (11), Landsteiner and Harte (12), Meyer, Smyth and Palmer (13), and Morgan and King (4)) is discussed at length in a communication (14) in which we report the comparative

TABLE 1

*The serological response of rabbits to immunization with a partially purified blood group A specific substance obtained from hog gastric mucin**

| RABBIT NUMBER‡ | DESCRIPTION OF SERUM | RATIO OF CONTENT OF SHEEP CELL LYSINS IN SERUM AFTER IMMUNIZATION TO THAT IN NORMAL SERUM† | | | | VOLUME OF SERUM REQUIRED TO GIVE 100% HEMOLYSIS | VOLUME OF SERUM REQUIRED TO GIVE PERCEPTIBLE AGGLUTINATION‡ OF HUMAN CELLS | | RATIO OF AGGLUTININ CONTENT IN SERUM AFTER IMMUNIZATION TO THAT IN NORMAL SERUM§ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Observed at | | | Average ratio observed | | | | | |
| | | 100% hem. | 50% hem. | 25% hem. | | | Group A cells | Group O cells | Group A cells | Group O cells |
| | | | | | | *ml* | *ml* | *ml* | | |
| 476 | Normal¶ | | | | | ≫500 | 25 | 45 | | |
| | 1st immune‖ | | | 24 | 24 | 25 | 0.4 | 60 | 60 | 0.7 |
| | 2nd immune** | | | 8.1 | 8.1 | 100 | 2.0 | 90 | 10 | 0.5 |
| | 3rd immune†† | | | 9.1 | 9.1 | 200 | 4.5 | 95 | 6 | 0.5 |
| 477 | Normal | | | | | 275 | 45 | 95 | | |
| | 1st immune | 1.8 | 2.3 | 2.1 | 2.1 | 150 | 12 | 375 | 4 | 0.3 |
| | 2nd immune | | 1.2 | 1.1 | 1.1 | >500 | 45 | 375 | 1 | 0.3 |
| | 3rd immune | | 4.2 | 4.6 | 4.4 | >500 | 65 | 150 | 0.7 | 0.6 |
| 478 | Normal | | | | | 500 | 25 | 45 | | |
| | 1st immune | 2.9 | 1.8 | 1.6 | 2.1 | 175 | 95 | 75 | 0.3 | 0.6 |
| | 2nd immune | 2.5 | 1.8 | 1.7 | 2.0 | 200 | — | — | | |
| | 3rd immune | | 2.4 | 2.3 | 2.3 | >500 | 45 | 95 | 0.6 | 0.5 |
| 482 | Normal | | | | | 425 | 6 | 6 | | |
| | 1st immune | 2.8 | 4.4 | 4.2 | 3.8 | 150 | 35 | 25 | 0.2 | 0.2 |
| | 2nd immune | | 2.2 | 2.1 | 2.2 | >500 | — | — | | |
| | 3rd immune | | 2.8 | 2.3 | 2.6 | >200 | 35 | 25 | 0.2 | 0.2 |

* The partially purified preparation of A-substance used in this work was obtained as follows: An aqueous suspension of hog gastric mucin granules (Wilson Lab. Item No. 443) was centrifuged in the open bowl of the Sharples (20,000 rpm) at pH 4.8. The supernate was then electrodialyzed. Fraction 112 precipitated during the course of this latter procedure and this material was lyophilized. A suspension of fraction 112 was then adjusted to pH 7.4 by the addition of NaOH, the solution filtered through a Seitz pad, and the filtrate lyophilized. A solution, containing 5 mg/ml of the A-substance (fraction 112A) in 0.9% NaCl, was used for immunization.

† This ratio was calculated by dividing the volume of normal (pre-immunization) serum required to give, e.g. 50% hemolysis, by the volume of serum after immunization which gave this same percentage of complete hemolysis. In each case the test was carried out in the same total volume and with the same amount of guinea-pig serum as a source of complement and the same volume of sheep cells. The volumes of sera which produced exactly the indicated degrees of hemolysis (25%, 50%, and 100%) were estimated by graphical interpolation on a curve of colorimetrically determined degrees of hemolysis *vs.* microliters of serum present in the test system.

‡ The technic used in carrying out the agglutination test has been described elsewhere (15).

§ This ratio was calculated by dividing the volume of normal serum required to give microscopically perceptible agglutination (15) of pooled human A cells or pooled human O cells by that required after immunization to effect this same degree of agglutination.

¶ In the case of all sera, normal and immune, the sample tested was pooled from two samples of serum obtained on successive days. The rabbits were always bled from the

effectiveness of all of these methods and of others in concentrating blood group A specific substance from a variety of natural sources. We have found that the serological activity of the antigen (fraction 112A) in inhibiting hemolysis of sheep cells by an A cell-immune rabbit serum and in inhibiting the isoagglutination of human A cells is about three times less than that of one of the most active materials (fraction 66) from mucin which we have yet examined and whose preparation is described in (14).

The data in table 1 show that the 60-fold increase in agglutinin content and 24-fold increase in hemolysin content observed for the serum of rabbit number 476 were somewhat transient; these ratios decreased to one-tenth and one-third, respectively, of their initial values despite continued immunization during an additional two months period. However, we have also observed comparable decreases in the hemolysin and agglutinin contents of sera from rabbits immunized during similar time intervals with human erythrocytes of blood group A. The successful immunization of one rabbit out of four (see table 1) with the A substance preparation is not greatly different from the response which we and others (16) have obtained when rabbits, unselected for the presence of pre-formed A agglutinins, have been immunized with human erythrocytes of blood group A. Attention may also be called to the fact that in no rabbit was the content of sheep cell hemolysins observed to decrease following immunization, although a decrease in A agglutinin content and O agglutinin content occurred frequently. The precision of the hemolysis test is such as to make the ratios of hemolysin contents reported in table 1 significant.

Morgan and Watkins have presented data (5) which show that purified A-substance preparations from hog mucin and from human ovarian cyst fluids are unable to inhibit the lytic action on sheep cells of anti-guinea pig kidney or anti-sheep red cell immune rabbit sera. Such inability is evidence for the non-identity of hog mucin A-substance and the antigens of sheep erythrocytes and guinea pig organs. However, it does not exclude the possibility that these substances may be somewhat similar. Our data which are presented in table 1 and, in fact, also the data reported by Morgan and Watkins (5) indicate that the group A-specific substances must, indeed, be similar in important respects to the Forssman antigen of sheep cells, since sheep cell hemolysins were produced in our work by immunization with a partially purified A-substance from hog mucin alone, and in the work of Morgan and Watkins by immunization with an artificially constituted antigen containing A-substance from hog mucin or from human ovarian cysts. The similarity but non-identity of the group specific substance present

marginal vein of the ear. The samples of blood were allowed to stand overnight at 4 C and the serum decanted without breaking the clot. The serum was then centrifuged.

|| This sample of serum was removed 6 and 7 days after the last of 13 intravenous injections of 5 mg of A-substance each. These injections were administered at the rate of 3 per week during approximately one month.

** As in || above, except that an additional number of 10 injections of A-substance was administered to each rabbit during the course of another month.

†† As in || and ** above, except that an additional number of 9 injections of A-substance was given to each rabbit during the course of another month.

in human erythrocytes of blood group A and of the antigens of sheep erythrocytes was established by the work of Schiff and Adelsberger (17). Therefore, the data which are now available allow the hypothesis that three substances possessing blood group A specificity—the one present in hog gastric mucin, the one derived from some human pseudomucinous ovarian cyst fluids, and the agglutinogen A of human erythrocytes—have as a common property the possession of specific chemical groupings which render them similar to the Forssman antigen of sheep erythrocytes and to each other.

We wish to take this opportunity to thank Professor Dan H. Campbell of this Institute for his interest in and advice during this work.

## SUMMARY

The antigenic power in rabbits of a partially purified A-substance from hog gastric mucin has been observed in one instance. No added protein component was present in the antigen used. This observation suggests that some of the substances present with the A-specific amino acid-polysaccharide complex in mucin confer antigenic properties upon it, just as the conjugated protein component of the somatic antigen of *Bact. dysenteriae* (Shiga) has been observed to do.

The presence in the partially purified A-substance from hog gastric mucin of one or more substances which are antigenically similar to the Forssman antigen of sheep erythrocytes is indicated by the production of sheep cell lysins when rabbits are immunized with the A-substance. This observation establishes another point of similarity of the A-substances derived from hog mucin and from some human ovarian cyst fluids, of the human agglutinogen A, and of some component of sheep erythrocytes.

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# D. A Colorimetric Method for the Estimation of the Activity of Substances Inhibiting the Isoagglutination of Blood Group A Cells[1]

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## INTRODUCTION

Investigations on the isolation and characterization of blood group specific substances have relied exclusively on serological tests for the determination of the activities of fractions obtained by various procedures. Isoagglutination inhibition and hemolysis inhibition tests have been used and, more recently, a precipitin test has been developed by Kabat and Bezer (1). In the course of work on the isolation of blood group substances from various sources it was considered advantageous to study a color test which appeared suitable for the estimation of the approximate activity of these preparations. This test has proved useful in our work and is of interest since it emphasizes an intrinsic feature of the structure of A-substance.[2]

The test involves the reaction of Ehrlich's *p*-dimethylaminobenzaldehyde reagent with substances which have been previously treated with a hot alkaline solution; the procedure is essentially that described by Morgan and Elson (2) for the estimation of N-acetylglucosamine. This procedure has been found to give a characteristic red-purple color for A-substance isolated from hog gastric mucin (3) and from human

* Contribution No. 1005.

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the California Institute of Technology.

[2] In this paper A-substance refers to material which is capable of inhibiting the isoagglutination of human blood group A cells by serum from individuals of blood group B; evidence is available (5) that some materials which are active in isohemolysis tests may have differing properties.

ovarian cysts (4). The reaction appears to possess some specificity since colors are not given by other polysaccharides containing hexosamine such as chondroitin sulfuric acid and hyaluronic acid (3), or by tryptophan (2), which is known to react with Ehrlich's reagent under other conditions (6).

It has been possible, by means of this reaction, to correlate the serological activities of preparations of A-substance, determined by isoagglutination inhibition tests, with the intensities of colors produced with Ehrlich's reagent. The correlation has been applied to fractions obtained from hog gastric mucin by procedures using mild conditions. The empirical relationship derived between serological activity and color intensity then provides a method for the assessment of the activity of subsequent undegraded A-substance preparations simply from the results of the color test.

While the color test possesses the advantages of considerable convenience and speed and somewhat greater precision than the isoagglutination inhibition test, it is emphasized that the method is not general and must be applied with caution to fractions obtained by procedures involving degradation of A-substance. Furthermore, no attempt has been made to investigate exhaustively all factors influencing the color test, since the limited knowledge of the structure of A-substance inevitably restricts investigations of this kind at present.

## Experimental

### *Color Test*

*p-Dimethylaminobenzaldehyde (Ehrlich's) Reagent.* The reagent was prepared by dissolving 2 g. of Eastman White Label *p*-dimethylaminobenzaldehyde in a solution containing 100 ml. of glacial acetic acid and 2 ml. of 12 *N* HCl. The reagent was stored in bottles protected from light.

*Test Solutions of Fractions.* Solutions were prepared by dissolving weighed samples in volumetric flasks and diluting with 0.9% NaCl; the test material concentration was usually 1000 $\gamma$/ml. It was convenient to use saline as the same solutions could then be used for the serological tests.

*N-Acetylglucosamine Solutions.* Weighed amounts of dry N-acetylglucosamine were dissolved in 0.9% NaCl, approximately 0.1 saturated with chloroform. Dilute solutions with concentrations in the range 10–100 $\gamma$/ml. were prepared by diluting aliquots of more concentrated solutions. It was found that sodium chloride had no significant effect on the color produced with Ehrlich's reagent. N-Acetylglucosamine was prepared essentially according to the procedure of Breuer (7) from *d*-glucosamine hydrochloride. The hydrochloride was neutralized with sodium hydroxide before acetylation.

*Procedure for the Analysis of A-substance.* One ml. of A-substance solution[3] and 0.1 ml. of 0.25 *M* $Na_2CO_3$ were added to a 15 ml. calibrated centrifuge tube and the solutions mixed. Two controls were prepared: (I) a blank on the reagents (1 ml. of 0.9% NaCl and 0.1 ml. of 0.25 *M* $Na_2CO_3$) and (II) a blank on A-substance (1 ml. of A-substance solution and 0.1 ml. of 0.25 *M* $Na_2CO_3$). The second control was required as A-substance solutions gave a light yellow color after digestion and some solutions gave slight turbidities on final dilutions with acetic acid.

The solutions were heated in a boiling water bath for 15 minutes and cooled by immersion in tap water. The solutions were diluted to 9 ml. with glacial acetic acid and 1 ml. of the *p*-dimethylaminobenzaldehyde reagent added to the A-substance solution and to Control (I). One ml. of glacial acetic acid, 0.24 *N* in HCl (prepared by mixing 1 ml. of 12 *N* HCl and 50 ml. of glacial acetic acid), was added to Control (II). The colors were allowed to develop in a water thermostat at 25.0 ± 0.1°C. for 50–60 minutes. The color intensities were then measured with the Klett-Summerson Colorimeter using a green filter (Klett No. 54); the instrument was preliminarily set at zero against a tube of distilled water. The color intensities were all read in the same Klett tube by rinsing the tube with several portions of solution before adding the major portion. The colorimeter reading for a particular fraction was taken to be equal to the colorimeter reading for the A-substance solution minus the sum of the colorimeter readings for Controls (I) and (II).

*Procedure for the Analysis of N-Acetylglucosamine.* The procedure was the same as for A-substance except that the digestion with alkali was only for 5 minutes, the time recommended by Morgan and Elson (2). Further digestion with alkali produced very rapid decreases in the intensity of the color with Ehrlich's reagent. Control (II) was not necessary as no turbidities are produced with N-acetylglucosamine in the acetic acid solutions.

### *Confirmatory and Other Experiments*

In the following sections the results of investigations of some of the conditions influencing the color test are summarized.

*Conditions of the Alkaline Digestion.* One-tenth ml. of 0.25 *M* $Na_2CO_3$/ml. of A-substance solution gave a maximum color intensity with Ehrlich's reagent with most preparations after 15 minutes digestion at 100°C. About 60–70% of the maximum color intensity was produced after 5 minutes digestion, 90–95% after 10 minutes digestion and 90–100% after 20 minutes digestion. None of the A-substance samples prepared from hog gastric mucin by fractionation with alcohol (8), sodium sulfate (3) or phenol-alcohol (3), from pepsin by autolysis (8) or alcohol fractionation (9), or by treatment of A-substance with papain-HCN or formamide (8) gave any significant color with *p*-dimethylaminobenzaldehyde without prior alkaline digestion.

For a digestion time of 15 minutes, the concentration of sodium carbonate given above (0.1 ml. of 0.25 *M* $Na_2CO_3$/ml. of A-substance solution) produced the maximum color intensity for an A-substance preparation obtained by sodium sulfate fractionation. The color intensities were 95% and 90% of the maximum color intensity when,

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[3] In the present status of the procedure, it is desirable to standardize on a selected concentration of material, *e.g.*, 1000 γ/ml. (± 10%), due to the non-linear dependence of the color on the concentration of A-substance.

respectively, 0.05 ml. and 0.15 ml. of 0.25 *M* $Na_2CO_3$/ml. of A-substance solution were used. The pH of the solution after digestion with 0.1 ml. of 0.25 *M* $Na_2CO_3$ was 10–10.5; before digestion the pH was usually somewhat higher, 10.3–10.8. If a test solution is highly buffered, or contains acid, the proper pH will not be maintained during digestion and erroneous results will be obtained.

*p-Dimethylaminobenzaldehyde Reagent.* The reagent whose preparation is described above had a pale yellow-orange color which increased in intensity on standing; for this reason the reagent was prepared fresh about every 7–10 days. During the period the reagent was being used, the blank color in the analyzed solutions gave very low colorimeter readings in the Klett-Summerson Colorimeter using a green filter (Klett filter No. 54, approximate spectral range, 500–570 mμ). A reagent prepared from *p*-dimethylaminobenzaldehyde recrystallized according to the procedure of Adams and Coleman (10) gave slightly smaller blank colors which increased but little on standing. Furthermore, this reagent, when freshly prepared, gave color intensities for A-substance solutions which were 8–10% greater than those obtained with the unpurified reagent. However, for some reason not thoroughly investigated, this reagent appeared to be somewhat less stable than the reagent prepared from unrecrystallized *p*-dimethylaminobenzaldehyde with respect to the color produced with A-substance; successive analyses of A-substance solutions over a period of several days gave decreasing colorimeter readings. For the purposes of this investigation it was more convenient to use the more reproducible, but somewhat less sensitive reagent prepared from unrecrystallized *p*-dimethylaminobenzaldehyde.

It is apparent from these results that it would be desirable to standardize the procedure so that color intensities produced with A-substance would not reflect changes in the reagent. A few experiments have indicated that N-acetylglucosamine may be a suitable standard as it is stable, can be prepared quite pure, and appears to respond in a similar manner as A-substance to changes in the reagent. Comparisons of the colors obtained with A-substance and N-acetylglucosamine are described in the following sections.

*Conditions for the Color Development.* The concentration of HCl in the *p*-dimethylaminobenzaldehyde reagent is 0.24 *N;* this differs from the concentration, 0.6 *N* HCl, recommended by Morgan and Elson. The latter concentration was unsuitable; the maximum color intensities for A-substance preparations with the reagent occurred in 10 minutes and the color faded rapidly thereafter at the temperature of development, 25°C. The lower concentration of HCl gave a maximum color intensity in 50–60 minutes for both A-substance and N-acetylglucosamine and the colors were stable for at least 30 minutes longer. The marked effect of temperature on development as well as HCl concentration on the rate of color development was noted by Morgan and Elson, who used temperatures of 13–16°C. For this reason the temperature during development was controlled by immersing solutions in a water bath thermostat at 25.0 ± 0.1°C. Since HCl concentration influences the rate of color development, reliable results will be obtained only if the test solutions of preparations do not contain large amounts of buffering agents.

*Comparison of Colors Obtained with A-Substance and N-Acetylglucosamine.* Morgan and King (3) have suggested that the color reaction with Ehrlich's reagent is due to an N-acetylglucosamine rest in A-substance. A-substance from hog gastric mucin was found to give colors equivalent to 12–13% N-acetylglucosamine; this estimate

is approximately confirmed. The equivalent *per cent* N-acetylglucosamine of a number of purified A-substance fractions has been found to vary from 10–13%; these values were calculated for colors obtained with 1000 $\gamma$ of material and for a period of heating with alkali of 15 minutes for A-substance and 5 minutes for N-acetylglucosamine. The fractions were isolated from hog gastric mucin by fractionation with alcohol, sodium sulfate or phenol and alcohol, from pepsin by autolysis or alcohol fractionation, or by treatment of A-substance with papain-HCN or formamide. Morgan and King used results for shorter digestion times of A-substance (6 minutes) for their calculations; their test conditions also differ as has been discussed above. It is of interest that the equivalent *per cent* N-acetylglucosamine accounts for less than half of the 28–30% hexosamine found in A-substance (3, 8, 11).

It should be noted that calculations of equivalent N-acetylglucosamine content are subject to variation depending on the amount of A-substance used for analysis and on the time of digestion of A-substance and N-acetylglucosamine with sodium carbonate solution. A maximum color intensity occurs for most A-substance preparations after digestion at 100°C. with alkali for 15 minutes; a considerably sharper maximum is produced for N-acetylglucosamine after 5 minutes digestion with alkali.

*Serological Test (Inhibition of Isoagglutination)*

*Anti A-($\alpha$)-Agglutinin.* Pooled serum collected from persons of blood Group B was used. This serum was prepared by the Hyland Laboratories[4] and was selected for reasonable potency before its pooling. All of the serum used by us bore the same lot number. It was dyed with a preservative and stored at 5°C. in the original containers. The undiluted serum just produced incipient agglutination at a dilution of 1:128 when tested against pooled A cells by the method described below. The potency of the serum remained constant during the course of the experiments. The serum was diluted with 24 volumes of 0.9% NaCl solution just before use in the inhibition of isoagglutination studies. Thus, there were present 20 microliters of serum in each tube of a series, while 4 microliters of serum were sufficient to produce just microscopically visible agglutination of the volume of cells used in the inhibition system. This four-fold excess of serum over that required to give incipient agglutination was insufficient, however, to produce complete agglutination of the volume of pooled Group A cells (not selected with reference to subtype) used.

*Anti B-($\beta$)-Agglutinin.* Pooled serum from persons of blood group A was used. This serum, prepared by Hyland Laboratories, was similar to that containing anti A-($\alpha$)-agglutinin. The undiluted serum just produced incipient agglutination at a dilution of 1:256 when tested against pooled B cells by the method described below. As in the case of the anti A-($\alpha$)-agglutinin 20 microliters of group A serum were present in each tube of a series, with 2 microliters being sufficient to produce just microscopically perceptible agglutination of the group B cells present. Thus, there was employed a nine-fold excess of serum over that required for incipient agglutination. This amount was almost sufficient to produce complete agglutination of the volume of group B cells used.

*Group A Erythrocytes.* The cells used in each experiment were pooled from ten individuals, each of Group A. Since the source of the cells was a Red Cross blood bank

[4] Address: 4534 W. Sunset Ave., Los Angeles, California.

center, each batch was obtained from different groups of donors. The unwashed cells were preserved at 5°C. and used until a suspension of them showed microscopically perceptible autoagglutination. They were then discarded. In no case were cells used for longer than 5 days following their collection from the donors. Just before use the cells were washed 4–5 times by suspending them in 4 times their volume of 0.9% NaCl solution, stirring and centrifuging. For use in the inhibition studies a 1% suspension of the freshly washed cells was prepared by diluting an aliquot of the packed cells with 0.9% NaCl solution.

*Group B Erythrocytes.* The pooled cells from ten individuals of blood group B were treated as described for group A cells.

*Experimental Procedure for Determinations of A and of B Activity.* The following description applies as well to the procedure for the determination of the inhibition of isoagglutination of group B cells as it does to that for group A cells, with suitable changes in the kind of sera and cells used. It is specifically discussed with reference to A activity only. Solutions of the substances to be tested were prepared in 0.9% NaCl at a concentration of approximately 1 mg./ml. Serial dilutions by two of the test substances with normal saline were made in small culture tubes. In making these dilutions 1 ml. serological pipets were used to mix and then withdraw 0.5 ml. aliquots from each tube for transfer to the next. A fresh pipet was used for each tube in a series. The final volume of test substance solution in each tube was 0.5 ml. Into each tube of the series and into suitable controls 0.5 ml. of 1:25 Group III (B) serum was pipetted. The contents of the tubes were mixed by sharp stroking of the tubes and were then allowed to stand in a water bath at 20–25°C. for one hour. One-half ml. of a 1% suspension of Group II (A) cells in normal saline was then pipetted into each tube of the series. The contents of each tube was again mixed and then allowed to stand for 2 hours at 20–25°C. At the end of this time the tubes were centrifuged for 1 minute in a clinical centrifuge. The examination of the mixture for agglutination was made as follows. The centrifuged cells in each tube in a series were resuspended by stroking the tube, at the same time observing whether there was massive agglutination. In this way it was possible to eliminate certain tubes from further consideration. For the final selection of the first tube in a series which had agglutination the contents were examined under the low-power objective of a microscope. The stage of the microscope was tilted so that when a drop of a cell suspension was streaked across the slide, the cells moved slowly across the field. By observing whether cells which seemed to be aggregated as they entered the field remained so as they moved across it, it was possible to distinguish true agglutination from adventitious clumping. The endpoint of inhibition was taken as the mean of the smallest amount of A substance which entirely prevented clumping of cells (recorded as 0 agglutination) and of that amount which permitted several 2-cell aggregates to be observed in two to three streakings of the suspension across the field (recorded as ± agglutination). In some cases the first tube of a series to show agglutination exhibited it sufficiently strongly that 1–3 2-cell clumps were continually visible in the moving field (recorded as + 1 agglutination). In these latter cases, where there was no tube having ± agglutination, the endpoint was taken as the mean of the amounts of test substance present in the last tube which showed 0 agglutination and in the first tube with + 1 agglutination.

The titer of a preparation, *i.e.*, the microliters of serum neutralized/$\gamma$ of substance, was calculated by dividing 20 (the number of microliters of serum in the test system)

by the amount of material taken as the endpoint; the amount of serum which reacts with the red cells in the test system is neglected. The experiment described immediately below indicates that the titer of a preparation is essentially independent of the amount of serum used in the test system.

An experiment was performed to investigate the dependence of the inhibition titer of a test substance on the agglutinin concentration. Six sets of tubes were prepared, each set being of serial dilutions by two of an A-substance preparation in normal saline (final volume in each tube; 0.5 ml.). The test substance used in this experiment was material precipitated from hog gastric mucin by 30% $Na_2SO_4$ and soluble upon electrodialysis of the precipitate.

To each tube within a series, 0.5 ml. of serum, diluted in some cases with normal saline, was added. A different dilution of serum was used for each series. After mixing their contents the tubes were allowed to stand 1 hour in a water bath at 25°C. To each tube 0.5 ml. of a 1% suspension of group A cells in normal saline was added. The tubes were allowed to stand for 2 hours at 37°C. They were then examined for agglutination by the method described above. The results are presented in Table I.

TABLE I

*Dependence of Endpoint Upon Amount of Serum Used in Test*

| Amount of Serum Present in Each Tube (μl) | Amount of Test Substance In Tube Showing Indicated Degree of Agglutination (γ) | |
|---|---|---|
| | 0 | ± |
| 500 | 36.1 | 18.1 |
| 250 | 18.1 | 9.0 |
| 125 | 9.0 | 4.5 |
| 62 | 2.2 | 1.1 |
| 31 | 2.2 | 1.1 |
| 20 | 1.1 | 0.56 |

Any endpoint obtained by the inhibition test may be in error by a factor of two. This limit of error is fixed partly by the use of serial dilutions by two and partly by the unknown extent to which small differences in experimental conditions affect the phenomenon of agglutination. With these considerations borne in mind the data in Table I can be seen to illustrate the generally linear dependence of inhibition endpoint on agglutinin concentration.

## Results

The correlation of the serological activity and the color intensity obtained with Ehrlich's reagent for A-substance fractions prepared by mild procedures from hog gastric mucin[5] is shown in Fig. 1. The preparations were obtained by fractionation with sodium sulfate or

[5] Wilson Laboratories gastric mucin granules, Item No. 443.

phenol-alcohol according to the methods of Morgan and King (3), by alcohol fractionation, by aqueous extraction and by electrodialysis of some of the fractions derived from these procedures. The procedures do not subject A-substance to conditions which are known to degrade it, such as highly acidic or basic solutions at elevated temperatures (3).

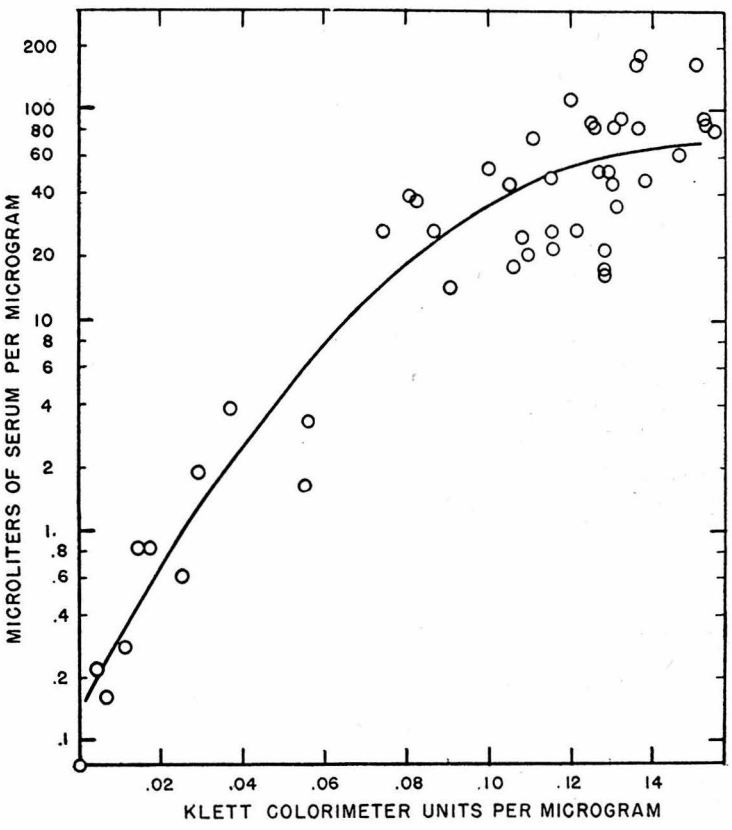


FIG. 1

Relation between Color Intensity with Ehrlich's Reagent and the Isoagglutination Inhibition Titer for A-Substance Preparations from Hog Gastric Mucin

This restriction permits a more accurate estimate of the degree of correlation between the color test and the serological test since degraded materials, which may differ substantially in behavior from undegraded materials in these tests, are omitted from consideration.

Data for 47 separate fractions are plotted in Fig. 1. The serological activity is reported as microliters of serum neutralized/$\gamma$ of test material. The value of the titer for a preparation was calculated from the mean of the amounts of material found by the serial dilution technique to produce minimal agglutination and complete inhibition of agglutination. Usually only single determinations of titers were made; the precision of the determination is estimated to be $\pm$ 50–100% over the whole range of titers.

The ability of the substance to give a color with Ehrlich's reagent is given on the abscissa of Fig. 1 as Klett Colorimeter units/$\gamma$ of test material. This value is not entirely independent of the amount of material utilized in the color test, usually about 1000 $\gamma$ with an over-all range of about 500–1500 $\gamma$. However, the values of the slopes (Klett Colorimeter units/$\gamma$) at the limits of this range were found to differ by less than 8% from the slope for 1000 $\gamma$ of material for a number of A-substance preparations. Since these deviations approach the precision of the color test and are small compared to errors in the serological test, it would be expected that the existence of a correlation between the two tests would not be obscured by neglecting these deviations. No corrections for these deviations have been applied to the data in this study. Such corrections would be a desirable refinement as the precision of the methods improve and when the nature of the color reaction is clearly established. The majority of the slopes reported are the mean of two determinations with an average deviation from the mean of 3% for highly active preparations and as much as 10–30% for weakly active preparations (less than 0.5 microliters/$\gamma$).

The curve shown in Fig. 1 is the least squares fit of a second-degree parabola to the data; the index of correlation for the curve is 0.95. The data show a normal distribution about the least squares curve; a semi-logarithmic plot of the data is convenient because of the wide range of titers of preparations. Since the precision of the color test is about ten times greater than the serological test (except at very low activities), the least squares solution has not considered the errors which may be involved in the colorimetric estimation. Because of the difficulty of estimating very faint colors at low activities, the estimated titers from the least squares curve below 0.17 microliters/$\gamma$ are not significant.

The curve in Fig. 1 shows that a significant relation exists between the color with Ehrlich's reagent and blood group A activity. This

TABLE II

*Comparison of Observed and Estimated Titers for Fractions Isolated from Hog Gastric Mucin**

| Compound No. | Procedure | Titer (μl serum/γ) | | Probability of observed deviation |
|---|---|---|---|---|
| | | Observed | Estimated | |
| R4-F4 | Alcohol fractionation (Landsteiner and Harte) | 0.085 | 0.17 | 0.27 |
| R5-F1 | | .29 | .20 | .56 |
| R4-F1 | | .30 | .27 | .87 |
| R5-F2 | | 20 | 19 | .94 |
| R10-F2 | | 8.2 | 19 | .18 |
| R8-F2B | | 44 | 27 | .43 |
| R4-F2 | | 29 | 28 | .96 |
| R5-F3 | | 111 | 44 | .14 |
| R8-F1 | | 54 | 54 | 1.00 |
| R4-F3 | | 63 | 56 | .85 |
| R11-F2 | | 154 | 59 | .13 |
| R8-F2A | | 42 | 61 | .56 |
| C-51 | Procedure of Meyer, Smyth, and Palmer | 6.1 | 2.2 | .11 |
| C-49 | | 5.7 | 8.0 | .59 |
| C-54 | | 22 | 27 | .75 |
| C-8 | | 27 | 35 | .68 |
| C-50 | | 21 | 39 | .33 |
| C-4 | | 53 | 44 | .77 |
| C-48 | | 33 | 65 | .28 |
| C-75 | Treatment with ammoniacal copper solution | 84 | 45 | .32 |
| C-76 | | 84 | 54 | .49 |
| C-77 | | 44 | 55 | .72 |
| R14-F2A | Treatment with alkaline pyridine | 48 | 38 | .71 |
| R14-F1A | | 53 | 53 | 1.00 |

* Notes to Table II.—*R4-F4:* Soluble after addition of 2 vol. EtOH to filtrate of aqueous suspension of mucin. *R5-F1:* Insoluble in aqueous suspension of mucin, pH 4.3, after heating at 90°C. *R4-F1:* Same as R5-F1, duplicate experiment. *R5-F2:* Insoluble on addition of ¾ vol. EtOH to filtrate of aqueous suspension of mucin. *R10-F2:* Obtained by dialysis of R5-F2 for two weeks at 5°C. *R8-F2B:* Residue on electrodialysis of R5-F3 for 5 days. *R4-F2:* Same as R5-F2, duplicate experiment. *R5-F3:* Precipitated between ¾ and 2 vol. EtOH from filtrate of aqueous suspension of mucin. *R8-F1:* Clear supernatant on electrodialysis of R5-F3 for 5 days. *R4-F3:* Same as R5-F3, duplicate experiment. *R11-F2:* Obtained by dialysis of R5-F3 for two weeks at 5°C. *R8-F2A:* aqueous washing of R8-F2B. *C-51:* Precipitated at pH 7 by zinc acetate. Electrodialyzed at pH 5.3, insoluble after electrodialysis. *C-49:* After

TABLE II (*Continued*)

| Compound No. | Procedure | Titer (μl serum/γ) | | Probability of observed deviation |
|---|---|---|---|---|
| | | Observed | Estimated | |
| R7-S1B | Treatment with $HCONH_2$ at 150°C. (Landsteiner and Harte) | .51 | 1.6 | .075 |
| R7-F3B | | 4.3 | 6.4 | .53 |
| R7-S2 | | 11 | 26 | .19 |
| R7-F2A | | 18 | 38 | .24 |
| R7-F2C | | 9.7 | 47 | .01 |
| R7-S3 | | 5.4 | 48 | .0006 |
| R7-F2B | | 11 | 54 | .01 |
| R7-F1 | | 29 | 64 | .21 |
| R7-F4A | | 7.1 | 64 | .0006 |
| R7-F5A | | 12 | 65 | .008 |
| R6-F4A | Treatment with papain-HCN (Landsteiner and Harte) | 6.9 | 56 | .001 |
| R6-F1A | | 37 | 59 | .46 |
| R6-F2 | | 48 | 60 | .72 |
| R6-F1B | | 24 | 63 | .13 |
| R6-F5A | | 25 | 65 | .13 |
| 1A | A-substance preparation by Kabat and Bezer | 87 | 64 | .63 |
| M-330 | A-substance preparation by Lilly | 83 | 60 | .61 |
| 960-GM-2 | A-substance preparations by Sharp and Dohme | 77 | 57 | .64 |
| 960-GM-1B | | 91 | 55 | .43 |
| 960-GM-1C | | 77 | 59 | .67 |

precipitation by 18 vol. glacial acetic acid, insoluble in water, insoluble after electrodialysis. *C-54:* As for C-51, except soluble after electrodialysis. *C-8:* Obtained after initial precipitation with 3 vol. EtOH, and after precipitation with 18 vol. glacial acetic acid, not dialyzed. *C-50:* As for C-49, except soluble after electrodialysis. *C-4:* Obtained after the initial precipitation with 3 vol. EtOH, but before acetic acid precipitation, not dialyzed. *C-48:* Obtained after precipitation by 18 vol. glacial acetic acid. Material soluble in water and precipitated at 30–35°C. by 30% $Na_2SO_4$, electrodialyzed. *C-75:* Extracted by phenol from mucin and treated for 1 hr. at room temperature with an ammoniacal copper solution (reagent contained 4.95 g. Cu/l. and was 5.2 *M* in $NH_4OH$). Adjusted to pH 5 with acetic acid. Dialyzed at room temperature. *C-76:* As for C-75, except treated 6 hr. with the reagent. *C-77:* As for C-75, except treated for 3 hr. with the reagent. *R14-F2A:* A-substance, isolated by $Na_2SO_4$ fractionation, treated with alkaline pyridine (0.002 *M* NaOH in 90% pyridine) for 2 days and then the material soluble in alkaline pyridine precipitated with acetone, dialyzed. *R14-F1A:* As for R14-F2A except material was insoluble in alkaline pyridine, dialyzed. *R7-S1B:* Soluble on addition of 2 vol. EtOH to formamide solution of

curve can be considered only as an approximation; more data, particularly at low and moderate activities, would be required for a more accurate relationship. Fig. 1 has been used for the estimation of the blood group A activity of, (a) fractions obtained from hog gastric mucin by somewhat more drastic conditions (Table II); (b) A-substances prepared by other investigators from hog stomach or hog gastric mucin (Table II) and (c) fractions obtained from sources other than hog stomach (Table III). Brief descriptions of the treatment and method of isolation of fractions are given in notes to the tables.

Table II shows a comparison of the observed and estimated titers for fractions isolated from hog gastric mucin[6] by alcohol fractionation of a previously heated mucin suspension (8), by treatment with ammoniacal copper solution, alkaline pyridine, formamide (8), papain-HCN (8) or by the procedure of Meyer, Smyth and Palmer (11), which involves a preliminary treatment of mucin with sodium carbonate at 70°C. Comparison of observed and estimated titers are also shown for A-substance isolated by Kabat and Bezer (1) from hog

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R5-F3. Dialyzed. Soluble in water on dialysis. *R7-F3B:* Soluble on addition of 4 vol. EtOH to solution of R7-F2B. *R7-S2:* Supernatant from first reprecipitation of R7-F1 from 66% EtOH, dialyzed. *R7-F2A:* Insoluble in 90% acetic acid solution of R7-F1. *R7-F2C:* Supernatant from the precipitation of R7-F2B from 90% acetic acid with acetone, dialyzed. *R7-S3:* Supernatant from second reprecipitation of R7-F1 from 66% EtOH, dialyzed. *R7-F2B:* Precipitated on addition of ½ vol. of acetone to supernatant of 90% acetic acid solution of R7-F1, dialyzed. *R7-F1:* Insoluble on addition of 2 vol. EtOH to formamide solution of R5-F3. Reprecipitated twice from 66% EtOH. *R7-F4A:* Insoluble on addition of 4 vol. EtOH to solution of R7-F2B. Reprecipitated from HCl solution with 4 vol. EtOH, dialyzed. *R7-F5A:* Precipitated from 90% acetone solution of R7-F4A. *R6-F4A:* Insoluble on addition of 4 vol. EtOH to solution of R6-F1A. Insoluble in 90% acetic acid solution. *R6-F1A:* Insoluble on addition of 1.5 vol. EtOH to filtered and dialyzed papain-HCN digest (7 days at 37°C.) of R4-F3. *R6-F2:* Supernatant from the precipitation of R6-F1A and R6-F1B with EtOH. *R6-F1B:* Same as R6-F1A except this fraction did not centrifuge readily from alcohol solution and was obtained by Seitz filtration. *R6-F5A:* Insoluble on addition of 4 vol. EtOH to solution of R6-F1A. Soluble in 90% acetic acid solution, precipitated by acetone, and dialyzed. *1A:* Prepared by alcohol fractionation, see reference (1). *M-330:* Prepared from hog stomach linings by peptic autolysis and alcohol fractionation. *960-GM-2:* Prepared from hog gastric mucin by phenol fractionation. *960-GM-1B:* Prepared by autolysis from hog gastric mucin. *960-GM-1C:* Prepared by tryptic digestion and alcohol fractionation from hog gastric mucin.

[6] Except where otherwise indicated, the hog gastric mucin was obtained from Wilson Laboratories (Item No. 443).

TABLE III

*Comparison of Observed and Estimated Titers for Fractions Isolated from Sources other than Hog Gastric Mucin**

| Compound No. | Source | Procedure | Titer (μl serum/γ) | | Probability of observed deviation |
|---|---|---|---|---|---|
| | | | Observed | Estimated | |
| C-105 | Horse stomach mucosa; (B activity, <.04 μl/γ) | | 0.21 | 0.33 | 0.48 |
| M-336C | Horse stomach prepared by Lilly; (B activity, 4.8 μl/γ) | | 3.2 | 9.8 | .08 |
| 960-P-HS-4 | Horse stomach linings prepared by Sharp & Dohme; (B activity, 5.6 μl/γ) | | .44 | 34 | .0000 |
| DM | Duodenal mucosa (Wilson) | Untreated source material | .87 | .22 | .03 |
| C-32 | Pepsin (Parke, Davis) | | .40 | .19 | .24 |
| C-33 | Pepsin (Wilson) | | 1.9 | .28 | .003 |
| C-5 | Pepsin | Autolysis procedure of Landsteiner and Harte | 2.0 | .66 | .08 |
| C-22 | | | 61 | 48 | .69 |
| C-44 | Pepsin | Alcohol fractionation (Landsteiner and Chase) | .049 | 0 | — |
| C-26 | | | 27 | 52 | .30 |

* Notes to Table III.—*C-105:* From fresh horse stomach mucosa treated according to U.S. Patent 1,829,270 (S. J. Fogelson) and then according to the $Na_2SO_4$ fractionation method of Morgan and King (3). *M-336C:* Prepared by peptic hydrolysis and alcohol fractionation. *960-P-HS-4:* Prepared by peptic-tryptic digestion and alcohol fractionation. *DM:* Wilson Laboratories duodenal mucosa preparation. *C-32:* Parke, Davis and Co. pepsin (1:3000). *C-33:* Wilson Laboratories pepsin (1:10,000), Item No. 414. *C-5:* From Wilson pepsin (see C-33), autolyzed, precipitated by 66% EtOH and then dissolved. Finally precipitated by 50% EtOH. *C-22:* From Wilson pepsin (see C-33), autolyzed, precipitated by 62% EtOH and then by 9 vol. glacial acetic acid and 5 vol. acetone; dialyzed and precipitated by 4 vol. EtOH plus sodium acetate. *C-44:* From Wilson pepsin (See C-33), precipitated by acetic acid from aqueous solution, washed. *C-26:* From Wilson pepsin (see C-33), obtained

TABLE III (*Continued*)

| Compound No. | Source | Procedure | Titer (μl serum/γ) | | Probability of observed deviation |
|---|---|---|---|---|---|
| | | | Observed | Estimated | |
| C-27 | Blood group A stromata | Procedure of Kossjakow | 2.4 | 6.8 | .10 |
| R1-F1 | Blood group A stromata | Procedure of Hallauer | <.008 | 0 | — |
| R1-F3 | | | <.008 | 0 | — |
| C-45 | | | .18 | 0 | — |
| R1-F5 | | | <.14 | .18 | — |
| R1-F2 | | | .35 | .19 | .34 |
| R1-F4 | | | .067 | .20 | .08 |
| C-30 | | | .067 | .26 | .03 |
| C-28 | | | .18 | .28 | .49 |
| C-73 | | | .77 | .56 | .61 |
| C-34 | Blood group A erythrocytes | Procedure of Hallauer | .011 | 0 | — |
| C-2 | | | <.09 | 0 | — |
| C-46 | | | .13 | 0 | — |
| C-57 | | | <.003 | 0 | — |
| C-3 | | | <.09 | .18 | — |
| C-56 | | | .92 | .21 | .02 |
| C-1 | | | .083 | .24 | .09 |
| C-113 | Pseudo-mucinous ovarian cysts (blood group A) | Alcohol fractionation | .42 | 0 | — |
| C-119 | | | 1.8 | 2.5 | .60 |

by two 65% alcohol precipitations at pH 8.5, dialyzed. *C-27:* Treated according to Kossjakow's procedure as far as one alcohol precipitation. *R1-F1:* Extract with 95% EtOH. *R1-F3:* First 25% EtOH extract. *C-45:* Boiling absolute alcohol extract. *R1-F5:* Extract with 10% EtOH. *R1-F2:* Extract with 50% EtOH. *R1-F4:* Second 25% EtOH extract. *C-30:* First 25% EtOH extract. One acetone precipitation. *C-28:* Second 25% EtOH extract. One acetone precipitation. *C-73:* Aqueous extract. Precipitated once with acetone. *C-34:* Extract with 10% EtOH. One acetone precipitation. *C-2:* First 25% EtOH extract. One acetone precipitation. *C-46:* Boiling absolute alcohol extract. *C-57:* Material precipitated by 66% EtOH from aqueous extract of material extracted by 50% EtOH according to Hallauer's procedure. *C-3:* Second 25% EtOH extract. One acetone precipitation. *C-56:* Material precipitated by 50% EtOH from aqueous extract of material extracted by 50% EtOH according to Hallauer's procedure. *C-1:* Extract with 50% EtOH. One acetone precipitation. *C-113:* Material precipitated by 50% EtOH from the native fluid of a pseudomucinous

gastric mucin and for A-substances isolated from hog stomach and hog gastric mucin by the Sharpe and Dohme and Lilly laboratories.[7]

Column 5 in Table II gives the calculated probability that the deviations between observed and estimated titers are due to a normal distribution of error. Probabilities less than 0.05 indicate deviations from the estimated titers which are significantly larger than deviations from the least squares curve shown in Fig. 1. The agreement between observed and estimated titers is satisfactory except for some of the fractions isolated by formamide and papain procedures. However, these two procedures are known to cause extensive degradation of A-substance (3, 8) and to decrease the isoagglutination titer of preparations. In these cases it appears that the degradation has disturbed the structure of A-substance to such a degree that the serological specificity is lost while the functional groups responsible for the color test are relatively unaffected. The other procedures of isolation apparently effect no substantial degradation of A-substance detectable by means of a comparison of the observed and estimated titers.

Table III summarizes the results of observed and estimated blood group A titers for fractions isolated from pepsin (8, 9), blood-group A erythrocytes and stromata (12, 13), horse stomach, and pseudomucinous ovarian cyst fluids of individuals of blood group A.[8] Materials isolated from horse stomach mucosa are often known to possess blood group B activity; titers against B cells are shown parenthetically in Table III for these preparations. In several cases unpurified materials were analyzed by the serological and color tests; comparisons of these results would not be expected to be as satisfactory as in those cases where some means has been resorted to for the separation of inactive materials which may interfere with the tests.

Probabilities are not calculated for those cases in which the estimated titer is below the limit of the colorimetric method, 0.17 microliters/$\gamma$, or in those cases where the observed titer is only known to be less than

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ovarian cyst removed from an individual of blood group A. Material dialyzed. *C-119:* Material soluble in 50% EtOH but insoluble in 65% EtOH from same cyst fluid as C-113. Material reprecipitated once and dialyzed.

[7] We are indebted to Dr. E. Brand for a sample of Kabat's preparation, and to Dr. R. H. Barnes of Sharpe and Dohme, Inc., and Dr. J. A. Leighty of Lilly Research Laboratories for other samples of A-substance and AB mixture.

[8] We are indebted to Dr. R. W. Hammack of the Hospital of the Good Samaritan, Los Angeles, California, for providing us with samples of fluids removed from ovarian cysts.

a given value. These results are of interest since they indicate that the colorimetric method is usually in qualitative agreement with the serological test for fractions of no, or very low, activity.

The results for material isolated from horse stomach are pertinent to the question of the behavior of B-substance isolated from this source in the color test. Recently Morgan and Waddell (14) have observed that B and O substances isolated from pseudo-mucinous ovarian cyst fluids give color tests after treatment with alkali. The agreement between the observed and estimated blood group A titers for the horse stomach preparations shown in Table III is satisfactory either when the B activity of the substance is low (Compound C-105) or when the serological test shows appreciable A activity (Compound M-336C). When a compound shows appreciable B activity but little A activity (Compound 960-P-HS-4), the color test still predicts a very high A activity, the agreement in this case being very poor. This evidence would appear to suggest that B-substance or some material associated with it in horse stomach also gives the color test or that the A-substance factor has been almost completely degraded by the peptic-tryptic digestion used in the preparation of this compound.

The deviations between observed and estimated titers for the remainder of the results in Table III have calculated probabilities which are usually greater than the value of 0.05 of the standard of significance. Some of the probabilities fall somewhat below 0.05 for preparations of low activities; this might be expected, as errors in the colorimetric procedure, which were not considered in the least squares solution, become appreciable at low color intensities.

## Discussion

The results which have been presented indicate that the functional groups responsible for the color reaction with Ehrlich's reagent are an inherent feature of the structure of A-substance. The groups appear to be common to the materials possessing A activity derived from human erythrocytes and pseudo-mucinous ovarian cyst fluids as well as from hog gastric mucin, pepsin and horse stomachs. It would not be expected that detailed differences in the A-substances isolated from these various sources could be revealed in this study due to the lack of precision in the correlation of the serological test and the color test.

Preliminary evidence from materials isolated from horse stomach would appear to indicate that B substance isolated from this source

also possesses similar functional groups; further investigation is required to establish these observations. It is evident that a knowledge of the specificity of the color reaction is important as a part of the general problem of interpreting serological specificity in terms of the chemical structure of the blood group substances.

While the colorimetric procedure has proved to be a useful supplement to serological tests in our work, it may be helpful to point out several difficulties in applying the procedure in its present form as a general method for the estimation of isoagglutination titers of A-substance preparations. First, the method requires a preliminary calibration with the aid of the relatively inaccurate serological test in case one is interested in interpreting the results in terms of serological titers. The empirical relation which is derived in this way will probably be difficult to obtain in a more direct manner until further investigation establishes the chemical structure of A-substance. Another feature restricting the usefulness of the colorimetric method is the fact that the estimated isoagglutination titers may be considerably in error if A-substance has been degraded significantly. This limitation imposes the necessity of determining by preliminary investigation whether the colorimetric method is suitable for the prediction of isoagglutination titers of fractions obtained by various procedures.

Finally, certain limitations in the color test restrict a routine application of the method. For example, solutions of preparations must be essentially neutral and contain no significant amount of buffer since the adjustment of the pH during the sodium carbonate digestion of A-substance as well as on addition of Ehrlich's reagent is critical. Occasionally preparations give turbidities in the final solutions and thus prevent accurate measurements of color intensity. Difficulties of this kind could probably be resolved by altering the experimental conditions of the color test.

## Summary

The intensity of the color produced by the reaction of A-substance with Ehrlich's *p*-dimethylaminobenzaldehyde reagent after preliminary treatment of A-substance preparations with alkali has been correlated with the isoagglutination inhibition activity of the original preparations. The use of this correlation to estimate the potency of A-substance preparations has been described. The color reaction with Ehrlich's reagent has been observed for blood group A specific preparations

isolated from hog gastric mucin, pepsin, blood group A erythrocytes and stromata, and human ovarian cyst fluids, and for blood group AB specific preparations from horse stomachs.

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# E. THE ELECTROPHORETIC PROPERTIES OF BLOOD GROUP A-SPECIFIC SUBSTANCE PREPARATIONS ISOLATED FROM HOG GASTRIC MUCIN[a,b]

by

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An A-substance preparation obtained from commercial hog gastric mucin by Morgan and King (1) was examined electrophoretically by Kekwick (2) who reported that the sample was essentially homogeneous at pH 4 and 8. Bendich, Kabat and Bezer (3) also found that two O-substance and two A-substance preparations isolated from individual hog stomach linings and an A-substance preparation obtained from commercial hog gastric mucin migrated with the same velocity at pH 7.4 and appeared to be electrophoretically homogeneous. In view of the fact that these investigators and others (4) appear to attach some significance to the apparent electrophoretic homogeneity of their preparations, it was thought advisable to examine the electrophoretic

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[a]This work was supported in part by a grant from the U. S. Public Health Service.

[b]A more complete discussion of the electrophoetic properties of blood group A-specific substance preparations isolated from hog gastric mucin is presented in (13).

properties of a large number of A-substance preparations of widely varying potency (5-8) and over a wide pH range. It was hoped that such a study would disclose the extent to which electrophoretic behavior is useful as a criterion of homogeneity, the possibility of further fractionation of inhomogeneous preparations by electrophoretic procedures and permit the determination of the apparent isoelectric points of A-substance preparations.

## Experimental

Buffer Solutions.- The compositions of the buffer solutions which were prepared were as follows: pH 8.00 (at 27°C): 0.0429 M in $Na_2HPO_4$, 0.00280 M in $NaH_2PO_4$. pH 6.00 (at 26°C): 0.0139 M in $Na_2HPO_4$, 0.0905 M in $NaH_2PO_4$. pH 3.90 (at 23°C): 0.0320 M in $CH_3COONa$, 0.178 M in $CH_3COOH$, 0.100 M in NaCl. pH 2.02 (at 25°C): 0.0320 M in $NaH_2PO_4$, 0.0427 M in $H_3PO_4$, 0.100 M in NaCl. The ionic strength calculated for each of the buffers is 0.13.

Preparation of A-Substance Solutions.- The desired weight of A-substance (5-8) previously dried in vacuo over $P_2O_5$, was dissolved in enough of the appropriate buffer solution to give a 0.50 per cent (w/v) solution. The solution was then dialyzed against 200 milliliters of buffer at 4°C for periods of time ranging from 18 to 115 hours previous to its transfer to the cell of the electrophoresis apparatus.

Electrophoresis Experiments.- No description will be given of the operations involved in carrying out a given experiment. Care was taken that the water bath had a constant temperature of 1.35°C during the experiment. A current of 15 milliamperes was passed through the cell for the duration of each experiment which was usually 3 hours. Boundary photographs were taken sometimes at 1 hour and 2 hours, and always at 3 hours. For photographing turbid solutions a red filter on the light source and Panchromatic plates were used.

Calculation of Mobilities of A-Substance Preparations.- Mobilities of the various preparations were calculated from distances of translation measured on photographic positives which had been enlarged three times (linear magnification) from the 5 x 7 inch plates exposed in the apparatus. By this technique small movements were rendered easily susceptible to measurement. This was important because, in general, the mobilities were small - of the order of $1 \times 10^{-5}$ $cm^2 . volt^{-1} . sec^{-1}$. In calculating the data only measurements made on the descending boundaries were used in accordance with the suggestion of Longsworth and MacInnes (9). In the case of the experiments at pH 2.0 and 3.9 small approximate corrections were made on the observed mobilities for the volume change during the experiment due to the electrode reactions and to transference of ions. This correction usually changed the observed mobility by only about $0.1 \times 10^{-5}$ $cm^2 . volt^{-1} . sec^{-1}$ or less. At pH 8.0 and pH 6.0

the available data on apparent molal volumes of the ion species present were too conflicting to allow useful corrections to be calculated. All mobilities given in Table 1 are the corrected values whenever such corrections could be calculated.

## Discussion

The results of our experiments indicate that, by and large, all of the A-substance preparations examined appeared to be essentially homogeneous irrespective of their activity or degree of purification. Although in several of the patterns the peaks had rather broad bases, in only two (Fraction 137 and 112, see Table 1) was there definite resolution into more than one main peak. In many cases a small amount (less than 10 per cent) of a fast moving component was observed, but this was clearly something foreign to the A-substance complex since it was never found present in any preparation that had been partially purified. The pattern for Fraction 115 in Figure 1 clearly shows the small peak due to this fast moving component. The type of patterns obtained for A-substance fractions is illustrated by the ascending boundary photographs reproduced in Figure 1. Here it can be seen that at pH 3.9 the various preparations give rise to only one peak. A similar result was obtained

Table I

THE ELECTROPHORETIC MOBILITIES OF SOME A-SUBSTANCE PREPARATIONS

| Fraction Number | Inhibition of Hemolysis Titer[a] (micrograms) | Electrophoretic Mobility[b] u x $10^5$ $cm^2$. $volt^{-1}$. $sec^{-1}$ at 1.35°C | | | |
|---|---|---|---|---|---|
| | | pH 8.00[c] Phosphate | pH 6.00[c] Phosphate | pH 3.90[c] Acetate | pH 2.02[c] Phosphate |
| R.18 F.10 | 0.05 | | | 1.0 | |
| R.18 F.11 | 0.05 | | | 1.1 | |
| R.20 F.1 | 0.060 | | | 1.5 | |
| R.20 F.2 | 0.051 | | | 1.0 | |
| 22 | 0.050 | 1.2 | | | |
| R.7 F.5A | 0.050 | 1.3 | | 0.7 | |
| 168 | 0.070 | 1.5 | | 2.7 | |
| R.1 F.2A | 0.075 | 1.4 | | 0.5 | |
| 66 | 0.080 | 1.8 | | 2.0 | |
| 87 | 0.093 | 1.4 | | 2.3 | |
| 69 | 0.10 | 1.4 | | | |
| 120 | 0.10 | 1.7? | | 1.2 | |
| 138 | 0.10 | 1.6 | | 0.6 | |
| 128 | 0.11 | 1.1 | 1.0 | 1.2 | 0.7 |
| 68 | 0.11 | 0.8 | | 0.3 | |
| 137 | 0.11 | 1.7[d]<br>1.3[e] | | | |
| 153 | 0.12 | 1.1 | | 1.9 | 1.3 |
| 71 | 0.12 | 1.2 | | | |

Table I (cont.)

| Fraction Number | Inhibition of Hemolysis Titer[a] (micrograms) | Electrophoretic Mobility[b] u x $10^5$ $cm^2$. $volt^{-1}$. $sec^{-1}$ at 1.35°C | | | |
|---|---|---|---|---|---|
| | | pH 8.00[c] Phosphate | pH 6.00[c] Phosphate | pH 3.90[c] Acetate | pH 2.02[c] Phosphate |
| 52 | 0.13 | | | -0.1 | 1.0 |
| 127 | 0.17 | 1.7? | 0.2 | 0.9 | 0.9 |
| 111 | 0.19 | 1.4 | 1.0 | -0.1 | 0.9 |
| 167 | 0.21 | 1.2 | | 0.3 | |
| 112 | 0.24 | 1.8[d] | 1.9[d]<br>1.6[e] | 1.5[d] | |
| 115 | 0.24 | 1.5? | | 0.3 | 1.0 |
| 123 | 0.24 | 1.5? | 0.3 | 0.6 | 0.8 |

Notes to Table I

[a] Micrograms of A-substance present in system in which erythrocytes are 50 per cent hemolyzed.

[b] Mobilities calculated from measurements on descending boundaries. Migrations are anodic unless otherwise indicated (by a minus sign).

[c] Each buffer had a calculated ionic strength of 0.13.

[d] Mobility of the faster moving component.

[e] Mobility of the slower moving component.

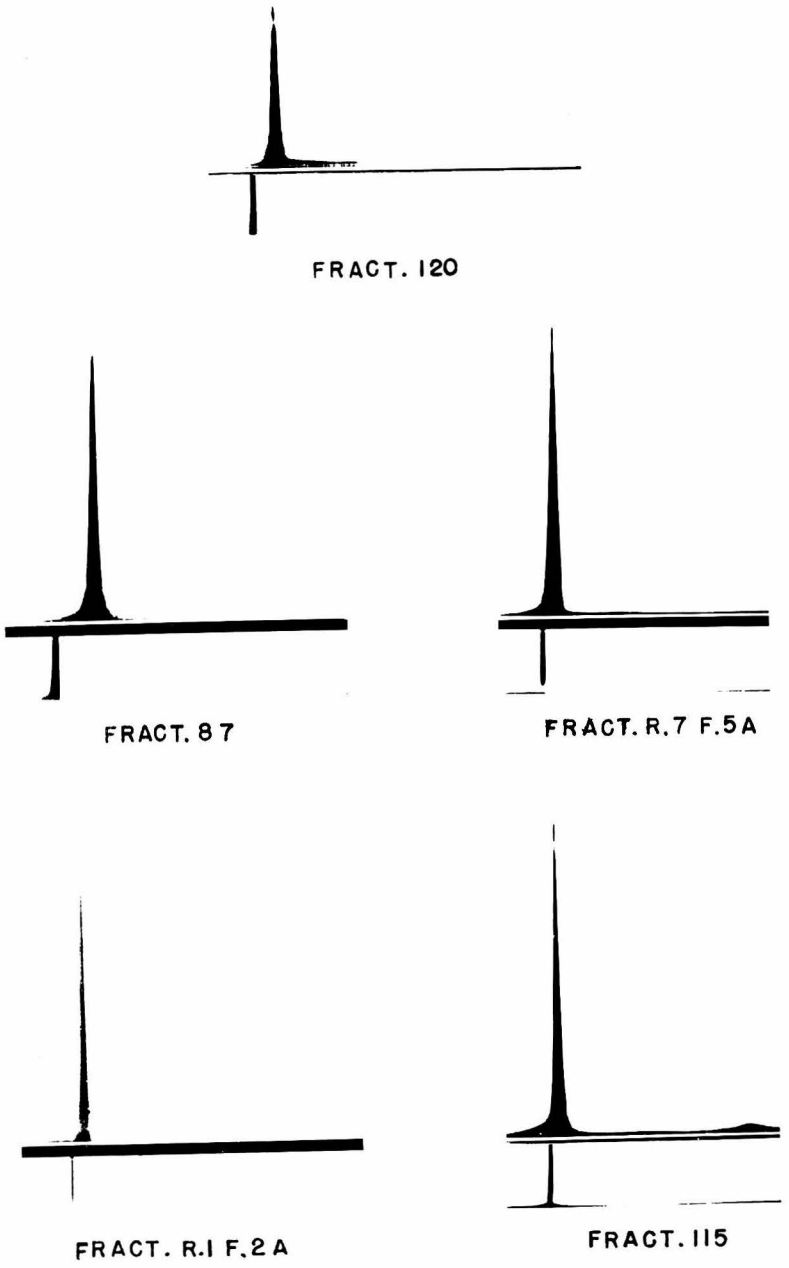


Fig. 1. Electrophoretic Patterns (initial and ascending boundaries after 3 hours at 1.35°) obtained for A-substance preparations isolated by different methods from commercial hog gastric mucin. Acetate buffer, pH 3.9. Fract. 120 and Fract. 115, alcohol fractionation. Fract. 87, electrodialysis and alcohol fractionation. Fract. R.7 F.5A, alcohol fractionation and formamide treatment. Fract. R.1 F.2A, sodium sulfate fractionation.

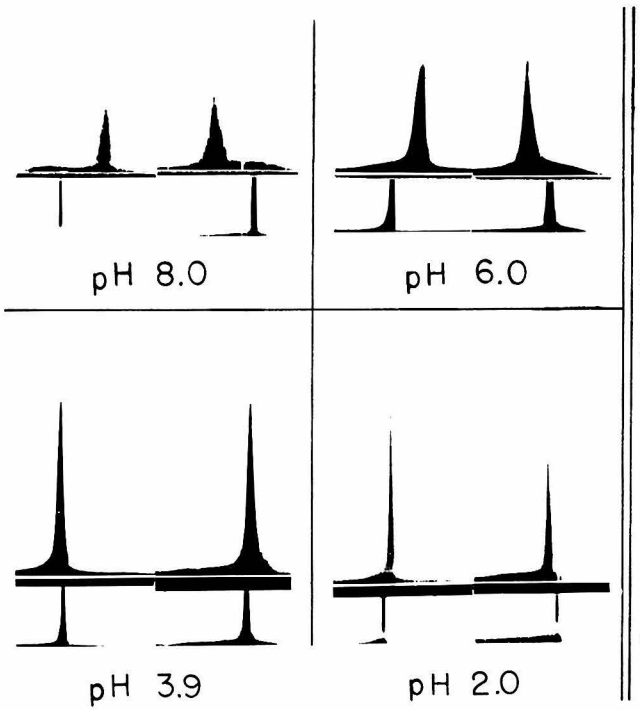


Fig. 2. Electrophoretic patterns obtained for an A-substance preparation (Fract. 111) at various hydrogen ion activities. Migration time: 3 hours at 1.35°. The initial, ascending (left), and decending (right) boundaries are shown.

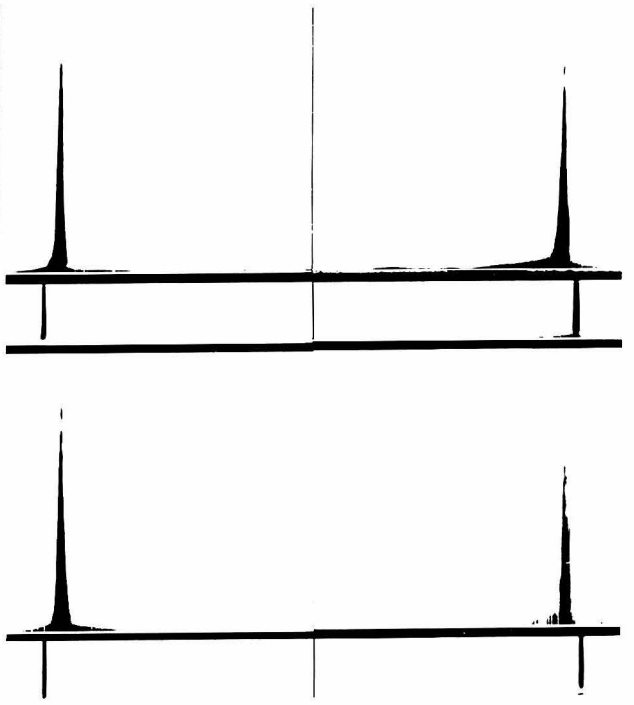

Fig. 3. Electrophoretic patterns for two preparations of A-substance (from hog gastric mucin) of greatly different properties. Upper: Initial and 3 hour boundaries of Fract. 127. Lower: initial and 3 hour boundaries of Fract. R.18 F.10.Acetate buffer pH 3.9.

at pH 2, 6, and 8. Figure 2 illustrates the qualitative similarity of the curves obtained for a typical fraction in experiments at pH 2.0, 3.9, 6.0 and 8.0. The type of broadening at the base that was occasionally observed for the less homogeneous fractions is shown in the pattern for Fraction 120 in Figure 1. This pattern suggests that a spectrum of minor components may be present, one differing from another only slightly in mobility. There is no apparent correlation between the electrophoretic mobility at any pH and the serological activity of a given A-substance preparation.

Figure 3 shows in a striking way the small degree of significance which can be attached to the finding that an A-substance fraction is electrophoretically homogenous. The patterns reproduced there are for two preparations which differ greatly in the amount of purification to which they have been subjected and in their serological activities. Fraction 127 is three to four times less potent in inhibiting hemolysis than is Fraction R.18 F.10 (8). The fractions differ markedly also in their absorption spectra (7,8) and in homogeneity with respect to solubility in alcohol-water solutions and in solutions of low pH and minimum ionic strength. The electrophoresis patterns for these fractions are essentially identical. It is to be noted that the similarity extends even to their mobilities. As mentioned above, Bendich, Kabat

and Bezer have reported (3) that A and O-substances have the same mobilities at pH 7.4. It is interesting that the magnitude of the mobilities found by these authors and those found by us at pH 8.0 (Table 1) are in excellent agreement.

Despite the finding that electrophoretic patterns are not generally useful in distinguishing among A-substance preparations, it is true that useful information can occasionally be obtained by this technique. A highly purified A-substance preparation, R.20 F.2, isolated by Holzman and Niemann (8) when examined electrophoretically at pH 3.85 was found to contain about 10 to 15 per cent of a second, somewhat faster moving component. Thus it is possible that with more highly purified A-substance preparations electrophoretic analysis may be useful.

Perhaps the most surprising feature of the experiments is the finding that no real isoelectric point for A-substance lies between pH 8.0 and pH 2.0 (see Table 1). Although we observed essentially no migration for two of the preparations at pH 3.9, the reproducibility of the rates of migration is not great. More important as evidence that anodic migration is a characteristic feature of A-substance or a complex thereof is the observation that considerable migration in this sense took place at pH 2.0, even in the case of those two fractions the sign of whose migration at pH 3.9 was doubtful (see Table 1). This is an unusual

situation. Silk fibroin is isoelectric at pH 2.0 to 2.4 (10) and is in this respect almost unique among the proteins that have been studied. Tiselius (11) and Herriott (12) have reported that pepsin from swine gastric mucosa does not seem to have an isoelectric point, in that it migrates anodically even in hydrochloric acid solution. Blood group A-substance from swine gastric mucosa appears to behave similarly.

## Acknowledgment

The authors wish to thank Drs. George Holzman and Stanley Swingle for their assistance during the course of this investigation.

## Summary

The electrophoretic behavior of a large number of A-substance preparations has been determined over a pH range from 2 to 8. Nearly all of the A-substance preparations were found to be electrophoretically homogeneous despite a five-fold variation in serological activity and other differences demonstrable by physical and chemical methods. In fact only with the lesser active and the most active preparations did electrophoretic analysis reveal a second component. In general the mobilities of the preparations were of the order of $1 \times 10^{-5}$ $cm^2 . volt^{-1} . sec^{-1}$ and there was no apparent correlation between the electrophoretic mobility of a preparation and its serological activity. All of the A-substance preparations migrated anodically over a pH range of 2 to 8.

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## F. SOME OBSERVATIONS ON THE REACTION OF BLOOD GROUP A-SPECIFIC SUBSTANCE WITH LEAD TETRAACETATE

### Introduction

Lead tetraacetate has the property of cleaving certain carbon to carbon bonds in α-glycols, α-hydroxy acids, and some nitrogenous compounds such as α-diamines, α-amino alcohols, and α-amino acids*.

Since blood group A-substance contains a large amount of carbohydrate (60-70%) and amino acids (40-30%), it was thought that it would be of interest to determine whether the A-substance preparations we had at hand would react with lead tetraacetate, and, if they did, what the rate of reaction and the nature of the products might be. At the same time Dr. Brown carried out a similar investigation with periodate (2); and it was thought that a comparison of the nature of the products obtained and the extent of reaction of these two oxidants would also be worthwhile, since, in many respects lead tetraacetate and periodate are similar in their oxidation specificity (1,3).

Lead tetraacetate functions best in a non-aqueous medium such as acetic acid or benzene since it hydrolyzes in

* Lead tetraacetate will also react with other classes of compounds than those described above (1), but the presence of many of these other classes of compounds in A-substance is extremely unlikely.

water relatively rapidly. On the other hand, A-substance was found to be relatively insoluble in acetic acid. Therefore the first factor which had to be investigated was the effect of water in acetic acid on the solubility of A-substance, and on the rate of decomposition of the lead tetraacetate by water itself. It was found that 83% acetic acid-17% water was the most satisfactory solvent in which to carry out the oxidation.

Another factor which was investigated was the variation of the amount of lead tetraacetate consumed with several A-substance preparations of widely different "purity". No significant difference was found although only a relatively few samples were tested. The limiting amount of lead tetraacetate consumed was generally about 3.2-3.3 millimoles/gram of A-substance. If it is assumed that the oxidation with which we are dealing is of the general type

$$\begin{array}{l} >C\text{-}OH \\ \quad | \\ >C\text{-}OH \end{array} + Pb(OAc)_4 \longrightarrow \begin{array}{l} >C{=}O \\ \\ >C{=}O \end{array} + \begin{array}{l} Pb(OAc)_2 \\ \\ 2HOAc \end{array}$$

the above quantity of lead tetraacetate would correspond to one pair of adjacent groups being oxidized per 300 molecular weight units. If the further assumption is made that A-substance is 60% carbohydrate of a unit weight of approximately 180, it would appear as a first approximation that on the average each carbohydrate residue has one pair of adjacent oxidizable -OH groups. The results of the periodate

oxidations presented in (2) are perhaps not so clear cut since in those experiments there was a rapid initial oxidation over a 5 to 15 minute period of about 2.5 millimoles of periodate/gram followed by a less rapid uptake for the next 4 hours in which a total of about 3.2-3.3 millimoles of periodate/gram were used, and then a still slower oxidation over the next 20 hours (see Table I).

The amount of non-dialyzable material after several oxidations with lead tetraacetate was determined; in one experiment this material amounted to 70-75% (by weight) of the originally non-dialyzable material taken; in another experiment it was 90%. With periodate oxidation the amount of recoverable material varied between 50 and 70%. In both oxidations the material which was recovered was characterized by a lack of solubility in water or 0.9% NaCl. This might be expected if hydroxyl groups were converted into aldehyde groups.

The reducing power of several of the products of oxidation were determined with the alkaline copper reagent of Shaffer-Hartmann (Somogyi modification) (4,5), and, as would be expected, a large increase in reducing power was obtained. Qualitatively the products obtained by tetraacetate oxidation were different than those obtained by periodate oxidation in that they were completely or nearly completely soluble in the hot alkaline reagent used, whereas the materials obtained

by even partial periodate oxidation were largely insoluble (2). The results of several tests to determine reducing power are shown in Table IV.

The observations reported in this section do not permit any conclusions to be drawn as to the structure of A-substance. In fact it is possible that the reaction observed with lead tetraacetate may be only a reaction with impurities, but this does not seem likely.

## Experimental and Results

Preparation of Reagents.- Lead tetraacetate was prepared by the method described in (6) from $Pb_3O_4$, acetic acid, and acetic anhydride. Glacial acetic acid (Grasselli C.P.) was purified by refluxing over 20 grams of lead tetraacetate and then distilling (in an all glass apparatus). The first 20% of the distillate was discarded. The next fraction of 1 liter was used for the preparation of solutions.

The standard lead tetraacetate solution was prepared by dissolving 15 grams of lead tetraacetate in 450 ml. of glacial acetic acid and then filtering through a fine sintered glass funnel to remove a small amount of solid $PbO_2$. The solution was standardized iodimetrically against standard sodium thiosulfate. The formality of the lead tetraacetate solution was about 0.07 F.

Experiment 1 was performed to determine the effect of water on the solubility of A-substance in acetic acid, on the rate

of oxidation of A-substance and on the rate of hydrolysis of lead tetraacetate. 200-250 mg. of A-substance (Sample 71*) was shaken with the appropriate small quantity of water or acetic acid (12-20 ml.) for several hours (in tightly stoppered glass bottles) and then the necessary amount of standard lead tetraacetate solution in glacial acetic acid was added. The initial concentration of the lead tetraacetate was 0.069 F. The solutions were shaken on the shaking machine for the duration of the experiment except for the time necessary to withdraw an aliquot to determine the amount of tetraacetate which had been consumed. The tetraacetate which had been consumed was determined iodimetrically with thiosulfate after the addition of KI. Controls were set up at the same time in exactly the same manner except that the A-substance was omitted. The temperature at which these experiments were carried out was 22-24°. The results which were obtained are presented below; duplicate experiments were done at 100% and 83% acetic acid (on different days). For comparison the results obtained by Dr. Brown for the oxidation of the same compound with sodium periodate are included in Table I (2).

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* Fraction 71 was a fraction which had been obtained from mucin by the phenol procedure, followed by sodium sulfate fractionation, followed by electrodialysis. It was insoluble upon electrodialysis (see section II-A for comparison with other fractions).

Table I

Oxidation of Fraction 71 with Lead Tetraacetate and Sodium Periodate

| Time Sample Taken After Addition of Oxidizing Agent | Glacial Acetic Acid[a] | | 83% Acetic Acid[b] | | 71% Acetic Acid[c] | Periodate Oxidation |
|---|---|---|---|---|---|---|
| | millimoles of Lead Tetraacetate Consumed per Gram A-Substance | | | | | Millimoles-$IO_4$/Gram A Substance |
| | Run I | Run II | Run I | Run II | Run I | |
| 5 min. | -- | -- | -- | -- | -- | 2.00 |
| 10 min. | -- | -- | -- | -- | -- | 2.40 |
| 15 min. | -- | -- | -- | -- | 1.88 | 2.60 |
| 30 min. | 0.03 | 0.19 | 1.81 | 1.78 | 2.46 | 2.68 |
| 1 hour | 0.12 | 0.33 | 2.44 | 2.40 | 2.98 | 2.86 |
| 2 hours | 0.18 | 0.52 | 2.91 | 2.90 | 3.26 | 3.30 |
| 3 hours | -- | -- | -- | -- | 3.26 | -- |
| 4 hours | 0.48 | 1.01 | 3.24 | 3.30 | 3.09 ? | 3.30 |
| 6 hours | 0.81 | 1.32 | 3.29 | 3.30 | -- | 3.52 |
| 8 hours | 1.08 | 1.57 | 3.21 | 3.30 | -- | -- |
| 24 hours | -- | 2.6 | -- | 2.86? | -- | 3.54 |

a. A slightly viscous suspension was obtained. Most of the material did not go into solution. A blank showed no change over the 8 hour period.

b. A turbid, viscous solution was obtained. The values are calculated using a small blank correction determined from a control.

c. A slightly viscous, turbid solution was obtained. The tetraacetate in the control blank decomposed more rapidly than that in the presence of A-substance. A satisfactory blank correction was obtained from that found for 83% acetic acid by assuming the rate of hydrolysis of tetraacetate was twice as rapid in 71% acetic acid as in 83% acetic acid.

From this experiment it was decided that 83% acetic acid was a satisfactory solvent in which to carry out the oxidations. The results were not reproducible in glacial acetic acid, undoubtedly due to the low solubility of A-substance in this solvent. In 71% acetic acid the rate of oxidation was very much more rapid, in fact it appeared to be almost exactly twice as fast as in 83% acetic acid, but the blank was large and it was difficult to obtain a satisfactory blank correction. 83% acetic acid appeared to be a very suitable solvent; the blank correction was nearly negligible up to 4 hours, and the results obtained on two runs were reproducible. The extent of oxidation obtained in 71% and 83% acetic acid with lead tetraacetate was equivalent.

Experiment 2 was performed to determine the effect that the concentration of lead tetraacetate would have upon the extent of oxidation and upon the rate of oxidation. Fraction 71 was used as the A-substance preparation. The solvent was 83% acetic acid. As before, aliquots were taken at intervals of time and the extent of oxidation was determined. (A) contained 225 mg. of fraction 71, 10 ml. of water and 50 ml. of 0.064 F $Pb(OAc)_4$; at the end of the experiment about 1/6 of the initial lead tetraacetate had been consumed. (B) contained 203 mg. of fraction 71, 10 ml. of water and 50 ml. of 0.032 F $Pb(OAc)_4$; at the end of the experiment about 45% of the original lead tetraacetate had been consumed. The data obtained are presented in Table II.

Table II

Effect of Amount of Lead Tetraacetate upon the Rate and Extent of Oxidation of A-Substance

| Time Sample Taken After Addition of Lead Tetra-Acetate | Millimoles of Lead Tetraacetate Consumed per Gram of A-Substance (Fraction 71) | |
|---|---|---|
| | A $I_o$ = 0.0533 F. | B $I_o$ = 0.0266 F. |
| 5 min. | 0.75 | 0.44 |
| 10 min. | 1.16 | 0.67 |
| 15 min. | 1.54 | 0.97 |
| 20 min. | 1.74 | 1.07 |
| 30 min. | 1.96 | 1.32 |
| 45 min. | 2.27 | 1.59 |
| 1 hour | 2.44 | 1.72 |
| 2 hours | 2.85 | 2.17 |
| 3 hours | 3.08 | 2.46 |
| 4 hours | 3.27 | 2.65 |
| 14 hours | -- | 3.34 |

The results of this experiment indicate that the rate of oxidation of A-substance depends upon the amount of lead tetraacetate which is present, but the extent of oxidation appears to be independent of the amount which is present (provided that an excess is present).

Experiment 3 was performed to determine the amount of lead tetraacetate which would be consumed by several preparations of A-substance, the amount of material which would be recovered after oxidation and dialysis against water, and the nature of the recovered material, particularly its reducing power. Four different samples of A-substance were used: Fraction 71, Fraction 66, Fraction 65 and Fraction 87. These fractions differed in solubility and method of preparation

as well as in serological activity (see section II-A)*.

The reduction capacities of several of the products of the lead tetraacetate oxidation of A-substance toward an alkaline copper solution were determined. These determinations were done by Dr. Brown. The procedure of Shaffer and Hartmann (4) as modified by Somogyi (5) was employed. The extent of reaction with the alkaline copper reagent was calculated with glucose as a standard and is expressed as "% reduction as glucose". The results of these experiments are shown in Tables III and IV.

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* Fraction 65 is not described in Section II-A. It was obtained by ethyl alcohol fractionation of a hog-gastric mucin solution in 90% phenol. It was precipitated in 80% phenol-10% water-10% ethanol solution. Subsequently it was precipitated by 65% aqueous ethanol, 50% aqueous ethanol, and then 65% aqueous ethanol. It was the insoluble residue upon electrodialysis. Its activity in the inhibition of isoagglutination test was low; i.e. it has a titer of about 20 $\pm$ 10. Fraction 66 was one of the more active preparations; its extinction coefficient in the range 250-280 mμ is low, $E^{1}_{1\%}$cm.(in water) = 0.9-1.0 (7,8).

Table III

The Amount of Lead Tetraacetate Consumed by Several A-Substance Preparations and The Amount Non-Dialyzable Material Remaining After Oxidation

| Time Sample Taken After Addition of Lead Tetra-Acetate | millimoles of Lead Tetraacetate Consumed per Gram of A-Substance and Percentage Non-Dialyzable Material Recovered (in parentheses) | | | |
|---|---|---|---|---|
| | Fraction 66* | Fraction 71* | Fraction 87** | Fraction 65** |
| 10 min. | 0.99 | 0.92 | -- | -- |
| 31 min. | 1.91 (83%) | 1.94 (85%) | -- | -- |
| 243 min. | 3.22 (75%) | 3.19 (71%) | -- | -- |
| 360 min. | 3.26 | 3.27 | -- | -- |
| 450 min. | | | 3.15 (90%) | 3.21 |

* 400 mg. of A-substance in 20 ml. of water and 100 ml. of 0.075 F $Pb(OAc)_4$ (in glacial acetic acid).

** 200 mg. of A-substance in 5 ml. of water and 25 ml. of 0.063 F $Pb(OAc)_4$ (in glacial acetic acid).

Table IV

Reduction Capacities of A-Substance Preparations after Oxidation with Lead Tetraacetate

| A-Substance Preparation | Time Oxidized with Lead Tetraacetate (minutes) | Lead Tetraacetate Consumed | % Reduction as Glucose |
|---|---|---|---|
| 66 | 0 | -- | 0.76<br>0.71 |
| | 31 | 1.91 | 8.35<br>8.54 |
| | 243 | 3.22 | 11.7<br>12.2 |
| 71 | 0 | -- | 0.79<br>0.87 |
| | 31 | 1.94 | 8.82<br>8.66 |
| | 243 | 3.19 | 11.2<br>11.4 |

The data obtained in this experiment indicate that (a ) several dissimilar A-substance preparations were oxidized to a very similar degree by lead tetraacetate, (b) a large proportion (70-75% minimum), of the product after oxidation is non-dialyzable, and (c) the reducing power or the non-dialyzable product as measured by the Sumogi test increases markedly.

The insolubility of the products in water or saline prevented the determination of inhibition of hemolysis or inhibition of iso-agglutination titers of the oxidized products.

## Summary

The oxidation of A-substance with lead tetraacetate has been investigated. 83% glacial acetic acid -17% water was found to be a satisfactory solvent in which to carry out the oxidation. The rate of oxidation was found to be dependent upon the water concentration and upon the concentration of lead tetraacetate. However, the extent of oxidation appeared to be independent of these variables. Several A-substance preparations of widely differing activity were found to be oxidized to a similar degree by lead tetraacetate. A maximum of 25-30% of the originally non-dialyzable A-substance becomes dialyzable after oxidation. The non-dialyzable product is insoluble in water and exhibits a large increase in reducing capacity as measured by the copper reduction test.

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# Systematic Qualitative Tests for Certain Acidic Elements in Organic Compounds

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**A system for the detection of nitrogen, chlorine, bromine, iodine, arsenic, sulfur, and phosphorus in a single 1-mg. sample of an organic compound and for carbon and fluorine in separate 1-mg. samples is described. The procedures are applicable to compounds whose boiling points are greater than approximately 60° C. and any of the above elements can be detected when present to the extent of 1 to 5% of the sample weight.**

ONE of the most sensitive and reliable qualitative tests for the presence of nitrogen in organic compounds is the modified Emich test (*1*, *4*, *5*) described by Johns (*3*). In this test the sample is pyrolyzed in the presence of calcium oxide and zinc and the liberated ammonia is detected with the aid of litmus. Because of the general applicability of this test it appeared desirable to exploit the pyrolytic technique, so that a single ignition could be used not only for the detection of nitrogen but also for the systematic qualitative identification of certain other acidic elements. Early in 1944 a system was developed in these laboratories which provided for the qualitative detection of nitrogen, chlorine, bromine, iodine, sulfur, phosphorus, and arsenic; an outline of this system is shown in Table I.

## SYSTEMATIC TESTS

**A. Combustion of Sample and Test for Nitrogen.** A combustion tube is prepared from a freshly cleaned 12-cm. length of Pyrex tubing 3 mm. in outside diameter by constricting a 2- to 3-mm. portion of the tube to an inside diameter of 0.5 to 0.7 mm. at a distance of 3 cm. from one end of the tube. Acid-washed and freshly ignited asbestos is introduced into the long arm of the combustion tube and is pressed firmly into the near side of the constriction with the aid of a clean glass rod until a plug 2 to 3 mm. in length is obtained.

A mixture of equal parts of calcium oxide, ordinarily prepared by the ignition of calcium oxalate, and 80-mesh zinc powder (sulfur- and arsenic-free) is introduced in small portions, and with tapping, into the long arm of the combustion tube until a 20-mm. column of the mixture is in position immediately adjacent to the asbestos plug. The liquid or solid sample contained in a 2- to 3-mm. segment of a 0.5-mm. inside diameter thin-walled capillary tube 10 to 15 mm. in length is placed on top of the calcium oxide–zinc column and the end of the long arm of the combustion tube is sealed with the aid of a forceps. The open end of the combustion tube is inserted into a 4-cm. length of 5-mm. outside diameter Pyrex tubing for a distance of approximately 1 cm. and in the other end of the sleeve is placed a strip of red litmus paper approximately 3 mm. in width. Since ordinary red litmus paper may not be sufficiently sensitive, strips of ordinary blue or neutral litmus paper are suspended in distilled water and just enough 1 *F* perchloric acid is added to change the color of the paper to pink. The papers are then washed with distilled water until a treated paper when pressed against a piece of neutral litmus paper will not turn the latter red. The prepared papers are pressed between soft filter papers and then stored in a moist condition in a sealed container.

The combustion tube assembly prepared as described above is placed on a wire gauze bearing a 10 × 30 mm. hole, in such a way that the portion of the tube bearing the calcium oxide–zinc mixture is over the hole. The asbestos plug in the combustion tube is first heated to a dull red glow with a small sharp flame from a Bunsen burner, and the burner is moved toward the sealed end of the tube so as next to heat the calcium oxide–zinc zone to glowing and, finally, the space bearing the sample. This heating operation need not consume more than 2 minutes. The presence

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**Table I. Systematic Detection of Nitrogen, Halogen, Arsenic, Sulfur, and Phosphorus in Organic Compounds**

A. Fuse 1-mg. sample with Zn and CaO. Test gas with litmus
Gas: $NH_3$ (blue litmus color: nitrogen present) Residue: $CaI_2$, $CaBr_2$, $CaCl_2$, $Ca_3(PO_4)_2$, CaO, $ZnI_2$, $ZnBr_2$, $ZnCl_2$, ZnS, $Zn_3As_2$, $Zn_3(PO_4)_2$, Zn
B. Leach residue with water
Solution: $Ca^{++}$, $I^-$, $Br^-$, $Cl^-$. Treat separate portions as indicated in B-1, B-2, and B-3

Residue: $Ca_3(PO_4)_2$, CaO, ZnO, ZnS, $Zn_3As_2$, $Zn_3(PO_4)_2$, Zn
C. Add $HClO_4$
Gases: $H_2S$, $AsH_3$
C-1 Collect gases separately in $HgCl_2$ and Pb $(C_2H_3O_2)_2$

Solution: $H_3PO_4$, $Zn^{++}$, $Ca^{++}$, $HClO_4$
D. Add portion to $(NH_4)_2MoO_4$ and $HNO_3$ on test paper. Add benzidine and $NaC_2H_3O_2$
Blue color: phosphorus present

| B-1. Add $AgNO_3$ and $HNO_3$ | B-2. Add chloramine-T, fluorescein, and acetic acid | B-3. Add starch, $HC_2H_3O_2$, and $NaNO_2$ |
|---|---|---|
| Ppt.: AgI, AgBr, AgCl (halogen present) | Pink color: eosin, tetraiodofluorescein (bromine and/or iodine present) | Starch-iodine color (iodine present) |

Ppt.: PbS (black, sulfur present), $HgAsH_2Cl$ (yellow, arsenic present)

of nitrogen in the sample is indicated by a change in color from pink to blue at the end of the litmus strip nearer the open end of the combustion tube. Any color obtained should be compared with that obtained from a blank test. Although exposed strips of litmus very quickly lose their blue color when allowed to stand in contact with air, comparisons may be made as long as 15 minutes after a combustion if the paper strips are placed between two microscope slides immediately after exposure.

**B-1. Tests for Halogen.** The short arm of the combustion tube is sealed and the long arm opened near the tip. The newly sealed end of the tube is warmed and as the tube cools several drops of water are added, so that the water is slowly drawn into the calcium oxide–zinc mixture. After 10 to 15 seconds the tube is centrifuged to collect the aqueous solution in the closed end of the tube. The combustion tube is cut about 5 mm. above the water meniscus and the aqueous solution reserved for subsequent tests. The solid residue contained in the other portion of the tube is carefully warmed to remove any water and set aside for subsequent operations.

In a 1- to 1.5-mm. thin-walled capillary a 4- to 5-mm. column of the aqueous extract is allowed to react with a 3- to 4-mm. column of a solution 0.5 *F* in silver nitrate and 3 *F* in nitric acid. The formation of a white or light yellow precipitate within 30 seconds indicates the presence of chloride, bromide, or iodide. With properly prepared reagents, blank tests will give only a very slight turbidity.

**B-2. Test for Bromide or Iodide.** If a positive test for halogen is obtained (B-1) a 5- to 6-mm. column of the aqueous extract is placed in a 1- to 1.5-mm. capillary tube and a 3- to 4-mm. column of freshly prepared 0.0035 *F* aqueous chloramine-T is added, followed by a 3- to 4-mm. column of a 50% aqueous ethanol solution 0.00075 *F* in fluorescein and 0.12 *F* in acetic acid and then another 3- to 4-mm. column of 0.0035 *F* chloramine-T. When viewed against a white background, the formation of a pink color is indicative of the presence of bromide or iodide. No color should be obtained in blank tests on properly prepared reagents.

**B-3. Test for Iodide.** If a positive test for bromide or iodide is obtained (B-2), a 3- to 4-mm. column of the aqueous extract is placed in a capillary tube and to it is added a 3- to 4-mm. column of a reagent freshly prepared by mixing equal columns of 0.5% starch solution, 1 *F* sodium nitrite, and 6 *F* acetic acid. The formation of a blue or black color indicates the presence of iodide. No color should be obtained in blank tests.

**C-1. Test for Sulfur and Arsenic.** Cut open the combustion tube near the middle of the fusion mixture and by gentle tapping transfer all the mixture into the 3 × 15 mm. depression of a culture-type microscope slide. A glass ring 18 mm. in inside diameter and 15 mm. high is then placed around the depression. With a wax pencil a line is drawn across the center of a cover slip and on one side of the line is placed a drop of 1 *F* lead acetate and on the other a drop of 0.1 *F* mercuric chloride. The cover slip is inverted and placed in position on the glass ring immediately after the addition of 4 drops of 9 *F* perchloric acid to the fusion mixture. The formation of a brown or black precipitate in the lead acetate drop after 2 to 3 minutes is indicative of the presence of sulfur; however, in the absence of arsenic the presence of sulfur may also be indicated by the formation of a white precipitate ($HgCl_2 \cdot 2HgS$) in the mercuric chloride drop.

The probable presence of arsenic is indicated by the formation of a yellow or brown precipitate in the drop of mercuric chloride. If the original sample contains tripositive or elementary

**Table II. Nitrogen Test**

Test Equivalent to That Given by Nitrogen, Taken as Ammonium Chloride

| | A. 5 to 10 γ N | B. 10 to 30 γ N | C. More than 30 γ N |
|---|---|---|---|
| Amines | Arsanilic acid | *n*-Heptylamine | 2-Amino-*n*-octane |
| | 2-Aminopyridine | *N*,*N*-diethylcyclohexylamine | *o*-Bromoaniline |
| | Quinoline ethiodide | Tris(β-chloroethyl)amine | 2-Amino-4-chlorothiophenol hydrochloride |
| | | *N*-ethylpyrrole | *N*-methyl-ethylamine hydrochloride |
| | | Pyridine | Dibenzylamine |
| | | 2-Chloropyridine | *N*-ethyl-diethanolamine hydrochloride |
| | | 2-Bromopyridine | *N*-methyl-bis(β-chloroethyl)-amine |
| | | 3-Bromopyridine | *N*-ethyl-bis(β-chloroethyl)-amine |
| | | Tetra-*n*-butylammonium iodide | *N*,*N*-dimethyl-*p*-bromoaniline |
| Amides | ... | *n*-Heptamide | Propionamide |
| | | *N*-acetyl-α-naphthylamine | *n*-Butyramide |
| | | *m*-Acetamidobenzoic acid | Chloroacetamide |
| | | *N*,*N*-diethylchloroacetamide | Trichloroacetamide |
| | | | *N*-acetyl-β-methoxyethyl-amine |
| | | Thioacetanilide | *N*,*N*-diethylacetamide |
| | | Ethyl *N*-phenylcarbamate | *N*,*N*-amylsuccinimide |
| | | Ethyl *N*,*N*-di-*n*-butylcarbamate | *n*-Butyloxamate |
| | | Benzenesulfonamide | *N*-methylurea |
| | | *o*-Toluenesulfonamide | *N*-acetylurea |
| | | | *N*-acetyl-*N*′-methylurea |
| | | | *N*,*N*′-di-*n*-butylthiourea |
| | | | Ethyl carbamate |
| | | | *n*-Propyl carbamate |
| | | | *n*-Butyl carbamate |
| | | | *n*-Amyl carbamate |
| | | | β-Ethoxyethyl carbamate |
| | | | Ethyl *N*-methylcarbamate |
| | | | Ethyl *N*-ethylcarbamate |
| | | | Ethyl *N*,*N*-diphenylcarbamate |
| | | | Ethyl *N*-phenyl-*N*-benzylcarbamate |
| | | | Sodium *N*,*N*-diethyldithiocarbamate |
| | | | *p*-Aminobenzenesulfonamide |
| | | | Dichloramine-T |
| Nitriles | ... | *n*-Butyronitrile | Acetonitrile |
| | | Chloroacetonitrile | Furonitrile |
| | | α-Bromobenzylcyanide | Succinonitrile |
| | | Benzoylacetonitrile | Methylcyanoacetate |
| | | | *p*-Nitrophenylacetonitrile |
| | | | β-Hydroxy-γ-chloro-*n*-butyronitrile |
| | | | Ethyl β-phenyl-α-cyanoacrylate |
| | | | *N*,*N*-di-*n*-butylcyanamide |
| | | | *N*,*N*-diallylcyanamide |
| | | | Dicyandiamide |
| | | | Cyanogen bromide |
| Oximes | ... | *n*-Butyraldoxime | *n*-Heptaldoxime |
| | | α-Benzil monoxime | Acetone oxime |
| | | | Cyclohexanone oxime |
| | | | Biacetyl monoxime |
| Nitro compounds | ... | Chloropicrin | Bromopicrin |
| | | | 2-Methyl-2-nitro-1-propanol |
| | | | *o*-Dinitrobenzene |
| | | | 4,4′-dinitrobiphenyl |
| | | | *m*-Nitrophenylarsonic acid |
| Nitroso compounds | *N*,*N*-diphenylnitrosoamine | Nitrosobenzene | 2-Nitroso-3-hydroxy-toluene |
| | | *N*-methyl-*N*-phenylnitrosoamine | *o*-Nitrosonitrobenzene |
| | | | *N*-nitrosopiperidine |
| | | | Ethyl *N*-nitroso-*N*-ethylcarbamate |
| Nitrates, nitrites | ... | Ethyl nitrate | Butyl nitrate |
| | | | Sodium nitrate |
| | | | Sodium nitrite |
| Miscellaneous heterocyclic compounds | Phenarsazine chloride | Benzothiazole | 2-Aminobenzothiazole |
| | | Benzoxazole | *o*-Benzoicsulfimide |
| | | | 1-Acetyl-2-thiohydantoin |
| Miscellaneous | Formyl diphenylamine | Benzalaniline | Ethylene thiocyanate |
| | Azobenzene | Benzalmethylamine | Potassium thiocyanate |
| | | Benzalazine | 1-Aminoguanidine sulfate |
| | | *N*,*N*-diphenylformamidine | |
| | | Diazoaminobenzene | |

phosphorus, some phosphorus may be present as calcium or zinc phosphide after fusion and when the perchloric acid is added to the fusion mixture phosphine may be liberated and may cause an orange-brown precipitate to form in the mercuric chloride. For this reason an orange or brown precipitate in the mercuric chloride drop is not necessarily proof of arsenic, though most of the tripositive phosphorus compounds examined do not give a detectable precipitate. With suitably selected zinc metal no significant amounts of sulfur or arsenic should be found in blank tests.

**D. Test for Phosphorus.** A test paper is prepared by placing a few drops of a solution 0.03 *F* in ammonium molybdate, $(NH_4)_6Mo_7O_{24}$, and 3.9 *F* in nitric acid on a Whatman No. 1 or similar filter paper and drying the paper at 80° to 90°. A 5- to 8-mm. column of the perchloric acid solution remaining after the operations described in C-1 and contained in a 1- to 1.5-mm. capillary tube is transferred to an impregnated section of the test paper. Then in succession are added a 5-mm. column of a solution, 0.0027 *F* in benzidine and 1.8 *F* in acetic acid, and an equal volume of saturated aqueous sodium acetate. The presence of phosphorus is indicated by the formation of a blue spot; this should always be compared with that obtained in a blank test.

### RESULTS OBTAINED WITH SYSTEMATIC SCHEME

**A. Nitrogen Test.** Experiments have shown that this test will detect as little as 2 to 3 micrograms of nitrogen taken as an ammonium salt or about 5 micrograms taken as a nitrate. With suitably prepared reagents a blank test will give at the most only a short zone of pale lavender color on the litmus paper.

A large number of compounds were tested for the presence of nitrogen in order to determine whether or not the test could be relied upon with nitrogen-containing compounds of widely varying structure.

In general a 1-mg. sample of the substance was taken and the test recorded as: (A) equivalent to test given by 5 to 10 micrograms of nitrogen taken as ammonium chloride; (B) equivalent to test given by 10 to 30 micrograms of nitrogen taken as ammonium chloride; and (C) equivalent to test given by more than 30 micrograms of nitrogen taken as ammonium chloride. The results of these tests are given in Table II.

In no case was a negative test obtained despite the relatively great variation in the types of the compounds tested. It therefore appears that the test described is generally applicable.

**Halogen Tests (B-1, B-2, B-3).** The tests for the halogens are based upon the assumption that only one halogen is present; if more than one halogen is present, only that one which has the higher atomic weight will be specifically detected. The sensitivity of the halide tests is limited by the purity of the reagents employed. However, no difficulty was experienced in preparing reagents which gave only a very slight turbidity with the silver nitrate–nitric acid reagent and no test for bromine and iodine. Satisfactory positive tests were obtained with 5-microgram quantities of chloride, bromide, and iodide in procedure B-1, with 5 micrograms of bromide and iodide in procedure B-2, and with 5 micrograms of iodide in procedure B-3. With a 1-mg. sample satisfactory halide tests were obtained with procedure B-1 for all halogen-containing compounds examined. In all cases where bromine or iodine was present a satisfactory test was obtained using procedure B-2, and when iodine was present a satisfactory test was obtained using procedure B-3. When bromine or iodine were absent the tests in procedures B-2 and B-3 were uniformly negative. The compounds listed in Table III were among those examined.

**Table III. Halogen Tests**

| | | |
|---|---|---|
| **Chlorine-Containing Compounds** | | |
| *n*-Butyl chloride | β - Chloroethyl - *p* - toluenesulfonate | Chloroform |
| 2-Chloropyridine | Dichloramine-T | 1,2-Dichloro-*n*-butane |
| 2,3-Dichlorodioxane | Bis(β-Chloroisopropyl) ether | Diphenylselenium dichloride |
| 1,1,1-Trichloroethane | Phenyl mercuric chloride | Tetrachloroethylene |
| *p*-Toluenesulfonyl chloride | Trichloroacetamide | *N*-methyl-bis(β-chloroethyl)amine |
| *N*-ethyl-bis(β-chloroethyl)amine | Tris(β-chloroethyl)amine | α-Chloroacetophenone |
| Bis(β-chloroethyl) sulfide | Bis(β-chloroethyl) sulfone | Chloropicrin |
| Ethyl dichloroarsine | β-Chloroethyl dichloroarsine | 1,1,2,2-Tetrachlorodinitroethane |
| **Bromine-Containing Compounds** | | |
| Benzalacetophenone dibromide | *o*-Bromoanisole | *N*,*N*-dimethyl-*p*-bromoaniline |
| Bromopicrin | 2-Bromopyridine | 3-Bromopyridine |
| 2-(β-butoxyethoxy)-ethyl bromide | Isobutylene bromide | 4,4′-Dibromodiphenyl sulfone |
| 1,2-Dibromoethylene | Epibromohydrin | Ethyl dibromoacetate |
| α-Bromoacetophenone | Phenylmercuric bromide | Sodium 2-bromoethanesulfonate |
| 4-Bromoacetylbiphenyl | α-Bromobenzylcyanide | Cyanogen bromide |
| Ethyl bromoacetate | | |
| **Iodine-Containing Compounds** | | |
| Iodoform | Iodosobenzene | *o*-Iodotoluene |
| Quinoline ethiodide | 2,4,6-Triiodobenzene | |

**Table IV. Sulfur Tests**

| | Test Equivalent to That Given by Sulfur, Taken as Sodium Sulfate | |
|---|---|---|
| | A. 20 to 40γ S | B. More than 40γ S |
| Mercaptans, sulfides, disulfides | 2-Mercaptobenzoxazole | Thioglycolic acid |
| | Phenyl sulfide | Thio-β-naphthol |
| | *p*-Tolyl ethyl sulfide | 2-Amino-4-chlorothiophenol hydrochloride |
| | *p*-Tolyl methyl sulfide | Thiodiglycol |
| | | Thiodiglycolic acid |
| | | Bis-(β-chloroethyl)sulfide |
| | | Benzyl sulfide |
| | | Isobutyl disulfide |
| | | Benzyl disulfide |
| | | Phenyl disulfide |
| | | Phosphorus sulfide ($P_4S_7$) |
| | | Phosphorus sesquisulfide ($P_4S_3$) |
| Sulfoxides, sulfones | *n*-Propyl sulfone | Phenyl sulfoxide |
| | Phenyl-*p*-tolyl sulfone | *n*-Butyl sulfone |
| | | Bis-(β-chloroethyl) sulfone |
| | | 4,4′-dibromodiphenyl sulfone |
| Sulfonic acids and derivatives | Sodium allylsulfonate | *o*-Toluenesulfonamide |
| | Sodium 2-bromoethanesulfonate | *p*-Aminobenzenesulfonamide |
| | | Dichloramine-T |
| | β-Chloroethyl toluenesulfonate | *o*-Benzoicsulfimide |
| | *p*-Toluene sulfonyl chloride | |
| | Benzenesulfonamide | |
| Sulfates, sulfites | 1-Aminoguanidine sulfate | *n*-Propyl sulfate |
| | | Sodium sulfate |
| | | Ammonium sulfamate |
| | | *n*-Butyl sulfite |
| Thiocyanates | Benzyl thiocyanate | Ethyl thiocyanate |
| | Potassium thiocyanate | *n*-Butyl thiocyanate |
| | | Ethylene thiocyanate |
| | | Perthiocyanic acid |
| Miscellaneous | Tri-*o*-cresyl thiophosphate | Thioacetanilide |
| | | *N*,*N*′-di-*n*-butylthiourea |
| | Triphenyl thiophosphate | Sodium diethyl dithiocarbamate |
| | Ethyl thioacetate | |
| | Trithiomethylene | Benzoyl disulfide |

**Sulfur Test (C-1).** The sensitivity of this test appears to be limited by the purity of the zinc metal used in the preparation of the combustion mixture. It was found that a sample of Baker's "zinc metal, granular 80-mesh" was sufficiently free of sulfur to permit the unambiguous detection of 10 micrograms of sulfur originally present as sodium sulfate. A number of sulfur-containing compounds were examined using 1-mg. samples and the intensity of the test was recorded as: (A) equivalent to test obtained with 20 to 40 micrograms of sulfur taken as sodium sulfate, and (B) equivalent to test obtained with more than 40 micrograms of sulfur taken as sodium sulfate. The results of these tests are given in Table IV.

In no case was a negative test obtained with a sulfur-containing compound and in view of the variety of compounds examined it appears that the sulfur test described is generally applicable.

**Arsenic Test (C-1).** If phosphorus is absent, the test for arsenic will provide for the unambiguous detection of as little as 5 micrograms of arsenic originally present as sodium arsenate. The presence of phosphorus may lead to spurious results. Therefore, if phosphorus is found to be present, caution should be

**Table V. Fluorine Test**

| A. Equivalent to 6 to 10 γ of Fluoride Ion | B. Equivalent to More than 10 γ of Fluoride Ion |
|---|---|
| Difluoroacetonitrile | *n*-Butylsulfonyl fluoride |
| β-Fluoroethyl chlorosulfonate | β-Chloroethyl fluoroacetate |
| β-Fluoroethylnitrite | Bis(β-chloroethyl) fluorophosphate |
| | Diethylamino phosphonyl fluoride |
| | *p,p'*-Difluorobiphenyl |
| | *N,N*-diethylfluoroacetamide |
| | Diisopropylfluorophosphate |
| | *N,N'*-tetramethylamino-phosphoryl fluoride |
| | β-Fluoroethanol |
| | Bis(β-fluoroethyl)carbonate |
| | *p*-Fluoroaniline |
| | *p*-Fluoroanisole |
| | β-Fluoroethylchloroformate |
| | β-Fluoroethylchloromethyl ether |
| | Bis(β-fluoroethyl)-β-fluoroethylphosphate |
| | Methyl fluoroacetate |

exercised in accepting a positive arsenic test as conclusive evidence of the presence of this latter element. Using *ca.* 1-mg. samples satisfactory tests were obtained with all arsenic-containing compounds examined, which included arsanilic acid, *m*-nitrophenylarsonic acid, phenarsazine chloride, phenarsazine cyanide, phenyldichloroarsine, ethyldichloroarsine, and β-chloroethyldichloroarsine.

**Phosphorus Test (D).** As little as 5 micrograms of phosphorus, originally present as sodium phosphate or phosphorus trichloride, can be detected with this test, provided care is taken to select reagents which are essentially free of phosphorus. Using a 1-mg. sample satisfactory tests were obtained with all phosphorus-containing compounds examined. These included red phosphorus, phosphorus sesquisulfide, phosphorus sulfide, phosphorus trichloride, sodium β-glycerophosphate, and tri-*o*-cresyl thiophosphate.

### QUALITATIVE TEST FOR CARBON

A method suitable for the detection of about 10 micrograms of carbon was developed which was based upon the wet combustion of a 1-mg. sample with a mixture of iodic, chromic, sulfuric, and phosphoric acids and subsequent detection of the carbon dioxide formed by precipitation as barium carbonate.

**Carbon Test.** A tip 0.2 to 0.3 mm. in inside diameter and 10 mm. long is formed on one end of a 15-cm. length of 1.5- to 2.5 mm. inside diameter soft glass thin-walled capillary tubing previously prepared from 30-mm. glass tubing thoroughly cleaned with a hot chromic acid–sulfuric acid mixture. The tube is then gently heated, in the flame of a microburner, at a point *ca.* 5 cm. from the tip, so as to cause the tube to bend by its own weight into a canelike form. A pipet is also prepared by forming a tip 0.5 mm. in outside diameter and *ca.* 8 cm. long on a piece of 1.0- to 1.5-mm. thin-walled capillary tubing. The combustion mixture used was prepared by carefully mixing 33 ml. of 85% phosphoric acid and 67 ml. of 30% fuming sulfuric acid in a 250-ml. g.s. flask and then adding 6.0 grams of chromium trioxide and 1.0 gram of potassium iodate. The contents of the unstoppered flask were heated, with gentle agitation, until the temperature reached 140° to 150°. The flask was then covered with an inverted beaker and allowed to cool to 25°, whereupon it was stoppered and contamination of the rim of the flask by dust prevented by inverting a beaker over the neck of the flask.

With the aid of the pipet described above a 25-mm. column of the combustion mixture is placed in the longer arm of the cane tube. The tip of the short arm of the cane tube is allowed to come in contact with a drop of a centrifuged solution 2 *F* in sodium hydroxide and saturated with barium chloride until a column 5 to 7 mm. in length is present in the larger part of the arm. The short arm of the cane tube is then sealed by rapidly passing the tip through the edge of a burner flame. The sample contained in a 0.5 mm. inside diameter capillary tube (3- to 4-mm. column = approximately 1 mg.) is inserted into the long arm of the cane tube and placed in position within a few millimeters of the column of combustion mixture. The long arm of the cane tube is then sealed at a point 10 to 20 mm. from the open end. The sealed cane tube is hung on the edge of a 15-ml. centrifuge tube and centrifuged gently to drive the sample and combustion mixture to the bottom of the tube. The portion of the cane tube containing the sample and combustion mixture is heated in a boiling water bath for 3 to 5 minutes. If a white crust is formed on the surface of the barium hydroxide solution the tube is taken from the bath and gently centrifuged until the precipitate is driven to the bottom of the short arm. The tube is replaced in the boiling water bath and after a minute a precipitate will reappear on the surface of the barium hydroxide solution if carbon is present.

Blank tests often may produce a small amount of precipitate on the surface of the barium hydroxide solution, but this precipitate does not reappear after one centrifuging. Positive tests for carbon in a 1-mg. sample of an organic compound will eventually lead to the formation of a heavy precipitate throughout the solution contained in the short arm of the cane tube, provided the cane tube is periodically centrifuged.

### RESULTS OBTAINED WITH CARBON TEST

A 1-mg. sample of bromopicrin, containing approximately 10 micrograms of carbon, gave an unambiguous positive test. However, acetic acid and compounds which yield acetic acid as the only carbon-containing compound upon hydrolysis may give only a poor test with 3 to 5 minutes' heating. In no case except that of acetic acid did a carbon-containing compound fail to give a satisfactory test, even though such difficultly oxidizable materials as benzene, pyridine, and graphite were examined. The other compounds tested were: acetylurea, arsanilic acid, benzyl chloride, *o*-bromoaniline, carbon tetrachloride, β-chloroethyl *p*-toluenesulfonate, 2-chloropyridine, diazoaminobenzene, dichloramine-T, 1,2-dichloro-*n*-butane, bis(β-chloroethyl) carbonate, dioxane, *N,N*-diphenylnitrosoamine, ethanol, fluorobenzene, methanol, *m*-nitrophenylarsonic acid, quinoline ethiodide, tetra-*n*-butylammonium iodide, tetrachloroethylene, tetralin, and triphenyl thiophosphate.

The technique used in the carbon test is generally applicable to all cases involving the liberation of a gas and its subsequent capture in a suitable reagent. For example, its application to the detection of α-amino acids by oxidation with ninhydrin to give carbon dioxide (*6*) is obvious.

### QUALITATIVE TEST FOR FLUORINE

The presence of fluorine in organic compounds can be detected by decomposing a 1-mg. sample with a mixture of iodic, chromic, sulfuric, and phosphoric acids, collecting the hydrogen fluoride in a hanging drop of water, and then examining the aqueous solution for the presence of fluoride by taking advantage of the insolubility of thorium fluoride and the intense red color of the lake of alizarin sulfonate adsorbed on thorium hydroxide. If fluorine is absent the lake forms and a red color is observed. If fluorine is present, thorium fluoride is formed and no color is observed.

**Fluorine Test.** Approximately 1 mg. of the liquid or finely powdered solid sample is placed in the 3 × 15 mm. depression of a culture-type microscope slide and a glass ring is placed around the depression as in the previously described arsenic-sulfur test. Ten drops of the iodic-chromic-sulfuric-phosphoric acid mixture are added to the sample and the ring is immediately covered with a 2.5-cm. watch glass, on the under side of which is a hanging drop of water. The assembly is allowed to stand for 5 minutes and then placed on a 7.5-cm. (3-inch) iron ring. Two drops of water are placed on the top side of the watch glass, to prevent subsequent evaporation of the hanging drop, and the bottom of the slide is carefully heated, with a small flame, until the mixture in the cell begins to fume and then for 30 seconds longer. The hanging drop is removed with a capillary pipet and transferred to a 10 × 75 mm. test tube. One drop of 6 *F* formic acid and one drop of 2% hydrazine hydrate are added and the mixture is heated to 50° to 60°. After 20 seconds 1 ml. of water, one drop of 3 *F* sodium hydroxide, and two drops of 0.12% ethanolic sodium alizarinsulfonate are added and the solution is thoroughly mixed. A similar solution is prepared for comparison purposes containing all the reagents used. To each tube one drop of 0.0005 *F* thorium nitrate is added, the solution is mixed by shaking the tubes gently, and the colors are compared. If fluorine is present the tube containing the hanging drop will be yellow and the comparison tube pink. One drop (0.05 ml.) of 0.0005 *F* thorium nitrate is equivalent to *ca.* 2 micrograms of fluoride and if fluorine is present the amount in the hanging drop can be estimated by the dropwise addition of the thorium nitrate solution.

Experiments have shown that, depending on the nature of the compound, 15 to 50% of the fluorine present in the sample is collected as fluoride in the hanging drop. If the sample does not contain bromine or chlorine, the procedure can be simplified by omitting the hydrazine reduction.

### RESULTS OBTAINED WITH FLUORINE TEST

One-milligram samples of a number of fluorine-containing compounds were examined and the result of the test was recorded as: (A) equivalent to test obtained with 6 to 10 micrograms of fluoride ion, and (B) equivalent to test given by more than 10 micrograms of fluoride ion. The results are given in Table V.

Negative tests were obtained with 1-mg. samples of β-chloroethyl chlorosulfonate, β-chloroethylisocyanide dichloride, and dimethylchloroarsine.

The system of qualitative analysis described above was used frequently during the war period for the rapid identification of elements in organic compounds and no difficulty was experienced in obtaining unambiguous results. During the past year the system has been used with equal success in a laboratory course given for senior and first-year graduate students.

### ACKNOWLEDGMENT

Several of the tests used in the systematic scheme have been based upon tests described by Feigl (*2*). The authors wish to express their appreciation of the assistance given by A. Briglio, D. Brown, G. Holzman, and T. Lee during the course of this work.

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# B. ELIMINATION OF INTERFERENCE BY CYANIDE ION IN SYSTEMATIC QUALITATIVE TESTS FOR CERTAIN ACIDIC ELEMENTS IN ORGANIC COMPOUNDS

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## Summary

Cyanide formed by the pyrolysis of certain nitrogenous compounds in the presence of calcium oxide and zinc may interfere in the halide tests described in a system for the qualitative detection of certain acidic elements present in organic compounds. A modified system is described in which this interference by cyanide is eliminated.

In a previous communication (1) a system for the detection of nitrogen, chlorine, bromine, iodine, arsenic, sulfur and phosphorus in a single one mg. sample of an organic compound was described. This system was based upon pyrolysis of the sample in the presence of zinc and calcium oxide (3), the detection of the evolved ammonia in the event that nitrogen were present, followed by subsequent

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tests for the other elements using the pyrolysis residue. For the detection of halides a portion of the residue was extracted with water and the aqueous extract tested for halide with silver nitrate, for bromide or iodide with fluorescein-chloramine-T, and for iodide with starch-nitrite.

During the past two years it has been noted that students will occasionally report the presence of halide in nitrogenous compounds containing no halogen. This spurious test has its origin in the fact that some nitrogenous compounds when pyrolyzed with zinc and calcium oxide will occasionally form cyanide as well as ammonia and the student observing the formation of a precipitate of silver cyanide will report the presence of halogen. While it is unlikely that an experienced observer would be misled, the fact that cyanide may also prevent the formation of eosin or tetraiodoeosin, as well as the starch iodine color, thus offering the possibility that bromine and iodine may be reported absent when actually present, suggested the desirability of modifying the system to avoid all possible difficulties.

The interference by cyanide has been provided for in the modified tests described below by the addition to the systematic scheme of a test for cyanide (2) in case both nitrogen and halide have been found to be present, and a procedure for the removal of cyanide if present.

## Addendum to Systematic Tests (1)

B-1 Tests for Halogen. In a 1- to 1.5 mm. thin-walled capillary a 4- to 5- mm. column of the aqueous extract of the pyrolysis residue is allowed to react with a 3- to 4- mm. column of a solution 0.5 F in silver nitrate and 3 F in nitric acid. The formation of a white or yellow precipitate within 30 seconds indicates the presence of cyanide, chloride, bromide, or iodide.

If nitrogen has been found to be absent (A), the test for bromide or iodide (B - 2) and the test for iodide (B - 3) are performed. If nitrogen has been found to be present the remainder of the aqueous extract is transferred with a capillary pipette to a 2 ml. beaker, 1 drop of a solution 0.1 F in sodium acetate and 0.1 F in acetic acid added, and the beaker covered with a circle of filter paper impregnated with 1 drop of a reagent freshly prepared by mixing equal volumes of 0.015 F aqueous cupric acetate and one-half saturated aqueous benzidine acetate (2). The appearance of a blue spot on the paper within a few seconds indicates the presence of cyanide. If cyanide is present, remove the filter paper and heat the mixture gently on a hot plate until the test for cyanide with a fresh circle of filter paper, impregnated with the cupric acetate-benzidine acetate reagent, is negative. Then repeat the test for halide (B - 1) and if positive (chlorine, bromine, or iodine present)

perform tests B - 2 (for bromide or iodide) and B - 3 (for iodide).

Results Obtained With Modified Systematic Scheme

It has been found that cyanide can be detected without difficulty with the cupric acetate-benzidine acetate reagent when as little as 1 microgram of cyanide is present in the aqueous extract of the pyrolysis residue. A more sensitive test for cyanide is not required because if less than 1 microgram of cyanide is present in the aqueous extract no significant interference by cyanide is observed. If cyanide is present in the aqueous extract of the pyrolysis residue, even in amounts as great as 100 to 200 micrograms, it can be removed by the recommended procedure to the point where no precipitate is obtained in the test for halide (B - 1) if halogens are absent and where bromide and iodide in amounts as low as 5 to 10 micrograms can be detected without difficulty. To date there has been no indication that the amount of cyanide that may be formed during a pyrolysis can cause any difficulty in the tests for sulfur, arsenic and phosphorus (1).

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# ISOLATION OF COMPOUNDS TOXIC TO TOMATO PLANTS FROM THAMNOSMA MONTANA

## Introduction

The presence of compounds toxic to certain plants species in the tissues of other plants has long been postulated to explain observations that certain plants are not associated in close proximity to other plants (for a review and historical background see (1)). It has been suggested that this is perhaps a particularly important characteristic of desert plants where the amount of nutrients, particularly water, may be very limited. Recently Gray and Bonner (2,3) isolated and identified 3-acetyl-6-methoxy-benzaldehyde as a toxic principle from Encelia farinosa. This compound caused death of young tomato seedlings grown in nutrient culture at a concentration of 250 mg/l. and caused 50% inhibition of growth at a concentration of about 125 mg./l. They also isolated another compound toxic to tomato plants from Encelia native to a different locality. Bonner and Galston (4) showed that guayule (Parthenium argentatum) when grown under laboratory conditions excreted a substance from the roots which was toxic to seedlings of the same species. This compound was identified as trans-cinnamic acid.

A survey made by Brown of eleven desert plant species native to southern California indicated that four of these species contained compounds which could be extracted by

water and which were highly toxic to tomato seedlings. Of these plants, Thamnosma montana yielded the most toxic extract and, accordingly, it was decided to attempt the isolation of the toxic compound or compounds present in this species.

By the methods described in the experimental section of this thesis three crystalline compounds have been isolated from Thamnosma montana. One of these compounds (m. p. 176-177°) will kill young tomato seedlings grown in nutrient culture within 1 week when given at a concentration of 12.5-15 mg./l.; and another (m. p. 121-122°) produces the same effect at a concentration of 125 mg./l. but causes 50% inhibition of growth at a concentration of about 20 mg./l. The third compound (m. p. 148-149°) is not sufficiently soluble in nutrient solution to obtain concentrations high enough to kill the tomato seedlings, but does produce definite inhibition of growth at concentrations of 10 mg./l. Analytical data indicate that these compounds have the empirical formulas $C_{17}H_{16}O_6$ or $C_{17}H_{18}O_6$, $C_{13}H_{10}O_5$, and $C_{17}H_{18}O_7$, respectively. Their ultra-violet spectra indicate that they are closely related.

The methods of isolation, analyses, and spectra are discussed in detail in the experimental section.

## Experimental

### A. SURVEY OF DESERT PLANTS FOR THE PRESENCE OF COMPOUNDS TOXIC TO TOMATO PLANTS

Plant specimens were collected in the Panamint Valley and Death Valley areas on April 5 and 6, 1947 by Dr. and Mrs. James Bonner, Dr. David Brown, and the author. The toxicity experiments to determine if toxic constituents were present in any of the plant specimens collected were done by Dr. Brown with the author's assistance and are included herein to provide a record of the results.

The first experiment was performed by making aqueous extracts of the plant specimens by grinding the plants in a Waring blender with a known volume of nutrient solution*. The extract was removed by suction filtration through a pad of the ground plant material and the resulting filtrate was centrifuged. In many cases the extracts were still not clear. 1/3 serial dilutions of the extracts were then made in nutrient solution and the toxicities of the extracts

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*Prepared by diluting greenhouse stock nutrient solution to 1/4 the initial concentration and adding 10 ml. of an iron and micronutrient-containing solution to 1 liter of the diluted solution.

to young tomato plants were then determined**.

On the basis of the first experiments, the results of which are shown in Table 1, it was concluded that Thamnosma montana, Prosopis juliflora, and Sarcobatus vermiculatus contained compounds which were capable of being extracted by water and which were highly toxic within 55 hours to tomato plants at a concentration of less than 2 mg/ml. of the crude extracted material. A second experiment in which tests were made with the same extracts at somewhat lower concentrations gave rather similar results except that Viguiera reticulata was concluded to be more toxic than Prosopis juliflora.

After these preliminary experiments had been made, the plant material was dried in a hot air oven at 75° for 5 days, and then ground in a meat grinder. The loss in weight upon drying was as follows: Encelia frutescens, 31%; Prosopis juliflora, 67%; Larrea tridentata, 32%; Thamnosma montana, 24%; Viguiera reticulata, 39%; Sarcobatus vermiculatus, 76%, and Franseria dumosa, 34%.

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** In all the experiments reported in this work, the toxicities of the extracts and compounds have been determined using young tomato seedlings as the test plant (1,2,3,4). The method has in general been the same. San Jose or Marglobe tomato plants grown in sand and with the primary leaf 1/8 to 3/8 inches long were used. These plants were 12 to 22 days old; in winter and early spring three weeks were required to obtain plants of the desired size; in the warmer seasons of the year plants 12 days old were of proper size. These young tomato plants were removed from the sand flats in which they had been germinated, washed with tap water, and transplanted to 15 ml. vials containing the solution to be tested. They were held in place in the vials with corks and cotton. The vials were placed in racks and placed in the air-conditioned

Known amounts of the ground, dried plant material were extracted with distilled water, first in the Waring blender, and then by standing at 4° for 2 days. The solutions were cleared by filtration and dried by lyophilization. The toxicity tests were made as previously described. Tests were made also of the lyophilizate. The results are shown in Table II.

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** greenhouse at 80° F. 2 to 5 plants were planted at each concentration to be tested. In general the plantings were made in the evening. Observations were made of the plants daily or semi-daily for a period up to 8 days, and additional distilled water was added to the vials if necessary to replace that lost by transpiration.

In the experiments reported subsequently in this thesis with "Thamnosa toxic" fractions, little or no significant variation in the results obtained was noted with season, although the experiments were carried out over a period of approximately 1 year. The effect by a toxic sample at a given concentration was relatively uniform, and 2-3 plants at each concentration were generally sufficient to determine the toxicity of a fraction. No significant difference was noted between the effects produced on Marglobe and San Jose tomato plants, although no detailed study was made of such variables as variety, age, size of plants, etc.

Table I

| Plant Material<br><br>Place of Collection<br><br>Plant Parts Extracted | Wet Weight of Plant Extracted per ml. of Nutrient Solution (grams) | Calc. Dry Weight of Plant extracted per ml. of Nutrient Solution* (grams) | Concentration of Extracted Material in Extract (calc.)** mg./ml. | Appearance of Tomato Plants after: 55 hrs. | 123 hrs |
|---|---|---|---|---|---|
| Encelia frutescens | 0.033 | 0.023 | 4.8 | 0/5 dead | 2/5 dead 3 stunted |
| Panamint Valley | 0.010 | 0.007 | 1.5 | 0/5 dead | 0/5 dead 5 stunted |
| stems, flowers, fruits, leaves. | | | | | |
| Prosopis julliflora | 0.015 | 0.005 | 1.8 | 5/5 dead in 43 hrs. | --- |
| Panamint Valley | | | | | |
| mostly leaves and flowers, a few stems. | | | | | |
| Chrysothamnus nauseosus | 0.05 | --- | --- | 5/5 dead | --- |
| Panamint Valley | 0.015 | --- | --- | 1/5 dead 4 stunted | 3/5 dead 2 stunted |
| Leaves and stems. | | | | | |
| Larrea tridentata | 0.05 | 0.033 | 7.6 | 5/5 dead | --- |
| | 0.015 | 0.010 | 2.4 | 0/5 dead 5 stunted | 4/5 dead 1 stunted |
| Panamint Valley | | | | | |
| stems, flowers, leaves, and fruits | | | | | |

* The dry weights of the plants have been calculated from a later experiment.
** The amount of soluble material in the extracts has been calculated from a later experiment.

Table I (cont.)

| Plant Material<br>Place of Collection<br>Plant Parts Extracted | Wet Weight of Plant Extracted per ml. of Nutrient Solution (grams) | Calc. Dry Weight of Plant Extracted per ml. of Nutrient Solution (grams) | Concentration of Extracted Material in Extract (calc.) mg./ml. | Appearance of Tomato Plants after: 55 hrs. | 123 hrs. |
|---|---|---|---|---|---|
| Parasela fremontii | 0.033 | --- | --- | 0/5 dead 2 stunted | 5/5 dead |
| Summit State Mountains stems, leaves. | 0.010 | --- | --- | 0/5 dead | 1/5 dead 4 stunted |
| Allenrolfia occidentalis | 0.050 | --- | --- | 0/5 dead 4 stunted | 4/5 dead 1 stunted |
| Panamint Valley stems and fruit mostly. | 0.015 | --- | --- | 0/5 dead | 3/5 dead 2 stunted |
| Thamnosma montana | 0.010 | 0.0075 | 1.7 | 5/5 dead in 43 hrs. | |
| Aguereberry Canyon stems, flowers, fruit. | | | | | |
| Viguiera reticulata | 0.033 | 0.020 | 4.2 | 3/5 dead 2 stunted | 5/5 dead |
| Panamint Valley leaves, a few flowers. | 0.010 | 0.006 | 1.3 | 0/5 dead 2 stunted | 3/5 dead 2 stunted |
| Ephedra viridis | 0.10 | --- | --- | 5/5 dead | --- |
| | 0.033 | --- | --- | 0/5 dead | 0/5 dead |
| Aguereberry Canyon mainly stems and leaves. | | | | | |

Table I (cont.)

| Plant Material<br>Place of Collection<br>Plant Parts Extracted | Wet Weight of Plant Extracted per ml. of Nutrient Solution (grams) | Calc. Dry Weight of Plant Extracted per ml. of Nutrient Solution (grams) | Concentration of Extracted Material in Extract (calc.) mg./ml. | Appearance of Tomato Plants after: 55 hrs. | 123 hrs |
|---|---|---|---|---|---|
| Sarcobatus vermiculatus | 0.06 | 0.014 | 7.6 | 5/5 dead in 43 hrs. | --- |
| Panamint Valley<br>leaves, stems, fruits, flowers (also galls). | 0.018 | 0.005 | 2.7 | 4/5 dead<br>1 stunted | 5/5 dead |
| Franseria dumosa | 0.033 | 0.022 | 4.8 | 0/5 dead<br>4 stunted | 5/5 dead |
| Panamint Valley<br>stems, leaves, flowers, and fruit. | 0.010 | 0.0067 | 1.5 | 0/5 dead | 3/5 dead<br>2 stunted |

Table II

| Plant Extracted | Percentage of Dry Weight of Plant Which Was Extracted by Water | Concentration of Extract in Nutrient Solution mg./ml. | Appearance of Tomato Plants After 171 hours |
|---|---|---|---|
| Thamnosma montana | 22 | 3.6 | 5/5 dead |
| | | 1.8 | 5/5 dead |
| | | 0.6 | 5 very stunted |
| | | 0.2 | 5 stunted |
| Viguiera reticulata | 21 | 5.25 | 5/5 dead |
| | | 1.75 | 5 subnormal |
| | | 0.60 | 5 almost normal |
| Franseria dumosa | 22 | 11 | 5 very stunted |
| | | 5.5 | 5 somewhat stunted |
| | | 1.85 | 5 nearly normal |
| | | 0.60 | 5 normal |
| Larrea tridentata | 24 | 18.0 | 5/5 dead |
| | | 6.0 | 5 very stunted |
| | | 2.0 | 5 stunted |
| Encelia frutescens | 21 | 10.0 | 1 dead; 4 very stunted |
| | | 5.25 | 5 subnormal |
| | | 1.75 | 5 almost normal |
| Sarcobatus vermiculatus | 53 | 7.00 | 5/5 dead |
| | | 3.50 | 5/5 dead |
| | | 1.75 | 2 dead; 3 subnormal |
| | | 0.85 | 5 nearly normal |
| Prosopis juliflora | 37 | 10.0 | 5/5 dead |
| | | 5.0 | 5/5 dead |
| | | 2.5 | 3 dead; 2 very stunted |
| | | 1.25 | 2 dead; 3 subnormal |

From the results shown in Table II of this experiment it was concluded that Thamnosma montana yielded the most toxic aqueous extract. This material caused death of young tomato plants at a concentration of about 1 mg./ml. within 7 days. From Sarcobatus vermiculatus and Prosopis juliflora the next most toxic substances were obtained; these were toxic at about 2.5 mg./ml. Viguiera reticulata produced only slightly less toxic material. The large amount of soluble material in Sarcobatus makes the actual toxicity of the plant parts relatively somewhat larger. Lyophilization of the above plants indicated that one or more of the following species contained volatile toxic substances: Encelia frutescens, Sarcobatus vermiculatus, or Prosopis juliflora. From certain other available data, it seems most likely that the latter plant contains the volatile toxic material. However, the amount of toxic material volatilized in lyophilization is small.

On the basis of the experiments described above it was decided to study in detail the toxic substance (or substances) in Thamnosma. These experiments are described below.

B. Isolation from Thamnosma montana of Compounds Toxic to Tomato Plants

Plant material.- With the aid of Dr. David Brown and Dr. Harold Garner, Thamnosma montana was collected on the hillsides near the road from White Water Junction to 29 Palms on April 3, 1948. Only the green growing parts of the plant were collected and these consisted mostly of stems with small leaves and a few flowers and seeds.

The Thamnosma was ground in a Wiley Mill using a screen with 4 mm. holes. The material ground readily; 2415 grams wet weight of ground Thamnosma were obtained. It was dried by "lyophilization" for 24-36 hours (200 grams of material in each 800 ml. flask). The loss in weight was approximately 40% and 1413 grams of dried Thamnosma montana were obtained. A small amount of volatile oil was collected in the lyophilizate, but it was toxic only at concentrations greater than 1 mg./ml. and was therefore discarded. The dried plant material was reground in the Wiley Mill using a 2.5 mm. screen; subsequently, it has been stored in a brown screw cap bottle at 4°.

Extraction.- Preliminary experiments were made to determine the most suitable solvent for extraction. 3.0 grams portions of the dried plant were placed in Soxhlet extractors and extracted with the following solvents for 24-48 hours: ether (peroxide free), acetone, water, benzene, and ether-sulfuric acid (a slurry was made of the Thamnosma with 6 ml. of 2 F $H_2SO_4$ and a small amount of shredded asbestos). The

organic solvents were removed by distillation at reduced pressure and the residues were taken up in nutrient solution. Another extract was made by allowing 3.0 grams of Thamnosma to stand with 100 ml. of water for 3 days at room temperature. Toxicity experiments indicated that all of the solvents tested were effective in removing toxic substances from the plants, and that hot or cold water and ether and sulfuric acid were the most effective. These extracts killed tomato plants in 5 days at a concentration of 2 mg. of Thamnosma extracted/ml. of test solution. Benzene, ether, and acetone were only slightly less effective. Subsequently water has been used to extract toxic substances from the dried plant tissue.

Preliminary experiments indicated that the toxic substances could be extracted from water by ether, and approximately a 5-fold increase in toxicity on a weight basis could thus be obtained. In this procedure, a large amount of highly colored material remained in the aqueous phase. Preliminary experiments also indicated that the toxic substances could be slowly sublimed at 150° at the minimum pressure obtained by an oil vacuum pump.

The "distribution coefficient of toxicity" was determined between ether and several solvents. It was found to be 2 for ether/water, ether/0.2 F HCl, and for ether/0.2 F $NaHCO_3$. 0.2 F NaOH extracted essentially all of the toxic material into the basic phase from ether.

As a result of further preliminary experiments not reported in detail here, the following method of extraction was used for the first large scale isolation of toxic substances from Thamnosma montana.

Isolation I.- This isolation is described in some detail since it was the first isolation of an appreciable quantity of crystalline, highly toxic material from Thamnosma montana.

101 grams of dried Thamnosma were placed in a large Soxhlet extractor which had been assembled in such a manner that it could be operated at reduced pressure. The plant material was extracted with water for 35 hours at a boiler temperature of 50-60° (100-150 mm. pressure). The extracted solution (approx. 500 ml.) was very dark brown in color, but the last extracts were colorless and tests indicated that no further toxicity was being extracted. A second extraction was made of 100 grams additional plant material in the same manner. The aqueous extracts were than extracted in 250 ml. portions with ether for 4-5 days in a continuous liquid-liquid extractor. At the end of this time the boiler contained a small amount of water (F-B) which had been carried over (very viscous and highly colored), and a light yellow ether phase (F-A). The ether phase contained 3.2 grams of crude material, toxic at 20 mg./l. = 160 t. u.* The water

* A "toxicity unit" (t. u.) is that amount of toxic material which, when dissolved in 1 l. of nutrient, will kill tomato plants in about 1 week under the conditions of the test.

phase (F-B) was dissolved in a total of 210 ml. of hot acetone. It contained 2.8 grams of crude toxic substances, toxic at 25 mg./l. (110 t. u.). Thus a total of 6.0 grams of highly toxic substances (270 t. u.) had been obtained from 200 grams of Thamnosma. This represented a 3% yield of materials toxic at 20-25 mg./l.

The ether solution of F-A was dried over Drierite, filtered and the ether distilled. Two phases were obtained upon concentration. They were dissolved in acetone, and filtered to remove a small amount of insoluble material, then evaporated to 5 ml. To the warm solution 25 ml. of benzene were added. It was allowed to cool slowly to room temperature but no immediate oil or turbidity formed. It was then placed in the cold room for 10 days, and a small amount of semi-crystalline precipitate was obtained. The solution was decanted from the precipitate. The precipitate was re-crystallized from 1 ml. of acetone. After standing in the cold room for several hours, the precipitate which had formed was centrifuged, washed with two 0.5 ml. portions of cold acetone and dried. Obtained 68 mg. (F-A-1) m. p. 170-174° (corr.).

To the main solution, 10 ml. of 60-70° ligroin were slowly added. The solution became turbid but no precipitate formed. After 1 hour 20 ml. of additional ligroin were added and the solution was allowed to stand several days at 4°. The supernatant (F-1A-3) was decanted from the oil

(F-1A-2). F-1A-2 contained about 500 mg. of material and by repeated crystallization from small volumes of acetone 50 mg. of a substance m. p. 121-122° and other fractions with wide melting ranges were isolated.

Additional ligroin was added to F-1A-3 and 520 mg. of yellow crystalline solid were obtained after long standing at 4°, m. p. 95-113°; toxic at 25 mg./l. No more fractionation was attempted with this fraction; and it was later chromatographed with other fractions which had wide melting ranges (see below).

F-B was filtered from the Drierite and the solution was concentrated to 5 ml.; 15 ml. of benzene were slowly added. The solution became turbid immediately and it was allowed to cool slowly. By vigorous scratching the precipitate was obtained in a moderately crystalline form. It was filtered and washed with benzene, but it became very gummy. The precipitate (F-B-1) was dissolved in 5 ml. of warm acetone and the solution was placed in the cold room overnight.

The filtrate from F-B-1 was evaporated to dryness and the residue was dissolved in 10 ml. of warm acetone and 20 ml. of redistilled benzene added. It was turbid when warm. Upon cooling slowly a precipitate formed. It was allowed to stand in the cold room overnight (F-B-2).

F-B-1 was filtered but the precipitate got very sticky and gummy upon washing with 66% benzene-34% acetone. A more

crystalline precipitate formed in the filtrate (vol. 13 ml.) and after standing several hours this precipitate was centrifuged and washed several times with acetone. Obtained 180 mg. of a crystalline compound, m. p. 174-176° (corr.). From the filtrate and washings there were obtained 200 mg. of additional crystalline product, m. p. 174-176° (corr.) and 420 mg. of a brown oil active at 70 mg./l.

One of these crystalline fractions was recrystallized and analyzed (F-B-2-A), m. p. 175.2-176.4° (corr.) (see below for summary of analyses and spectrum). It was toxic to tomato plants at 12.5-15 mg./l. Thus, approximately 25 t. u. had been isolated as a pure crystalline compound. Experiments indicated that it was extracted by dilute NaOH from ether, and that the partition coefficients of the pure compound between ether/water, ether/0.2 F $NaHCO_3$, and ether/0.2 F HCl were about 2.

The precipitate in F-B-2 also became gummy upon filtering and when combined with the similar precipitate from F-B-1 weighed 230 mg. No further work was done with this material. The filtrate was allowed to stand in the cold room but no further precipitate formed. The supernatant from F-B-1 was added and after standing for a much longer time, an oil film plus some dense clusters of crystals were obtained on the bottom of the flask. From these crystals there was finally isolated by recrystallization from acetone 125 mg. of additional toxic compound m. p. 174-175° and 120 mg. m. p.

165-170°. From the supernatant 60 mg. of the compound with a m. p. 121-122.5° and 220 mg. with a m. p. 75-145° were isolated.

In all, there were isolated from this extraction of 200 grams of Thamnosma, about 700 mg. (45 t. u.) of toxic compound, m. p. 175°, 110 mg. m. p. 121-122°, and approximately 2 grams of other solid fractions with wide melting ranges. The fractionation procedure is summarized in Outline I.

Isolation II.- 400 grams of dried Thamnosma montana were extracted in 4 portions in the Soxhlet extractor with water under reduced pressure. Each extraction was for 2 days; the total volume of the extracts was about 2 1/2 liters.

The combined extract was placed in a continuous liquid-liquid extractor with 500 ml. of additional water and 150 ml. of acetone used to dissolve some insoluble oily material in the extraction flask. The solution was extracted with 1 1/2 liters of ether (peroxide free) for 7 days. On cooling to room temperature 3 phases were present in the ether boiler:

A. Ether phase (light yellow color).

B. Water phase (brown color-49 ml.).

C. A heavy oil phase (small in volume).

The main aqueous solution was extracted with additional ether for 3 days. The ether boiler contained two phases:

D. Ether phase (light yellow).

E. Water phase (light brown-33 ml.).

OUTLINE I

Isolation I of Toxic Materials from *Thamnosma montana*

200 Grams Dried *Thamnosma montana*

Extracted with Water

Aqueous Extract Extracted with Ether

Ether Phase

F-A
3.2 grams
160 t. u.

Recrystallized from Acetone-Benzene

Ppt. F-A-1 70 mg. m.p. 170-174°

Supernatant

Ligroin added

Supernatant F-1-A-3

Ligroin added

Ppt. 520 mg. m.p. 95-113° toxic at 25 mg./l.

Oil F-1-A-2 500 mg.

By Repeated Crystallization 50 mg. obtained with a m.p. of 121-122°

Small Phase Extracted by Ether but Insoluble in Ether

F-B
2.8 grams
110 t. u.

Redrystallized from Benzene

Gummy Ppt. F-B-1

Recrystallized from Acetone-Benzene

Ppt. 230 mg.

From Filtrate Obtained 380 mg. m.p. 174-176°, toxic at 15 mg./l., and 420 mg. oil active at 70 mg./l.

Supernatant Concentrated and Recrystallized from Acetone-Benzene

From Supernatant on Standing, Oil and Crystals Deposited

Recrystallized Several Times

More Insoluble 125 mg., m.p. 174-175°, and 120 mg., m.p. 165-170°

More Soluble 60 mg., m.p. 121°, and 220 mg., m.p. 75-148°

The extracted aqueous phase was F.

Aliquots of the fractions were taken and the amount of material present and the toxicity of each fraction were determined.

Table III

Summary of Fractions Obtained in Isolation II of Toxic Materials from Thamnosma montana

| Fraction | Total Solids | Toxicity | Toxic Units | Toxicity/ 100 grams Thamnosma |
|---|---|---|---|---|
| A | 11.5 grams | 20 mg./l. | 580 | 145 |
| B | 2.0 grams | 50 mg./l. | 40 | 10 |
| C | 1.1 grams | 60 mg./l. | 17 | 4 |
| D | 0.24 grams | 50 mg./l. | 5 | 1 |
| E | | | | <2 |
| F | 86 grams | 2 gr./l. | Probably toxic due to salt present | |
| Total | 100 grams | | 642 | 160 |

15 grams

extractable by ether

Thus, 100 grams (25%) of soluble materials were extracted by water from 400 grams of dried Thamnosma. About 15 grams (15%) of this was extracted from water by ether. Fraction A was obtained in about 3% yield from dried Thamnosma; the other fractions were discarded.

The ether solution (A) was allowed to stand in the cold room for several days, whereupon a crystalline precipitate began to form. The separatory funnel in which the solution was contained was unstoppered and allowed to evaporate at room temperature for one week to a volume of 350 ml; then it was placed in the cold room for several days. The light yellow-brown precipitate which had been obtained was filtered and washed with a small amount of acetone. Precipitate (A-1) weighed 3.6 grams, m. p. 100-160°; toxic at 25 mg./l. (140 t. u.). The filtrate and washings were allowed to evaporate to 11 ml. in the cold room. The oily solid (A-2) present was separated. (A-2) weighed 3.0 grams, toxic at 25 mg./l. (120 t. u.). The remaining product (A-3) was obtained as an oil: (A-3) weighed 6.3 grams, toxic at 25 mg./l. (250 t. u.). Thus, from (A) there had been obtained 12.9 grams of material, all toxic at 25 mg./ml., or a total of 510 t. u.

(A-1) was dissolved in 35-40 ml. of hot acetone and was filtered hot to remove a small amount of insoluble material. The filtrate was evaporated to 20 ml. and allowed to stand at room temperature for several hours. Then it was filtered on a centrifuge funnel and washed with several small portions of acetone. The precipitate (A-1-1) weighed 1.2 grams, m. p. 120-167°. The filtrate and washings were combined and stored at 4° overnight. The precipitate (A-1-2) was separated and washed with a small volume of cold acetone. It weighed 0.75 grams, m. p. 99-103°. The filtrate and washings from (A-1-2)

were allowed to evaporate to 5 ml. and were stored in the cold room for several weeks. The precipitate (A-1-3) was separated on a centrifuge funnel. It weighed 0.70 grams, m. p. 100-155°, mostly 145-150°. The filtrate (A-1-5) was used in chromatographic experiments described below.

(A-1-1) was recrystallized from 25 ml. of acetone. After standing several days at room temperature, a nearly white, dense, crystalline precipitate was obtained. The precipitate (A-1-1-1) weighed 0.72 grams after washing with a small amount of acetone and drying, m. p. 175-177.5° (corr.). This fraction was toxic at 15 mg./l. (50 t. u.) and definite stunting and inhibition of root growth was observed at 6 mg./l.

(A-1-2) dissolved readily in 6 ml. of warm acetone. It was allowed to cool to room temperature and a yellow precipitate (A-1-2-1) formed slowly. (A-1-2-1) weighed 0.24 grams, m. p. 110-122, mostly 118-122° (corr.). The filtrate was allowed to stand in the cold room in an unstoppered centrifuge tube for 2 days. The precipitate (A-1-2-2) weighed after washing and drying 0.28 grams, m. p. 119-122° (corr.). This fraction was toxic at 125 mg./l. The leaf length of tomato plants was 50% of normal at a concentration of (A-1-2-2)/18 mg./l. and few roots had grown. Only slight inhibition of root growth was observed at 9 mg./l. Only fractions with wide melting ranges were obtained from the filtrate from (A-1-2-2). A portion of (A-1-2-2) was recrystallized from an acetone-water mixture for analyses and for spectrum (see below).

(A-2) (3.0 grams) was dissolved in acetone and allowed to evaporate to 15 ml. The precipitate (A-2-1) was centrifuged out on a centrifuge funnel and washed with a small volume of acetone. It weighed 0.48 grams, m. p. 138-175°, mostly 138-143° (corr.). The filtrate and washings were allowed to evaporate slowly at 4° to 10 ml. The precipitate (A-2-2) was centrifuged and washed with a small volume of cold acetone. (A-2-2) weighed 0.37 grams, m. p. 140-145° (corr.). The filtrate and washings from (A-2-2) were allowed to evaporate to 5 ml. and an additional solid fraction (A-2-3) weighing 0.26 grams, m. p. 128-141° (corr.) was obtained. The filtrate (A-2-4) contained 1.9 grams of material. A small portion was sublimed at 150° and the minimum pressure obtainable by an oil vacuum pump. Highly toxic materials (toxic at 12.5 mg./l.) were sublimed under these conditions, but they could not be crystallized. The spectra of the sublimed fractions indicated that they were similar to the crystalline fractions which had been obtained. Most of (A-2-4) was chromatographed (see below).

(A-2-1), (A-2-2), and (A-2-3) were recrystallized from small volumes of acetone. A total of 800 mg. of a crystalline compound, m. p. 148-149.5 was obtained in several fractions (A-2-1-1-A, A-2-2-1-A, A-2-2-1-B, etc.). This compound is not very soluble in water and consequently could not be tested at concentrations of greater than 40 mg./l. The effect of this compound at concentrations of 40 mg./l. was to kill the cotyledons of the tomato plants and to produce

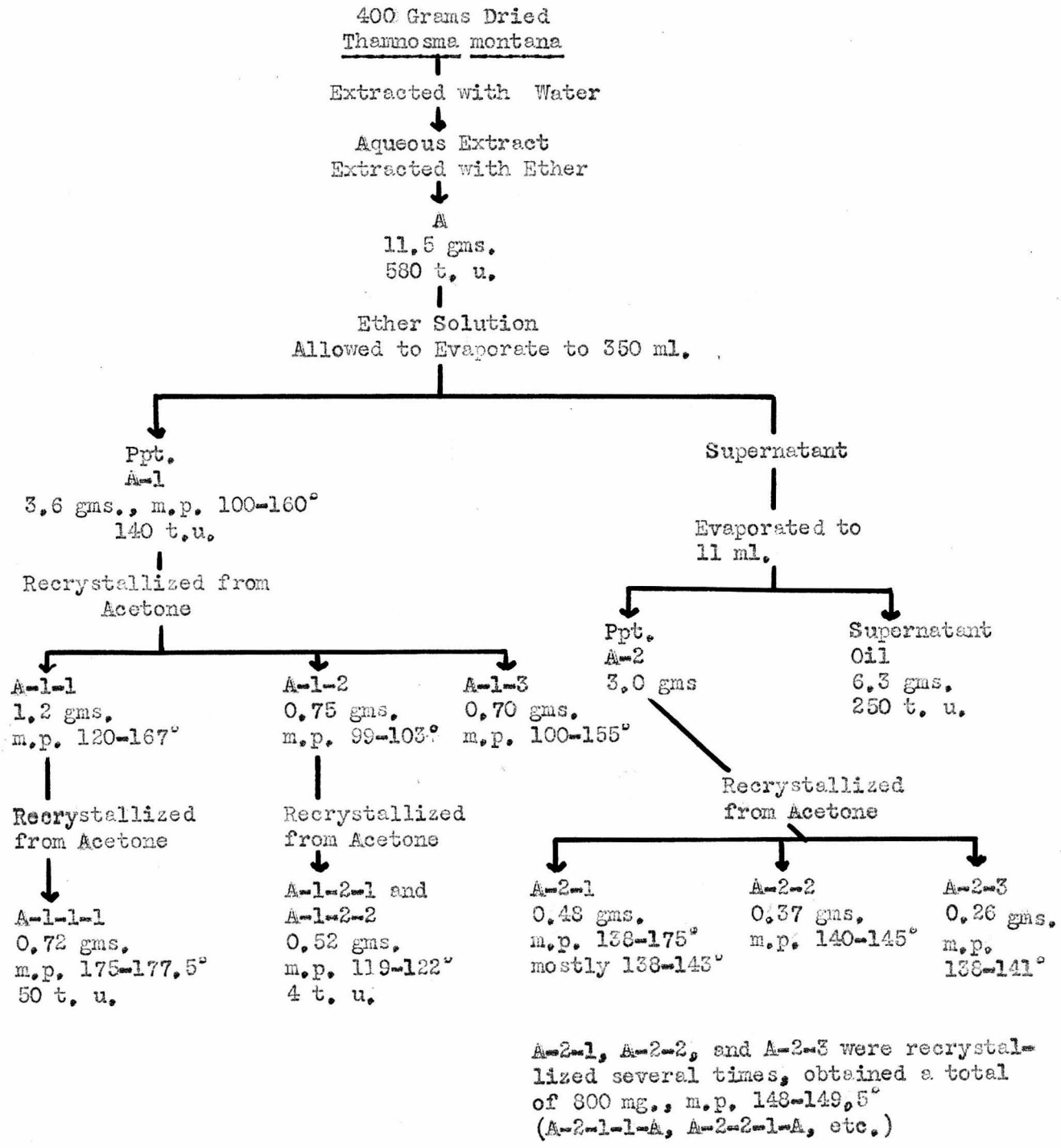
necrotic spots in the primary leaves of the tomato plants. The primary leaf was very stunted. Inhibition of growth was caused at a concentration of the toxic of 9 mg./l. The analysis and spectrum of this compound are discussed in a subsequent section.

Thus from 400 grams of Thamnosma montana there was obtained by the above fractionation 720 mg. of pure toxic compound, m. p. 175-177.5° (corr.) (0.18% from dried plant) (50 t. u.); 520 mg. of toxic compound, m. p. 120-122° (corr.), and 800 mg. of a toxic compound, m. p. 148-149° (corr.). The total weight of material recovered as reasonably sharp melting solids was 2 grams out of 12 grams which had been extracted by ether. The total number of toxic units was 50-55 out of an original total of about 500. By the chromatographic methods discussed below an additional 1 gram of material has been obtained in solid form. Most of these fractions have wide melting ranges, but, tentatively, 300 mg. probably contain the toxic material m. p. 148-149° while 700 mg. contain the 175° toxic compound or other very toxic substances melting in the same range. In all, an additional 50-60 t. u. have been recovered in solid form.

This isolation is summarized in Outline II.

OUTLINE II

Isolation II of Toxic Materials from *Thamnosma montana*



Isolation III.- Since, by the previous isolation methods, only about 10-20% of the total toxic units had been recovered in crystalline form and since initial chromatographic experiments gave promising results, it was decided to attempt an isolation based mainly on chromatographic methods.

300 grams of dried Thamnosma montana were extracted in three 100 gram portions in the Soxhlet extractor as previously described. The aqueous extract was centrifuged to remove a small amount of insoluble material and then it was extracted for two days in the continuous liquid-liquid extractor with ether. On the second day, the boiler became "dry" and the extracted material was inadvertently heated with a small amount of water at 100° for about 2-3 hours. The extract (C) was not readily soluble in 200 ml. of ether; it was finally dissolved in 200 ml. of ether and 300 ml. of acetone. (C) contained 6.8 grams of crude toxic material, toxic at 20 mg./l. (340 t. u.). The aqueous extract of Thamnosma was again extracted with a fresh portion of ether for 4 days. The ether extract contained 2.4 grams of material (D), toxic at 25 mg./l. (100 t. u.). In all, a total of 440 t. u. was obtained from 300 grams of Thamnosma (150 t. u./ 100 grams). Since this is about equal to the number of toxic units obtained in isolation II, it was concluded that the heating at 100° had not destroyed any of the toxicity of the extract.

Chromatography.- (C) and (D) were combined and the ether-acetone was distilled off. The residue was dissolved in 50 ml. of acetone (redistilled), and 450 ml. of benzene (redistilled) were added. Some insoluble oil precipitated out. The supernatant was placed on a No. 6 chromatographic column containing 25 cm. of 2/1 silicic acid-Celite. The column had been prewashed with 200 ml. of 10% acetone-90% benzene. The oil which had been precipitated was dissolved in 10 ml. of acetone and 90 ml. of benzene were added; the supernatant was decanted onto the column. This was repeated twice more after which nearly all of the oily material was on the column.

The column was developed with 1 liter of 10% acetone-90% benzene, followed by 1 liter of 15% acetone-85% benzene, 1 liter of 20% acetone-80% benzene, 500 ml. of 50% acetone-50% benzene, and finally 500 ml. of acetone.

Approximately 200 ml. fractions of the eluent were collected. In general these fractions appeared yellow, although the shade and intensity varied, and the later fractions which were obtained were more highly colored (golden-brown).

Each fraction was concentrated under reduced pressure to 1-2 ml; then it was dissolved in acetone and transferred to a weighed centrifuge tube and allowed to evaporate slowly. In some cases an oil was obtained, in others, semi-crystalline solids. The oily material often crystallized when 1-2 ml. of acetone was added. In general, the fractions were placed

in the cold room for several days after the addition of the acetone and allowed to crystallize. The precipitate (C-X-A) was centrifuged out on a centrifuge funnel and washed with a small volume of acetone. In general only an oil could be obtained from the filtrate from this first precipitate, but in several cases an additional small amount of solid material was obtained from such fractions. A number of the fractions were tested for toxicity to tomato plants. A description of the fractions obtained is contained in Table IV.

In all, a minimum of 8.0 grams out of 9.0 grams taken for the chromatographic experiment were recovered. Of this, a total of 2.2 grams was obtained as solid fractions, about 350 mg. of which was the toxic compound melting at 148°, 190 mg. were the nearly pure toxic compound melting at 175°, and 1.1 grams were obtained as fractions with m. p. of 158-176°. A total of about 100 toxic units was obtained in solid form, and 180 toxic units in the form of active oils. (It is believed that this is a conservative estimate of the actual number of toxic units recovered.).

A second large scale chromatogram was done using fractions which had been obtained as oils from Isolations I and II. In all an estimated total of 7.7 grams was used containing 240 toxic units. A No. 6 column containing 24 cm. of 2/1 silicic acid-Celite which had been prewashed with 500 ml. of 12.5% acetone-87.5% benzene was used. The crude fractions were dissolved in 50 ml. of acetone and 400 ml. of benzene and put on the column. The small amount of material

Table IV

Summary of Fractions Obtained by Chromatography of Crude

Thamnosma montana Extracts on Silicic Acid-Celite

| Fraction | Weight (mg.) | Fraction | Weight (mg.) | Melting Point (corr.) | Minimum Toxic Conc.* (mg./l.) | Fraction | Weight (mg.) | Minimum Toxic Conc.* (mg./l.) |
|---|---|---|---|---|---|---|---|---|
| C-1 | discarded | | | | | | | |
| C-2 | 85 | C-2-A | 20 | 144-147° | | C-2-B | 65 | |
| C-3 | 1400 (min.) | C-3-A | 210 | 145-148° | 150 | C-3,4-B | 1050 (min.) | >100 |
| C-4 | | C-4-A | 125 | 135-170° (mostly by 146°) | | | | |
| C-5 | 625 | C-5-A | 60 | 171-173° | | C-5-B | 565 | 100 |
| C-6 | 1450 | C-6-A | 610 | 158-176° | 15 | C-6-B | 840 | |
| C-7 | 740 | C-7-A | 300 | 158-176° | 15 | C-7-B | 440 | |
| C-8 | 540 | C-8-A | 200 | 158-176° | 15 | C-8-B | 340 | |
| C-9 | 300 | C-9-A | 75 | 173-176° | | C-9-B | 220 | |
| C-10 | 270 | C-10-A | 55 | 175-177° | | C-10-B | 215 | |
| C-6 to C-10-B were combined and recrystallized from acetone. | | | | | | | | |
| | | C-6-10-B | 530 | 105-130° | 40 | C-6-10-C | 1510 | 15 |

Table IV (cont.)

| Fraction | Weight (mg.) | Fraction | Weight (mg.) (corr.) | Melting Point Conc.* (mg./l.) | Minimum Toxic Conc.* (mg./l.) | Fraction | Weight (mg.) | Minimum Toxic Conc.* (mg./l.) |
|---|---|---|---|---|---|---|---|---|
| C-11 and C-12 | 1230 | C-11-12-A | 40 | 295-300° | | C-11-12-B | 1190 | 15 |
| C-13 | 120 | | | oil | | | | |
| C-14-20 | 270 | | | oil | 100 | | | |
| C-21-25 | 1050 | | | oil | 100 | | | |

* Toxicity is expressed as the minimum concentration of material at which the tomato plants were killed in 8 days.

which had precipitated out of this solution was again dissolved in 10 ml. of acetone, 90 ml. of benzene were added, and it was put on the column. This was repeated twice more after which nearly all of the material was on the column. The small amount remaining was discarded. The column was developed with 2.2 liters of 12.5% acetone-87.5% benzene, then with 1 liter of 25% acetone-75% benzene, then 1 liter of 50% acetone-50% benzene, and finally with 750 ml. of acetone. 200 ml. fractions were collected in the eluent. They were treated in a manner similar to that described for the chromatographic experiment above. The results are summarized in Table V.

In all a total of 8.0 grams of material was recovered. About 300 mg. of this material was obtained as the nearly pure toxic compound melting at 148°, about 500 mg. was obtained as solid fractions with a melting point of 156-172°. In addition there were obtained 2.6 grams of a highly active oil (toxic at 10-12.5 mg./l. or less). Also there was 100 mg. of a very high melting component obtained. This compound is orange and is probably a plant pigment. It is too insoluble in water to test for toxicity. It was estimated that a total of 325-340 toxic units were recovered.

A third large scale chromatogram was carried out using an estimated 8.2 grams of Thamnosma toxic fractions. The fractions used included those which had been obtained by

Table V

Summary of Fractions Obtained by Chromatography of Some

Crude Thamnosma montana Fractions on Silicic Acid-Celite

| Fraction | Weight (mg.) | Fraction | Weight (mg.) | Melting Point (corr.) | Minimum Toxic Conc. (mg./m/.) | Fraction | Weight (mg.) | Min. Toxic Conc. |
|---|---|---|---|---|---|---|---|---|
| B-1 | 70 | | | | | | | |
| B-2 | 745 | B-2-A | 165 | 142-145° | | B-1,2,3-B | 1580 | 50 |
| B-3 | 1060 | B-3-A | 125 | 141-144° | | | | |
| B-4 | 520 | | | oil | 100 | | | |
| B-5 | 325 | | | oil | | | | |
| B-6 | 735 | B-6-A | 265 | 156-172° | est. 12.5 | | | |
| B-7 | 995 | B-7-A | 275 | 156-172° | est. 12.5 | B-6,7,8-B | 1800 | 10 |
| B-8 | 665 | B-8-A | 35 | 169-175° | est. 12.5 | | | |
| B-9 | 520 | | | | | B-9,10-B | 860 | 12.5 |
| B-10 | 340 | B-10-A | 75 | 190-192°? | | | | |
| B-11 | 205 | | | | 50 | | | |
| B-12 | 120 | B-12,13-A | 25 | 300-305° | not tested | B-12,13,14-B | 200 | 100 |
| B-13 | 64 | | | | | | | |
| B-14 | 38 | | | | | | | |
| B-15 to B-19 | 420 | B-15-19-A | 75 | 300-305° | not tested | B-15-19-B | 340 | 100 |
| B-19 to B-28 | 1300 | | | | > 200 | | | |

the previous two chromatograms as oils and which were active at concentrations of 15 mg./l. or less and also a few fractions obtained from chromatograms not described herein. An estimated 580 toxic units were contained in these combined fractions with an average toxicity of 14 mg./l. The compound was placed on a No.6 column containing 24 cm. of 2/1 silicic acid-Celite in the same manner as described for the chromatogram above. However, only a very small amount of material was insoluble in the 500 ml. of 12.5 acetone-87.5% benzene in which the compound was initially dissolved. The column was developed with 2800 ml. of 12.5% acetone-87.5% benzene, then with 1 liter of 25% acetone-75% benzene, then with 500 ml. of 50% acetone-50% benzene, and finally with 1 liter of acetone. 200 ml. fractions were collected and recrystallized in a manner similar to that described for the first chromatogram. The results are summarized in Table VI.

From the last described chromatogram there was recovered 7.8 grams of material. Approximately 2.0 grams of this were solid fractions, most of which had wide melting ranges. Approximately 1.7 grams were in the form of inactive oils (not active at concentrations of 50 mg./l. or less), while 3.9 grams were recovered as highly active oils(active at concentrations of 20 mg./l., most of the fractions were active at concentrations of 10 mg/l.). In all an estimated 520 toxic units were recovered. The figure of 10 mg./l. is a

Table VI

Summary of Fractions Obtained by Chromatography of Some Crude Thamnosma montana Fractions on Silicic Acid-Celite

| Fraction | Weight (mg.) | Fraction | Weight (mg.) | Melting Point (corr.) | Min. Toxic Conc. (mg./ml.) | Remarks |
|---|---|---|---|---|---|---|
| D-1 | 145 | | | | | Combined D-1, D-2-B, and D-3,, obtained D-3-A. |
| D-2 | 245 | D-2-A | 25 | 139-146° | | |
| D-3 | 375 | D-3-A | 140 | 90-160 mostly by 120° | | |
| | | D-3-B | 600 | oil | 125 | |
| D-4 | 225 | | | oil | 200 | |
| D-5 | 640 | D-5-A | 60 | 157-170° | | |
| | | D-5-B | 580 | oil | 50 | |
| D-6 | 1600 | D-6-A | 120 | 170-175° | | |
| | | D-6-B | 395 | 100-150° | | |
| | | D-C-C | 1080 | oil | 10 | |
| D-7 | 1335 | D-7-A | 75 | 174-176° | | |
| | | D-7-B | 120 | 98-104° | | |
| | | D-7-C | 185 | <95-140° | | |
| | | D-7-D | 950 | oil | 20 | |
| D-8 | 1070 | D-8-A | 200 | 92-158° | | |
| | | D-8-B | 35 | 90-110° | | |
| | | D-8-C | 830 | oil | 10 | |
| D-9 | 785 | D-9-A | 60 | 98-108° | | |
| | | D-9-B | 195 | <90-150° | | |
| | | D-9-C | 530 | oil | 10 | |
| D-10 | 515 | D-10-A | 90 | 105-180 mostly 155-180° | | |
| D-11 | 335 | D-11-12-A | 20 | 300-305° | | D-11 and D-12 combined. The filtrate from D-11-12-A was combined with that from D-10-A |
| D-12 | 170 | | | | | |
| | | D-10-12-B | 375 | 80-120° mostly 100-120° | | |
| | | D-10-12-C | 520 | oil | 10 | |
| D-13-25 | 390 | | | oil | 50 | |

conservative estimate of the activity of several of these oil fractions and it seems to the author that it is very likely that they contain a toxic compound (or compounds) which has not as yet been isolated in a crystalline form. The active oils and a number of the solid but not pure toxic fractions should be purified further by appropriate means. It is believed that by further chromatographic experiments additional purification can be achieved, since the experiments described above the columns were overloaded and the emphasis has been placed on separating on a large scale the highly active from the more inactive components.

The overall yield of products from the three isolations is approximately as indicated below in Table VII.

Table VII

Summary of Fractions Obtained from Thamnosma montana

| Description of Fractions | Approximate Yield | Estimated Toxicity Units | Comments |
|---|---|---|---|
| m.p. 170-175° | 1.8 gr. | 150 | This compound is toxic at 12.5 mg./l. |
| m.p. 158-175° | 1.7 gr. | 120 | This fraction is toxic at 12.5-15 mg./l. |
| m.p. 140-148° | 1.5 gr. | -- | Inhibits growth at a concentration of 10 mg./l. |
| m.p. 118-122° | 0.6 gr. | 5 | This compound is toxic at 125 mg./l and inhibits growth at 20 mg./l. |
| Wide melting in range 80-160° | 3.8 gr. | 250 | Estimated toxic units, most fractions have not been tested. |
| Oils (active) | 3.9 gr. | 390 | These oils are active at a concentration of 10 mg./l. or less. |
| Oils (inactive) | 8.2 gr. | | Not toxic at concentrations of 50 mg./l. Most fractions not active at concentrations of 100-200 mg./l. |
| Total | 21.5 gr.* | 915 | |

* The total amount of material which had been initially extracted into ether from the aqueous extracts and which was used for the above isolation was 28 grams from 900 grams of Thamnosma montana and contained an estimated total of 1290 toxic units. The yields given have not been corrected for the material which was used for toxicity experiments or treated in other miscellaneous ways not described in this thesis.

C. Analyses of Toxic Compounds from Thamnosma montana.-

Qualitative tests using the zinc-calcium oxide fusion method described in part III of this thesis indicated that the three crystalline compounds which had been isolated did not contain nitrogen, halogen (including fluoride), sulfur, arsenic or phosphorus. Presumably they contain only carbon, hydrogen, and oxygen.

Carbon, hydrogen, methoxyl, and Rast molecular weight determinations have been made on several different samples of each of the three toxic substances which were obtained as pure compounds. They are summarized below.

| Fraction | m.p. (corr.) | Analysis | Analyst |
|---|---|---|---|
| F-B-2A-1 | 176-177° | C, 64.84; H, 5.80 | Oppenheimer |
| F-B-2-B | 176-177° | C, 64.80; H, 5.99<br>$OCH_3$, 9.98%<br>M. W. (Rast) 272 | " |
| F-B-2-B | " | C, 64.58; H, 5.79 | Elek |
| A-1-1-1 (Recrystallized) | " | C, 64.78; H, 5.78<br>$OCH_3$, 9.87% | Elek |
| | Average | C, 64.75; H, 5.84<br>$OCH_3$, 9.92% | |

On the assumption that the compound contains one methoxyl, a molecular weight of 313 is obtained. Using this as a basis $C_{17}H_{18}O_6$ or $C_{17}H_{16}O_6$ are calculated as the most reasonable tentative formulas. The latter formula is preferred.

Anal. Calcd. for $C_{17}H_{18}O_6$ (318.3): C, 64.14; H, 5.70; $OCH_3$, 9.75

$C_{17}H_{16}O_6$ (316.3): C, 64.54; H, 5.10; $OCH_3$, 9.81

| Fraction | m.p. (corr.) | Analysis | Analyst |
|---|---|---|---|
| A-2-2-1-A | 148-149° | C, 63.73; H, 4.52 | Swinehart |
| | | $OCH_3$, 25.50% | " |
| | | M. W. (Rast) 227 | |
| " | " | C, 63.54; H, 4.17 | Elek |
| | | $OCH_3$, 24.53% | |

On the assumption that the compound contains two methoxyl groups, a molecular weight between 243 and 253 is obtained. Using this as a basis, $C_{13}H_{10}O_5$ is calculated as the most reasonable formula.

Anal. Calcd. for $C_{13}H_{10}O_5$ (246.2): C, 63.41; H, 4.09; $CH_3O$, 25.20%.

The formula for this compound may also be written tentatively as $C_{11}H_4O_3(OCH_3)_2$; therefore, it is highly unsaturated.

| Fraction | m.p. (corr.) | Analysis | Analyst |
|---|---|---|---|
| A-1-2-2-R | 121-122° | C, 61.38; H, 5.46 | Elek |
| | | $OCH_3$, 9.15 | " |
| | | M. W. (Rast) 357 | Swinehart |

On the assumption that the compound contains one methoxyl group, $C_{17}H_{18}O_7$ has been calculated as the most reasonable formula.

Anal. Calcd. for $C_{17}H_{18}O_7$ (334.3): C, 61.07; H, 5.43; $OCH_3$, 9.28.

It is interesting to note that this formula differs from the preferred formula for the 177° compound by one $H_2O$.

Independent estimates of the molecular weight ratios of these compounds have been obtained from their spectra.

D. Spectra of Toxic Compounds from Thamnosma montana.-

The ultra-violet absorption spectra of the three crystalline compounds were obtained in methanol using the Beckman Model DU spectrophotometer. The absorption curves are shown in Figure I. $E^{1\%}_{1\ cm.\ max.}$ and $E^{1\%}_{1\ cm.\ min.}$ are summarized in Table VIII. Also calculated are $\varepsilon_{max.}$ and $\varepsilon_{min.}$ using the tentative formulas assigned above.

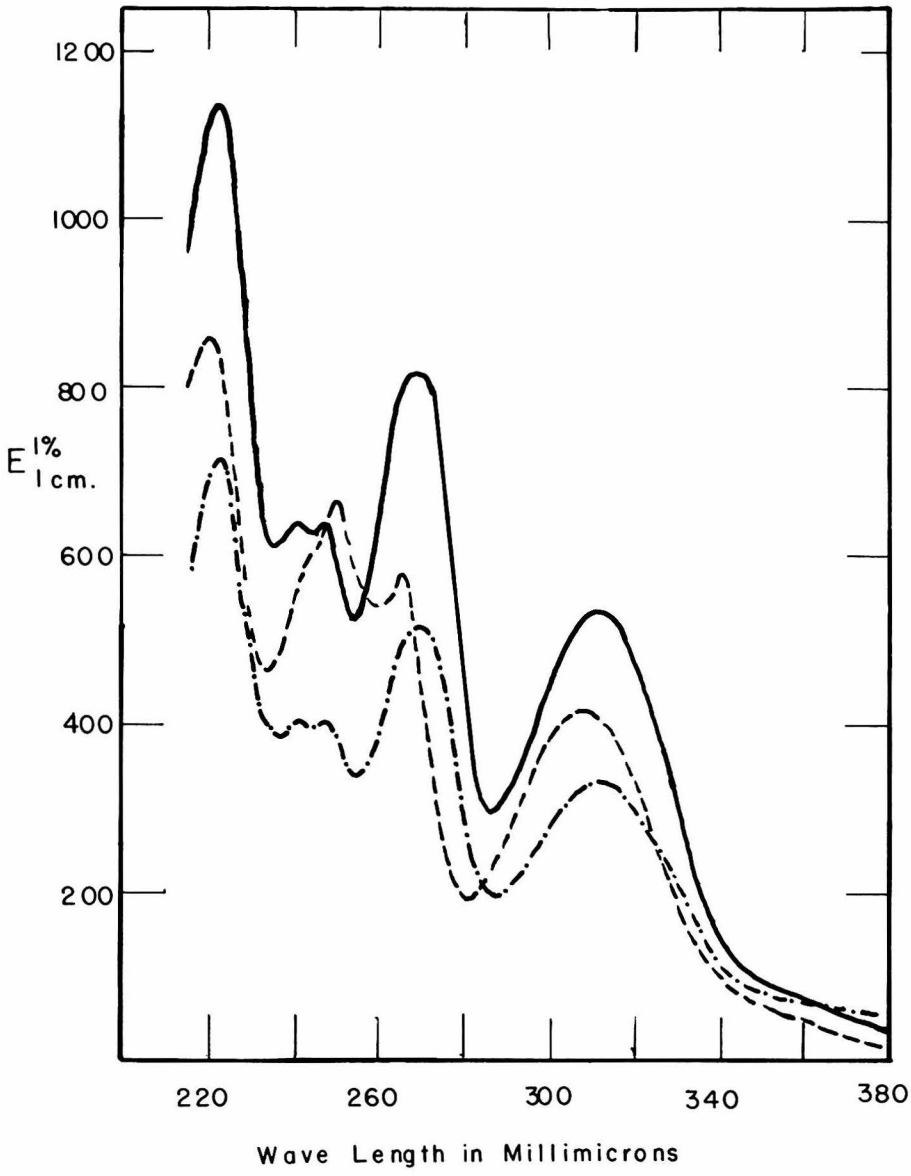



Figure 1. The Ultra-Violet Absorption Spectra of Crystalline Toxic Compounds Isolated from Thamnosma montana.

m. p. 148-149.5°

m. p. 176-177° - - - - - - - -

and m. p. 121-122° -.-.-.-.-.-.-.-

Table VIII

$E^{1\%}_{1\ cm.\ max.}$ and $E^{1\%}_{1\ cm.\ min.}$ and Tentative Values for $\varepsilon_{max.}$ and $\varepsilon_{min.}$ for the Crystalline Compounds Isolated from Thamnosma montana (in methanol)

| Fraction | $\lambda$ (mμ) | $E^{1\%}_{1\ cm.}$ | $\varepsilon$ (tentative) |
|---|---|---|---|
| m.p. 176-177° | 308 (max.) | 420 | 13,300 |
| | 281 (min.) | 200 | 6.300 |
| F-B-2-B or | 266 (max.) | 580 | 18,300 |
| A-1-1-1 | 261 (min.) | 545 | 17,200 |
| | 251 (max.) | 670 | 21.200 |
| | 234 (min.) | 470 | 14,800 |
| | 221 (max.) | 875 | 27,600 |
| m.p. 148-149° | 312 (max.) | 545 | 13,400 |
| | 287 (min.) | 300 | 7,400 |
| A-2-1-1-A or | 270 (max.) | 830 | 20,400 |
| A-2-2-1-A | 254 (min.) | 525 | 12,900 |
| | 248 (max.) | 640 | 15,700 |
| | 244 (min.) | 620 | 15,300 |
| | 242 (max.) | 635 | 15,600 |
| | 236 (min.) | 610 | 15,000 |
| | 222 (max.) | 1150 | 28,300 |
| m.p. 121-122° | 312 (max.) | 340 | 11,400 |
| | 288 (min.) | 200 | 6,700 |
| A-1-2-2 | 270 (max.) | 530 | 17,700 |
| | 254 (min.) | 345 | 11,500 |
| | 248 (max.) | 415 | 13,900 |
| | 244 (min.) | 410 | 13,700 |
| | 242 (max.) | 420 | 14,000 |
| | 236 (min.) | 400 | 13,300 |
| | 223 (max.) | 750 | 25,000 |

The absorption spectra of the three crystalline compounds indicate that the compounds with melting points of 121-122° and 148-149° are very closely related in structure since their spectra are identical in shape, differing only in intensity (when calculated as $E^{1\%}_{1\,cm.}$). The molar extinction coefficients calculated at the maxima and minima on the basis of the formulas assigned from the analytical data do not agree closely. This may be an indication that one or both of the assigned formulas are in error. The absorption spectrum of the toxic with melting point 176-177° shows maxima in the 310 mμ, 266 mμ and 222 mμ region similar to those obtained for the other two compounds. In addition it has a distinguishing peak of high intensity at 251 mμ. The molar extinction coefficients calculated for this compound on the basis of the tentative formula assigned agree very well with those calculated for the compound with a m. p. of 148-149°.

In the absence of additional data on the structure of these compounds it is difficult to interpret their absorption spectra. It appears fairly certain that conjugated chromophores are present, however; perhaps the maximum in the 220 mμ region is the E (ethylenic) absorption of benzene shifted toward the visible by the presence of chromophores in conjugation with it (5). It is to be noted that all of the absorption maxima observed in the region 220-315 mμ are relatively intense.

The absorption spectrum of the toxic compound with melting point 176-177° is essentially the same in water as it is in methanol. Its spectrum remained unchanged when dissolved in water or dilute hydrochloric acid (pH 2.6) for 100 days (stored in the dark).

The change in the spectrum of the 176-177° toxic compound was investigated in dilute base. The spectrum in water at pH 5.6, at pH 12 after 130-140 minutes, and pH 12 after 22 hours are shown in Figure II. Other experiments at pH 11.2 indicated that similar changes occurred at this pH but less rapidly. The absorption at 308 mμ in the presence of dilute base decreased more rapidly in intensity than the absorption at the shorter wave lengths. On acifification of a solution of the toxic compound which had been allowed to stand at pH 12.5 for 3 hours, the maxima and minima in the region 220-280 mμ characteristic of the original compound were present as soon as the data could be obtained, (within 2 to 4 minutes). However, the maximum at 308 mμ was restored much more slowly,-between 15 to 40 minutes being required for it to obtain its final value. In this experiment, the intensity of absorption of the final curve was about 10% less than the initial curve, but this may have been within the experimental error. The experiment was not repeated.

The behaviour in dilute base and acid of this compound may indicate that the group (or groups) which cause the absorption in the 308 mμ region are slowly base labile, while

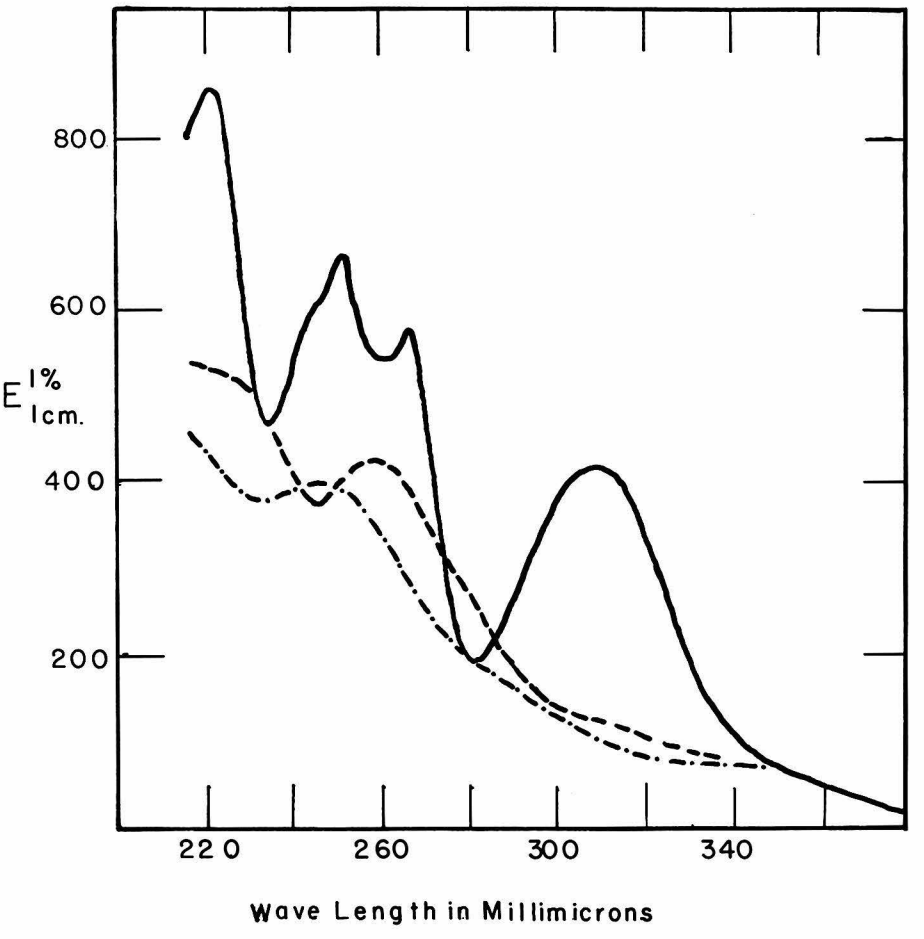



Figure 2. The Ultra-Violet Absorption Spectra of toxic compound from Thamnosma montana, m. p. 176-177°
pH 5.6 (water) _____,
pH 12.0 (water) after 130-140 minutes- - - -,
and after 22 hours.-.-.-.-.-.-.-.

the chromophoric groups which cause absorption at shorter wave lengths are changed in the presence of base after the group causing absorption at 308 mμ has been modified. In acid, the chromophores causing absorption at the lower wave lengths rapidly return to their original structure, followed by a slow restoration of the chromophoric group or groups causing absorption at 308 mμ.

The infra-red spectrum of A-1-1-1 (recrystallized) was obtained in the region 2.6 to 13.3 microns*. The spectrum was taken in a mineral oil mull using a recording Beckman infra-red spectrophotometer. The absorption at 3.49, 6.95 and 7.39 microns is due to the mineral oil. Strong absorption was obtained at 2.99 microns indicating the presence of -OH groups (6,7). The absorption at 5.98 microns is undoubtedly indicative of a carbonyl group such as an aldehyde or a ketone. Absorption in the 12 to 14 micron region is often characteristic of substituted phenyl derivatives.

The infra-red spectra of the other crystalline fractions which have been obtained would possibly give further indication of the manner in which these compounds differ, particularly after some functional group tests and some derivatives have been made.

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* The infra-red spectrum was obtained by Mr. S. Burket.

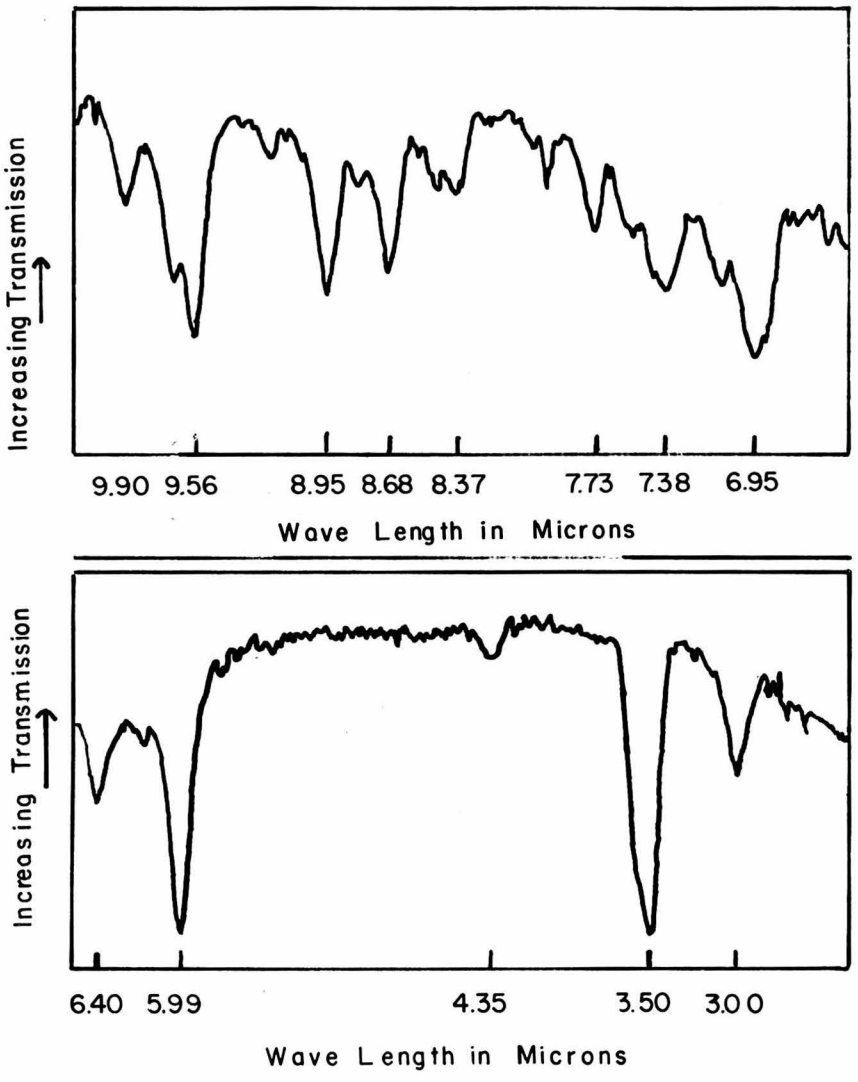



Figure 3A. Infra-Red Spectrum of Crystalline Toxic Compound, m. p. 176-177° (A-1-1-1) from Thamnosma montana (in mineral oil mull).

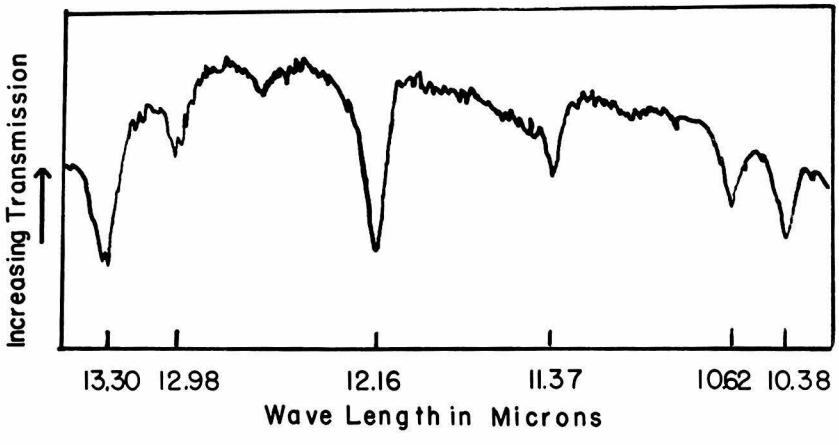



Figure 3B. Infra-Red Spectrum of Crystalline Toxic Compound, m. p. 176-177° (A-1-1-1) from Thamnosma montana (in mineral oil mull).

E. Chemical properties of the Toxic Substances in Thamnosma montana.-

As yet no systematic investigation of the functional groups present in these toxic substances has been made. It has been noted above that these toxic compounds are extractable by water and by organic solvents. The compound with a m. p. of 176-177° is less soluble than the other two compounds in acetone. The toxic compound with a m. p. of 148-149° is only slightly soluble in water, and the compound with a m. p. of 121-122° has only limited solubility. The highest melting compound is the most soluble of the three compounds in water. Evidence has been obtained that the toxic compounds are sublimable and no loss of activity seems to occur in subliming in vacuo at 150°. The toxicity of the extracts of Thamnosma does not seem to diminish with time, thus indicating that the compounds are stable. Also, the spectrum of the 176-177° toxic did not change over a long period of time when dissolved in dilute acid or water. The spectrum of the highest melting toxic fraction is reversibly changed in acid and base. The spectra of the other two compounds have not been investigated under similar conditions. All three of the compounds contain methoxyl groups and are probably unsaturated. Using the Calandra formula for the structure of organic compounds*, it can be calculated that the sum of the

* $r + d + 2t = 3n + 1 - e/2$
where r = the number of rings, d = the number of double bonds, t = the number of triple bonds, n = the number of atoms forming complete octets, and e = the total number of available electrons.

rings, double bonds, and two times the triple bonds is 9 for $C_{17}H_{18}O_6$, $C_{17}H_{18}O_7$ and $C_{13}H_{10}O_5$ and 10 for $C_{17}H_{16}O_6$.

The toxic compound with a melting point of 176-177° does not give a phenol test (ferric chloride) but it does reduce Tollen's reagent at room temperature (reduction begins within 1 minute).

No attempt has been made to prepare derivatives or to do oxidative or other types of degradative studies on the pure compounds which have been obtained.

## Summary

A survey of 11 desert plants for the presence of compounds toxic to tomato plants indicated that highly toxic extracts were obtained from *Thamnosma montana*, *Sarcobatus vermiculatus*, *Prosopis juliflora* and *Viguiera reticulata*.

Three crystalline toxic compounds have been isolated from *Thamnosma montana*. These compounds have been assigned the tentative empirical formulas $C_{16}H_{15}O_5(OCH_3)$ or $C_{16}H_{13}O_5(OCH_3)$, $C_{11}H_4O_3(OCH_3)_2$, and $C_{16}H_{15}O_6(OCH_3)$. The ultra-violet absorption spectra of these compounds indicate that they are probably very similar in structure.

The most toxic of the three crystalline compounds which has been isolated will cause seedling tomato plants grown in nutrient culture to die in one week at a concentration of 12.5-15 mg./l. Non-crystalline oils have also been obtained which are equally or even more toxic, indicating that other highly toxic materials may be present in *Thamnosma montana*.

## References

1. Gray, R., Ph. D. Thesis, Calif. Inst. of Technology, (1948).

2. Gray, R. and Bonner, J., Am. J. Bot., 35, 52 (1948).

3. Gray, R. and Bonner, J., J. Am. Chem. Soc., 70, 1249 (1948).

4. Bonner, J. and Galston, A., Bot. Gaz.,106, 185 (1944).

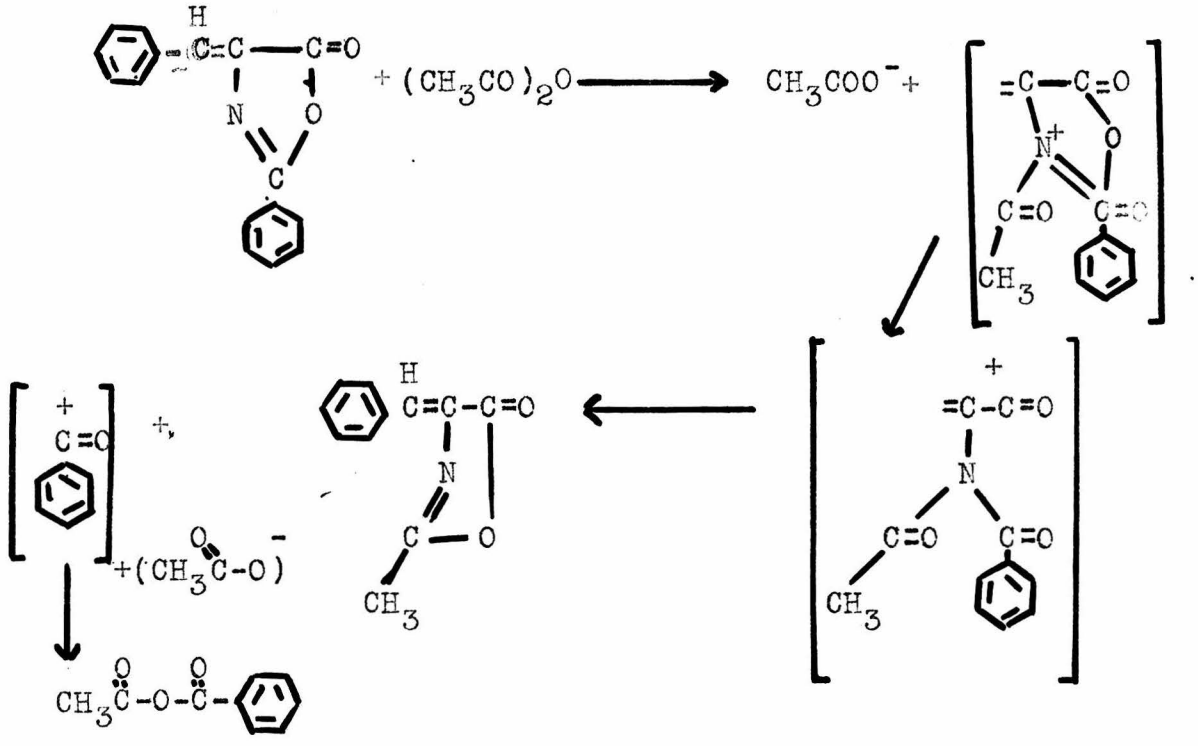
5. Braude, E., Chem. Society, Annual Reports, 42, 105 (1945).

6. Barnes, R., Gore, R., Stafford, R. and Williams, V., Analytical Chem., 20, 402 (1948).

7. Anderson, J. and Seyfried, W., Analytical Chem., 20, 998 (1948).

PROPOSITIONS

1. Trans-acylation has been observed in the preparation of oxazalones (section 1-A, Thesis). The following mechanism is proposed for such trans-acylation:



2. It is proposed that methyl cresyl ether and substituted methyl cresyl ethers can be brominated in the side chain by N-bromosuccinimide when the reaction is catalyzed by peroxide catalysts or by ultra-violet light. The resulting compounds would be useful in syntheses of tyrosine and substituted tyrosines.

Djerrassi, C., Chem. Rev., 43, 271 (1948).

3. a. It is sometimes desirable to isolate aldehydes as bisulfite addition compounds. It is proposed that such compounds after drying, would be suitable to use directly in the Erlenmeyer azlactone synthesis without preliminary regeneration to the aldehyde.

b. It is proposed that oxazalones will be rapidly converted to the corresponding amino acid ester in alcoholic solution in the presence of ultra-violet light.

4. It is proposed that electrophoresis on paper would be feasible on a preparative scale using an apparatus similar in design to that used by Mitchell for paper chromatography but modified so that an E. M. F. can be applied.

Mitchell, H., Science, submitted for publication.

Haugaard, G. and Kroner, T., J. Am. Chem. Soc., 70, 2135 (1948).

5. The following method of synthesis of chloromycetin (D-threo-2-(dichloroacetamido)-3-(p-nitrophenyl)-1,3-propanediol) (1) is proposed:

a.) N-acetyl-erythro-DL-phenylserine is prepared from trans-cinnamic acid (2).

b.) The erythro-isomer so obtained is converted into the threo-isomer by treating the former with diazomethane, and reacting the ester with excess thionyl chloride at room temperature (3). The product (2-methyl-5-phenyl-4-carbomethoxyoxazaline hydrochloride) is hydrolyzed with

dilute acid to yield N-acetyl-threo-DL-phenylserine.

c.) N-acetyl-threo-DL-phenylserine is resolved into D- and L-isomers with papain and the D-isomer is recovered unchanged. This is hydrolyzed to the optically active free amino acid, reduced with $LiAlH_4$ to the corresponding amino alcohol, and subsequently converted by the methods used by Crooks et al to chloromycetin (or chloroamphenicol).

1. Chem. and Eng. News, p. 1135, April 18 (1949).

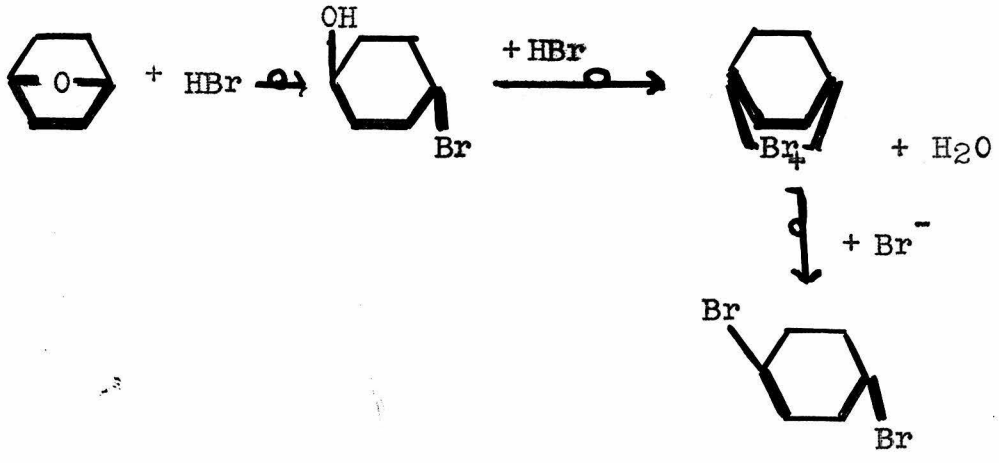
2. Carter, H. and Zirkle, C., J. Biol. Chem., 178, 709 (1949).

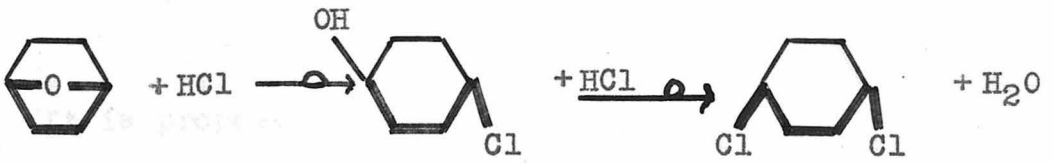
3. Pfister, K., Robinson, C., Shabica, A. and Tishler, M., J. Am. Chem. Soc. 70, 2297 (1949).

6. Evidence is presented (section I-D, Thesis) that the dichlorocyclohexane which is isolated by treatment of 1,4-epoxyclohexane with concentrated HCl is cis-1,4-dichloro-cyclohexane. The trans-dibromide has been obtained by treating 1,4-epoxycyclohexane with concentrated HBr (1).

The following mechanism is proposed to account for the difference in configuration of the products of the two reactions:

a.)



b.) 


This mechanism is consistent with earlier observations (2) that the chlorine atom has less tendency to form cyclic intermediates than does the bromine atom.

b. 1,4-Epoxycyclohexane is proposed as a starting material to make pure trans-4-chlorocyclohexanol.

1. Olberg, R., Pines, H. and Ipatieff, V., J. Am. Chem. Soc.,66, 1098 (1944).

2. Lucas, H. and Gould, C., J. Am. Chem. Soc., 63, 2541 (1941).

7. The solubilities of cesium and rubidium dichloroiodides in hydrochloric acid solutions were recently determined by gravimetric methods. It is proposed that such determinations could be made with greater ease by the use of iodometric methods.

Bender, P. and Strehlow, R., J. Am. Chem. Soc., 70, 1995 (1948).

8. a. It is proposed that the toxic compound (m. p. 175°) isolated from Thamnosma montana (section IV, Thesis) is a lactone.

b. It is proposed that this and other compounds which have been isolated from Thamnosma montana will have antibiotic properties.

Haynes, L. J., Quarterly Reviews, 2, 46 (1948).

9. a. It is proposed that fluorooxaloacetic acid can be synthesized from ethyl oxalate and ethyl fluoroacetate.

b. This compound may be an inhibitor in the Krebs cycle and may facilitate the study of certain steps in the cycle.

10. It has recently been demonstrated that N-acetyl-p-methoxyphenylalanine and N-benzoyl-p-methoxyphenylalanine do not form normal acid chlorides, while N-carbobenzoxy-p-methoxyphenylalanine and N-toluenesulfonyl-p-methoxy-phenylalanine do form acid chlorides. It is proposed that a correlation will be found between the acylated amino acids which do not form normal acid chlorides and the specificity of resolution by papain.

Carter, H. and Hinman, J., J. Biol. Chem., 178, 403 (1949).

11. It is proposed that an effort be made to improve the appearance of the California Institute of Technology campus by suitable plantings of annuals and shrubs which are more floriferous than those which are at present planted on the campus.