Studies upon the Isolation and Identification of Auxins in Plant Materials

A Thesis

Submitted by

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F O R E W O R D

In presenting the results of these investigations, I wish to express my appreciation to all those who have been of assistance in my years of graduate study. My gratitude especially is due Professor Arie J. Haagen-Smit for his unstinting effort and sympathetic interest in the direction of my studies.

To my associates in the laboratory, particularly Doctor Arthur N. Prater, Doctor David M. Bonner and Doctor John R. Raper, I express my thanks.

S U M M A R Y

SUMMARY

1. A survey was made of plant materials in search of a suitable new source material for auxin isolation.

2. The existing methods of isolation were considered as to their usefulness and point of application in the procedure. An alkali acetone purification and an exhaustive ligroin extraction were introduced.

3. It was found that indole-3-acetic acid rendered uncertain the interpretations of separations made in the final isolation steps. Heretofore, it has been considered that all of the indole-3-acetic acid is completely destroyed by the esterification procedure preliminary to high-vacuum distillation. This has been shown not to be the case.

4. The phenomenon of the increased yield of auxin from wheat and corn by alkali treatment was investigated by isolation. Both pseudo-auxin-a and indole-3-acetic acid were isolated and identified, which marks the first time that this last substance has been found in a higher plant material. The evidence obtained indicates that about 90% of the increased amount of auxin obtained by the alkali hydrolysis is due to indole-3-acetic acid.

5. A substance giving a positive curvature in the Avena test was discovered. This substance is a growth inhibitor.

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SECTION I

INTRODUCTION TO THE DETERMINATION, ISOLATION AND IDENTIFICATION OF AUXINS

Introduction to the Determination, Isolation and Identification of Auxins

The term plant hormone (phytohormone) has been defined in the following way: "A hormone is a substance which, being produced in any one part of the organism, is transferred to another part and there influences a specific physiological process". (1)

The phytohormones concerned in the present work are the auxins, a group of substances regulating cell elongation. The customary use of the term "auxin" is generic rather than specific, since the term is applied to any naturally occurring substance which produces a negative curvature in a standardized physiological test, the Avena test. Three of these auxins have been isolated and their structures determined. It is quite possible that other auxins, of related or different type structures, occur in nature.

The first indication of the occurrence of growth-promoting substances in quantity outside of the plant was given in 1925 by the experiments of Seubert (2), who found that malt extract, saliva, diastase, and pepsin contained a substance (apparently not an enzyme) which was active in causing negative Avena curvatures. Gorter (3) also found growth-promoting activity in numerous enzyme preparations. In 1928, Went (4), in a report on his investigations upon the growth substance secreted by the Avena coleoptile tip, showed that physiological activity remains after boiling. Consequently the active substance was not enzymatic in character.

Also in 1928, Nielsen (5) showed that a growth substance which influences cell elongation in Avena is formed by Rhizopus suinus and by Absidia ramosa (two pathogenic fungi). Subsequently, studies were made on isolation methods by various investigators. In 1932 Dolk and Thimann (6) in the United States published a report on investigations in which they had succeeded in effecting large purifications of the active substance from a liquid Rhizopus culture medium. Simultaneously and independently Kögl and Haagen-Smit (7) in Holland made preliminary purifications upon extracts of the growth-substances formed by cultures of Rhizopus reflexus, yeast, and Bacterium coli. At the same time they found a growth substance to be present in large quantities in human urine.

When urine was carried through an isolation process by Kögl, Haagen-Smit and Erxleben (7 and 8), a small amount of a very active acid finally was obtained. This acid ($C_{18}H_{32}O_5$) was called auxin-a. The extent of purification from evaporated urine was 20,000 to 50,000 times.

Subsequently Kögl, Erxleben, and Haagen-Smit (9), by the use of a similar separation procedure, isolated another physiologically active substance from malt and from corn germ oil. This substance ($C_{18}H_{30}O_4$), also an acid, was called auxin-b. It proved to be isomeric with the lactone formed from auxin-a, and it had the same activity as auxin-a.

Both auxin-a and -b spontaneously lose their physiological activity upon standing for one to two months (8). The inactivation goes on even in the crystalline state, and no way has been found of inhibiting the change. No oxidation is

is involved, for the process may take place in vacuo, and in the dark. Many methods of storage of the substance have been tried, but the inactivation goes on at the same rate under all conditions. That the change must be one of isomerization is proved by the fact that the inactivation does not involve a change in composition or in molecular weight, and further investigation (10) showed that on inactivation an allyl rearrangement takes place. The inactive product is called pseudo-auxin.

Subsequent to the isolation of auxin-a and -b, another active substance was isolated from urine and identified by Kögl, Haagen-Smit, and Erxleben (11). This substance, often called hetero-auxin, proved to be identical with indole-3-acetic acid.

Next, Kögl and Kostermans (12) investigated yeast plasmolysate, a material on which preliminary studies had been made by Kögl and Haagen-Smit (7), and isolated the same substance, indole-3-acetic acid. They showed that this substance represented a considerable part of the original activity of the yeast.

Thimann (13), continuing earlier work of Dolk and Thimann, succeeded in isolating the active substance from Rhizopus cultures. Although the material isolated was not of such purity that a correct melting point of indole-3-acetic acid was found and no mixed melting point could be taken, the substance isolated showed the sensitivity to acids and the color reactions of indole-3-acetic acid.

Until the present time, it has been considered that auxins-a- and -b are associated with the higher plants and that hetero-auxin (indole-3-acetic acid) is to be found in the lower plants only. The presence of indole-3-acetic acid in human urine has been ascribed to decomposition of tryptophane.

A tremendous amount of work has been done on the physiology and chemistry of the auxins. The subject matter of the field has been covered in the book "Phytohormones" by Went and Thimann (1937), and in many review articles, the latest of which are by Haagen-Smit (14) and Went (15).

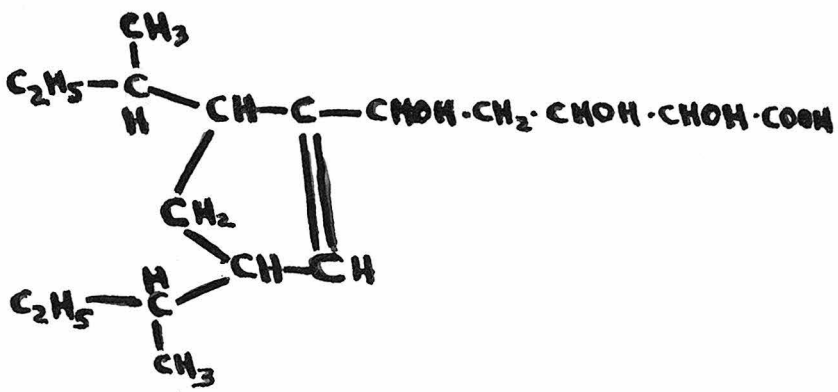
Structure of the Auxins

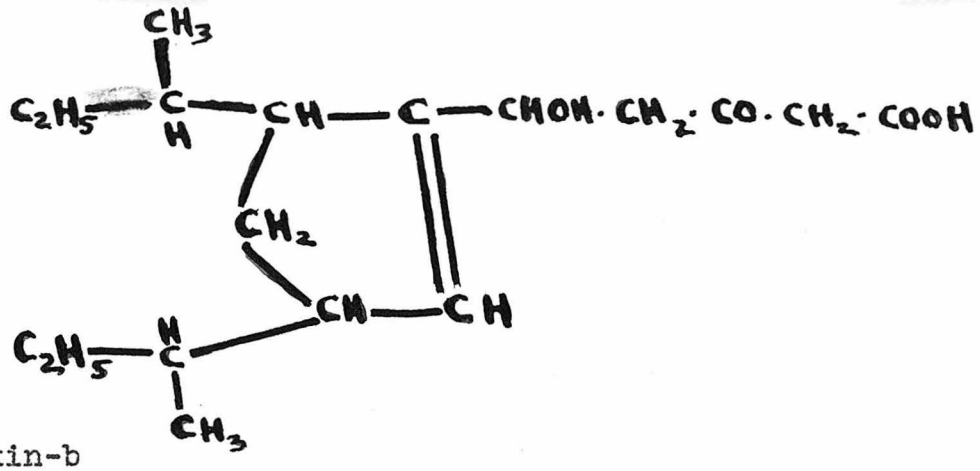
Auxins-a and -b

Although a total of only 700 milligrams of crystalline material was available, the structures of auxins-a and -b were determined in a series of researches by Kögl and his associates (16, 17, 18). Auxin-a was found to be a cyclopentene derivative. Auxin-b was found to be closely related to auxin-a. The difference in the structures is brought out by the following structural formulas:

Auxin-a

(Auxentriolic Acid)

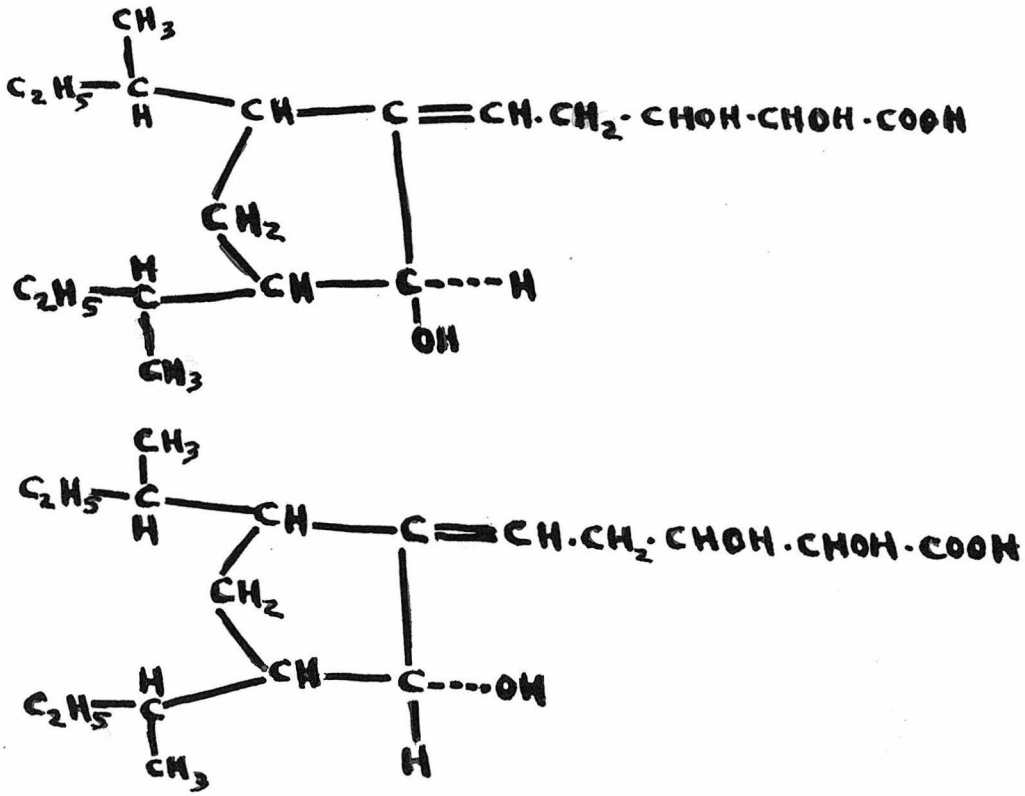




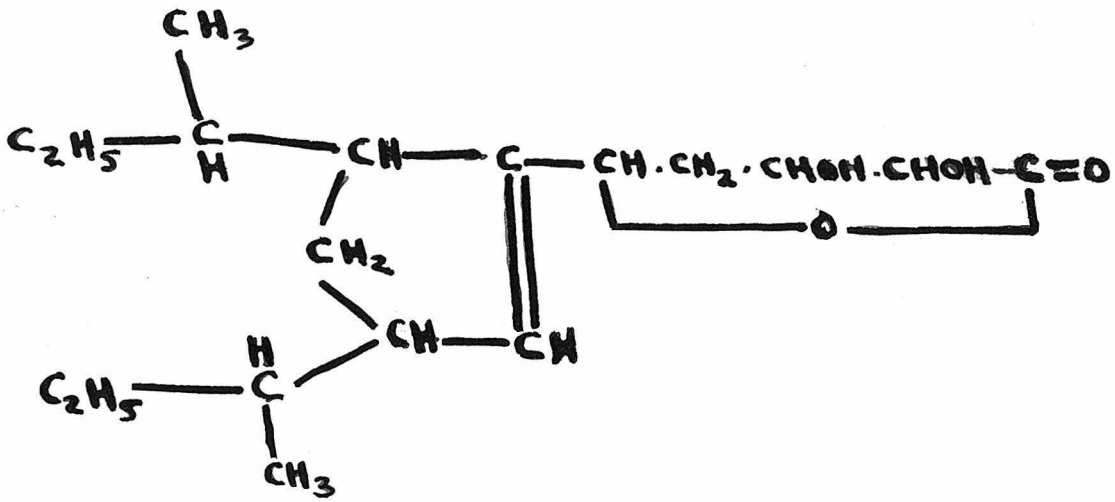
Auxin-b

(Auxenolonic Acid)

Pseudo-auxin-a was found to be the isomer of auxin-a formed by the shift of the double bond from the ring to the side-chain, the delta-hydroxyl group shifting to the ring (10). An asymmetric carbon atom is thus formed, providing for two optically active pseudo auxins from auxin-a.



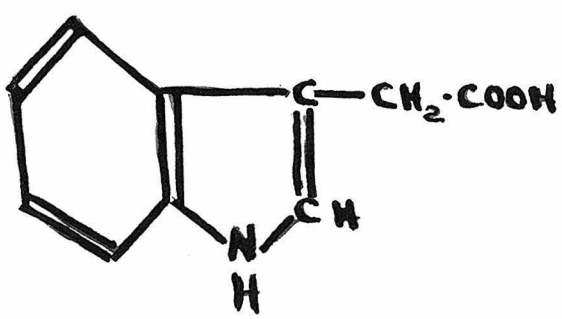
Pseudo-auxin-a_1 and -a_2



Auxin-a-lactone

Under the influence of ultraviolet light, auxin-a-lactone rearranges into lumi-auxin-a-lactone. This product has been shown not to be a true lactone but to be identical with the pseudo-auxin arising from the self-inactivation of auxin-a-lactone (19).

Indole-3-acetic acid



As has been mentioned before in discussions concerning growth substances in plants, the term auxin is used in a non-specific way. At the present time this usage is necessary, since a great deal of study still must be done on

the problems of the identification of auxins and of the state of auxins in plant materials.

Although various tests have been devised for differentiating between auxins in plant tissue extracts, the only certain method of identification is still the actual isolation in pure condition of the physiologically active substances.

Among the differentiation tests devised, one frequently used has been that of Kögl, Haagen-Smit, and Erxleben (9), which depends upon the different stabilities of auxin-a, auxin-b, and indole-3-acetic acid during boiling with acid and boiling with base. In most cases the application of this method has been to such relatively impure extracts that uncertainty regarding the validity of the results is inevitable.

Another differentiation method which has been applied is the calculation of molecular weight from diffusion experiment date (9). While valuable indications are obtained with this procedure, there can be no certainty as to the molecule involved. Furthermore, recent investigations have shown that inhibitor substances affect the diffusion experiments (20).

The sensitivity of auxin-a and -b to light has also been used by van Overbeek as a test to differentiate between these substances and indole-3-acetic acid (21). When this rearrangement reaction test is used upon crude extracts, uncertainties are introduced due to the possible interfering effects of the various substances present together with the active material.

Color tests, principally based on reactions with ferric chloride, have been applied to the recognition of indole-3-acetic acid (9, 22). However, these color tests are known to be very unreliable due to the great number of substances with which ferric chloride forms colored compounds.

In order to determine the identity of a growth substance or to estimate its activity, it is first necessary to extract the active substance from the plant material and transfer it to a medium which conveniently can be used for an isolation procedure or for physiological testing. For some time it has been clear that the procedure used for the extraction has an important relation to the amount of active substance extracted.

There are four essentially different ways in which the extraction of the auxin may be carried out.

(1) Diffusion from the plant medium into an artificially prepared medium such as agar (4).

(2) Extraction with a solvent.

(3) Extraction with a solvent following chemical or enzymatic treatment <u>in vitro</u> by:

  (a) sodium ethylate hydrolysis (23)

  (b) lipase (24)

  (c) chymotrypsin (Private communication from W. D. Leech, and 25)

(4) Biological digestion (24), in which the plant material is ingested by an animal, and the active substances are recovered from the urine.

Investigations in which the use of the different types of extraction methods has been examined under various conditions have led to the conclusion that auxin exists in plant tissues in more than one form. Some auxin is readily removable by diffusion, and the remainder is obtainable only by exhaustive diffusion or by the use of other methods, the total amount recovered being dependent upon the method and the conditions employed.

Thimann and Skoog (26) have distinguished in the materials that they studied (Lemna, Nicotiana callus, Phaseolus nodules, Avena) between different types of behavior, and they have made a tentative classification of the forms in which they consider the auxin to be present in tissues, namely:

1) Free, readily diffusible.
2) Naturally Bound, apparently an auxin-protein complex.
3) Reversibly Fixed, becomes non-extractable on drying and again extractable upon rewetting.

In addition to the above, there should be added still another classification, that of "Irreversible Fixation". However, it should be noted that this effect may be due to the destruction of an enzymatic system and may not be conclusively indicative of a separate form or condition of auxin in the plant material.

One of the complications in considerations regarding the state of auxin in plants is that of the precursor. Skoog (27) clearly showed in Avena seedlings the existence of an auxin precursor, an inactive substance from the seed which is activated into auxin at the coleoptile tip. Van Overbeek (28)

has studied the relative amounts of auxin obtainable from Zea and Avena coleoptile tips by exhaustive diffusion, as compared to that removed by exhaustive extraction, and he has found that the "potential" auxin, as measured by exhaustive diffusion, is considerably greater than that obtainable by complete extraction. This "potential" auxin he ascribes to continued formation from precursor.

Went (29) has studied the movement of precursor to the tip end of a decapitated coleoptile and has found an increasing accumulation of the precursor. This together with the evidence of Skoog and of Van Overbeek indicated that the precursor is something different than a non-motile auxin-protein complex.

It should be noted that different plant materials may behave differently in regard to the extraction of auxin and that no simple quantitative extraction method has as yet been devised. For example, Thimann and Skoog found that repeated solvent extraction upon Lemna continued to remove auxin but that this was not the case with Avena coleoptiles. They conclude, as did van Overbeek, that solvent extraction recovers only a part of the auxin which the tips would have produced had they remained alive.

Another aspect of the problem of the forms of auxin in plant materials has been introduced by the work on the inhibitor substance. Redemann (private communication) has shown that an alkaline hydrolysis of the inhibitor produces a substance effecting negative curvatures in the Avena test.

In summary of the various phases of investigations on the forms of auxin in plants, it may be said that it is certain that auxin does occur in more than one form but that the nature of the forms is still a matter of tentative classification and is subject to much more experimental study.

In the present investigation, the attention has been directed more to the identity of the auxin extracted from a plant material than to the forms in which it exists in the plant.

Went (4), and Kögl, Haagen-Smit, and Erxleben (9) determined molecular weights of the readily diffusible auxin by diffusion experiment methods. Went obtained the value 376, and Kögl, Haagen-Smit, and Erxleben obtained 346. The value given by pure auxin-a in the same procedure was 376. It seems likely that the "free" auxin is auxin-a.

The biological digestion method of Kögl, Haagen-Smit, and Erxleben (24), while of necessity limited in its application, is capable of giving the total hormone content. In this procedure the human organism is used to extract the growth substance from the plant material, the extracted auxin being found in the urine.

The biological digestion experiments arose from the observation that about two hours after each meal, the urine showed a peak in growth substance activity. During days of fasting, the auxin activity of the urine remained constant. This showed that the rise in auxin activity after meals was connected with the ingested food. Experiments were done in

which various types of foodstuffs were ingested in separate testing periods. It was found that the foods which led to the immediate rise in the auxin activity of the urine were those which themselves contained auxin activity. Consequently, it was concluded that the increase in the activity of the urine soon after the ingestion of auxin-containing foods was due to auxin extracted from the foods.

Among the foods giving the increase were oils and fats such as corn oil and butter. To eliminate the possibility of the effect being due to a stimulation of the organism by the foodstuff, hydrogenated (and consequently auxin inactivated) fats were employed in control experiments. No rise in the urinary auxin activity was obtained.

Among the results obtained with this method was the observation that in most cases the increase in urinary auxin was several times more than could be accounted for by assay of the original food. Evidently this additional amount of auxin originated from a "bound" auxin (precursor or otherwise) in the food.

In this laboratory, experiments were carried out on wheat by Leech towards increasing the auxin yield in vitro. Among the various enzymes tried, only chymotrypsin produced a substantial increase and the additional amount of auxin found was only a fraction of that found on biological digestion. This effect also was observed by Skoog and Thimann (25). Upon examination of the effect of varying pH upon the yield of

auxin, the observation was made that standing at the optimal pH, 10.5, for forty-five hours, increased the auxin yield up to ten-fold the amount obtained from control experiments under neutral conditions.

The conditions for liberating auxin from wheat established by Leech offered a promising basis for investigating the identity of the liberated auxin. Accordingly, isolation work was begun by the present author using an alkali hydrolysis treatment as an early step in the procedure.

SECTION II

PROGRAM OF PRESENTED WORK

Program of Presented Work

Previous isolation work on auxins was performed upon only two materials from higher plants, namely corn oil and malt. It was therefore highly desirable to investigate the possibility of other auxins being present. This was especially so, since it had been shown that, by the extraction methods used by Kögl and his associated on the above mentioned materials, only a minor portion of the auxins present could be identified as auxin-a and -b. In the earlier work the readily extractable auxins were isolated, whereas the work of Leech showed that in many cases this represents only a fraction of the total quantity of auxin present. A study was therefore made on the nature of the growth promoting substance present after alkaline hydrolysis.

In beginning this isolation study, it was necessary to make a more extended search for possible sources of auxins, and at the same time an investigation was made of methods which could improve the auxin isolation procedure, since the methods previously used had been chosen to fit the immediate needs of earlier investigators. Further, it was considered important to study the isolation procedure as a whole and to examine the possibility of improving yields.

SECTION III

Studies on Auxin Sources

Studies on Auxin Sources

For the estimation of auxin, there was used the Avena test as described by Went and Thimann (ref. 1, pp 21-51). The testing conditions briefly were as follows:

1) The tests were performed in a darkroom which was maintained at a temperature of 25°C. and at a relative humidity between 82 and 85 percent.
2) A filter, OG2 Jena, was used over the light source to cut out the phototropically active wave lengths.
3) The seeds at first used were Victory oats. Due to the present war conditions, the Swedish source of supply of Victory oats was curtailed during the course of the present investigations. Because of the need in this and other laboratories for a steady and uniform supply of oat seeds for the Avena test, van Overbeek (36) investigated a large number of American strains finally finding a satisfactory substitute for the Victory strain. The seedlings from the new source adopted are more sensitive than were those formerly used. The new seeds were used in the latter part of the present investigation.
4) For the preparation of the test plants the Avena seeds were soaked in water for one hour, beginning at 9 A.M., three days before the test and were planted at 4 P.M. the following day.

5) The seedlings were decapitated for the first time at 10 A.M. on the testing day and for the second time at 1 P.M.

6) The test blocks (1x2x2 mm., made from 2% agar) were soaked for one hour in the test solution before application to the plant.

7) Shadowgraphs of the test plants were made 100 minutes after the application of the blocks.

8) The relative sensitivity of the test plants was estimated each day by testing three standard indol-3-acetic acid solutions; 4 mgms. of indole-3-acetic acid in 25 liters, 75 liters and 225 liters of water respectively. The concentrations of these standards were chosen so as to obtain a correlation factor when the plants were relatively high or low in sensitivity compared to the usual normal level.

9) All dilutions of test solutions were made with tap water to which had been added 0,2 ml of glacial acetic acid per liter (7).

In order to prepare from each material examined a solution for testing, an extraction was done by leaving the material stand overnight in the cold room covered with peroxide-free ether. The ether extract, together with any expressed water, was drained off, and the ether was removed by distillation. The residue was made up to a definite volume with water, and the resulting solution was used as

the stock solution for testing.

In the preparation of the agar blocks for the Avena test, the simpler soaking method was used in the place of the more refined melted agar method, since any effects of significance for isolation work would have to be of considerable magnitude.

Many different types of plant materials were tested. Data is given in the accompanying tables.

TABLE I

The Auxin Activity of Dried Plant Materials

Material	Yield*
Carrots	0.0
Horse Radish	0.0
Radish	0.0
Squash	0.03
Irish Moss	0.0
Potatoe Peeling	0.04
Onion	0.0
Alfalfa	0.04
Sweet Potato	0.05 - .07
Tomato	0.06
Lettuce	0.0 - 0.02
Parsley	0.0
Lemon	0.0
Pumpkin	0.0
Orange	0.0
Cucumber	0.0
Sweet Corn	0.0
Turnip	0.008
Parsley	0.0
Turnip leaf	0.003
Cauliflower	0.020
Rhubarb	0.0

* Computed as mgm. Indole-3-acetic acid per kilogram.

TABLE I
(continued)

Material	Yield*
Carrot Leaves	0.0
Swiss Chard	0.0
Leaks	0.02
Celery Leaves	0.0
Mustard Greens	0.0
Mint	0.0
Watercress	0.040
Endive	0.0
Dulse	0.0
Broccoli	0.0
Kale	0.0
Pacific Kelp Powder	0.0 - 0.30
Japanese Keop Powder	1.00
Red Cabbage	0.0 - 0.080
White Cabbage	0.03
Egg Plant	0.0
Cucumber	0.03
Garlic	0.0
Asparagus	0.0
Jerusalem Artichoke	0.0
String Beans	0.0 - 0.04
Beets	0.0

* Computed as mgm. Indole-3-acetic acid per kilogram.

TABLE I
(continued)

Material	Yield*
Rhubarb Root	0.0
Lentil	0.0
Celery Stalk	0.01
Spinach	0.003 - 0.04
Dill	0.03
Okra	0.07 - 0.10
Green Peas	0.02
Parsnip	0.0
Beet Leaves	0.002

* Computed as mgm. Indole-3-acetic acid per kilogram.

TABLE II

Auxin Yields from Seed Materials

Seed	Whole	Oil	Cake
Corn	0.2-0.7	---	---
Cotton	---	0.09	0.0-0.004
Linseed	---	0.0	0.0
Peanut Kernal	0.1	0.0	---
Lumbang	---	0.04	0.03
Sesame	---	0.05	---
Jojoba	0.0	---	---
Chia	1.1	---	---
Nabo	1.1	---	---
Coconut	(milk 0.0-0.1)	0.0-0.4	0.4-0.8

Auxin yield calculated as milligrams, indole-3-acetic acid per Kg.

TABLE III

Auxin Yields from Sprouted Barley Seed

Material	Activity*
1. Unsprouted seed	0.78
2. Seed sprouted but not dried	0.33
3. Seed sprouted and dried	0.00
4. Sprouts only - dried	1.10
5. Sprouted seeds without sprouts	0.22
6. Malt - Riker #1	0.90
7. Malt - Riker #2	1.80

*Computed as mgm. Indole-3-acetic acid per kilo

TABLE IV

Auxin Yields of Fresh Fruits and Vegetables

Material	Yield*
Lettuce	0.1
White Cabbage	0.0-0.01
Spinach	0.02
Red Cabbage	0.0
Brussel Sprouts	0.0
Watercress (fresh)	0.01
Mustard Greens	0.0
Endive	0.5-0.01
Romaine	0.5-0.01
Lettuce	0.01-0.1
Radish	Positive curvatures due to inhibitor
5 day old etiolated pea seedlings	0.09
4 week old pea plants, (greenhouse)	0.3-0.6
Clover	0.1
String Beans	0.2
Sweet Potato	0.0
White Potato	0.01
Cucumber	0.0
Tomato	0.0
Apple	0.0
Persimmon	0.0
Banana	0.0
Pear	0.0

*Computed as milligrams indole-3-acetic acid per kg.

The principal result of the study, other than the discovery of a plant-growth-inhibitor substance (see Section VI) was that no particularly rich source of auxin was available, the richest source material being young pea plants. However, this material is not suitable for laboratory isolation purposes since a yield of only 0.1 milligram auxin-a per kilogram of plant material can be expected. Thus, to obtain an initial crude extract containing 100 milligrams of auxin, 1000 kilograms of young pea seedlings would be necessary. This represents considerably more acreage than can be handled on a laboratory scale.

In the present investigation special interest was given to seeds and seed oils. Kögl and his associates (23) had available for their work a supply of a malt from Czechoslovakia and of a corn oil of unknown origin, both of which contained 10 milligrams of Auxin-a per kilogram. The supply of these materials was not consistent, however, and efforts to determine the exact source of these products failed. It is not known what caused the high auxin levels in these materials.

No sample of a material of similarly desirable properties could be obtained on the American market.

SECTION IV

INVESTIGATIONS OF ISOLATION METHODS

Investigations of Isolation Methods

The difficulties encountered in the isolation of physiologically active substances such as the auxins are due to their presence in extremely small amounts. The investigator has the task of systematically removing the inactive material, and many steps are required to obtain a chemically pure material.

In the case of the auxins, which show solubility properties different from those of the higher molecular products such as cellulose, hemi-cellulose, and proteins, a solvent extraction was indicated.

Kögl, Haagen-Smit, and Erxleben used (23), therefore, in the isolation of the auxins from malt, a water extraction which left behind a considerable amount of the water insoluble material of the malt. In the case of the isolation from corn oil, advantage was taken of the difference in solubility properties between fats and auxin. The result of this initial purification was a solution of auxins in water, a very small amount of active material in a very large amount of water. It was therefore, necessary to reduce this volume appreciably. Whereas this problem finds a simple solution in industry, it constitutes a formidable obstacle in the laboratory since only small scale equipment is available.

Considering that large scale evaporating equipment was not available for the present work, attention was given to the problem, and a modified spray type evaporator of suitable

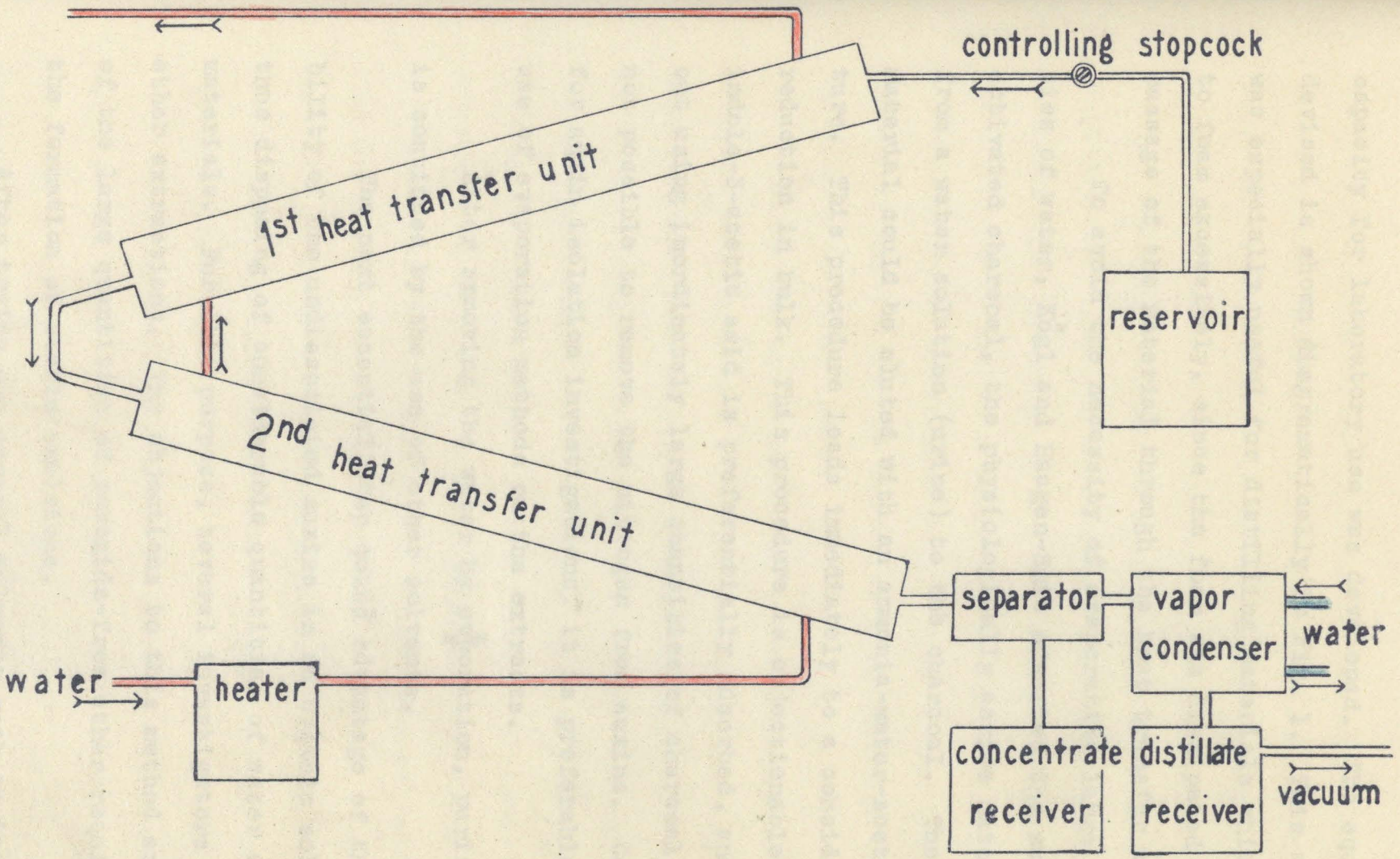


FIGURE 1
SCHEMATIC DIAGRAM OF MODIFIED SPRAY TYPE EVAPORATOR

capacity for laboratory use was developed. The equipment devised is shown diagramatically in Fig. 1. This apparatus was especially useful for distilling materials which tended to foam excessively, since the foam was dissipated during passage of the material through the heat transfer units.

To avoid the necessity of evaporating large quantities of water, Kögl and Haagen-Smit adsorbed the auxins onto activated charcoal, the physiologically active material going from a water solution (urine) to the charcoal. The active material could be eluted with an ammonia-water-acetone mixture. This procedure leads immediately to a considerable reduction in bulk. This procedure is objectionable in that indole-3-acetic acid is preferentially adsorbed, and, without using inordinately large quantities of charcoal, it is not possible to remove the nitrogen free auxins. Consequently for auxin isolation investigations, it is preferable to make use of evaporation methods on the extracts.

After removing the water by evaporation, purification is continued by the use of other solvents.

The next essential step takes advantage of the solubility of the undissociated auxins in an organic solvent, thus disposing of considerable quantities of water soluble materials. For this purpose, several investigators have used ether extractions. The objections to this method are those of the large quantities of peroxide-free ether required and the formation of stable emulsions.

After trying out several solvents such as isobutyl

alcohol and tetrachloreothylene, acetone was found to have satisfactory qualities. Since acetone is miscible in all proportions with water, it is necessary to saturate the acidified solution with a salt such as ammonium sulfate or sodium chloride in order to salt out the acetone phase. This procedure has the added advantage that the high salt concentration prevents, to a large extent, emulsification, and it seems to effect a reduction in emulsification in the subsequent ether extraction step. This method has been successfully applied to the isolation from cornmeal (Section V) as well as to other cases.

Since the above mentioned solvents dissolve auxin, solvents were next used in which the auxin was not soluble. Many solvents were tried, but the most useful was found to be low boiling petroleum ether (30° to 60° C.),which removed large quantities of inactive material, consisting chiefly of benzoic acid. It was found that higher boiling petroleum ether had to be used with caution, since the active material was partially soluble in this solvent. Exhaustive extraction with ligroin (90°-120° C.) was found to remove the activity into the soluble fraction. This behavior has been used for fractionation in isolations on cornmeal (Section V).

Another separation process based on partition between solvents was used by Kögl and Haagen-Smit. This step, usually referred to as the "benzene fractionation", could not be improved upon and repeatedly was found to give satisfactory purifications. This method was used in all of the present

purification processes.

Another separation step employed, was adsorptive precipitation. Among the possible reactions used in this investigation, the precipitation of calcium hydroxide was the most satisfactory. With this treatment, the enrichment was very low, but it served to remove from the fraction treated, tarry inactive material difficult to dispose of in any other way. The precipitation of lead hydroxide was also used, but it was unsatisfactory in that it often resulted in the activity being split equally between the precipitate and the filtrate.

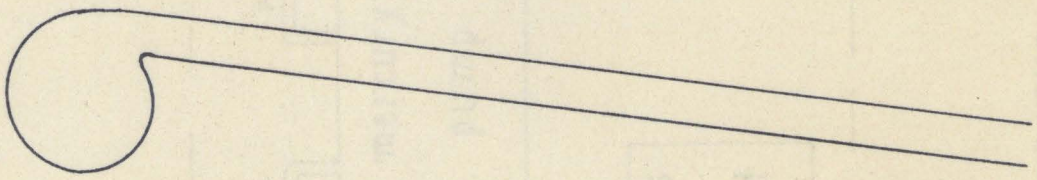
Since the auxins are acids, a segregation of the acidic materials was made by a fractionation with sodium bicarbonate, a customary procedure. This method was employed as an early step in every isolation and, it was also carried out once or twice subsequently.

Most of the various steps mentioned were used repeatedly, as often as a further purification was effected.

The remaining major step involved the formation from the acidic substances of lactones and esters, in order to be able to effect the final separation, by high-vacuum distillation (0.001 mm. pressure), of the active substances from inert materials. The equipment used is shown in Figures II and III.

The need was felt for the introduction of another step into the isolation process, one which would effect a more specific separation than those already in use.

FIGURE 2
TYPES OF MICRO-ALEMBICS
FOR DISTILLATION



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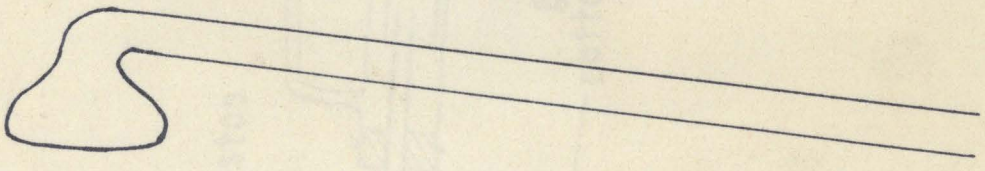

B. flat bottom

full scale

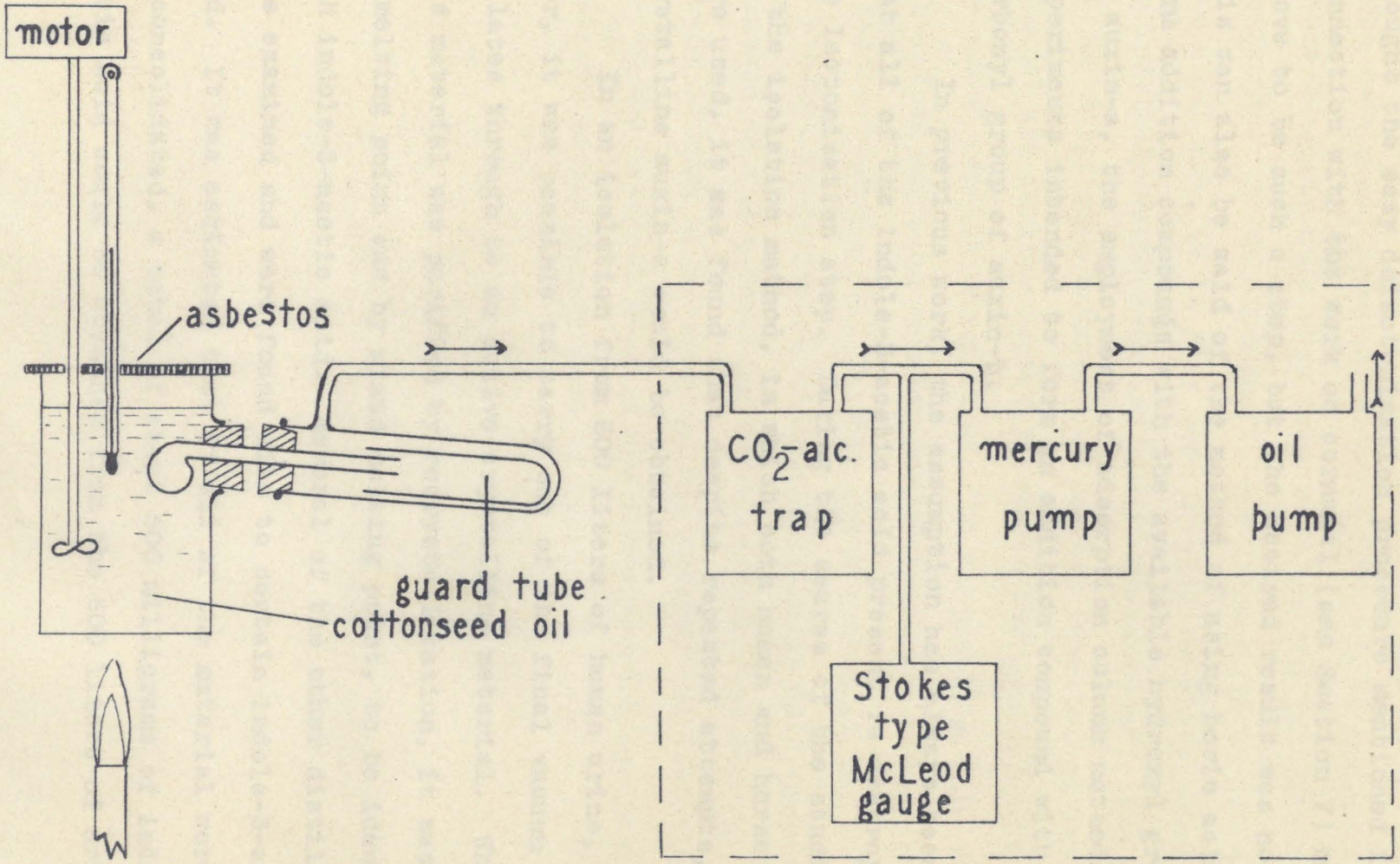

FIGURE 3
HIGH VACUUM DISTILLATION APPARATUS

Several experiments carried out in this direction showed no increase in the purity of the fractions. It was thought the easy delactonization procedure mentioned in connection with the work on cornmeal (see Section V) might prove to be such a step, but the desired result was not had. This can also be said of the method of using boric acid to form addition compounds with the available hydroxyl groups in auxin-a, the employment of adsorption column methods, and experiments intended to form an addition compound with the carbonyl group of auxin-b.

In previous work, the assumption has always been made that all of the indole-3-acetic acid present is destroyed by the lactonization step. During the course of the studies on the isolation method, in which both human and horse urine were used, it was found that despite repeated attempts, no crystalline auxin-a could be obtained.

In an isolation from 600 liters of human urine, however, it was possible to carry one of the final vacuum distillates through to an active crystalline material. When this material was purified by recrystallization, it was found, by melting point and by mixed melting point, to be identical with indole-3-acetic acid. Several of the other distillates were examined and were found also to contain indole-3-acetic acid. It was estimated that if all of the material were to be consolidated, a total of about 500 milligrams of indole-3-acetic acid could be obtained from the 600 liters of urine.

In view of the assumptions of earlier investigators, this was a surprising development. Consequently, experiments were done, in which weighed portions of crystalline indole-3-acetic acid were treated under the lactonization conditions. These experiments are summarized in Table I. From these data it is seen that as much as sixty percent of the indole-3-acetic acid may remain undestroyed. The reason for the difference between the present results and the results of the earlier investigators in the isolation work upon urine remains unexplained. It is possible that dietary differences may lead to much larger average concentrations of indole-3-acetic acid in this country than in Holland. The average concentraion of indole-3-acetic acid in the urine collected in the laboratory for this work from healthy individuals is very high, since it was possible to actually isolate the material despite the fifty percent destruction indicated in the experiments of Table I.

TABLE I

Destruction of Synthetic Indole-3-acetic Acid by 1.5% Hydrogen Chloride in Anhydrous Methyl Alcohol.

1) 10 ml. of HCl-MeoH reagent plus weighed amount of crystalline synthetic indole-3-acetic acid.
2) Refluxed for 60 minutes on boiling water bath.
3) Reagent removed in vacuum.
4) Residue made up to 100 ml. (stock solution for Avena test).

Trial	mgm. I-3-A taken for treatment	mgm. I-3-A in 100 ml. stock soln.		% Remaining after heating with the reagent
		Without treatment	Observed after treatment	
1	1.0	1.0	0.64	64
2	2.5	2.5	0.80	31
3	10	10	5.6	56
4	20	20	8.0	40

SECTION V

ISOLATIONS OF AUXINS FROM CORNMEAL

Isolations of Auxins from Cornmeal

In the present section there will be considered investigations which were carried out upon the isolation of auxins from cornmeal. This work was based on experiments with wheat subjected to an alkaline hydrolysis treatment (30) to increase the auxin yields.

A. Source Material

At the beginning of this work, it was intended to use wheat as the material for isolation. Consequently, tests were made on available wheat supplies to determine a suitable source.

Wheat kernels (1 gram) were soaked for four hours in 20 ml. sodium hydroxide solution at pH 10.5, ground, and allowed to stand at room temperature for 24 to 26 hours covered with toluene, the pH being maintained at 10.5. The aqueous supernatant liquid then was tested. Twenty-four hours was found to be a suitable hydrolysis time for comparison tests. (However, sixty hours was used in isolation work to obtain maximal yield.)

Representative results are given in Table I.

It was found that an optimum yield of auxin depended upon maintenance of the pH at 10. to 10.5. A steady change in the acid direction occurred during the first eight hours. This change was compensated at two hour intervals for the first eight hours, and another adjustment was made eight hours later.

TABLE I

Alkaline Treatment of Wheat

Wheat Source	Protein Type	Conditions of Hydrolysis	mgm./kilo*
Australia	Low	Neutral	0.85
"	"	Basic	2.4
"	"	"	1.3
Globe Mills	"	Neutral	0.24
"	"	Basic	0.69
"	"	"	0.59
Ambler Mills	Medium	Neutral	0.06
"	"	Basic	0.50
Capitol Mills	Low	Neutral	0.24
"	"	Basic	2.5

* In terms of indole-3-acetic acid

While the maximum rate of hydrolysis was found to occur between thirty five and forty degrees Centigrade, almost the same yields were obtained at room temperature. Consequently, in view of the practical considerations involved in large-scale isolation work, the investigations were carried out at room temperature.

Contamination by infection and fermentation was prevented by the addition of toluene (one percent of the total amount of solution). Storage of the mixture in the cold-room during the hydrolysis period could not be utilized, for it was found that test mixtures allowed to stand at zero degrees Centigrade gave a yield of auxin only fifty percent of that obtained from duplicate mixtures at room temperature.

After the above preliminary experiments had been made, it was decided to use corn rather than wheat for isolation, since the available supply of corn had a greater potential auxin content and had more uniformity than the available wheat. Although test experiments, made by Leech with wheat, in which tryptophane had been added, did not show any increase in auxin yield beyond the level of the controls, it was felt nevertheless that the tryptophane content of wheat might lead to disturbing secondary effects. Corn is known to be low in tryptophane.

Since cornmeal was more convenient for use than whole corn, preliminary experiments were carried out on this material to determine the optimal extraction conditions. The tests gave similar results to those on wheat.

Representative data are given in Table II.

There were considerable variations in auxin yields between cornmeals obtained from different mills and some differences between different samples from the same mill. White cornmeal gave, in general, a lower value than yellow cornmeal.

After testing cornmeals from various sources, yellow cornmeal from the Globe Grain and Milling Company of Los Angeles was selected as the source material for isolation, both because of good yields and because of the ease of obtaining a large, uniform supply fresh from milling.

B. Preliminary Extraction Method

The obvious method to try in separating the auxin from the bulk of the cornmeal was one which is essentially an extension of the testing procedure, namely: the use of the same dilute aqueous basic solution for extracting the auxin as for effecting the hydrolysis.

Two variations of this method were used in separate isolations. Difficulties were encountered in extending the small scale experiments to large scale operations.

In the first isolation the amount of solvent (dilute base) to cornmeal was so adjusted that a paste-like mass was formed. The amount taken was such that the mixture could be stirred readily but also such that no great amount of supernatant liquid would separate. The original plan was to squeeze this mass through a cloth bag in order to effect

TABLE II

Alkaline Treatment of Cornmeal

Source	White or Yellow	Conditions of Hydrolysis	mgm./Kilo*
I. Treatment with water as compared to treatment with base-			
Globe Mills	Yellow	Neutral	3.8
" "	"	Basic	33.0
" "	White	Neutral	
" "	"	Basic	17.0
Capitol Mills	Yellow	Neutral	3.7
" "	"	Basic	14.0
" "	White	Neutral	4.6
" "	"	Basic	11.0
II. Treatment with base -- no water control --			
Globe Mills	Yellow	Basic	24.0
Capitol Mills	White	"	19.0
Globe Mills	Yellow	"	19.0
" "	White	"	8.5
Capitol Mills	Yellow	"	16.0
" "	White	"	10.0
Ambler Mills	Yellow	"	11.0

* In terms of indole-3-acetic acid

the separation of the liquor from the residual solid material. When, at the end of the hydrolysis period, this procedure was carried out, it was found to be very unsatisfactory. During the hydrolysis, large amounts of starch and of oil had appeared. On squeezing through cloth, considerable quantities of the starch and of the oily material went into the filtrate. Furthermore, it proved very difficult to wash the gummy residue, with the result that an incomplete auxin recovery was had. As much as possible of the starch and oil was removed from the filtrate and washings by decantation, centrifugation, and filtration. This initial extract was saved for further treatment.

In the second isolation, a different sort of water extraction was adopted, in which the amount of solvent was made large in comparison to the amount of cornmeal (5 liters water per kilogram cornmeal). It was hoped that under such conditions the extract could be decanted without the complications with starch and with oil had in the first trial.

The extraction was carried out in five-gallon bottles. Every eight hours the mixture was adjusted to pH 10.5 and was well shaken. At the conclusion of the hydrolysis period (60 hours), the supernatant liquid was decanted off with a siphon. The residue was washed three times with water by decantation. The combined extracts (supernatant liquid and washes) were taken for the isolation.

The second type of aqueous extraction did not prove satisfactory either. The starch did not settle completely to

the bottom, and overnight standing before decantation had to be adopted to allow each wash to settle. The finely divided starch remaining in the combined filtrates could not be completely centifuged or filtered out.

In both isolations, the initial extracts were neutralized and evaporated, and the distillation residues were extracted with acetone. In both cases, evaporation of the initial extracts was made difficult by excessive foaming which was not readily controllable by the usual methods.

After the experience with the first two isolations, it was decided that another method of extraction must be found. The preliminary isolations mentioned above were started with five and ten kilograms of cornmeal respectively. The difficulties encountered in the extraction of these relatively small quantities would be insuperable in an isolation begun with the much larger quantity of cornmeal necessary to secure a reasonable amount of a final product.

C. Final Extraction Method

A convenient method for the extraction of the auxin from cornmeal would be the use of acetone. However, it is also necessary to consider the factor of alkaline hydrolysis. A possible course of procedure would be to carry out the hydrolysis in an alkaline water-acetone mixture, the solvent mixture serving both for the hydrolysis and for the extraction.

Preliminary experiments were carried out. It was found that a fifty percent by volume mixture of the two

solvents was suitable. A mixture containing seventy-five percent by volume acetone gave a lower yield of auxin than did the fifty percent; when the solvent contained only twenty-five percent acetone, a considerable quantity of free starch appeared. The fifty percent mixture is apparently sufficiently aqueous to effect the hydrolysis but does not provide suitable conditions for the breaking up of starch granules.

The preliminary experiments were done on 200 gram lots of cornmeal, a hydrolysis time of twenty-four hours being used. The solvent-cornmeal ratio was adjusted to form a pasty mass, but still sufficiently thin to permit good stirring. The pH was adjusted initially to 10.5 (with 6 N sodium hydroxide,Beckman glass electrode pH meter) and was readjusted to pH 10.5 every few hours.

At the conclusion of the hydrolysis period, the mass was pressed in a small hand-press, and the residue was washed with a fifty percent by volume water-acetone mixture. The amount of wash solvent taken was gauged in the same way as the amount of initial solvent. It was found that some auxin was still being washed out in the third wash, but the washing was stopped at this point in view of practical considerations in dealing with large quantities in a full-scale isolation.

After separation, the combined filtrates were acidified with acetic acid and then were saturated with sodium chloride to salt out the acetone phase. The resulting two

phases were separated. The aqueous phase then was washed several times with acetone to completely extract all of the auxin. The acetone extract was purified further.

The initial steps of the large scale isolations were performed in essentially the same way as the preliminary experiments. The total quantity of cornmeal for each isolation was divided into portions, each of which was estimated as of a size to be pressed and washed in one day (40-50 kg. per portion). The mixtures were made up in large crocks,covered, and allowed to stand at room temperature.

Every eight hours, the mixture was stirred, and the pH was adjusted to 10.5.

The acetone was purified before use by refluxing with alkaline potassium permanganate, followed by distillation in the presence of an excess of permanganate.

At the conclusion of the hydrolysis period, the mass was transferred batchwise to the cylinder of the "28 Ton" press (described elsewhere) and pressed as dry as possible. The residue from the treatment of each lot was mixed with 50% by volume acetone-water solution and left to stand. After all of the day's portion had been pressed, the press residue-wash mixture was re-pressed; the pressed out liquid was added to the first filtrate, and the residue was discarded. Only one washing was carried out due to the practical difficulties involved in the handling of so much material.

The combined press liquors were acidified with acetic acid and then were treated with sodium chloride in

order to cause the formation of a two phase system. In the small-scale experiments, this separation took place without difficulty. In the large-scale isolation work much difficulty was had at first. In trying to saturate the solution with salt, an excess of salt was added to the solution, and vigorous shaking of the five-gallon containers was resorted to. This resulted in the formation of an emulsified suspension and not in the separation of an acetone solution. The simple effects due to temperature and pH dependency were examined, but no solution of the difficulty was reached in that direction. Next, the acidified filtrate was passed through a 3 foot thick bed of salt in a large column one foot in diameter. Separation did occur, but stoppage of the salt bed by material suspended in the filtrate rendered passage of the solution by gravity head too slow for practical use. When suction was resorted to, channeling of the salt bed took place.

The final solution to the difficulty proved to be relatively simple. Salt was added to the bottles, and the bottles were shaken, but not sufficiently vigorously to set up an emulsion. The partially saturated salt solution was then passed slowly through a layer of coarse salt on the perforated section of a ten gallon, earthenware filter. Often separation occurred on the first passage, but sometimes the solution had to be passed repeatedly through the bed before the acetone layer separated out.

This method depends for its effectiveness upon

having the solution partially saturated with salt before using the salt bed and upon using for the bed, salt sufficiently coarse to avoid stoppage of the filter bed by any gummy material in the solution. No filter paper may be used.

Some trouble was had with starch and gummy materials going through the press into the filtrate, especially as noted above in connection with the salting out step, but the acetone separation procedure as finally used served to remove most of this material. Much of it remained with the salt, and the rest remained with the aqueous phase, leaving a clear acetone solution.

The aqueous phases were apportioned among five-gallon bottles, about three gallons being placed in each. Each lot was washed by decantation with five separate, three liter portions of acetone, the acetone wash being added to the main acetone solution. At the conclusion of the washing, the aqueous residue was found to be practically free of auxin and was discarded.

At the time of the initial separation into two phases, a small amount of a very stable emulsion collected at the interface in each bottle. This emulsion was combined into one lot in a five gallon bottle. All of the acetone used to wash the aqueous layers was poured through this bottle before use as wash solvent, in order to clear the emulsion of auxin.

At the time of acidification of the first press liquor with acetic acid, a light yellow cloudiness appeared

in the solution. This probably was caused by the precipitation of free fatty acids and likely was the origin of the stable emulsion mentioned above.

The acetone extract obtained from the separation of the two phases was distilled at atmospheric pressure, and the residue was further evaporated in vacuum to a suitable working volume for ether extraction.

D. Isolation Steps

Two complete isolations starting with an alkaline aqueous acetone treatment of cornmeal were carried through. In the first of these (designated as Cornmeal Isolation #3) 270 kilograms of cornmeal were taken as the starting material, and in the second 45 kilograms were used. After the completion of the initial extraction, the isolation process was begun by the use of the methods previously described.

The first of these isolations showed that it was not possible to apply the whole process used in the isolation of auxin-a from urine and from corn oil (23, 8). Several adaptations and new steps had to be included. In Isolation #3 it was found that there was:

1) A substantial partition in activity between the soluble and insoluble fractions when an exhaustive ligroin separation (21 hot extractions) was carried out.
2) Occurrence of pseudo-auxin-a in the final stages of the operations upon the ligroin soluble fraction.
3) A large drop in activity occurring upon lactonization of the ligroin insoluble fraction.

Consideration of the above points led to the conclusion that auxin-a, or more likely its lactone, can be washed out with ligroin, if the treatment be thorough enough, for it was assumed that since pseudo-auxin-a was found, auxin-a was originally present. Further, it was considered that the drop in activity upon lactonization of the ligroin insoluble fraction was due to the destruction of a physiologically active acid, possibly indole-3-acetic acid.

Accordingly, an exhaustive ligroin extraction was performed early in the next isolation. The customary lead precipitation now was omitted; first, because it was known that an important partition of activity occurs and second, because it was felt that the results of an exhaustive ligroin extraction might replace the purification usually effected with the lead precipitation.

Larger portions of ligroin were used for Isolation #4 than for #3. Also, a longer extraction time was employed. In each case, the extractions were done by refluxing the fraction being extracted with ligroin (90-120 degree Centigrade boiling range) on a boiling water bath. The conditions may be summarized as follows:

Isolation	#3	#4
Wgt. of fraction extracted (gms.)	10.	4.0
Ml. ligroin per portion	500	800
Extraction time per portion (min.)	20	30
Number of separate portions used	21	20

After this separation, the insoluble fraction (Isolation #4) was found to be almost inactive.

After lactonization of the ligroin soluble fraction, the high vacuum distillation was carried on until a bath temperature of 125° C. was reached. Test showed that the bulk of the activity went into this first distillate.

The vacuum distillation first fraction (472 milligrams) was allowed to stand overnight with five milliliters of a fifty percent by volume water-acetone solution in the presence of an excess of solid sodium bicarbonate. The distillate, an oily syrup, did not dissolve completely, but was partly in the form of fine suspended droplets. A separation with ether was made, and it was found that all of the activity remained with the bicarbonate insoluble ether soluble, neutral fraction.

It was expected that auxin-a-lactone, if present in the syrup, would readily hydrolyze under the mild alkaline conditions, that the free acid, auxin-a, formed would dissolve in the bicarbonate as the salt, and that the less reactive material (esters) would be taken up by the ether.

This did not happen, and consequently it was decided that the most likely explanation for the physiological activity of the high vacuum distillate would be to ascribe the activity to an active ester formed by methylation of an active acid. Kostermans (1935) had previously found the methyl ester of indole-3-acetic acid to be physiologically active.

Acid-base destruction tests (9) were made on ten milligram samples of the neutral syrup fraction. Boiling with acid (1 N.) for two hours destroyed the activity, but boiling with base (1 N.) did not. This result indicates that the active acid is indole-3-acetic acid.

The neutral syrup fraction was then hydrolysed by treatment at room temperature with two milliliters of 3 N. potassium hydroxide. The oil completely dissolved. The remainder of the procedure was directed at the isolation of the active acid formed by the basic hydrolysis.

CHART A

SCHEMATIC DIAGRAM OF CORNMEAL ISOLATION #3

(The first figure in the bracket following the fraction is the weight of the fraction, the second is the amount of auxin calculated as indole acetic acid)

Yellow Cornmeal (270 kg.: 5400 mgm.)

↓ Alkaline hydrolysis in 50% acetone-water (350 L.)

Pressing out of filtrate - followed by washing of the residue with 50% acetone-water (250 L.)

Extract (375 L.)

↓ Salting-out - followed by acetone washing of the saturated aqueous phase.

Acetone Phase (550 L.)

↓ Removal of acetone - followed by vacuum concentration of residue.

Concentrated Residue (10 L.)

↓ Extraction with ether

Ether Soluble Fraction

↓ Fractionation with bicarbonate

Acid Fraction (Bicarbonate soluble) (386 gm.)

↓ Extraction with petroleum ether

Insoluble Fraction

↓ Fractionation with bicarbonate

Acid Fraction (Bicarbonate soluble) (39 gm: 580 mgm.)

↓

CHART A
(continued)

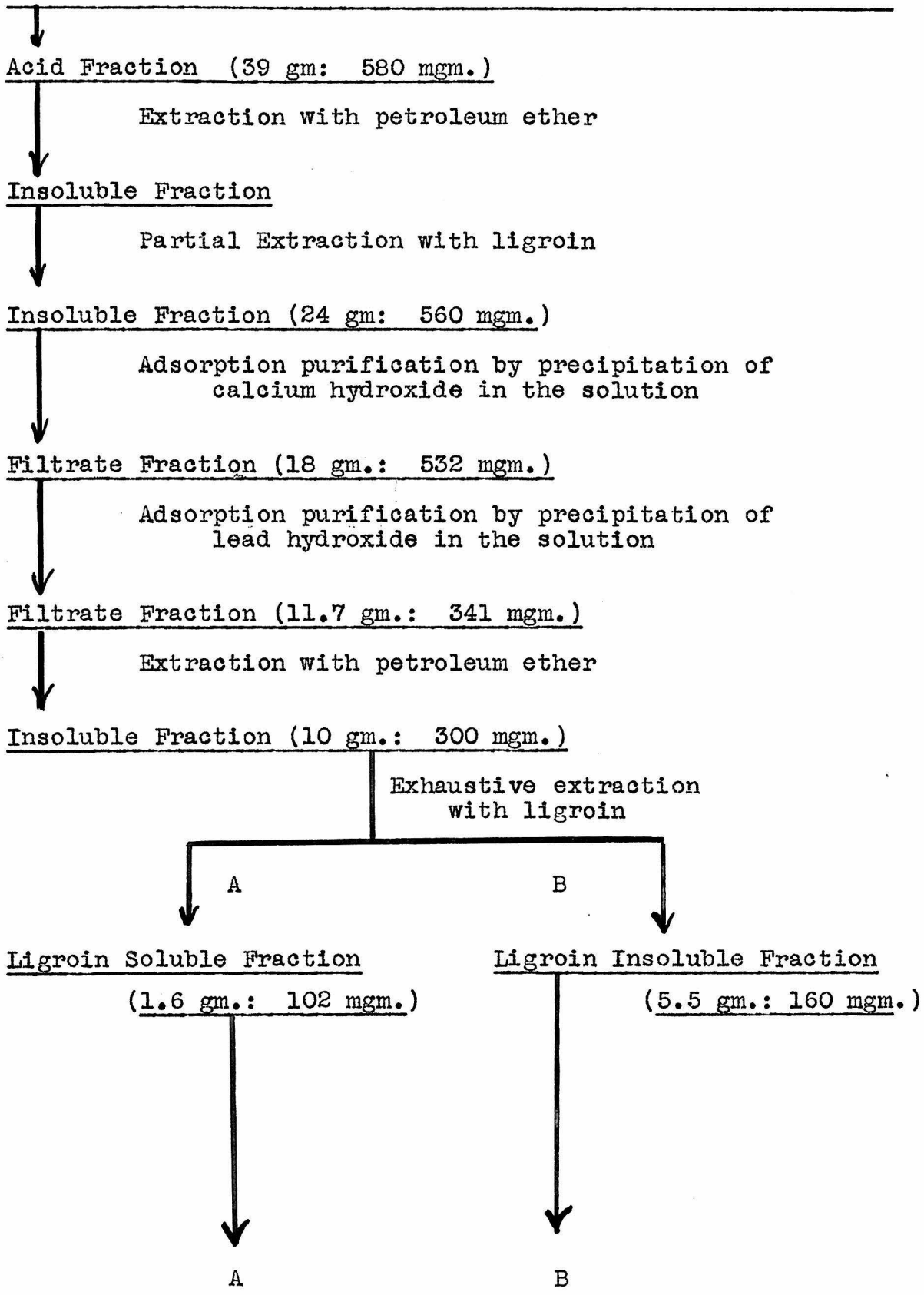

CHART A

(continued)

Sub-Isolation A ---

Ligroin Soluble Fraction (1.6 gm.: 102 mgm.)

Fractionation with benzene

Main Residue (0.96 gm.: 82 mgm.)

Lactonization -- (1.5% solution of hydrogen chloride in anhydrous methyl alcohol)

Ester-Lactone Fraction (.94 gm.: 30 mgm.)

Distill in high vacuum -- fractionation by temperature control at constant pressure

Crystals of Pseudo-Auxin-a

4 mgm., m.p. 196-7 C.

Obtained from the fraction distilling at 120-130 C.

Several slightly active and inactive syrup fractions

CHART A

(continued)

Sub-Isolation B --- (2/3 of total activity

↓

Ligroin Insoluble Fraction (5.5 gm.: 160 mgm.)

↓ Fractionation with benzene

Main Residue (2.2 gm.: 160 mgm.)

↓ Lactonization -- (1.5% solution of hydrogen chloride in anyhydrous methyl alcohol)

Ester-Lactone Fraction (2.03 gm.: 5 mgm.)

↓ Distill in high vacuum -- fractionation by temperature control at constant pressure

Several almost inactive syrups.

Some inactive crystals of unknown composition distilling, with sublimation, at 160-5 C.

(Note the large drop in activity upon lactonization)

CHART B

SCHEMATIC DIAGRAM OF CORNMEAL ISOLATION #4

(The first figure in the bracket following the fraction is the weight of the fraction, the second is the amount of auxin calculated as indole acetic acid)

Yellow Cornmeal (45 kg.)

↓ Alkaline hydrolysis in 50% acetone-water (60 L.)

↓ Pressing out of filtrate - followed by washing of the residue with 50% acetone-water (40 L.)

Extract (64 L.)

↓ Salting-out - followed by acetone washing of the aqueous layer

Acetone Phase (93 L.)

↓ Removal of acetone - followed by vacuum concentration of residue

Concentrated Residue (6 L.)

↓ Extraction with ether (20 L.)

Ether Soluble Fraction (123 gm.: 200 mgm.)

↓ Fractionation with bicarbonate

Acid Fraction (Bicarbonate soluble) (36 gm.)

↓ Extraction with petroleum ether

Insoluble Fraction (15 gm.)

↓ Fractionation with bicarbonate

Acid Fraction (Bicarbonate soluble) (12 gm.: 200 mgm.)

↓

CHART B
(continued)

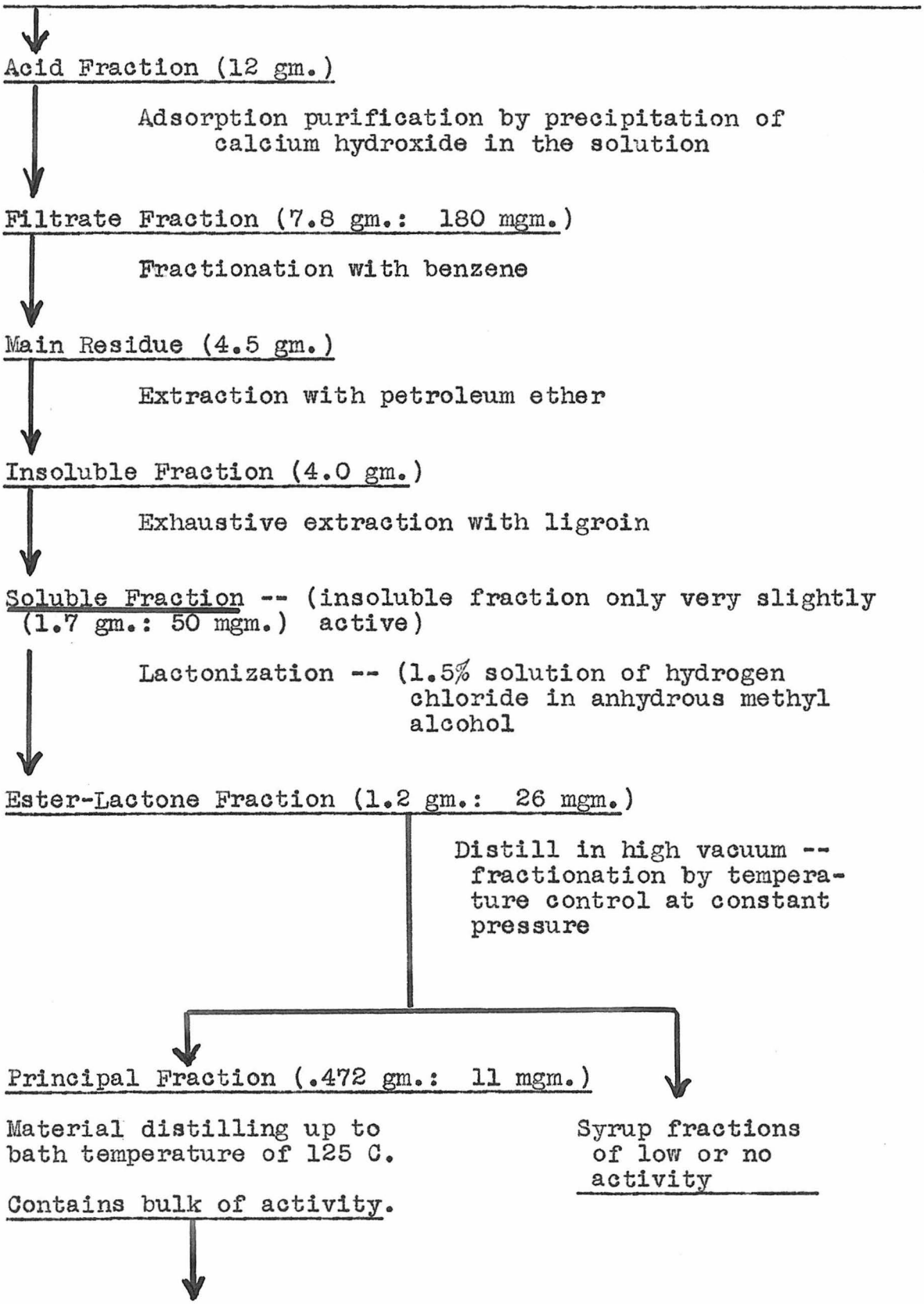

CHART B
(continued)

Principal Fraction from High Vacuum Distillation
(472 mgm.: 11 mgm.)

↓ Fractionation with bicarbonate in aqueous acetone

Neutral Fraction (326 mgm.: 10 mgm.)

↓ Hydrolysis at room temperature with 3N. KOH

Acid Fraction (250 mgm.: 7 mgm.)

↓ Extraction with xylene

Soluble Fraction (122 mgm.: 7 mgm.)

↓ Long standing, followed by recrystallization

Crystalline, colorless plates

M.p 164-5 C. No m.p. depression on mixing with synthetic indole-3-acetic acid (m.p. 164-5 C.)

Crystals identified as indole-3-acetic acid.

E. Identification of Indole-3-acetic Acid and of Pseudo-auxin-a

1) From Cornmeal Isolation #3 ---

Four milligrams of white crystals melting at 196-7°C., with sintering beginning at 173°C., were obtained. Kögl, Koningsberger, and Erxleben (10) give the melting point of pseudo-auxin-a as 193-4°C. (uncor.), with sintering beginning at 176°C. It was concluded that the crystalline material obtained in this isolation was pseudo-auxin-a.

2) From Cornmeal Isolation #4 ---

Crystalline material melting at 164-5°C., with no melting point depression on mixing with synthetic indole-3-acetic acid (m.p. 163-4°), was obtained. It was concluded that the crystalline material obtained in this isolation was indole-3-acetic acid.

F. Discussion of Results

The present investigation has led to the definite establishment for the first time of the presence of indole-3-acetic acid in a higher plant material by isolation.

By comparison of the four milligrams of pseudo-auxin-a obtained in the very large-scale isolation (#3, 270 kg.) with the seven milligrams of indole-3-acetic acid yielded by the small isolation (#4, 45 kg.), the conclusion is indicated that the predominant part of the physiological growth activity of the alkaline treated cornmeal is due to indole-3-acetic acid.

Since the amount of indole-3-acetic acid obtained was many times greater than that expected from an extraction performed without the alkaline treatment, the conclusion is indicated that the extra auxin liberated by alkaline hydrolysis of the cornmeal is, in part, indole-3-acetic acid.

Auxin seems to exist in such a variety of forms or conditions in the plant and, indeed, in such different forms in different plants, that it would be unlikely that a complete explanation can be based upon the isolation of one active substance from one source. However, in view of the isolation of indole-3-acetic acid from a higher plant material reported in this thesis, the presence of this auxin in free and in bound form in the higher plants must be considered in future investigations.

Apart from indole-3-acetic acid, it is probable that the substance dissolving in ligroin in the exhaustive

ligroin extraction is auxin-a-lactone rather than the free acid, for theformer is of a structure which could be more easily dissolved in this solvent. Since auxin-a is known to exist in equilibrium with its lactone, the auxin-a can continuously rearrange to the lactone if the lactone is continuously removed, a condition which the procedure adopted for the exhaustive ligroin extraction permits. All of the activity due originally to auxin-a will then appear in the ligroin soluble fraction.

The pseudo-auxin-a isolated may have had its origin in photo-inactivation of the auxin-a-lactone, a process known to occur readily (19). It is of importance that this possibility be considered in future investigations.

SECTION VI

THE INHIBITOR SUBSTANCE FROM RADISH PLANTS

The Inhibitor Substance From Radish Plants

In the course of the investigation of a variety of plant materials as possible auxin sources, young radish plants were examined.

Late in February and in March of 1938, radish seeds (variety Early Scarlet) were set out in a mixture of soil, sand, and leaf mold in trays in the greenhouse. During April the young plants were harvested, and the leaves and stems were tested for auxin.

The standard Avena test method used in the survey was employed. The plant material was left standing overnight in the cold room covered with ether. The ether extract was then poured off, together with the small amount of aqueous solution which had come out of the plant material during the time of standing with the ether. The ether was distilled off, and the aqueous residue was made up to a suitable volume with water. Agar blocks were soaked in serial dilutions of this stock solution and then were used in the Avena test according to the standard procedure.

The first such test was done on April 1, 1938. Surprisingly enough, the Avena seedlings showed a very definite positive curvature (in the direction of the agar block) rather than the negative curvature that would be obtained with auxin or the zero curvature that would be obtained if no growth active substance were present.

The tests were repeated on fresh samples of the

radish material. Positive curvatures were found consistently. Typical data are given in Table I.

Two possibilities were apparent:

1) The positive curvature being due to some poisonous material which killed the cells in proximity to the block.

2) The positive curvature being due to a substance which actually slowed down or inhibited the rate of cell elongation, on the side of the test plant nearest to the block, below the level of the normal growth rate.

The character of the curvature supported the latter possibility, since growth on the block-side of the plant still continued rather than ceased entirely. Furthermore, there was a relation between the dilution of stock solution taken and the positive curvature effect produced. The conclusion was justified that an actual substance was involved rather than a poisoning of some sort. While positive curvatures have been reported previously Stark (31), Nielsen (32), Seubert (2), these were very likely due to deficiencies in testing procedure. The present instance is the first case of unimpeachable evidence for a definite inhibitor substance).

TABLE I

Experiments on the Extraction of Young Radish Plants

Date of Planting	Date of Assay	Grams Sample	Ml. Test Soln.	Test Angle	Standard Angle
2/24/1938	4/1/1938	25	10	$+3^{\circ}$	-9°
3/23/1938	4/7/1938	4	2	$+2^{\circ}$	-9°
"	"	4	6	0°	-9°
"	4/19/1938	4	2	$+1^{\circ}$	-1°
"	4/21/1938	4	2	$+4^{\circ}$	-5°
"	"	4	20	0°	-5°

The test angle is the curvature produced on Avena plants by blocks soaked in the test solution.

The standard angle is the curvature produced on Avena plants by blocks soaked in a solution of indole-3-acetic acid (4 mgm./75 liters).

Since the experiments described here showed the presence of an actual inhibitor substance in the young radish plant, it was decided that a complete investigation of the substance should be carried out. Mr. W. S. Stewart, who had observed the phenomenon independently, began to work on the physiological aspects of the problem, and C. E. Redemann in this laboratory began a study of the chemical properties of the subtance with a view towards eventual isolation.[1] Redemann devised a standard test for the study of the distribution on fractionation, found a suitable extraction method, and established some of the properties of the substance, namely:

1. Extractability with organic solvents.
2. Neutral character of the molecule--no acidic or basic groups.
3. Ease of hydrolysis to yield a substance giving negative curvatures in the Avena test.

Redemann and Schmauss (33) also obtained indications of the growth promoting substance set free upon hydrolysis being indole-3-acetic acid. Isolation work and a study of the properties of the inhibitor is still in progress in this laboratory.

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[1] A copy of the first publication upon the inhibitor substance from radish plants (Stewart, Bergren, and Redemann) is included as Addendum I.

It should be noted that the inhibitor substance has been found in a number of cotyledons. Stewart (**37**) lists, as plant materials in which this substance (or one showing the same effect) has been found: the cotyledons of six varieties of radish, the cotyledons of turnip, rutabagas, mustard, and nasturtium. Further, it has been reported in the fruit of the pepper tree by A. J. Haagen-Smit (34).

It has been interesting to note that subsequent to the time of this work, investigators in other laboratories have reported indications of definite inhibitor substances. Goodwin (20) obtained some results suggesting that discrepancies in diffusion constant data calculations of the molecular weight of auxins are due to inhibitor substances. Larsen (35) reports finding evidence for the presence of several different types of inhibitors in different sorts of extracts. The existence of definite inhibitor substances is generally recognized and is considered in interpretations of growth substance investigations.

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Diss. Utrecht, 1935

A D D E N D A

A

Copy of a Publication

by

W. S. Stewart, W. R. Bergren, and C. E. Redemann

Entitled

"A Plant Growth Inhibitor"

Science 89 185-6 (1939)

Reprinted from SCIENCE, February 24, 1939, Vol. 89, No. 2304, pages 185–186.

A PLANT GROWTH INHIBITOR

DURING the course of physiological investigations on the plant hormone relationships in radish, strain French Breakfast, an ether extraction was made of 4.935 grams fresh weight of cotyledons from seven-day-old seedlings. These plants had been grown in the open in rich, loamy soil. The extraction was carried out according to the simplified auxin extraction method of Van Overbeek.[1] On testing the extract by the Avena test (Went and Thimann[2]) positive curvatures of from 17 to 23 degrees were found instead of the usual negative ones. (If the substance being tested is growth-promoting then the Avena plant will grow more rapidly on the side on which the substance is applied. Thus, because of this unsymmetrical growth, the plant will become curved in a direction away from the side on which the substance is applied. This is known as a negative curvature. If, however, the material causes an inhibition of growth then the plant will likewise grow unsymmetrically, but now the resulting curvature will be in a direction toward the side of application of the substance. This is known as a positive curvature.)

The relation between the concentration of the extract and degrees of positive curvature was investigated. In determining the amount of positive curvature 48 Avena plants were used at each dilution value. The inhibitor, extracted as above, was taken up in 1½ per cent. agar and cut into blocks $1.6 \times 2 \times 2$ mm for application to the test plants. The standard Avena technique for auxin determination was used except that the curvature—positive in this case—was measured 150 minutes after applying the inhibitor instead of after

[1] J. Van Overbeek, *Proc. Nat. Acad. Sci.*, 24: 42, 1938.
[2] F. W. Went and K. V. Thimann, "Phytohormones," Macmillan Company, New York, 1937.

90 minutes, as is customary when testing growth-promoting substances. The results are seen in Fig. 1. This graph shows that positive curvatures between 3 to 13 degrees are proportional to the concentration of the inhibitor.

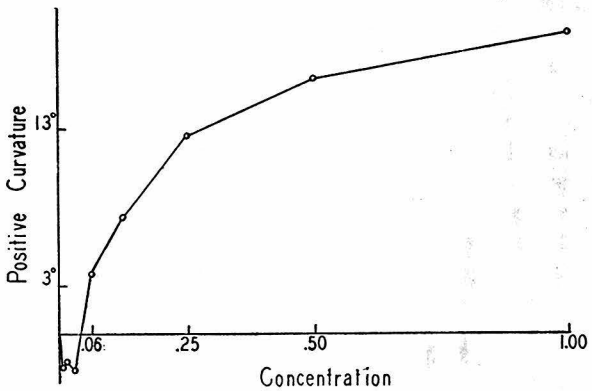

FIG. 1. Relation between positive curvature of Avena plants and two-fold dilutions of inhibitor substance.

Using the method given by Schneider and Went[3] a Photokymograph test was made of the reaction time of the coleoptile to growth inhibitor. The results are presented in Fig. 2. It is observed that a negative curvature is initiated which rapidly changes between the first and second hour to a positive curvature, reaching its maximum three hours after the application of the inhibitor. It is interesting to note that the reaction rate for this negative curvature is different from the negative curvature caused by auxin. This is shown by the control run made at the same time as the inhibitor test but by using a growth-promoting substance, indole –3–acetic acid, .05 mg per liter. Each point on the graph is the average of twelve Avena test plants.

[3] C. L. Schneider and F. W. Went, *Bot. Gaz.*, 99: 470, 1938.

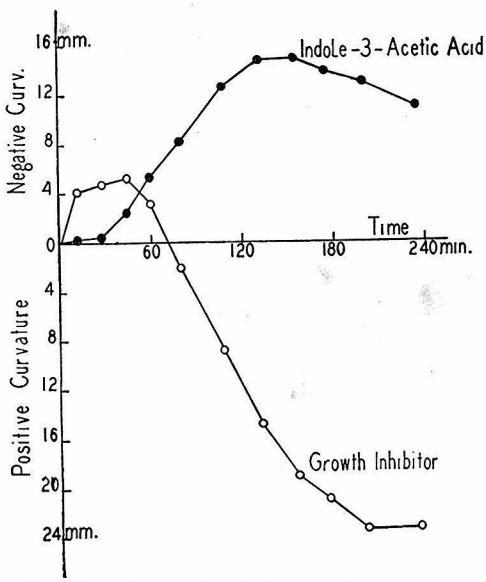

FIG. 2. Curvature rate of Avena plants upon application of: (*a*) .05 mg/liter, indole–3–acetic acid, and (*b*) growth inhibitor. (Ordinate values given as mm deviation of the extended coleoptile from the vertical position.)

Inhibitor was found in the cotyledons of radish plants grown in the light or dark, but it was not found in the hypocotyl in either case.

The inhibitor substance is of neutral character. Accordingly as one would expect on the basis of Went's[4] potential gradient theory of auxin transport, it should be transported acropetally as well as basipetally. Experiments prove this to be the case, as was shown by equal amounts of inhibitor passing through normal and inverted 4 mm long sections of Avena coleoptiles. Similar experiments show there is likewise no inhibitor transport polarity in radish hypocotyl sections.

[4] F. W. Went, *Jahrb. wiss. Bot.*, 76: 582, 1932.

In conclusion, it may be said that the positive curvatures resulting from the application of the inhibitor are not to be considered the same as the positive curvatures resulting from the retardation of the physiological tip regeneration in the Avena coleoptile because these are usually of slight magnitude, and furthermore they are never preceded by a negative curvature during the first hour.

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B.

Copy of the Manuscript of a Paper for Publication in

Science

May, 1941

by

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Estimation, Isolation, and Identification of Auxins in Plant Material

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The separation and concentration of auxin extracts by workers interested in the estimation of auxin have made use of many of the usual methods of assaying for the growth hormones. These methods are of five general patterns, namely: (1) the diffusion method [1], (2) the extraction process [2], (3) the enzymatic method (lipase[3], chymotrypsin[3a]), (4) the alkaline alcoholic hydrolysis[4], and the biological digestion method[3].

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(1) Went, F. W.; Rec. Trav. Bot. Neerl. 25, 1-116 (1928)

(2) Avery, G. S.; Am. Jour. of Bot. 26, 679 (1939)

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(3a) Skoog, F. and K. V. Thimmann: Science 92, 64 (1940)

(4) Kögl, F., A. J. Haagen-Smit, and H. Erxleben: Z. physiol. Chem. 225, 215-229 (1934)

The biological digestion method of Kögl, Haagen-Smit, and Erxleben as performed by feeding experiments on humans is capable of giving the total hormone content, the free hormone, and the precursor form (or bond form) as well. By necessity this method is limited in its application.

Using the results obtained in the biological digestion method as a criterion for the assay of the total growth hormone set free in dormant tissues, the following more practical method was developed, whereby one is able to remove an amount which is equivalent to the amount of the growth promoting substances obtained by the biological method. This modified procedure has been in use in this laboratory for the past two years in the investigation of auxin-like substances in high and low protein wheats and in the isolation work from corn.

The procedure is briefly as follows: dry whole wheat kernels are soaked for four hours in sodium hydroxide solution at pH 10.5, ground in a glass mortar with clean sand, allowed to stand at 20° C. for 45 hours (together with one ml. of tuolene) with the pH maintained at 10.5. At the end of this period, the mixture is centrifuged, and the supernatant liquid is tested by the standard Avena procedure.

Table I illustrates a typical experiment. The value obtained at pH 10.5 compares favorably with the amount of auxin recovered by the biological digestion method.

TABLE I

pH	Auxin per kilogram of wheat *
4.	0.37 mgm.
7.	2.25
10.5	6.96
11.5	1.50

* In terms of indole-3-acetic acid

Temperature effects were also considered. Above 60° C. losses began to occur. The maximum rate of hydrolysis was found to lie between 35° and 40° C. However, since the rate was almost the same at 20° C., the investigation was carried out at room temperature in view of practical considerations involved.

Tests upon samples of corn showed the same effects of increased auxin activity upon standing at pH 10.5 as did the wheat. Yellow cornmeal, fresh from the mill, was found to be more convenient for isolation purposes and accordingly was used.

It was found that a 50% acetone-water mixture could be satisfactorily used for the hydrolysis. The ratio of solvent to cornmeal was adjusted to form a thick paste-like mass, and the pH was maintained near 10.5 for a period of 45 to 60

hours. After the hydrolysis period, the mass was pressed, and the residue was washed with a 50% acetone-water mixture. The filtrate, combined with the washings, formed the initial extract for the isolation. Addition of sodium chloride gave rise to the formation of a separate acetone layer; after separation, the aqueous residue was washed with acetone. The acetone solutions were distilled, and the distillation residues were combined and extracted with ether. The isolation proceeded from the ether-soluble material according to the general procedures already described in the literature for isolation from other materials [4,5].

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(5) Kögl, F., A. J. Haagen-Smit, and H. Erxleben: Z. physiol. Chem. 214, 241-261 (1933)

The isolations gave as an end product a syrup which was physiologically active and which gave a color reaction with ferric chloride similar to that given by indole-3-acetic acid. Upon long standing this syrup crystallized. A portion of the material, when recrystallized, showed a melting point of 164-5° C. and no melting point depression on mixing with synthetic indole-3-acetic acid.

This is proof of the identity of the isolated material with indole-3-acetic acid. This is the first time that indole-3-acetic acid has been isolated from higher plants. Since the amount of the isolated acid is many times greater than the auxin amount indicated by the normal extraction process, it undoubtedly represents a large part of

the auxin present in bound form.

In addition to the indole-3-acetic acid, there was obtained a small amount of crystalline pseudo-auxin-a which melted at 196-7° C., with sintering beginning at 173°. Kögl, Koningsberger, and Erxleben[6] give the melting point of pseudo-auxin-a as 193-4° C., with sintering beginning at 176°. This shows the presence of some auxin-a in the original starting material, which rearranged to pseudo-auxin-a during the isolation procedures.

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(6) Kögl, F., C. Koningsberger, and H. Erxleben: Z. physiol. Chem. 244, 266-278 (1936)

C.

Copy of a Publication by

W. R. Bergren and C. A. G. Wiersma

Entitled

"Chemical Changes in the Adductor Muscle of the Cheliped of the Crayfish in Relation to the Double Motor Innervation"

Journal of General Physiology 22 193-206 (1938)

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CHEMICAL CHANGES IN THE ADDUCTOR MUSCLE OF THE CHELIPED OF THE CRAYFISH IN RELATION TO THE DOUBLE MOTOR INNERVATION

BY W. R. BERGREN AND C. A. G. WIERSMA

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena)

(Accepted for publication, August 7, 1938)

Work from this laboratory has shown that a number of the striated muscles of the legs of the crayfish show a typical motor innervation (1). Such muscles are innervated by two motor fibers only, each causing a different type of contraction. One of these, resulting from stimulation of the thicker fiber, is termed the "fast" contraction, while the other is called the "slow." Both anatomical evidence and physiological experiments indicate that each muscle fiber is innervated by these two axons and that both contractions occur in the same muscle fibers (2, 3).

The most striking difference between the two contractions is found in the adductor muscle of the cheliped of the crayfish. In this case, a single impulse in the thicker motor fiber causes a twitch-like contraction, and the system behaves in almost all respects as does a single motor unit of a vertebrate muscle: single shocks of different strengths give twitches of a considerable strength and a constant height, and during tetanic contraction the action currents are all of the same magnitude. In contrast to this, single shocks given to the thinner fiber produce no visible response in the muscle, but faradic stimulation causes action currents which grow in height and a contraction with a long latent period. The action currents in this case are always much smaller than those of the fast contraction.

The chemical changes occurring in the adductor muscle of the cheliped of the crayfish *Cambarus clarkii* during these two types of contraction were investigated in order to obtain evidence on two possible mechanisms by which the two contractions might occur in the same muscle fiber. In the first place, two contractile substances

might be present, a "phasic" and a "tonic" one, analogous to those supposed by Botazzi (4) in the vertebrate striated muscle, or the same substance might contract in both cases, the difference between the contractions being due solely to differences in the transmission mechanism between the nerve impulse and the contractile substance.

Methods were devised for the rapid removal of the stimulated cheliped to liquid air and for the analysis of the frozen muscle tissue. The changes in phosphate and in lactic acid content were used as indices of the chemical changes occurring.

Methods

Animals with paired normal chelipeds were used in this work. The chelipeds were cut from the animal at the ischiopodite and kept in the physiological solution described by van Harreveld (5). One cheliped served as a control for the experimental treatment of the other.

The motor nerve fiber giving the slow contraction, or that giving rise to the fast, was prepared in the meropodite in the manner described by van Harreveld and Wiersma (1). All of the motor and inhibitor fibers going to the other muscles in the cheliped were cut, as well as the unwanted motor fiber to the adductor muscle. Usually a small sensory bundle was kept intact to strengthen the bridge between the two thicker parts of the nerve bundle. The stimulating electrodes were always placed on the thicker proximal part of the nerve bundle in the meropodite.

Since it is impossible in preparing the fiber to avoid stimulation altogether, a rest period of between 45 and 120 minutes was allowed between the conclusion of preparation and the beginning of an experiment. The prepared cheliped was then fastened upon a wooden block fitted with flexible silver wire stimulating electrodes and with a rod for mounting upon a heavy support stand. The dactylopodite was tied to an isometric lever attached to the same stand. The electrodes were connected by flexible leads to a thyratron stimulator giving rectangular shocks of 0.5σ duration at various determined frequencies. The mechanical effect of the contraction resulting from the stimulus was recorded, and then the cheliped, still mounted on the block, was plunged into liquid air. Stimulation was continued during the period of removal to liquid air, this time (1–3 seconds) being included in the total time of stimulation. The control cheliped was also frozen at about the same time.

The frozen cheliped was opened, and the tissue of the adductor muscle was separated out (free of chitin and tendon) and dropped into liquid air. The tissue

fragments, together with a small amount of liquid air, were placed into the barrel of a small crusher, a greatly simplified form of that described by Graeser, Ginsberg, and Friedemann (6). It is essential to keep the tissue fragments and the pellets obtained by crushing constantly cooled with liquid air, since it was found that any thawing produces irregularities in the resting values between the muscles of paired, unstimulated chelipeds.

Phosphate Determination

A method for the removal of proteins with 25 per cent trichloracetic acid was developed, and the molybdate-stannous chloride method of Kuttner and Cohen (7) was used for the determination of phosphate in the deproteinized solution. The values are reported as milligrams per cent of phosphorus.

The frozen muscle pellet (300–500 mg.) is rapidly weighed on a torsion balance and dropped into 5.0 ml. of ice cold 25 per cent trichloracetic acid. The flask is stoppered and vigorously shaken for 15 seconds, and then ice cold water is added to make a total dilution to 25.0 ml., allowance being made for 80 per cent water content of the tissue. The diluted mixture is well shaken and set aside in an ice bath. Within 6 to 10 minutes aliquots are taken for colorimetric analysis. A red filter (Eastman Wratten filter 25A) is used in the colorimeter to avoid errors arising from the slight difference in color between standard and unknown.

It was found that, due to some property of the muscle proteins, the ordinary concentrations of 5 per cent or 10 per cent trichloracetic acid failed to give complete precipitation, a flocculent precipitate appearing in the final color mixtures. When the pellets were shaken with 25 per cent trichloracetic acid, however, the final colored solutions were clear. Before the colorimetric determination, the mixture had to be diluted to only 5 per cent trichloracetic acid in order to avoid interference in the color development. The use of ice cold water was found to minimize the effect of hydrolysis of phosphagen and so to eliminate the necessity of an extrapolation to a zero time value. No catalysis of the hydrolysis of the phosphagen by acid-molybdate was observed.

Analyses carried through the complete procedure gave an average recovery of 99 per cent of added phosphate. The method of protein precipitation described has also been found suitable for dealing with *Astacus trowbridgii* and with *Pagurus ochotensis*.

Lactic Acid Determination

The protein precipitation procedure used was the modification of the zinc hydroxide method of Somogyi described by Graeser, Ginsberg, and Friedemann (6), with the exception that, since no sugar determinations were made, glucose was not added to the zinc sulfate-sulfuric acid solution. For the removal of bisulfite binding substances the copper-lime precipitation was used.

For the determination of lactic acid in the copper-lime filtrate a modification of the direct distillation procedure of Friedemann and Graeser (8) was made. Following the work of Avery, Kerr, and Ghantus (9), reduced volumes of sample and of reagents were used. The distillation apparatus was modelled after the micro-distillation apparatus of Lehnartz (10). The manganous sulfate-sulfuric acid reagent of Friedemann and Graeser was found to be as effective for the conditions adopted as the stronger reagent of Avery, Kerr, and Ghantus, and potassium permanganate was found satisfactory as the oxidizing agent.

An average recovery of 96 per cent was obtained with known lactic acid solutions carried through the entire procedure.

RESULTS

Examination of Possible Interfering Factors

The suitability of the use of one cheliped to obtain the resting value for the other was investigated. In Table I are summarized the results of a number of experiments made to compare the resting value levels of phosphate and of lactic acid in the adductor muscles of paired unstimulated chelipeds. The difference between the chelipeds of one animal is small, so it is clear that one cheliped may be used as the control for the other.

There are several possibilities of production of phosphate and of lactic acid from stimuli other than those controlled in an experiment. The factors considered have been preparation of the nerve, rest in physiological solution, and the freezing of the cheliped in liquid air. The most important of these, in view of the unsymmetrical stimulation involved, is the preparation of the nerve bundle.

A series of experiments were performed in which the nerve bundle of one of a pair of chelipeds was given the maximum stimulation encountered in preparation. Both were then allowed to rest for the usual time, frozen, and analyzed. The same differences were found

TABLE I

Difference in Phosphorus and Lactic Acid Values between the Adductor Muscles of Paired, Unstimulated Chelipeds

Analysis for.....	Phosphorus			Lactic acid		
Milligrams per cent found in.....	Left muscle	Right muscle	Difference	Left muscle	Right muscle	Difference
Values found	51	57	±6	19	26	±7
	49	54	5	26	18	8
	40	39	1	34	29	5
	53	52	1	17	14	3
	55	53	2	19	20	1
	47	45	2	28	30	2
	51	49	2	25	30	5
	55	49	6	28	25	3
	51	48	3	14	15	1
	44	46	2	14	20	6
	47	44	3	17	22	5
	60	56	4	20	22	2
	58	60	2			
	51	51	0			
	60	58	2			
	59	59	0			
	44	48	4			
	51	53	2			
	48	53	5			
Mean value.....	51.1 ± 1.2	51.2 ± 1.2		21.8 ± 1.9	22.2 ± 1.5	
No. of values.....	19			12		

as are recorded in Table I for paired unstimulated chelipeds; therefore, the phosphate or lactic acid formed has disappeared on resting.

In a series of experiments in which one cheliped was dropped into liquid air immediately upon cutting from the animal, with the other being allowed to stand in physiological solution for periods up to 2 hours, there was found only a small

increase in phosphate and lactic acid in the latter. This amount is within the limits of variation noted for the resting values between paired unstimulated chelipeds.

It was observed that an unstimulated cheliped would sometimes have the dactylopodite in the closed position after liquid air treatment and sometimes not (the stimulated chelipeds were always closed). For many of the experiments on control values (Table I), a record was kept of this position of the dactylopodite. No correlation with the analytical results was found; and it was concluded that if the closing of an unstimulated cheliped is indicative of a real contraction, this contraction adds no measurable amount to the production of phosphate or of lactic acid. It is more likely that the closed position is indicative of some mechanical effect of freezing. Although Meyerhof and Lohmann (11) are of the opinion that freezing of the tissue causes a large breakdown of the phosphagen, the present work shows either that such an effect does not enter or that it is small and so uniform as not to enter into the present considerations. It may be that some thawing occurred in the work of Meyerhof and Lohmann, a condition conducive to high results.

Stimulation of the Isolated Motor Axons at Determined Frequencies

It is of special importance to note that at stimulation frequencies of 50 shocks per second for the fast contraction and at 200 for the slow, the resulting contractions are very much alike. The maximum tensions developed in different preparations are in the main very similar and are reached within 2 seconds, and both kinds of contractions have a similar fatigue. This is in accord with the findings of Wiersma and van Harreveld (3), who obtained evidence that in one cheliped these two contractions are almost identical in strength and in speed. In contrast to this, the slow contraction at 50 shocks per second shows a much more gradual rise in tension before the maximum is reached (about 15 seconds); and the maximum tension, though of the same order, is on the whole less. A few attempts were made to investigate the fast contractions arising at a stimulation frequency of 200 per second, but the very quick drop in tension after the maximum had been reached made impracticable their use for this investigation.

In Table II are summarized the results of the stimulation experiments. The string to the lever was cut as soon as the contraction began to drop from the maximum. In the case of the slow contractions obtained by stimulation at a frequency of 50 shocks per second,

fatigue is very much postponed, and it was possible to continue these for much longer periods than the fast at 50 or the slow at 200 shocks per second. It will be seen from the table that although the slow contraction at 50 shocks per second was of much longer duration, no significant increase in the amount of phosphate, and only a small increase of the lactic acid, was found. The increases in the amount of phosphate during contractions arising from stimulation of the fast system at 50 per second and of the slow at 200 for the same period were closely alike, both showing the same very definite rise above the base level. The lactic acid formation was also definitely larger than in the slow at 50 per second, but there was a difference which may be significant in that the slow at 200 seems to form less than the fast at 50 per second.

It is to be seen that practically all of the contractions of the fast at 50 and of the slow at 200 per second done for the lactic acid measurements are of a duration of less than 15 seconds. These experiments were performed at another time of the year than those on phosphate, and all preparations showed at this time a quicker fatigue. A similar difference is present in the slow contractions at 50 per second: in the lactic acid determinations, the average duration of contraction is 30 seconds, whereas the mean value for the phosphate determinations is 80 seconds.

No way could be found of correlating the tensions produced in the contractions of the muscles of the different animals with the chemical changes found. The nature of the attachment of the adductor muscle to the chitin and to the tendon makes extremely difficult a complete isolation of the liquid air frozen muscle. Thus the estimation of any value for the size of the muscle, a factor of great importance with regard to the total tension developed, was not possible.

A factor which is not clear from the tables presented is the relation between phosphate and lactic acid and the stimulation time. In order to show that such a relation is indeed present under our experimental conditions, the chemical changes after a determined number of twitches were measured. No preparation of the nerve fibers was necessary in these experiments (although carried out in a number of cases), since stimulation of the slow fiber at these frequencies has hardly any effect mechanically and (as is evident from the determina-

TABLE II

Formation of Phosphate and of Lactic Acid during Slow and Fast Contractions

Preparation		Analysis for							
		Phosphate (as phosphorus)				Lactic acid			
Fiber	Frequency of stimulation (shocks per second)	Stimulation time	Milligrams per cent found in			Stimulation time	Milligrams per cent found in		
			Control muscle	Stim. muscle	Difference		Control muscle	Stim. muscle	Difference
		sec.				*sec.*			
Slow	50	51	49	43	−6	32	28	24	−4
		62	58	55	−3	17	23	21	−2
		122	59	57	−2	22	17	19	+2
		32	58	61	+3	15	15	18	+3
		92	49	53	+4	27	23	26	3
		62	51	56	5	31	24	28	4
		47	48	53	5	17	28	33	5
		92	61	67	6	62	12	22	10
		122	38	45	7	38	13	26	13
		122	45	52	7	62	26	41	15
						16	18	34	16
						21	24	42	18
Mean value.....			51.5±2.1	53.9±2.1			21.0±1.6	28.0±2.2	
Difference in mean values.....			2.4±3.0				7.0±2.7		
Fast	50	12	55	59	+4	8	17	24	+7
		11	57	65	8	11	13	20	7
		9	41	51	10	10	21	28	7
		12	56	67	11	4	30	43	13
		17	56	68	12	7	21	36	15
		18	61	74	13	12	23	42	19
		17	57	71	14	8	21	41	20
		17	59	74	15	8	19	40	21
		18	44	63	19	9	16	39	23
		15	40	62	22	8	27	53	26
		10	55	78	23				
		32	51	75	24				
		17	56	85	29				
Mean value.....			52.8±2.1	68.5±2.4			20.5±1.5	36.7±2.9	
Difference in mean values.....			14.7±3.0				16.2±3.3		

TABLE II—*Concluded*

Preparation		Analysis for							
		Phosphate (as phosphorus)				Lactic acid			
Fiber	Frequency of stimulation (shocks per second)	Stimulation time	Milligrams per cent found in			Stimulation time	Milligrams per cent found in		
			Control muscle	Stim. muscle	Difference		Control muscle	Stim. muscle	Difference
		sec.				*sec.*			
Slow	200	17	54	64	10	7	17	18	1
		17	58	70	12	8	12	16	4
		17	56	69	13	10	16	21	5
		17	41	60	19	18	17	24	7
		16	60	82	22	8	18	30	12
						11	18	31	13
						11	26	41	15
						9	22	39	17
						7	31	49	18
						6	21	47	26
Mean value.....			54.1±3.3	69.7±3.4			19.9±1.6	31.7±3.3	
Difference in mean values.....			15.6±4.8				11.8±3.7		

TABLE III

Phosphate and Lactic Acid Formation on Single Shock Stimulation

(Frequency = 1, 3, 6, or 12½ shocks per second)

Total number of twitches	Milligrams per cent increase in			
	Phosphorus		Lactic acid	
	No. of values	Average value	No. of values	Average value
15– 50	3	7	9	7
50–100	8	11	7	8
100–150	2	13	4	12
150–200	3	23	2	20
200–400	3	23	—	—

tions upon slow preparations at 50 per second) certainly causes no appreciable chemical changes. The results are given in Table III. It will be seen that the production of phosphate gradually rises with

an increasing number of twitches and that the lactic acid formation is somewhat similar, although not so clearly demonstrated as that of the phosphate.

Phosphagen Phosphate

Since the amount of phosphate found in the stimulation experiments was, on the average, no more than 16 mg. per cent phosphorus (highest value, 29 mg. per cent) above the resting level, it was of interest to

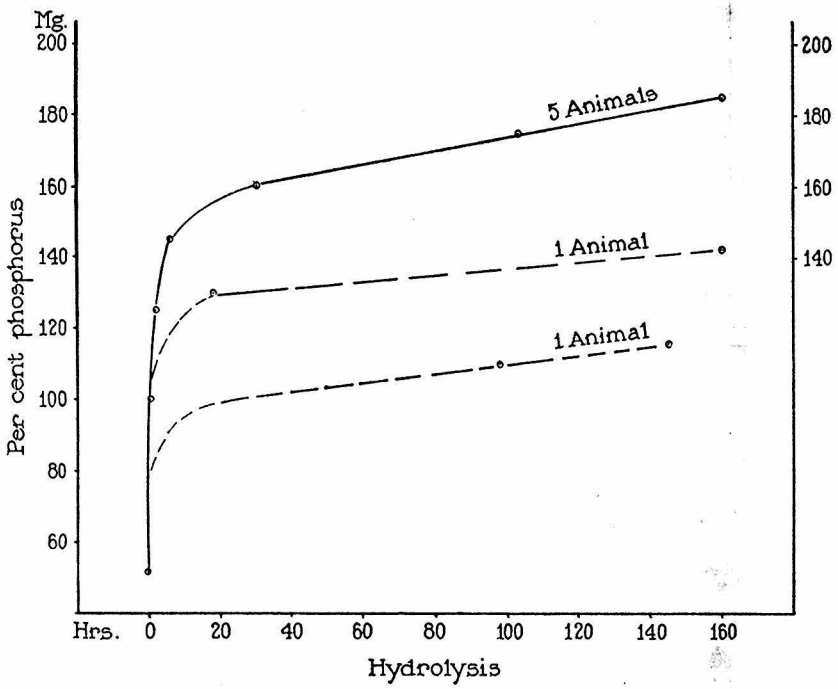

FIG. 1. Hydrolysis of phosphate compounds in the adductor muscle.

determine the fraction of the total available phosphagen represented by this amount. A number of experiments were made in which pairs of chelipeds were frozen in liquid air, with or without stimulation of one of the pair. The initial phosphate was determined, and the mixture of tissue and 5 per cent trichloracetic acid was left standing at 35°C., determinations being made at intervals.

The results are plotted in Fig. 1, the initial portion of the curve for one experiment being shown in detail in Fig. 2.

In the group of five animals giving closely concordant data, it will be seen that during the first 2 hours the hydrolysis proceeds rapidly, a level of 130–135 mg. per cent phosphorus being reached. The rate then decreases, and after about 30 hours (level of 160 mg. per cent) a new, much slower rate is followed. At the end of 160 hours the final levels for the five pairs of chelipeds are 198,197; 181,176; 189,185; 176,174; 184,184: the average is 185 mg. per cent.

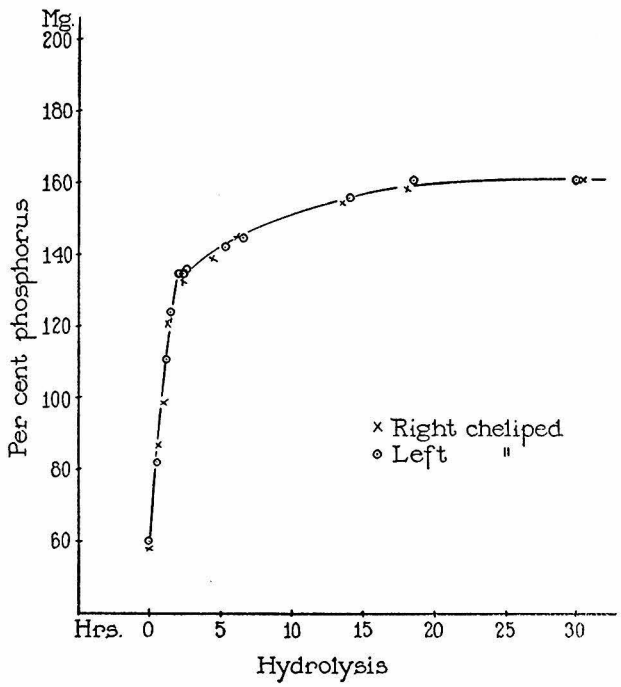

FIG. 2. Production of phosphate on hydrolysis of the tissue from the paired claws of one animal.

If the hydrolysis during the first 2 hours is considered as being that of the phosphagen and the remainder is assumed to be that of other more or less readily hydrolyzable phosphate compounds, the representative phosphagen level should be about 140 mg. per cent. With a resting level of phosphate at 50 mg. per cent phosphorus, the amount of available phosphagen is in the order of 90 mg. per cent phosphorus.

Thus, on the average, less than one-fifth of the available phosphagen was found hydrolyzed on contraction, at the most one-third.

Experiments in which one of the pair of chelipeds was stimulated showed at the beginning of the hydrolysis the accustomed difference in the phosphorus level, but this difference had disappeared at the end of the first 2 hour period.

Of the two deviating animals, one had a phosphagen level at about 110 mg. per cent and a final level at 142 mg. per cent phosphorus; the other, only 90 and 116 mg. per cent respectively. Also in these cases, the paired chelipeds gave closely similar figures.

DISCUSSION

The present investigation was undertaken in order to compare the chemical changes in the fast and the slow contractions and to correlate this information, if possible, with existing physiological data. The results obtained clearly show that the chemical changes involved in the two types of contraction are of the same order if the mechanical effects are comparable, and that they are distinctly different if the stimulation is such as to produce quite different mechanical effects. Since equivalent changes accompany equivalent action, it is necessary to assume that the two types of contraction do not differ in the mechanism of the chemical changes involved, which is strongly indicative of the presence of only one contractile substance.

The evidence obtained thus strengthens the conclusions arrived at by anatomical and physiological experiments (2, 3); namely, that each muscle fiber is innervated by both the fast and the slow axon and that both contractions occur in the same muscle fiber. The difference between the two contractions then lies in the modes of transmission at the neuromuscular junction.

For the present considerations more emphasis has been placed upon the phosphate values, for it is generally considered that the phosphate stands in a more directly integral position in the cycle of chemical changes in muscle than does the lactic acid. That only a fraction of the available phosphagen is found broken down to phosphate after contraction is of significance towards understanding the individual variations observed between animals. This fraction is probably more

apparent than real, its exact magnitude depending upon the balance between breakdown and resynthesis. Also, the supply of total phosphate and of available phosphagen is variable between animals.

The fact that no catalysis of the hydrolysis of the phosphagen in acid-molybdate solution was observed in the course of the phosphate determinations indicates that the phosphagen of *Cambarus clarkii* is arginine phosphoric acid rather than the creatine compound. This conclusion is substantiated by the results of previous investigators (Kutscher (12), Eggleton and Eggleton (13), Meyerhof and Lohmann (14), Schutze (15)) on the isolation of the phosphagen of crustaceans.

The resting value for phosphate of Schutze on *Astacus fluviatilis* (about 90 mg. per cent phosphorus) is higher than that found in the present work on *Cambarus clarkii*, as are his maximal values (240 mg. per cent). The high resting value probably is due to injury of the muscle tissue during preparation for analysis. Meyerhof and Lohmann (11) give a much lower resting value (about 35 mg. per cent) for *Astacus*, but their maximal values are only about 100 mg. per cent. The discrepancy in the breakdown phosphate values no doubt lies in the conditions of the hydrolysis, and that the values of Meyerhof and Lohmann are lower than those of the present work may be due to a difference between *Astacus* and *Cambarus*. In this connection it is of interest that the available phosphagen for the latter amounts to 65 per cent of the sum of resting phosphate plus that from phosphagen, a figure in agreement with the 60–75 per cent noted by Meyerhof and Lohmann for *Astacus*.

The very high resting lactic acid values of Schutze (average 322 mg. per cent for 9 values) are not in accord with the present data. However, the few values given by Meyerhof and Lohmann are of the same order as the present ones, and it may be that the analytical method used by Schutze gave apparent high results.

SUMMARY

An investigation has been made of the phosphate and lactic acid changes in the adductor muscle of the cheliped of the crayfish *Cambarus clarkii* upon stimulation of the isolated axons for the fast and slow contractions at determined frequencies. The data obtained point to the following conclusions:

1. When the mechanical effects of the two types of contraction are the same, the chemical changes are of the same order. If the mechanical effects are different, the chemical changes likewise are not equivalent. This is especially to be seen in the case of stimulation at 50 shocks per second: a slowly rising, long continued, strong slow contrac-

tion takes place with no apparent change in the phosphate content; a quickly rising fast contraction occurs with a large increase in the phosphate.

2. Since equivalent chemical changes accompany equivalent mechanical action, the two types of contraction do not differ in the essential mechanism of the chemical changes involved, and only one type of contractile substance is present.

3. Even when a contraction has taken place to the maximum extent obtainable, only enough phosphate is found to correspond to one-fifth to one-third of the available phosphagen.

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