I Biological Synthesis of Creatine

II Corneal Vascularity and Ariboflavinosis

III Root "Bleeding"

by

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The candidate wishes to express his sincerest appreciation for the opportunity of working with Dr. H. Borsook in the work herein described and for the invaluable experience it has given him.

In the following section, a series of reprints is presented of research carried out with Dr. Borsook on the biological synthesis of creatine and its precursors.

SUMMARY

It has been established that liver slices can convert glycocyamine to creatine.

Of the large number of compounds tested, only methionine can methylate glycocyamine to form creatine.

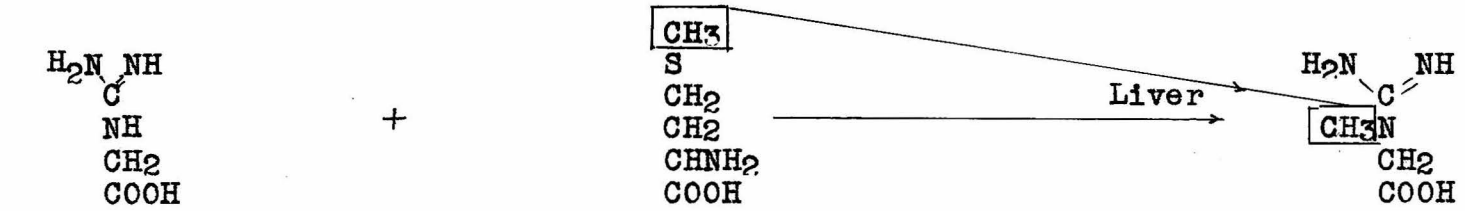
The following animals were able to methylate glycocyamine: cat, dog, guinea pig, frog, pigeon, rabbit, and rat.

In order to study the formation of glycocyamine a more rapid and accurate method was developed for this compound.

It was shown that glycocyamine is formed by cell free extracts of kidney by the transfer of the amidino group of arginine to glycine. Beef, cat, dog, guinea pig, pigeon, rabbit, rat, and sheep kidney can carry out this reaction.

It was shown that a similar mechanism exists in man, for there is a rapid rise in the amount of glycocyamine of the urine after the ingestion of arginine and glycine.

A method was developed which permitted the simultaneous determination of both arginine and glycocyamine. With this method it was shown that the arginine for the above reaction was formed by the transfer of the amino group of glutamic acid to citrulline.



Glycocyamine Methionine Creatine

CONVERSION OF GLYCOCYAMINE TO CREATINE

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THE FORMATION OF CREATINE FROM GLYCOCYAMINE IN THE LIVER

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The study of the precursors of creatine in animals has been beset by two difficulties principally. One has been the lack of really adequate biological material; the other, the lack of a specific, and at the same time sensitive analytical method. Experiments hitherto have consisted in attempts to change the urinary excretion of creatine and creatinine, or the creatine content of the tissues of intact animals or of isolated perfused organs. The normal, *i.e.* uncontrolled, fluctuations in tissue composition and urinary excretion are relatively large compared with the changes induced experimentally; it is often impossible to distinguish when experimental effects are observed, whether these have arisen from changes in the processes of excretion or synthesis; there may be variations in the water content of the tissues, thereby affecting their percentile composition; all of these have stood in the way of firm conclusions being drawn.

Some of these difficulties have been avoided in perfusion experiments on isolated organs (1). These experiments are extremely laborious, time-consuming, and costly. The experimentally induced change in creatine content is at the most—about 30 per cent—a small deviation from the normal. The normal base-line is not constant but varies with the age and weight of the animal. Since the same specimen cannot be used for the experiment and control, a large number of animals must be used first to establish the normal base-line, encompassing the variations of the normal, and then an equally large number for each single experiment in order to obtain a body of data sufficiently large for a statistical conclusion which may be significant.

In 1935 we published some observations of a slight increase in in "apparent" creatine when rat liver slices were incubated with a protein hydrolysate (2). Granted that such liver slices could synthesize creatine, most of the difficulties in working with whole animals or whole organs are avoided. With tissue slices the one specimen of tissue provides material for controls and for testing a variety of experimental variants simultaneously. This was the reason that the observation of even a slight synthesis of creatine by tissue slices seemed promising.

We were faced here, however, with the second difficulty mentioned above, uncertainty whether the material which is augmented by the liver slices is really creatine. A number of substances give a positive test with the Jaffe reagent. The amount of the material in question formed by the liver slices was too small to be identified by isolation.

Accordingly further study of this problem was postponed until a more specific analytical method which could be adapted to submicro scale was available. Such a method was provided by Dubos and Miller (3). They discovered and succeeded in culturing a soil bacterium which specifically destroys creatine and creatinine.

We again took up the problem, employing tissue slices and this new adjuvant to our former submicromethod for creatine (4). We have found that liver slices of the cat, rabbit, and rat are able to convert glycocyamine to creatine. In the experimental conditions we have observed, the increase is 5 to 20 times the amount originally present in the slices. The difficulties and uncertainties which exist in conclusions resting on statistical analysis of small differences have therefore been overcome. The liver is well suited to this study, because its normal creatine content and therefore the control or blank value is very low.

We have found further that when methionine is present in the Ringer's solution, the amount of creatine formed from the added glycocyamine was on the average about 50 per cent greater than when methionine was not added. We have tested thirty-two other substances including amino acids, methylated amines and a purine, and betaine. All of these were negative in this respect. It seems reasonable to conclude therefore that rat liver slices transfer the methyl group of methionine to glycocyamine, thus converting the latter substance to creatine.

Technique

The tissue slice technique used is only slightly modified from that described by Warburg (5). The details of the reaction vessels and the manner in which they are mounted are described in a previous communication (6).

The blank controls and each experimental variant were carried through in triplicate. We have had twenty to thirty reaction vessels, each containing three slices from the same liver, running simultaneously. It was necessary to make the sampling of the slices as uniform as possible, because the capacity to synthesize creatine may vary significantly between the lobes. The procedure we finally adopted was to arrange the reaction vessels in three rows (when each experimental variant is carried through in triplicate), one vessel in each row for each variant. Consecutive slices were transferred to a Petri dish containing Ringer's solution, one slice for each vessel in the row. After 3 minutes soaking in this vessel, with gentle agitation, the slices were transferred to the reaction vessels. The same was done for the other two rows. The whole procedure was then repeated according to the number of slices wanted in each vessel. The reason for the 3 minutes soaking in the Ringer's solution before transfer to the reaction vessels is that more creatine was formed than when the slices were transferred directly with only momentary rinsing in Ringer's solution.

At the end of the reaction period the contents of the vessels, including the slices, were transferred to test-tubes, and the bottles and transfer pipette washed with two 1 ml. portions of 0.02 N HCl. The test-tubes were placed in a boiling water bath for 10 minutes with occasional stirring or shaking. After this time the test-tubes were cooled and the volumes made up to 6 ml. with 0.02 N HCl. These test-tubes are marked at 6 ml. Usually only 0.1 to 0.2 ml. was needed to bring the volumes to the mark. The suspensions were then filtered. The coagulated slices with the coagulated protein in each vessel were transferred to small glass dishes and the weight determined after drying at 100°.

The analytical procedure was essentially that described previously (4). Some minor details were varied from time to time in addition to employment of the bacterial digestion procedure of Dubos and Miller. Table I is a protocol of an experiment in

which the complete analytical procedure was used. Instead of triplicate reaction vessels, six were used for each experimental variant. The boiled extracts in pairs of vessels were joined and filtered. To 8 ml. of this filtrate were added 2 ml. of 0.25 M phosphate buffer at pH 7.0. This solution was divided into two portions. In one-half the creatine was determined directly. The creatine in the other half was destroyed by a suspension of NC soil organisms prepared according to the prescription of Dubos and Miller. The difference between the color developed by the Jaffe reagent with and without this digestion gave the true creatine (and creatinine) in the original solution.

The bacterial digestion was carried out as follows: 5 ml. of the solution neutralized with phosphate were transferred to a 250 ml. Erlenmeyer flask. To this was added 1 ml. of a suspension of the NC soil organisms. The necks of the flasks were covered with squares of Parafilm and set away in an air bath at 38° for $\frac{1}{2}$ to $\frac{3}{4}$ of an hour. At the end of this time 1 ml. of 0.5 N HCl was added to each flask. The contents were then centrifuged. 6 ml. of the clear supernatant solution were taken for analysis. The length of time the bacteria were allowed to react on the experimental solutions was based on a prior determination of the potency of the bacterial suspension used. It was the time required by 1 ml. of the bacterial suspension to destroy completely the creatine in 5 ml. of a 2 mg. per cent solution. This was a larger amount of creatine than in any of the tissue extracts submitted to digestion.

To the 5 ml. of tissue extract containing the phosphate buffer, but which had not been digested by the bacteria, were added 1 ml. of water and 1 ml. of 0.5 N HCl. 6 ml. were then taken for analysis. From this point on the analytical procedure was identical for the solutions which had been digested with bacteria and those which had not. Both sets were carried through to the completion of the analysis simultaneously.

The 6 ml. aliquots were transferred to thick walled Pyrex test-tubes with internal dimensions of 125 × 12 mm. The tubes were covered with parchment paper caps and autoclaved for 20 minutes at 125°. After they were cooled, a small amount of Lloyd's reagent was added to each. We have found that the amount of the Lloyd's reagent may vary from 10 to 60 mg. without affecting the final result. The test-tubes were now shaken for 7 minutes

on a shaker of the type devised by Fisher and Wilhelmi (7). The tubes were then centrifuged, the supernatant solution discarded with the last drop at the rim of the test-tube taken up with filter paper, and the Lloyd's reagent resuspended in 2 ml. of 0.01 N HCl. The tubes were centrifuged again, the supernatant solution again discarded, and the adherent moisture on the walls of the test-tube carefully taken up with filter paper. 3 ml. of a sodium picrate solution were added to each tube. This solution consists of 10 parts of saturated picric acid (purified) and 1 part of 10 per cent NaOH, these being mixed immediately before use. The test-tubes were again shaken for 7 minutes and centrifuged. The color was measured on a spectrophotometer with light of approximately 0.525 μ wave-length. With concentrations ranging from 0 to 2 mg. per cent there is a strictly linear relation between concentration of creatine and the intensity of color measured in this manner.

The following controls were taken through the above identical procedure including digestion by the bacteria: Ringer's solution alone, Ringer's solution containing the same concentration of glycocyamine used in the experiment, and Ringer's solution containing glycocyamine and methionine. Each of these was carried out in triplicate. In addition 1 ml. of the bacterial suspension alone and bacterial suspension plus 5 ml. of 2 mg. per cent creatine were incubated with the experimental solutions and carried through the same analytical procedure, performed in duplicate. Finally a set of five standard creatine solutions with concentrations ranging from 0.1 to 2 mg. per cent was treated in exactly the same manner as the experimental solutions except that they were not submitted to bacterial digestion. The amounts of creatine in the experimental solutions and controls were determined by interpolation from the straight line given by the readings of these standard solutions. The above controls and the standards were carried through afresh in every experiment with the experimental solutions.

It is a testimony to the convenience of this analytical method that we have frequently carried through more than 60 individual analyses from the bacterial digestion to the final spectrophotometer reading in less than 5 hours.

We found in experiments with rat and rabbit liver that essen-

TABLE I

Formation of Creatine by Rat Liver Slices in 6 Hours at 37.5° from Glycocyamine

Treatment	Composition of medium			Dry weight of tissue	Apparent creatine				Residual chromogenic material after bacterial digestion	True creatine			
	Ringer's solution	Glycocyamine	*dl*-Methionine		Total	Total amount formed	Amount formed from glycocyamine	Q (creatine) × 100 from glycocyamine		Amount present	Total amount formed	Amount formed from glycocyamine	Q (creatine) × 100 from glycocyamine
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
	ml.	*mg.*	*mg.*	*mg.*	*mg. per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
Analyzed immediately	4			40.8	0.12				0.07	0.05			
" "	4			41.5	0.14				0.07	0.07			
" "	4			59.8	0.17				0.10	0.07			
Incubated 6 hrs.	4			34.1	0.21	0.12			0.14	0.07	0.03		(0.15)
" 6 "	4			40.2	0.26	0.14			0.18	0.08	0.03		(0.13)
" 6 "	4			38.0	0.22	0.12			0.14	0.08	0.03		(0.15)
" 6 "	4	0.25		35.4	1.20	0.60	0.38	1.9	0.70	0.50	0.45	0.42	2.1
" 6 "	4	0.25		45.0	1.54	0.94	0.66	2.5	0.72	0.82	0.77	0.74	2.8
" 6 "	4	0.25		35.5	1.31	0.71	0.49	2.4	0.72	0.59	0.54	0.51	2.5
" 6 "	4	0.25	1.45	32.5	1.54	0.90	0.70	3.7	0.72	0.82	0.78	0.75	3.9
" 6 "	4	0.25	1.45	37.8	1.54	0.90	0.64	2.9	0.70	0.84	0.79	0.76	3.4
" 6 "	4	0.25	1.45	35.5	1.53	0.89	0.67	3.2	0.71	0.82	0.77	0.74	3.6
" 6 "	4	0.25			0.61				0.61				
" 6 "	4	0.25			0.60				0.60				

Incubated 6 hrs.	4	0.25		0.60			0.61			
" 6 "	4	0.25	1.45	0.65			0.64			
" 6 "	4	0.25	1.45	0.66			0.63			
" 6 "	4	0.25	1.45	0.61			0.64			
1 ml. bacterial suspension incubated 45 min. at 37°							0.01			
0.1 mg. creatine incubated with 1 ml. bacterial suspension 45 min. at 37°							0.007			
							0.006			
							0.006			

The factor converting mg. per cent to Q(creatine) is $4/100 \times 1.5 \times 171 \times 1/6 \times 1/$(dry weight of tissue): 4/100, since there were only 4 ml. in each reaction vessel for the amount of tissue indicated; 1.5 because these 4 ml. were diluted to 6 ml. in the transfer from the reaction vessels to the test-tubes in which they were boiled; 171, because 1 mg. of creatine would occupy 171 c.mm. at S.T.P. if it were a gas; 1/6, because the experiment was run for 6 hours. Q(creatine) is therefore the amount of creatine formed, expressed as if it were a gas in c.mm. at S.T.P., per mg. of tissue (dry weight) per hour.

tially the same result was obtained whether bacteria were used or not. Accordingly we later dispensed with the use of the bacteria, except as indicated.

Results

Table I is a condensed protocol of a typical experiment. The figures in Column 6 are the spectrophotometer readings converted to mg. per cent by interpolation from the standard curve. The figures in Column 7 are obtained by subtracting from those in Column 6 the values of the glycocyamine or glycocyamine plus the methionine blank, and the amount present in the tissue at zero time. The glycocyamine blank value is quite large. It arises from the conversion of glycocyamine to glycocyamidine during the autoclaving. It was essential that the glycocyamine blanks be treated exactly the same as the experimental solutions through all the operations from immersion in the water bath at 37.5° for the same length of time to the final development of the color. The figures in Column 8 are obtained by subtracting from those in Column 7 the amount of chromogenic material formed by the tissue in the Ringer's solution without glycocyamine. Q(creatine) (Columns 9 and 14) is the amount of creatine formed, expressed as if it were a gas in c.mm. at S.T.P., per mg. of tissue (dry weight) per hour. The figures in Column 11 are the differences between those in Columns 6 and 10. From the figures for the blanks in Columns 6 and 10, it is seen that the bacteria digested none of the glycocyamine. The figures in Columns 9 and 14 are not significantly different. They are a little higher in Column 14 than in Column 9 because in Column 7 a correction should have been applied to the glycocyamine blank value for the glycocyamine converted to creatine, from 10 to 16 per cent. If this had been done, the figures in Column 8 would have been increased by 0.06, which would have made them the same, within experimental error, as those in Column 13.

In a previous determination we have found that there was practically no creatinine in the tissue extracts.

Table I shows that the liver slices convert glycocyamine to creatine. This, as far as we know, is the first time the biological conversion of glycocyamine to creatine has been demonstrated by an unequivocally specific analytical method for creatine, and in which

the tissue used both in the controls and in the experiment came from the identical organ specimen. The increases in the experimental vessels were from 10 to 15 times the amount present in the tissue at the beginning of the experiment. The figures in Columns 9 and 14 show that with added methionine there was 40 per cent more creatine formed than when methionine was not added.

Most of the chromogenic material in the liver slices at the beginning and end of the experiment was not true creatine. This is in accord with the findings of Baker and Miller (8). There is, however, a slight increase in true creatine in the liver slices suspended in Ringer's solution containing no glycocyamine. We have found this repeatedly. Most of this non-creatine chromogenic material found in the tissue blanks is also not glycocyamine. This was ascertained by a direct determination for glycocyamine.

The values for Q(creatine) are much smaller than those found with liver slices for the formation of urea, amino acids, or hippuric acid (6). Nevertheless, this rate, small though it is, is more than sufficient to account for the total creatine plus creatinine excretion in the rat. Thus, an adult rat with a liver weighing 12 gm. might excrete 9 mg. of creatine plus creatinine in 24 hours (9). A Q(creatine) of 0.02 would correspond in such an animal to the formation of 7 mg. of creatine in 24 hours.

The following compounds and combinations of compounds were tried instead of glycocyamine to determine whether they could serve as precursors of creatine: arginine, arginine plus glycine, arginine plus glycolic acid, choline, glycine, glycine plus urea, glycolic acid, and methionine. Each of these has been tested on both rat and rabbit liver slices several times. The results have been consistently negative.

Table II is a summary of most of our experiments with glycocyamine and methionine. In the course of these experiments we have used glycocyamine from two different commercial sources, and two specimens of *dl*-methionine, one prepared in this laboratory and one obtained commercially. A different animal was used for each pair of figures.

The data in Table II show the increase in creatine formation invariably obtained when methionine was added to the glycocyamine. Approximately the same relative increase occurred regardless of age, sex, and nutritional condition of the animal, and

TABLE II

Formation of Creatine by Rat Liver from Glycocyamine with and without Added Methionine at 37.5°

From glycocyamine alone	From glycocyamine and methionine	Duration of incubation	Age and sex	Nutritional condition
Q(creatine) × *100*	*Q(creatine)* × *100*	*hrs.*		
3.2	6.2	1	Adult. ♂	Normal nutrition
6.1	9.0	2	" ♂	" "
4.0	5.5	3	" ♂	" "
4.0	6.5	3	" ♂	" "
5.7	8.1	3	" ♂	" "
1.9	3.4	3	3 mos. ♂	" "
4.2	7.4	3	3 " ♂	" "
6.6	7.9	3	3 " ♀	" "
6.5	9.0	3	3 " ♀	" "
4.6	6.6	4	Adult. ♂	" "
8.5	11.3	4	" ♂	" "
6.1	8.5	4	" ♂	" "
7.3	10.6	4	" ♂	" "
7.7	11.1	4	" ♂	" "
3.1	5.8	4	1 mo. ♂	" "
2.3	4.5	4	1 " ♂	" "
1.5	2.3	4	Adult. ♂	Fasted 66 hrs.
1.5	2.4	4	" ♂	" 66 "
4.1	6.4	4	" ♀	Normal nutrition
4.4	6.9	4	" ♀	" "
2.7*		6	" ♂	" "
2.8*	4.8*	6	" ♂	" "
2.5*	3.6*	6	" ♂	" "
2.4	3.3	6	" ♂	" "
3.1		6	" ♂	" "
2.5		6	2 mos. ♂	" "
2.2		6	Adult. ♀	" "
2.7		6	" ♀	" "

Each of the above figures is the average of a triplicate determination. Each reaction vessel contained 20 to 40 mg. (dry weight) of liver, 4 ml. of Ringer's solution containing 0.25 mg. of glycocyamine, and in the methionine series in addition 1.49 mg. of the amino acid.

* Values obtained with the employment of bacteria in the analysis.

regardless also of the duration of the experiment. The over-all average value for Q(creatine) × 100 from glycocyamine alone is 4.1, and from glycocyamine plus methionine 6.6.

We have no explanation as yet for the large variations in the rate of creatine formation in different experiments.

It will be noted that most of the results in Table II were obtained without the employment of bacteria in the analysis. Three experiments with rat liver and three with rabbit liver were carried out in which bacteria were used. The values for Q(creatine) were, as in Table I, essentially the same with and without the use of bacteria. We feel therefore that the results obtained without the use of bacteria with this tissue and under these experimental conditions are as reliable indices of true creatine as those obtained with bacteria.

TABLE III

Rate for Formation of Creatine from Glycocyamine with and without Added Methionine

Time	Without methionine		With methionine	
	Amount of creatine formed per 100 mg. (dry weight) of tissue	Q(creatine) × 100	Amount of creatine formed per 100 mg. (dry weight) of tissue	Q(creatine) × 100
hrs.	*mg.*		*mg.*	
1	0.018	2.8	0.036	6.2
2	0.070	6.0	0.101	8.6
4	0.095	4.6	0.150	6.4
6	0.144	4.5	0.203	5.8

Each of these figures is the average of a triplicate determination. The composition of the Ringer's solution and amount of tissue were the same as in the experiments of Tables I and II. Two lobes of liver were used. Consecutive slices were placed alternately in the vessels with and without methionine. The comparison at each hour, therefore, is of the activity of immediately adjacent sections of liver. Bacteria were not employed in the analyses here.

Table III contains the results in more detail of an experiment in which the rate of creatine formation from glycocyamine, with and without methionine, was studied. The figures show that the methylation of glycocyamine proceeds unchecked for at least 6 hours. The maximum at 2 hours in each series is accidental. It did not occur in other similar experiments.

The absolute amount of glycocyamine methylated was not increased by a 10-fold increase in the initial concentration of gly-

cocyamine. We have not yet explored thoroughly the effect of changing the concentration of methionine. In one experiment 0.0025 M methionine was as effective as 0.01 M. The fact that addition of so much methionine increases the rate of methylation only 40 or 50 per cent leads us to suspect that the methylating agent in the liver itself may not be methionine. Another piece of evidence which points in this direction is that the ratio of the rates of creatine formation with and without methionine is nearly the same throughout the whole period of from 1 to 6 hours (Table III). In other words, the effects of the methionine and of the methylating agent in the tissues were additive. It is possible that the methylating agent in the tissues is derived from methionine.

The following compounds were tested with rat liver as possible methylating agents of glycocyamine. All gave negative results: acetylcholine, *d*-alanine, *d*-arginine, *l*-asparagine, *l*-aspartic acid, betaine, caffeine, choline, *l*-cysteine, *l*-cystine, ethanolamine, *d*-glutamic acid, *d*-glutamine, glycine, glycolic acid, guanidine, *l*-histidine, *dl*-isoleucine, *l*-leucine, *d*-lysine, mono-, di-, tri-, and tetramethylamine, *d*-ornithine, *l*-hydroxproline, *dl*-phenylalanine, *l*-proline, *dl*-serine, *d*-threonine, *l*-tryptophane, and *l*-tyrosine. The final concentration of glycocyamine in the Ringer's solution in these experiments was always approximately 0.0005 M, and 0.0025 M of the compound whose methylating possibilities were being tested. The significance of the positive effect invariably obtained with methionine is heightened obviously by the fact that all of the above compounds were negative.

Some experiments have been made with the kidneys of the cat, rabbit, and rat. Slices of the cortex with and without methionine either failed to methylate glycocyamine or the slight positive results were within the experimental error. These experiments are part of a survey not yet completed of the organs of a number of animals. The details of these experiments will be published later.

Minced liver of the rat or rabbit failed to give any measurable increase in creatine in 6 hours at 37.5° on incubation with glycocyamine, with or without methionine.

Similarly negative results were obtained with slices of heart

and sartorius. But the cell structure is not preserved in slices of these muscular structures as it is in slices of liver.

Until conditions are discovered in which positive results are obtained with minced liver, we feel that no significance can be attached to negative results with sections whose cell structure has been broken or to extracts of other organs.

DISCUSSION

Beard, Boggess, and Pizzolato (10) proposed that glycine and urea condense to form glycocyamine, which is then methylated by more glycine or glutamic acid. We have observed neither this condensation nor the proposed methylating reaction with rat or rabbit liver slices. The conclusions of Beard *et al.* are based largely on experiments on the rat. The negative results in our experiments with glycine and urea we believe are significant in view of the positive results with glycocyamine and methionine. Experiments on the synthesis of urea, amino acids, and hippuric acid have shown that results obtained with tissue slices afford reliable qualitative information, at least, regarding the potentialities of the intact tissue *in situ*.

Bodansky (11) fed glycocyamine to normal rats and at 3, 6, 12, and 24 hours after feeding measured the glycocyamine and creatine concentrations in the liver, muscle, heart, and kidney. Bodansky's interpretation of the data obtained in these experiments was that significant increases in creatine content occurred only in the kidney, and that the increases in the liver were not sufficiently clear cut to be significant. Bodansky concluded that his findings suggest "that methylation of the guanidoacetic acid may have occurred in the kidneys. . . In view of the occurrence of guanidoacetic acid in large amounts in the liver and the failure to show an increase in creatine, it is surmised that the liver plays an insignificant rôle, if any, in creatine production."

This surmise is in direct conflict with our observations. Bodansky's experimental observations, however, and ours are not in conflict. Thus the creatine content of the liver in two controls in Bodansky's experiments was 16.0 and 20.4 mg. per 100 gm. of tissue, and in three experiments with glycine, 18.6, 16.8, and 19.0 mg. After glycocyamine feeding the figures are 21.7 mg. in 3 hours

and 23.3 in 6 hours, and in a second experiment 30.5 in 3 hours and 22.3 in 6 hours. The relative increases over the control values in the liver with glycocyamine were of the same order of magnitude as those found in kidney. The increases in true creatine in the liver were probably relatively much larger, since most of the chromogenic material in the liver with the Jaffe reagent is not creatine (Table I, and also Baker and Miller).

There is another physiological factor which must be taken into account in interpreting the data obtained by Bodansky. This factor is that the kidney is better able to store creatine than the liver. The analyses of Baker and Miller and our own show that the true creatine content of the kidney is 4 or 5 times that of the liver. Bodansky found that when creatine was fed the creatine content of the liver was twice the control value at the 3rd hour but had declined to the control value by the 6th hour, whereas in the kidney the concentration was 70 per cent above the control value at the end of the 6th hour.

Our observations show conclusively that glycocyamine can be methylated by rat liver. The rate of methylation by kidney slices is much slower than in liver, if it is not absolutely negative.

All these observations are brought into accord by the hypothesis that in the experiments of Bodansky the creatine synthesized from glycocyamine in liver was quickly removed by the blood and stored for a relatively long period in the kidney. We have no reliable data of our own at present on the possible conversion of glycocyamine to creatine in other organs or in the muscles.

Fisher and Wilhelmi found that when isolated male rabbit heart was perfused there was an increase in creatine when arginine was added to the perfusate. No increase in creatine was observed under these conditions in the hearts of prepubertal animals. Davenport, Fisher, and Wilhelmi (12), extending these observations, found that glycolic acid was essential for the methylation of glycocyamine. They suggested the following mechanism of creatine formation in the rabbit heart. Arginine is broken down to glycocyamine and glycolic acid; the glycolic acid then methylates the glycocyamine to form creatine.

In rat and rabbit liver slices the results with arginine, with and without glycolic acid or glycine, and with the two acids alone did

not yield detectable amounts of either glycocyamine or creatine. The only substance we have yet found which is capable of methylating glycocyamine is methionine.

We have attempted to repeat the observations of Fisher *et al.* with slices of rabbit heart. These experiments were unsatisfactory because of the difficulties of obtaining uniform sampling and because the experimental effects were small compared with the amount of creatine initially present. For the reason stated above we do not attach any significance to these experiments.

The difference between the observations on the perfused heart and ours on the liver stand, for the time being at least, either as an unresolved discrepancy or as indicating important differences in the mechanism of creatine formation in the heart and in the liver.

SUMMARY

1. Liver slices of cat, rabbit, and rat convert glycocyamine to creatine.

2. This methylation is accelerated in rat liver by methionine, (other animals are now being studied).

3. Methionine is the only substance we have yet found among a large number of amino acids, methylated amines, and other compounds which is able to effect this methylation in rat liver.

The authors wish to thank Mr. Y. Tajima for the assistance he gave them throughout this work, and Dr. H. W. Davenport for advice and assistance in the construction of the shaker used here. They are indebted to Dr. R. Dubos, and wish to thank him for specimens of the bacteria used in these experiments and valuable information on the culture details.

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CREATINE FORMATION IN LIVER AND IN KIDNEY*

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We reported recently (1) the formation of creatine from glycocyamine by rat liver slices; and that 40 to 50 per cent more creatine was formed when methionine was added with the glycocyamine to the Ringer's solution in which the slices were immersed. Among some thirty odd amino acids, methylated amines, a methylated purine, and betaine only methionine gave this increased rate of methylation. The rate of creatine formation under these conditions is sufficient, if it is of the same order of magnitude *in vivo*, to make good the entire loss as urinary creatinine.

If the methylation of glycocyamine in the liver is important both as a mechanism and as a site of creatine formation in animals, one may expect to find it in the livers of most other animals. We accordingly examined the livers of a number of animals for their ability to carry out this reaction. At the same time the possibility of its occurring in their kidneys was investigated also.

The findings in this survey, summarized in Table I, were that glycocyamine can be methylated by the liver of every animal examined; the stimulating effect of methionine observed with rat liver was found with some but not all the other animals; the kidney appears to be unimportant (except in the pigeon) for the methylation of glycocyamine without or with methionine.

The experimental and analytical procedure was the same as was described in our previous communication, except that the experiments with frog liver and kidney were carried out at 25° instead

* Presented before the meeting of the American Society of Biological Chemists at New Orleans, March 13–16, 1940 (*Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **133**, p. xv (1940)).

of 38°. All the creatine figures given represent differences in chromogenic material in the Jaffe reagent before and after digestion with the NC bacteria of Miller and Dubos (4).

Baker and Miller (3) have reported that creatine is formed from unidentified precursors in rat liver and kidney (slices and mash). Our findings on this point are that small amounts of creatine (beyond the experimental error) were formed from pre-

TABLE I

Formation of Creatine in Liver and Kidney Slices

Temperature 38°; glucose-Ringer's solution. The creatine is given as mg. per 100 gm. of wet weight of tissue.

Animal	Creatine in liver				Creatine in kidney			
		After 4 hrs. incubation				After 4 hrs. incubation		
	Initially present	In Ringer's solution alone	With 6.25 mg. per cent glycocyamine	With 6.25 mg. per cent glycocyamine and 40 mg. per cent *dl*-methionine	Initially present	In Ringer's solution alone	With 6.25 mg. per cent glycocyamine	With 6.25 mg. per cent glycocyamine and 40 mg. per cent *dl*-methionine
Cat	12.0	12.2	21.1	23.2	1.9	2.5	2.4	2.3
Dog	2.9	2.9	3.5	10.3	17	26	26	26
Guinea pig	1.7	3.4	12.4	12.1	3.0	4.5	8.5	6.6
Frog (25°)	2.2	2.2	4.8	8.4	36	44	47	45
Pigeon	1.0	2.0	11.8	16.0	10.8	10.7	17.9	22.3
Rabbit	5.8	5.7	26.4	12.0	9.4	15.2	15.0	15.4
Rat	1.4	2.8	20.6	28.4	10.8	18.6	16.3	18.2

The initial values are lower than those given by Baker and Miller (2, 3) for rat liver and kidney. In our procedure the slices are washed in a large volume of Ringer's solution for at least 3 minutes before they are transferred to the reaction vessels or submitted to analysis. Preformed creatine undoubtedly diffuses out of the slices during this washing.

existing precursors in the liver slices of the guinea pig, pigeon, and rat. Kidney slices gave significant increases in every case except in the pigeon.

After the cell structure of rat liver and kidney was destroyed, no creatine was formed from preexisting precursors nor from glycocyamine without or with methionine. The homogenizing procedure of Potter and Elvehjem (5) was used to disrupt the cells. It would seem that the creatine formed by chopped rat liver and

kidney from preexisting precursors (Baker and Miller (3)) must be ascribed to the intact cells in the mash rather than to a liberated enzyme.

It is difficult to account for the large (absolute) amounts of creatine formed from preexisting precursors in the kidney slices in every case except in the pigeon, in view of the inability of cat, dog, rabbit, and rat kidney slices to methylate added glycocyamine, even in the presence of added methionine. The one animal which was negative in this respect—the pigeon—was the only animal whose kidney gave a positive effect with glycocyamine and a further increase with methionine.

When the Ringer's solution contained glycocyamine, there were large increases in creatine with the liver slices of every animal except the dog. In the interpretation of this negative effect in the case of the dog the positive effect observed when methionine was added with the glycocyamine must be taken into account. One possible interpretation is that the preexisting concentration of the glycocyamine-methylating agent is very low in dog liver—lower than in the livers of any of the other animals investigated.

With kidney slices the added glycocyamine was not methylated or was methylated only to an insignificant extent in every case except in the guinea pig and pigeon.

Bodansky (6) found an increase in the creatine content of rat kidney after glycocyamine was fed. Our interpretation of this increase was that it arose from temporary storage in the kidney of creatine synthesized in the liver from the glycocyamine fed (1). This interpretation is based on the finding that rat kidney slices do not methylate glycocyamine under conditions in which methylation readily occurs in liver. Bodansky concurs in this interpretation (personal communication).

Methionine accelerated the methylation of glycocyamine by liver slices of the dog, frog, pigeon, and rat. This stimulation was not observed with the cat, guinea pig, and rabbit. Two possible reasons for this failure are that the livers of these animals contained a plethora of the methylating agent, or that the relatively high concentration of the unnatural isomer in the *dl*-methionine used was inhibitory (as in the rabbit liver slices).

The pigeon was the only animal whose kidney slices were stimulated by methionine in their methylation of glycocyamine.

The pigeon is exceptional as far as creatine formation is concerned in that its kidney appears to share this function with its liver and resembles in this respect the livers rather than the kidneys of the other animals.

The negative effect of methionine with the kidney slices of all the animals tried except the pigeon is a little unexpected. Oxidative deamination is more active in kidney than in liver slices (7); and after oxidative deamination the lability of the S-methyl group is increased (8). It might have been expected therefore that the methylation of glycocyamine would be more active in kidney than in liver. This is not the case.

Our finding in this survey, that methylation of glycocyamine is an active function of the livers of all the animals examined, strengthens the conclusion that creatine is formed normally in the liver by the methylation of glycocyamine. It would be surprising if this is a fortuitous coincidence of little or no physiological significance. Other mechanisms and other sites of creatine formation are, of course, not excluded by these findings.

The significance of methionine here is less clear. Our findings suggest that it is the methylating agent of glycocyamine in liver or its precursor. We are hesitant at present to accept methionine itself as the methylating agent because its effect is small considering the concentration used. It may be that the actual methylating agent is the product of the oxidative deamination of methionine. This and other possibilities are now under investigation.

Du Vigneaud and coworkers (9) have shown that the presence of choline in the diet enables the animal to utilize homocystine or homocysteine in place of methionine. They have suggested that choline enables the body to methylate the sulfhydryl group of homocysteine by furnishing the required methyl group, and further that methionine may furnish the methyl groups of choline. The reality of the latter relationship is now established by direct proof recently presented by du Vigneaud.[1]

Our observations suggest that the S-methyl group of methionine may participate also in the formation of the N-methyl group of creatine. In this connection and in view of our findings with dog liver slices it is interesting that Stekol and Schmidt (10) found

[1] Verbal communication at the meeting of the American Society of Biological Chemists at New Orleans, March 13–16, 1940.

in the dog an increase in urinary creatinine after *dl*-methionine was fed, and none after glycine, glutamic acid, or *l*-pyrrolidonecarboxylic acid.

SUMMARY

1. A survey was made of the possibility of creatine formation *in vitro* by slices of the livers and kidneys of the cat, dog, guinea pig, frog, pigeon, rabbit, and rat.

2. Evidence of the existence of small amounts of creatine precursors in the livers of the guinea pig, pigeon, and rat, and in the kidneys of all of the above animals except the pigeon was found.

3. This finding appears to be much less significant quantitatively than the much more active glycocyamine-methylating function of the liver slices of all animals investigated. Only guinea pig and pigeon kidney slices possessed this ability.

4. The above findings are interpreted as indicating that creatine normally is formed by the methylation of glycocyamine in the liver.

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A MICROMETHOD FOR THE DETERMINATION OF GLYCOCYAMINE IN BIOLOGICAL FLUIDS AND TISSUE EXTRACTS

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In the two following communications (1, 2) evidence is presented that glycocyamine is a normal precursor of creatine in the animal body. These studies required a satisfactory micromethod for the determination of glycocyamine. The most reliable method described in the literature consists in adsorption on Lloyd's reagent in acid solution, elution with baryta, removal of arginine from the eluate by repeated adsorption on permutit, and colorimetric determination of the remaining glycocyamine by means of the Sakaguchi reaction. There are only two substances which are common in biological fluids and which give an intense color in the Sakaguchi reaction. These are arginine and glycocyamine. This method was first introduced by Weber (3) and was modified by Bodansky (4) and by Davenport and Fisher (5).

In our hands even the latest version of the method, that described by Davenport and Fisher, had the following shortcomings: it was laborious and time-consuming, the adsorption of the glycocyamine on the Lloyd's reagent was incomplete, further losses of glycocyamine occurred in the repeated treatment with permutit (Davenport and Fisher report losing only 10 per cent in three adsorptions; with the permutit available to us we lost 80 per cent), and the color developed was unstable. Furthermore, the amount of glycocyamine lost on the permutit varied according to the amount of arginine present, the less arginine the greater the loss of glycocyamine.

All these disadvantages have been removed in the method described below. It is the first method in which glycocyamine

added to blood or urine can be determined quantitatively, even in concentrations as low as 0.1 mg. per cent. 2 to 5 ml. are sufficient for an analysis. An indication of the speed and convenience of the method is that twenty to forty analyses can be carried through simultaneously in about 2 hours.

Reagents—

0.02 M sodium-potassium phosphate solution at pH 7.0 (Sørensen buffer); or 0.3 per cent sodium chloride solution.

Permutit.

A stock solution of 0.2 per cent α-naphthol in absolute alcohol, diluted with water to 0.04 per cent before use.

40 per cent solution of urea in water.

Hypobromite solution made as follows: 0.66 ml. of liquid bromine (specific gravity 3.0) in 100 ml. of 5 per cent sodium hydroxide.

Procedure

Urine is diluted 5 to 10 times, according to its concentration. Blood may be deproteinized either by adjusting the pH to 6.0, 5-fold dilution with water, boiling and filtration, or by precipitation of the proteins with sodium tungstate and sulfuric acid according to the procedure of Folin and Wu, with the modification that for each ml. of blood, 2 ml. of water and 1 ml. each of the 10 per cent sodium tungstate and 0.66 N sulfuric acid are used. Tissue extracts are diluted with water until the final solution contains 1 gm. of tissue (fresh weight) in 40 ml. of suspension. The pH is adjusted to 6.0, and the suspension immersed in a boiling water bath for 10 minutes, cooled, and filtered. The analysis is carried out on the deproteinized filtrate.

Any arginine which may be present is removed by sending the solution through a column of permutit contained in the stem of a funnel made of two pieces of glass tubing. The dimensions are as follows: upper piece 100 mm. long, 15 mm. external diameter; lower piece 100 mm. long, 7 mm. external diameter (5 mm. internal diameter). The bottom end of the funnel is constricted slightly.

The dimensions of the lower narrow tubing should be as close as possible to those given above. If the tubing is narrower, the filtration will be slow; if it is much wider the reduced height of the

column of permutit may allow some arginine to escape adsorption. The lower end of the funnel is stoppered loosely with a small piece of absorbent cotton just sufficient to hold back the permutit. 0.9 gm. of permutit is poured in and tapped gently to remove entrapped air. It should form a column about 85 mm. tall. A test-tube graduated at 10 ml. is placed under the end of the funnel.

5 ml. of the solution to be analyzed, obtained as described above, are pipetted into the funnel. After all of this solution has passed below the top of the permutit column, 5 ml. of either the 0.02 M phosphate buffer or of the 0.3 per cent sodium chloride solution are pipetted in above the permutit. Each 5 ml. portion of solution takes about 15 minutes to pass through the column. When the second 5 ml. portion has passed through, the filtrate in the graduated test-tube below is made up to the 10 ml. mark with water and then shaken.

Washing the permutit column with the phosphate or sodium chloride solution removes the small amount of glycocyamine which remains in the permutit column after the 5 ml. of the solution under analysis have passed through. The dilute phosphate buffer and the sodium chloride solution are equally effective. We prefer the sodium chloride solution, because it is simpler to make up and also because a faint turbidity is sometimes obtained in the final filtrate when the phosphate solution is used.

Different batches of permutit may differ in their affinity for arginine and glycocyamine. We have mentioned above that Davenport and Fisher lost only 10 per cent of the glycocyamine present in three successive treatments with fresh permutit, whereas we lost 80 per cent. It may be advisable therefore before a fresh batch of permutit is used in the procedure described to ascertain the height of the column and amount of permutit necessary to remove all the arginine without loss of glycocyamine. The prescription we have given allows some latitude and it may cover all the variations in permutit now sold in this country.

The permutit can be regenerated after it has been used by the following procedure. It is washed several times with tap water, thrown on a Buchner funnel, an equal volume of 25 per cent sodium chloride solution passed through, and then washed with distilled water until the wash water is chloride-free.

The development of the color by means of the Sakaguchi reaction

and the colorimetry may be carried out with any convenient aliquot so long as the proportions of the reagents are kept the same. The quantities of reagents given below are for 2 ml. aliquots which we have been using. Twenty to forty analyses can be carried through simultaneously. Test-tubes containing the aliquots of the solutions to be analyzed, inserted in a rack, are

TABLE I

Determination of Glycocyamine in Aqueous Solutions Containing Different Amounts of Interfering Substances

All values are concentrations measured in mg. per cent.

The concentrations of the added substances were those in the final mixtures before they were sent through the permutit columns. The final filtrates contained only half the concentrations of glycocyamine shown. Accordingly the standards were diluted 1:1 with 0.02 M phosphate buffer solution.

Arginine added	Creatine added	Glycocyamine added	Glycocyamine found by analysis
35	10	3.0	3.02
35	5	3.0	3.01
35	1	3.0	3.01
35	0	3.0	3.00
35	10	1.0	1.01
35	5	1.0	1.02
35	1	1.0	1.00
35	0	1.0	1.00
35	10	0.1	0.10
35	5	0.1	0.10
35	1	0.1	0.10
35	0	0.1	0.10
70	25	0.1	0.10
35	25	0.1	0.10
18.5	25	0.1	0.09
0	25	0.1	0.09

placed in an ice water bath. In this bath also are the α-naphthol (0.04 per cent), urea, and hypobromite solutions. After 5 to 10 minutes 0.4 ml. of the α-naphthol solution is added to each tube, the contents shaken, 0.4 ml. of the chilled urea solution added, and the contents shaken again. After another 5 minute interval 0.2 ml. of the chilled hypobromite solution is added, the contents immediately mixed by vigorous shaking, and the tube replaced in the ice bath.

The color develops slowly, attaining its maximum intensity in about 20 minutes, after which (so long as the solution is in the ice bath) it remains constant for at least 1 hour. Before the color is measured, the solution is warmed to room temperature by shaking in water for ½ minute and the dissolved gas removed by vigorous tapping for 20 seconds. If the colored solution is left at room temperature for ½ hour or more, the color slowly fades.

TABLE II

Determination of Glycocyamine in Ringer's Solution and in Rat Heart and Kidney Extracts to Which Different Quantities of Arginine and Glycocyamine Were Added

All values are concentrations measured in mg. per cent.

The concentrations of added glycocyamine and the experimental values given were those in the final filtrates; *i.e.*, after they had passed through the permutit and were diluted with an equal volume of washing solution. The concentrations of added arginine were those before the solutions had passed through the permutit.

Arginine added	Glycocyamine added	Glycocyamine					
		Ringer's solution		Kidney extract		Heart extract	
		Found	After subtracting blank value	Found	After subtracting blank value	Found	After subtracting blank value
0	0			0.04	0	0.08	0
35	0	0	0	0.04	0	0.08	0
35	1.50	1.53	1.53	1.59	1.55	1.56	1.48
0	0.75	0.765	0.765	0.79	0.75	0.85	0.77
35	0.75	0.80	0.80	0.82	0.78	0.84	0.76
35	0.375	0.41	0.41	0.44	0.40	0.45	0.37
35	0.075	0.08	0.08	0.11	0.07	0.15	0.07

With each group of analyses the color is developed simultaneously in four standard solutions containing 0, 0.25, 0.5, and 1.0 mg. per cent of glycocyamine.

To measure the color we have used a spectrophotometer or a colorimeter. With either instrument the best light is that at approximately 0.525 μ. There is a linear relationship between the concentration of glycocyamine up to 1 mg. per cent and the intensity of the color when it is measured with light near to 0.525 μ.

Tables I to IV indicate the sensitivity and reliability of the method described above. Table I gives some typical results

with prepared mixtures, in aqueous solution, containing varying amounts of glycocyamine and of two interfering substances, arginine and creatine.

Table II summarizes some tests of the method with Ringer's solution and two tissue extracts. These tests were necessary, even after the results in Table I were obtained, because the equilibrium relation between the glycocyamine adsorbed on permutit and that remaining in solution is affected by the concentration of

TABLE III

Determination of Added Glycocyamine and of Added Arginine in Human Urine (Diluted 5-Fold with Water)

All values are concentrations measured in mg. per cent.

The concentrations of added glycocyamine and the experimental values found were those in the filtrates; *i.e.*, after they had passed through the permutit and were diluted with an equal volume of washing solution. The concentrations of added arginine were those before the solutions had passed through the permutit.

Glycocyamine added	Arginine added	Glycocyamine	
		Found	After subtracting amount originally present
0	0	0.21	0
0	22	0.21	0
1.0	0	1.26	1.05
0.5	0	0.72	0.51
0.5	30	0.71	0.50
0.5	22	0.70	0.49
0.5	10	0.69	0.48
0.25	0	0.46	0.25

salt. The reliability of most of the experimental results described in the next communication (1) depends on the accuracy of this analytical method. The data in Table II show that the agreement between theoretical and experimental values is satisfactory.

The determination of glycocyamine in urine is one of the most severe tests to which an analytical method for glycocyamine can be subjected. Urine contains relatively high concentrations of interfering substances such as urea, ammonia, creatinine, and amino acids including arginine. The rigor of the test was in-

creased by adding to the urine varying amounts of arginine and glycocyamine. Table III shows that the same satisfactory agreement is obtained between theoretical and experimental values as in simple aqueous solutions. This is also the case with blood (Table IV).

TABLE IV

Determination of Glycocyamine Added to Human Blood

All values are given in mg. per cent.

Varying amounts of a 10 mg. per cent glycocyamine solution and of a 20 mg. per cent arginine solution were added to 4 ml. aliquots of whole human blood. To these mixtures water was added so that the final dilution of the blood was the same in each case; *i.e.*, 4 ml. of blood and 8 ml. of water or of the combined glycocyamine and arginine solutions. 4 ml. of 10 per cent sodium tungstate followed by 4 ml. of 0.66 N H_2SO_4 were then added to each mixture. The analyses were carried out on the filtrates. The concentrations of glycocyamine and arginine in these filtrates were further reduced by one-half in the analytical procedure.

Concentration ÷ 2 in Folin-Wu filtrate		Concentration of glycocyamine in Folin-Wu filtrate	
Glycocyamine added	Arginine added	Found	After subtracting amount originally present
1.0	0	0.99	0.95
0.5	0	0.55	0.50
0.2	0	0.24	0.19
0.1	0	0.15	0.10
0	0	0.05	0
0.5	2.0	0.55	0.50
0.5	1.5	0.55	0.50
0.5	1.0	0.54	0.49
0.5	0.5	0.54	0.49
0	2.0	0.05	0

SUMMARY

A micromethod is described for the determination of glycocyamine in biological fluids and tissue extracts. The advantages of this method over those previously described are that added glycocyamine is recovered quantitatively; it is faster and more convenient.

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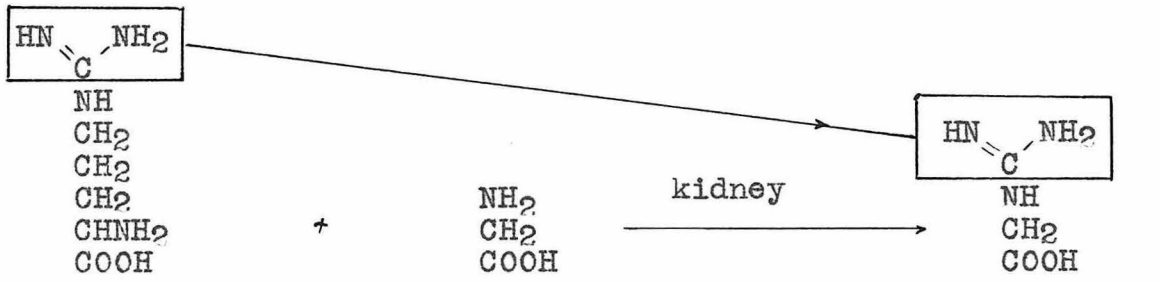

Arginine Glycine Glycocyamine

The F ormation of Glycocyamine

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THE FORMATION OF GLYCOCYAMINE IN ANIMAL TISSUES*

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It was shown in preceding communications that glycocyamine is converted into creatine by surviving liver slices (1). Our findings indicated that the methylating agent is methionine or a derivative of methionine. Liver slices can methylate glycocyamine rapidly enough to permit assignment to the liver alone, if necessary, of the task of making good the loss of creatine and creatinine in the urine. This holds for the livers of all mammals studied. We found no evidence of this methylating mechanism in any other tissues, except possibly slight activity in the kidney. In the pigeon the kidney is as effective in this respect as the liver.

These experimental facts were corroborated (as far as the rat is concerned) by experiments on living animals with tracers. Bloch and Schoenheimer, using N^{15}, found that glycocyamine is readily converted to creatine (2). Du Vigneaud and his collaborators fed rats methionine with deuterium in the methyl group; after only 3 days a relatively large quantity of deuterium was found in the muscle creatine (3).

Glycocyamine has had a favored position among the possible precursors of creatine. It is nearer to creatine structurally than any other precursor which has been proposed; and its convertibility to creatine in the living organism has been proved. The case against glycocyamine rested on two arguments: (1) that only a small fraction, 5 to 15 per cent, of administered glycocyamine is methylated, and (2) that glycocyamine had not been found as a normal constituent of animal tissues. It has, however, been isolated by Weber from human and dog urine (4).

* A summary of this work has appeared (*Science*, **91**, 551 (1940)).

The findings with surviving liver slices and the tracer studies reinstated glycocyamine, more firmly than before, as a possible normal precursor of creatine. The quantity of creatine synthesized daily is so large that, if glycocyamine is its normal precursor, an active mechanism for the formation of glycocyamine must exist. We undertook a search for this mechanism.

The first necessity was an adequate analytical method. Such a method is described in the preceding communication (5).

With it we have found that kidney slices rapidly form glycocyamine from arginine and glycine. All other tissues tested are negative in this respect.

This interaction of arginine and glycine is also catalyzed by thoroughly macerated cell-free kidney tissue suspended in a phosphate buffer solution.

The formation of glycocyamine from arginine and glycine is a new biochemical reaction which may be called "transamidination." We propose that the enzyme be designated "glycine-transamidinase." The discovery of this reaction provides direct proof that arginine and glycine are precursors of creatine.

Bloch and Schoenheimer fed ammonia containing N^{15} to rats and later found the isotope in the amidine nitrogen of creatine. After glycine containing N^{15} was fed, the isotope was found in creatine in the sarcosine nitrogen. In a later communication the same authors presented more direct evidence (again obtained by the use of N^{15}) which confirmed the findings we had reported that glycocyamine is formed by the transfer of the amidine group from arginine to glycine. They fed $l(+)$-arginine with N^{15} in the amidine group to rats (6); afterwards the creatine in the muscles had a far higher isotopic content than after the administration of isotopic ammonia, urea, or any other amino acid except glycine. It was so high that they considered that the amidine group of creatine must have originated from arginine.

The present communication contains the details of our experimental procedure, our findings on the effects of a fairly large number of amino acids and derivatives of arginine and of glycine, surveys of the capacity for glycine transamidination of the organs of a number of common experimental animals, and the results of some studies of the effect of concentration of reactants, pH, and time on the rate of transamidination.

Technique and Results

The tissue slice technique used and the details of the reaction vessels are described in a previous communication (7).

At the end of an experiment the contents of the reaction vessels were transferred with the slices to test-tubes graduated at 20 ml.; the vessels were washed with three 2 ml. portions of 0.02 M phosphate buffer solution at pH 6.0 and the washings added to the main solution. The pH was adjusted finally to 6 with a drop of 0.5 N hydrochloric acid. The test-tubes containing the slices, the main solutions, and washings were kept in a boiling water bath for 10 minutes, after which they were cooled to room temperature, and made up to the 20 ml. mark with water and mixed by shaking. These solutions were then filtered. 5 ml. of the clear protein-free filtrate were analyzed for glycocyamine by the procedure described in the preceding communication (5).

The coagulated slices and protein in each test-tube were transferred to small glass weighing dishes, heated overnight at 105°, and, after cooling in a desiccator, were weighed.

Table I is the detailed protocol of a typical experiment. A significant amount of glycocyamine is formed when arginine alone is added to the Ringer's solution. Glycine without arginine also leads to a slight increase in glycocyamine. When both amino acids are added together, the increase in glycocyamine is more than 10 times the increase with arginine alone. The increases obtained with arginine alone and with glycine alone indicate either the presence of these amino acids in the free state (more glycine than arginine) or their formation in small amounts by autolysis within the slices.

In the experiment whose results are recorded in Table I, and in a number of others, creatine analyses were carried out with the specific bacterial (NC) enzyme of Dubos and Miller (8, 9). No evidence of creatine formation was found.

The steps in the proof that the substance we were measuring was glycocyamine were as follows: Autoclaving in acid solution produced a substance which was adsorbed by Lloyd's reagent and gave a positive test with the Jaffe reagent. This chromogenic material was not digested (before autoclaving) by the NC bacteria of Dubos and Miller under conditions in which creatine and creatinine were completely digested. A strongly positive Saka-

guchi test was obtained in the unautoclaved solution after all the arginine was removed by exhaustive adsorption on permutit. The depth of color which the unknown solution gave with the Jaffe reagent (after autoclaving) corresponded, assuming it to be glycocyamine, to the intensity of color it gave in the Sakaguchi reaction after removal of the arginine. A liter of solution was

TABLE I

Protocol of Typical Experiment Showing Formation of Glycocyamine from Arginine and Glycine by Rat Kidney Slices

Ringer's solution, 38°, 4 hours.

Dry weight of slices (1)	Ringer's solution (2)	0.02 M arginine in Ringer's solution (3)	0.04 M glycine in Ringer's solution (4)	Concentration of glycocyamine in aliquot taken for analysis (5)	Glycocyamine found per 100 gm. fresh tissue (6)
mg.	*ml.*	*ml.*	*ml.*	*mg. per cent*	*mg.*
26.4	4			0.02	6
30.4	4			0.04	11
22.5	3	1		0.10	36
21.0	3	1		0.11	42
20.0	3		1	0.04	16
22.0	3		1	0.05	18
23.6	2	1	1	1.03	349
21.4	2	1	1	0.97	363

The figures in Column 6 are obtained by multiplying those in Column 5 by 8000 and dividing them by the dry weight of the tissue in mg. (Column 1). The figure 8000 is obtained as follows: the solution is diluted 5-fold before analysis; in the course of analysis it undergoes a further 1:1 dilution; the results in Column 5 expressed as mg. per cent must be divided by 25, since there were only 4 ml. of the original reaction solution; to express the results on the basis of 100 gm. of fresh tissue, the factor $100{,}000/5 \times W$ is used, W being the dry weight in mg. given in Column 1. The factor therefore is $5 \times 2 \times 1/25 \times 100{,}000/(5 \times W) = 8000/W$.

now collected in which kidney slices had acted upon arginine and glycine, and which contained, according to analysis, about 50 mg. of glycocyamine. The glycocyamine was isolated by adsorption on Lloyd's reagent in acid solution, elution with baryta, removal of the arginine by repeated adsorption with permutit, and crystallization from glacial acetic acid as glycocyamine acetate. These crystals were the characteristic needles and thin prisms (10).

The free glycocyamine was regenerated from the acetate by boiling in dilute aqueous solution and crystallized by evaporation of the water. 25 mg. of crude glycocyamine were thus obtained. It was thrice recrystallized from water, with a final yield of 11 mg. of the pure dry material which was analyzed. It gave the following figures.[1]

Observed.	C 30.8, H 5.95, N 35.8
Theoretical for glycocyamine.	" 30.8, " 6.0, " 35.9

TABLE II

Formation of Glycocyamine by Rat Kidney Slices from Arginine and Glycine or Glycine Derivatives

Glucose-Ringer's solution, 3 hours, 38°. Concentration of arginine 0.005 M; glycine or derivatives 0.01 M.

Arginine	Glycine or glycine derivative	Glycocyamine found per 100 gm. fresh tissue
		mg.
−		8
+		35
−	Glycine	19
+	Betaine	44
+	Glutathione	296
+	Glycine	382
+	" anhydride	8
+	Glycylglycine	436
+	Glycolic acid + ammonia	6
+	Hippuric acid	35
+	Leucylglycine	254
+	Sarcosine	109
+	" anhydride	6

Table II summarizes the relative effectiveness of glycine and some glycine derivatives as precursors of glycocyamine. The effect of the glycine peptides is accounted for on the hypothesis that these are first hydrolyzed and that it is the free glycine which reacts with arginine to form glycocyamine. The argument is as follows: The rate of glycocyamine formation is proportional to the concentration of free glycine (Table VIII). When glycine

[1] We are indebted to and wish to thank Dr. A. J. Haagen-Smit for these analyses.

peptides were the source of the glycine, the concentration of free glycine was initially 0 and only in the course of the 3 hour experimental period did it approximate 0.01 M, whereas when glycine itself was added the initial concentration was 0.01 M. Hence smaller amounts of glycocyamine were formed from glutathione, leucylglycine, and hippuric acid than from the same initial concentration of glycine. Glycylglycine gave a higher value than glycine, because on hydrolysis it yields 2 molecules of glycine and as a result the concentration of glycine rose well above 0.01 M before the end of the 3 hour period.

It follows, if the above is the correct explanation of the effect of glycine peptides, that rat kidney contains a dipeptidase for leucylglycine, an enzyme which liberates glycine from glutathione, and no enzymes capable of forming free glycine at a significant rate from betaine, glycine anhydride, or hippuric acid.

The results with sarcosine indicate that this substance is demethylated without deamination in rat kidney. Separate analyses showed that no creatine was formed; this proved that demethylation of the sarcosine had occurred prior to the transamidination. The kidney contains, therefore, an enzyme which demethylates sarcosine.

These findings with sarcosine are complemented by those of Bloch and Schoenheimer (2, 11) who, using N^{15} as a tracer, found that sarcosine is converted to glycine *in vivo* and that in the course of the demethylation the glycine nitrogen originally attached to the carbon chain is not replaced. Their experiments therefore excluded intermediate deamination of sarcosine in the course of its conversion to glycine.

Analogous to its inability to hydrolyze glycine anhydride the kidney is unable to hydrolyze sarcosine anhydride.

The negative result shown in Table II with glycolic acid and ammonia indicates that rat kidney is unable to form glycine from these two substances at a significant rate.

The experiments summarized in Table III revealed that rat kidney evidently is able to synthesize arginine from citrulline. The other possible amidine donators which were tested, guanidine, ornithine, and urea, were negative.

The positive result with citrulline was retested in a number of more adequately controlled experiments. A group of typical

TABLE III

Formation of Glycocyamine by Rat Kidney Slices from Glycine and Arginine or Other Possible Donators of Amidine Group

Glucose-Ringer's solution, 3 hours, 38°. Concentration of glycine 0.01 M; of arginine or other amidine donators 0.005 M.

Glycine	Amidine donator	Glycocyamine formed per 100 gm. fresh tissue
		mg.
−		35
−	*l*(+)-Arginine	68
+	"	498
+	*l*(+)-Citrulline + ammonia	330
+	Guanidine	16
+	*d*(−)-Ornithine + ammonia	12
+	Urea	33

TABLE IV

Formation of Glycocyamine from Glycine and Citrulline by Rat Kidney Slices and by Cell-Free Macerate of Rat Kidney

Slices in glucose-Ringer's solution, macerate in 0.1 M phosphate buffer, pH 7.0, 3 hours, 38°. Glycine 0.01 M; amidine donator (ammonia, arginine, citrulline, or ornithine) 0.005 M.

	Glycine	Amidine donator	Glycocyamine formed per 100 gm. fresh tissue
			mg.
Kidney slices	−		22
	+		31
	−	Ammonia	18
	−	Arginine	68
	−	Citrulline	58
	+	Arginine	357
	+	Citrulline	200
	+	" + ammonia	227
	+	Ornithine + "	8
Cell-free macerate	−		30
	+	Arginine	120
	+	Citrulline + ammonia	59

results is shown in Table IV. Citrulline was only slightly less effective than citrulline plus ammonia.

Included in Table IV are some typical results obtained with a

cell-free macerate of kidney. This suspension was less active than an equivalent amount of kidney tissue in the form of slices but qualitatively the results were in every respect the same.

The suspension was made by the homogenizing procedure of Potter and Elvehjem (12). The kidney was stripped of its capsule, split down the longitudinal axis, the pelvic fat and the medulla cut away, the remainder homogenized with 4 times its weight of 0.01 M phosphate buffer at pH 7.0, and the resulting suspension passed through gauze. Such a suspension retains all its transamidinase activity for at least 2 months if kept in an ice box.

The optimum pH of glycine-transamidinase is in the neighborhood of pH 7.0 (Table V).

TABLE V

Effect of pH on Activity of Glycine-Transamidinase

38°, 4 hours. Glycine 0.01 M; arginine 0.005 M.

pH	Glycocyamine formed per 100 gm. fresh tissue
	mg.
6.0	214
6.5	259
7.0	320
7.5	278
8.0	246

The activity of glycine-transamidinase in cell-free solution is unaffected by 0.001 M potassium cyanide or by carrying out the reaction *in vacuo*. For example, one extract under the same conditions as those described above formed 67 mg. of glycocyamine per 100 gm. of fresh tissue; in the presence of 0.001 M potassium cyanide it formed 80 mg. and anaerobically, 72 mg.

The following amino acids and amides were tested with rat kidney slices and arginine as possible precursors of the glycine radical in glycocyamine. The initial concentration in every case was 0.005 M. They were all negative: *d*-alanine, *dl*-alanine, *l*-asparagine, *l*-aspartic acid, *l*-cysteine, *l*-cystine, *d*-glutamic acid, *d*-glutamine, *l*-hydroxyproline, *l*-histidine, *dl*-isoleucine, *l*-leucine, *d*-lysine, *dl*-methionine, *dl*-norleucine, *l*-proline, *dl*-phenylalanine, *dl*-serine, *d*-threonine, *l*-tryptophane, *l*-tyrosine,

and *d*-valine. These negative results indicate that under the conditions of these experiments none of these amino acids is a precursor of glycine.

Glycine-transamidinase activity was found in the kidney of every animal tested except the frog (Table VI). Whenever activity was found in kidney slices, it was also found in the cell-free extract. The beef and sheep kidneys were used at least 24 hours after the animals were slaughtered; they were obtained in a butcher shop. In the cases of all the other animals the kidneys were removed immediately after the animals were killed and the extracts made soon afterwards. The negative results with extracts of frog kidney call for further study.

TABLE VI

Formation of Glycocyamine from Glycine and Arginine by Kidney Slices and by Cell-Free Macerate of Kidney of Various Animals

Slices in glucose-Ringer's solution, macerate in 0.1 M phosphate buffer, pH 7.0. Glycine 0.01 M; arginine 0.005 M. 38°, 3 hours.

Animal	Glycocyamine formed per 100 gm. fresh tissue by	
	Kidney slices	Cell-free macerate
	mg.	*mg.*
Beef		190
Cat	93	32
Dog	281	480
Frog		0
Guinea pig	38	14
Pigeon	27	16
Rabbit	187	160
Rat	357	120
Sheep		160

Liver slices and cell-free extracts of heart and of muscle of all the animals listed in Table VI were tested for glycine-transamidinase activity. Except in the case of the pigeon (see below) they were all negative. The blood, brain, intestine, and spleen of the rat were also examined; they were negative. Rat liver slices also gave negative results with glycine plus arginine, urea, or guanidine.

Before the advent of Weber's method no glycocyamine could be demonstrated in animal tissues. With this method glyco-

cyamine was detected in urine (4), in intestine, testes, and kidney (13).

Using our more sensitive method, we found glycocyamine to be widely distributed in the tissues of the rat. The concentrations (mg. per 100 gm. of fresh tissue) were blood 0.5 to 1; brain, heart, liver, skeletal muscle, and spleen 3 to 6; small intestine 10; kidney (cortex) 15 to 30.

The question arose whether transamidination in the kidney can be sufficiently rapid under physiological conditions to account for the total production of creatine in the body as indicated by the

TABLE VII

Rate of Glycocyamine Formation by Rat Kidney Slices at 38° from Arginine Initially 0.005 M and Glycine 0.01 M

Time	Glycocyamine per 100 gm. fresh tissue		
	Found	Formed	Average rate of formation per hr.
hrs.	*mg.*	*mg.*	*mg.*
0	32		
0.5	95	63	126
1.0	164	132	132
2.0	315	283	142
4.0	505	473	118
6.0	741	709	118

daily excretion in the urine of creatine and creatinine. A number of experiments were carried out to obtain what information we could on this point.

Table VII shows that the glycine-transamidinase in rat kidney slices remained practically unimpaired for 6 hours. This is in accord with the stability of the enzyme in cell-free extracts.

In Table VIII are some figures on the effect of the concentration of the reactants, arginine and glycine, on the rate of transamidination. With equal arginine and glycine concentrations from 0.001 to 0.0001 M the rate was approximately linear with respect to concentration.

We can estimate what the rate of glycocyamine formation in the kidney must be to make good the loss of tissue creatine which appears in the urine as creatinine. Two human kidneys

weigh approximately 300 gm. An average figure for the creatinine excreted in the urine in 24 hours is 1.7 gm. To make good this loss the average hourly production of glycocyamine which is necessary must be approximately 25 mg. per 100 gm. of tissue per hour.

This rate of glycocyamine formation would have been obtained in the experiments of Table VIII with concentrations of glycine and arginine between 0.0005 and 0.001 M. This is probably the concentration range of these amino acids in kidney. The basis of this estimate is as follows: The arginine concentration in dog

TABLE VIII

Variation in Rate of Glycocyamine Formation by Rat Kidney Slices with Different Concentrations of Arginine and Glycine

Ringer's solution, 4 hours, 38°.

Initial concentration		Glycocyamine formed per 100 gm. fresh tissue	Initial concentration		Glycocyamine formed per 100 gm. fresh tissue	Initial concentration		Glycocyamine formed per 100 gm. fresh tissue
Arginine	Glycine		Arginine	Glycine		Arginine	Glycine	
mole per l.	*mole per l.*	*mg.*	*mole per l.*	*mole per l.*	*mg.*	*mole per l.*	*mole per l.*	*mg.*
0.02	0.01	190	0.005	0.02	617	0.005	0.005	360
0.01	0.01	590	0.005	0.01	533	0.0025	0.0025	310
0.005	0.01	533	0.005	0.005	361	0.001	0.001	134
0.0025	0.01	535	0.005	0.0025	252	0.0005	0.0005	78
0.00125	0.01	303	0.005	0.00125	201	0.0001	0.0001	20
0.000625	0.01	201	0.005	0.000625	103			

blood is between 2.6 and 3.9 mg. per cent (14); we have found 2.0 to 5.0 mg. per cent in human blood. This range corresponds to 0.0001 to 0.00025 M. The concentrations of these amino acids in the kidney are certainly much higher than in blood, since the total free amino nitrogen in kidney and other tissues is 10 times or more that in blood (15, 16). The rates of glycocyamine formation recorded in Table VIII are sufficient therefore to make good the total loss of creatine from the tissues. This estimate is, of course, based on the assumption that the rate of glycocyamine formation in human kidney *in vivo* is of the same order as in rat kidney slices *in vitro*, which seems not unreasonable.

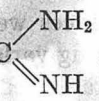
DISCUSSION

The experimental results given above, confirmed and complemented as they now are by the findings in studies with tracers, make a strong case for the following mechanism of creatine formation in animals: (*a*) arginine and glycine in the kidney form glycocyamine; (*b*) in the liver glycocyamine and methionine form creatine. The mechanisms for these reactions are widely distributed in mammals. The quantitative aspects of the data show that the transamidination mechanism for the formation of glycocyamine is fast enough to replace all the creatine lost from the body. Other mechanisms of creatine formation are not excluded; but the tracer studies indicate that the arginine-glycine-methionine mechanism is quantitatively the most important one *in vivo*.

The argument *contra* glycocyamine, that it had never been found as a constituent of animal tissues, antedated the development of suitable analytical methods. This argument is now completely disposed of by the detection of glycocyamine in practically all tissues.

The controversy whether arginine is a precursor of creatine (17, 18) may be considered as settled in favor of the affirmative. The path of its conversion to creatine, however, is different from what was generally believed. It was proposed that arginine was converted to glycocyamine by way of deamination and β oxidation; thus all of the nitrogen was derived directly from arginine. This made it necessary to explain away the evidence which suggested that glycine played some part in creatine formation, unless it be in the methylation of glycocyamine.

Bergmann and Zervas were the exception. They observed that "triacetyl anhydro arginine" and glycine ethyl ester reacted in the absence of water to give a fairly good yield of diacetyl glycocyamine ethyl ester (19); and the same arginine derivative with sarcosine ethyl ester gave diacetyl creatine ethyl ester (20). Their view was that, "der wesentliche Punkt der biologischen Kreatinbildung in einer direkten Umsetzung eines reaktionslustigen Argininabkömmlings mit einem Aminoäthanderivat beruht." This clear statement that the guanidine group of creatine arises *in vivo* by transamidination needs now to be modified in two respects: that an enzyme, glycine-transamidinase, and not a

reactive derivative or split-product renders the  group in arginine labile as it is in "triacetyl anhydro arginine." The enzyme arginase exerts a similar influence. The other modification is that glycine itself and not a derivative is the acceptor of the amidine group in the biological transamidination reaction.

We may now infer that one of the reasons for the essential character of arginine and methionine in the rat is that they participate in the formation of creatine. These amino acids, of course, also serve other functions, for example the rôle of arginine in urea formation and of methionine in other methylation reactions (3), in addition to their participation in the constitution of tissue protein.

The recent findings on essential amino acids for the chick indicate that arginine and glycine are required for creatine formation in this animal. Arnold *et al.* (21) reported that arginine is essential for rapid growth. This was confirmed by Klose *et al.* (22) who showed further that arginine is necessary for maintenance as well as growth. Recently Klose and Almquist reported that citrulline is as effective as arginine, whereas ornithine alone or with urea is ineffective (23). We have found (Tables III and IV) that glycocyamine is formed from citrulline and glycine, the citrulline presumably being first converted to arginine, while ornithine, with or without added ammonia, is completely negative.

The parallel between the amino acid requirements for creatine formation and for growth was extended further when Almquist *et al.* found that glycine is essential for the growth of the chick (24) and that creatine as a substitute for glycine is even more effective than glycine itself. Glycolic acid and betaine could not replace creatine (25).

We have examined the organs of the pigeon for transamidinase activity. Activity was found not only in the kidney but also in heart, liver, and skeletal muscle. The limiting amino acid appeared to be glycine; *i.e.*, nearly as much glycocyamine was formed when glycine alone was added as from glycine and arginine together. The differences between experimental and control were, however, small in absolute terms compared with those found in the kidneys of other animals. Although these

differences were greater than could be ascribed to analytical or sampling variations, we cannot consider our findings in the pigeon as established until more determinations have been made. For this reason the detailed figures are not presented here. We hope that other workers with facilities for a study such as this on birds may undertake the investigation. Our laboratory does not at present possess such facilities.

SUMMARY

1. Beef, cat, dog, guinea pig, pigeon, rabbit, rat, and sheep kidney form glycocyamine from arginine and glycine. This reaction is catalyzed by cell-free extracts of kidney as well as by surviving kidney slices.

2. It is proposed that this reaction be designated "transamidination," and the enzyme "glycine-transamidinase." The optimum pH of this enzyme is about 7.0. It is not affected by potassium cyanide nor by anaerobiosis.

3. Transamidination does not occur in the liver, heart, or skeletal muscle of the animals mentioned above; the blood, brain, and spleen of the rat were tested also and found inactive. It is possible that in the pigeon a low glycine-transamidinase activity resides in liver, heart, and skeletal muscle as well as in kidney.

4. Glycocyamine is also formed in the kidney from glycine and citrulline. Glycine plus ornithine (with or without ammonia), urea, or guanidine is negative in this respect.

5. A large number of amino acids, several amides, and anhydrides were tested as possible precursors of the glycine radical of glycocyamine. They were all negative, as was also glycolic acid plus ammonia.

6. Glycocyamine is formed from arginine and sarcosine. Evidence is presented that the sarcosine is first demethylated, thus being converted to glycine, indicating the presence of a demethylating enzyme in kidney. Sarcosine anhydride is negative.

7. The above findings, complemented by the tracer studies in the laboratories of Schoenheimer and of du Vigneaud, and in conjunction with our previous findings, prove the existence of the following mechanism of creatine formation in animals: arginine and glycine form glycocyamine in the kidney; the glycocyamine is methylated in the liver by methionine (or a derivative of methionine) to form creatine.

8. Quantitative aspects of the data indicate that all of the creatine formed in animals may normally be formed by this mechanism.

9. Evidence of the generality of transamidination is seen in the close parallel between the above findings and those on amino acids essential for the growth of the chick.

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THE FORMATION OF GLYCOCYAMINE IN MAN AND ITS URINARY EXCRETION

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Glycocyamine was first isolated from human and dog urine and identified by Weber (1–3). He supported the view that glycocyamine is a normal precursor of creatine and that its appearance in urine (2) is "an overflow phenomenon of an intermediate metabolic product . . ." He expressed no views on the mechanism of its formation.

Glycocyamine is formed by the interaction of arginine and glycine in the kidney (and probably only in that organ) of the cat, dog, guinea pig, rabbit, and rat (4). In order to ascertain whether this reaction occurs in man we have studied the effect of arginine and glycine, ingested separately and together, on the glycocyamine content of urine and blood.

The subjects were seven normal men. The methods used for the determination of glycocyamine in urine and in blood have been described (5).

The first experiments consisted in comparing the urinary excretion of glycocyamine during 24, later only 12, hours in which no protein was ingested with that on a day in which gelatin was ingested. Gelatin consists of about 25 per cent glycine and 9 per cent arginine (6). Each experiment ran for 2 days. On the evening preceding the 1st day the subject had a normal meal at 6 p.m. and 300 ml. of water in the course of the evening. During the following day he drank 1700 ml. of orange juice and an additional 300 ml. of water. This was the "non-protein" day in Fig. 1. Next day the subject drank 875 ml. of orange juice, and 1000 ml. of water containing 275 gm. of sucrose. He ingested

65 gm. of gelatin between 8 and 9 a.m. The gelatin contained 16 gm. of glycine and 5.8 gm. of arginine. This was the "gelatin" day.

A typical result is given in Fig. 1. It shows the rapid rise in the urinary excretion of glycocyamine after the ingestion of gela-

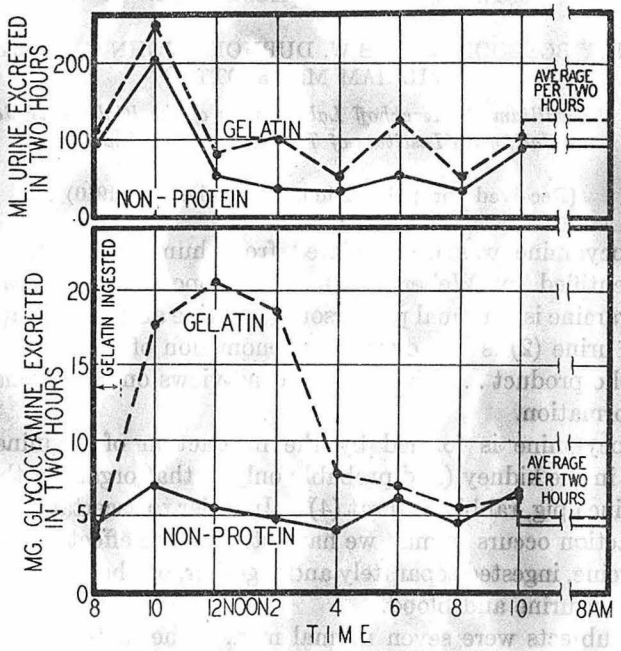

FIG. 1. Glycocyamine excretion in urine. The ordinates represent the glycocyamine excreted in the urine in the 2 hour period ending at the time indicated; the abscissae, the time at which the urine was voided. 65 gm. of gelatin were ingested between 8 and 9 a.m. The horizontal right-hand ends of both curves represent the total excretion in the night urine (10 p.m. to 8 a.m.) divided by 5.

tin. The maximum was attained between 10 a.m. and 12 noon. By 4 p.m. this rate had subsided near to the value on the non-protein day, and remained so during the following 14 hours. This rapid rise and fall in the rate of glycocyamine excretion occurred both when the arginine and glycine were taken in the form of gelatin and as the pure amino acids (see Figs. 1 and 2).

It is improbable that the increased urinary excretion of glyco-

cyamine observed after the ingestion of gelatin represents glycocyamine "washed out" of the tissues. If that had been the case, the excretion during the last 14 hours of the gelatin day probably would have fallen below that during the same interval on the non-protein day; and the total amount of glycocyamine excreted over the 24 hour period would have been nearly the same on these 2 days. The 24 hour excretion of glycocyamine on the non-protein day was 65 mg. and on the gelatin day 117 mg. The lack of any relation in Fig. 1 between urine volume and the amount of glycocyamine excreted is in accord with this interpretation.

Table I is a summary of the findings in experiments similar to that in Fig. 1 in five other subjects. The data for the period from 6 a.m. to 6 p.m. only are given.

TABLE I

Effect of Ingestion of 65 Gm. of Gelatin on Urinary Glycocyamine

Subject No.	Non-protein day		Gelatin day	
	Urine volume	Glycocyamine	Urine volume	Glycocyamine
	ml.	*mg.*	*ml.*	*mg.*
1	475	43.7	890	87.5
2	1715	34.3	1785	114.0
3	370	34.0	600	60.0
4	1220	32.9	1520	88.2
5	470	24.4	1350	81.6

The figures for glycocyamine excretion on the non-protein days in Table I indicate that over 24 hours they would have been of the order of magnitude of 40 to 60 mg. Weber (3) reported average figures for 24 hours of 39.5 mg. for males and 75.1 mg. for females. Weber's values are lower, probably because the figures obtained with his analytical method represent only 75 per cent or less of the glycocyamine present.

A series of experiments was carried out on two subjects in which arginine and glycine, alone and together, were administered in the form of the pure amino acids. The two subjects remained on the identical diet for the 9 days of the experiment. Breakfast and luncheon consisted of orange juice and coffee; dinner was a normal meal in which the meat course was a weighed amount of Hamburger steak. During the day 200 ml. of water were drunk

every 2 hours. Urine was collected every 2 hours from 6 a.m. to 6 p.m. On the 3rd day of this régime the urine voided every 2 hours during the day was analyzed; the values obtained gave the "normal" graph in Fig. 2. On the 5th day the subjects ingested 16 gm. of glycine between 8.30 and 9.30 a.m.; the glycocyamine excretion on this day is designated in Fig. 2 as "glycine." On the 7th day between 8.30 and 9.30 a.m. the subjects consumed 16 gm. of glycine and 4.8 gm. of arginine, and on the 9th day 4.8 gm. of arginine. The glycocyamine excretion on these days shown in Fig. 2 is designated "glycine + arginine" and "arginine."

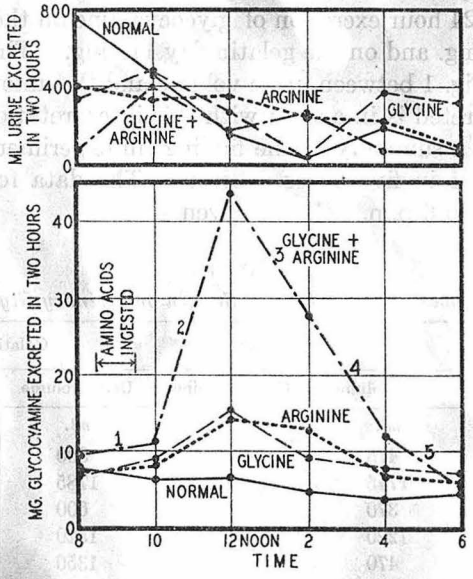

FIG. 2. Glycocyamine excretion in urine. The ordinates represent the glycocyamine excreted in the urine in the 2 hour period ending at the time indicated; the abscissae, the time at which the urine was voided. The amino acids were ingested, on each of the 4 days, in four portions between 8.30 and 9.30 a.m. The blood concentrations, at successive 2 hour intervals after the beginning of the experiment, on a day on which glycine and arginine were ingested together were *1*, 0.6, *2*, 0.4, *3*, 0.7, *4*, 0.8, and *5*, 1.0 mg. per cent.

Fig. 2 shows that after the ingestion of glycine alone more glycocyamine appeared in the urine. These increases were small,

however, compared to those obtained when the two amino acids were taken together. Thus on the normal day the glycocyamine excretion in 12 hours was 29.0 mg., on the glycine day 54.3 mg., on the arginine day 55.4 mg., and on the glycine + arginine day 99.0 mg. The sum of the increases over the normal day with glycine and arginine when these were ingested separately was 51.7 mg.; when ingested together it was 70 mg.

In the second subject the glycocyamine excretion on the normal day was 31.2 mg., on the glycine day 30.7 mg., on the arginine day 28.4 mg., and on the glycine + arginine day 61.8 mg.

The increase in glycocyamine in the urine after the ingestion of arginine and glycine was of the same order of magnitude as when the same amounts of these amino acids were ingested in the form of gelatin. The increase in urinary glycocyamine after the ingestion of gelatin may be ascribed to the arginine and glycine it contained.

These findings taken in conjunction with those obtained with surviving slices of kidney and with kidney extracts (4) may be considered as establishing the formation of glycocyamine by transamidination in man.

Blood was analyzed every 2 hours on the days on which the subjects ingested the glycine and arginine together. In the subject of Fig. 2 the concentrations were, in mg. per cent, 0.6, 0.4, 0.7, 0.8, and 1.0. The peak of the urinary excretion of glycocyamine occurred between the second and third of these values. There was no discernible relation between the glycocyamine concentration in the blood and its increased rate of excretion in the urine. The findings were essentially the same in the other subject.

We have made some preliminary observations on the excretion of glycocyamine by human subjects with kidney disease. On an ordinary diet they excreted less glycocyamine than normal individuals and the increase after the ingestion of arginine and glycine was less also. Thus in one subject who was diagnosed as having subacute glomerulonephritis the excretion of glycocyamine in the urine in an experiment similar to that of Fig. 1 was on the non-protein day 1.5, 3.0, 2.5, 2.6, 2.1, and 1.0 mg. in successive 2 hour intervals between 6 a.m. and 6 p.m.; and on the gelatin day 1.0, 3.1, 5.3, 2.4, 1.9, and 2.8 mg.

SUMMARY

1. When arginine and glycine are ingested together by human subjects, there is a rapid rise in the amount of glycocyamine excreted in the urine. This increase is greater than the sum of the increases which may occur when the same amounts of amino acids are taken separately.

2. A similar increase in glycocyamine excretion is observed after the ingestion of gelatin, which is rich in glycine and arginine. The order of magnitude of this increase is the same as that given by the quantity of arginine and glycine contained in the gelatin when these are administered as pure amino acids.

3. These findings indicate that in man glycocyamine is formed by transamidination.

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A MICROMETHOD FOR THE DETERMINATION OF ARGININE

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Micromethods for the determination of arginine based on the use of the Sakaguchi reagent have been described (1–4). This reagent gives a strong color with glycocyamine, arginine, and other monosubstituted guanidine derivatives.

In a previous communication (5) a method for the determination of glycocyamine was described based on the Sakaguchi reaction and the quantitative separation of glycocyamine from arginine by selective adsorption of the arginine on permutit. In the method outlined below the separated arginine is eluted from the permutit and determined independently.

A number of common non-chromogenic substances such as ammonia, urea, histidine, and creatine reduce the color obtained with the Sakaguchi reagent. A change in the usual order of addition of the color reagents similar to that previously described (5) decreases the interference by these compounds.

Reagents and Apparatus—

3 per cent sodium chloride.

0.3 per cent sodium chloride.

Permutit according to Folin. Permutit can be regenerated after use by allowing 3 per cent sodium chloride to percolate through and then washing with distilled water until chloride-free. This is most conveniently done in large batches on a Buchner funnel.

10 per cent urea in water.

0.2 per cent naphthol in absolute alcohol, diluted with 4 volumes of the 10 per cent urea solution before use.

Hypobromite solution. 0.66 ml. of liquid bromine are added

to 100 ml. of 5 per cent sodium hydroxide. Since this solution deteriorates, it should not be kept for more than 1 or 2 days, and is best kept in a refrigerator.

10 mg. per cent arginine in 0.1 N hydrochloric acid. Standard solutions are made on the day on which they are to be used, by diluting this stock solution with water.

Adsorption column. The permutit is contained in the stem of a glass funnel whose dimensions are: upper part 15 mm. external diameter, 100 mm. long; stem 7 mm. external diameter, 100 mm. long. The lower end of the stem is slightly constricted. A small amount of cotton is placed above the constriction. 0.9 gm. of permutit is poured in and tapped gently to settle the particles.

A 0.2 ml. micro pipette whose contents are delivered under pressure. The delivery time should be sufficiently short to insure rapid delivery and complete mixing (6).

Preparation of Solutions for Analysis—For complete separation of glycocyamine and arginine the salt concentration of the solution should not be over 0.5 per cent. If neither of these compounds is present in amounts over 2 mg. per cent, the salt concentration may be as high as 1 per cent. Urine is usually diluted 5 to 10 times with water. Blood filtrates may be prepared by deproteinizing according to Folin and Wu or by heat coagulation at pH 6 after 1:10 dilution with water. Tissue extracts are diluted to contain 1 gm. of tissue (fresh weight) in 40 ml. of suspension. The pH is adjusted to 6.0, and the suspension immersed in a boiling water bath for 10 minutes, cooled, and filtered. Analyses are carried out on the filtrates.

Procedure

5 ml. of the solution to be analyzed are passed through the permutit column and the small amount of glycocyamine remaining in the column is removed with 5 ml. of 0.3 per cent sodium chloride. The combined filtrate contains all the glycocyamine. A test-tube graduated at 10 ml. is now placed under the funnel, and the arginine is eluted by passing 10 ml. of 3 per cent sodium chloride through the column. The solution in the test-tube is made up to the 10 ml. mark and shaken.

A 2 ml. aliquot is taken for analysis. It is first cooled in an ice bath, then 0.5 ml. of the ice-cold naphthol-urea solution is added,

and after 2 minutes 0.2 ml. of ice-cold sodium hypobromite solution added by means of the micro pipette. This pipette is placed above the solution level and kept away from the sides of the test-tube to prevent contamination by urea in the solution. The color is simultaneously developed in a series of standard solutions containing 0, 0.25, 0.5, 1.0, and 2.0 mg. per cent of arginine.[1] After 20 minutes the color development is complete and remains stable for 2 hours if the solutions are kept in an ice bath. The tubes are shaken for a few seconds to remove excess gas, warmed by immersion in water at room temperature, and the intensity of

TABLE I

Elution of Arginine from Permutit Column

5 ml. of a 2 mg. per cent solution of arginine in 0.5 per cent sodium chloride were passed through the adsorption column followed by 5 ml. of 0.5 per cent sodium chloride. The arginine was then eluted by the salt solution as shown.

Sodium chloride eluting solution		Arginine recovered
Amount	Concentration	
ml.	*per cent*	*per cent*
10	0.5	0
5	1.0	2
10	1.0	40
5	2.0	60
10	2.0	100
10	3.0	100
10	5.0	100
5	5.0	88
10	10.0	88

the color measured in a spectrophotometer or a colorimeter, with light of approximately 0.525 μ (yellow-green).

DISCUSSION

As shown in Table I, the complete removal of arginine from the permutit column depends on the amount as well as the concentration of salt solution used. Under the conditions described, the separation of glycocyamine and arginine is complete over a wide

[1] If an appropriate correction factor is applied, glycocyamine standards which are stable may be used.

range of concentrations (Table II). With concentrations of arginine of over 2 mg. per cent the color is too intense to be read and must be diluted before the color is developed. The blanks are yellow instead of colorless as in the procedures described in the literature.

In these older procedures a large excess of urea is added after a given interval to prevent destruction of the chromogenic compound by the hypobromite. During this interval other substances may compete for the hypobromite and reduce or completely inhibit the formation of color. The advantage of the procedure described

TABLE II

Limiting Concentrations of Glycocyamine and of Arginine That Can Be Separated on Permutit Column

All values are concentrations in mg. per cent. The concentrations were those of the solutions passed through the permutit column. The final filtrates were diluted to bring the concentration to approximately 1 mg. per cent before the color was developed.

Arginine added	Glycocyamine added	Arginine found
0.0	2.0	0.0
0.0	2.5	0.0
0.0	5.0	0.0
0.0	10.0	0.0
1.0	0.0	1.0
2.0	0.0	2.0
5.0	0.0	5.0
10.0	0.0	9.6
20.0	0.0	18.4

above is that this interference is greatly reduced and for most purposes is negligible.

Table III shows the recovery of arginine added to urine, kidney extract, and blood.

Weber (2) has studied the influence of some non-chromogenic substances on color development. He found that 6 mg. per cent of ammonia, 12 mg. per cent of histidine dihydrochloride, 6 mg. per cent of tyrosine, 6 mg. per cent of tryptophane, 40 mg. per cent of creatine, or 160 mg. per cent of urea[2] "either prevents all color

[2] These are the concentrations in the final diluted filtrates.

formation or the color is so altered that even for qualitative purposes the test is worthless." With the method described in this communication, 60 mg. per cent of ammonia, 5 mg. per cent of histidine hydrochloride, 8 mg. per cent of tyrosine, 8 mg. per cent of tryptophane, 20 mg. per cent of creatine, and 2000 mg. per cent of urea are without influence on the recovery of arginine. If tryptophane and histidine are present in excess of the

TABLE III

Recovery of Arginine Added to Urine, Kidney Extract, and Blood

All the values are concentrations in the eluate measured in mg. per cent. The urine was diluted 1:4 with water. The kidney extract was made by grinding up 1 part by weight of kidney with 40 parts of 0.5 per cent sodium chloride, bringing the extract to pH 5.0, boiling 10 minutes, cooling, and filtering. The analysis was carried out on the protein-free filtrate. In both the kidney extract and blood, arginine was added before protein precipitation.

Arginine added	Arginine					
	Urine		Kidney extract		Folin-Wu blood filtrate	
	Found	After subtraction of blank value	Found	After subtraction of blank value	Found	After subtraction of blank value
0.0	0.13		0.15		0.19	
	0.13		0.14		0.20	
0.2	0.345	0.215			0.41	0.21
	0.34	0.210			0.43	0.22
0.5	0.61	0.48	0.65	0.505	0.68	0.48
	0.61	0.48	0.655	0.51	0.68	0.48
1.0	1.12	0.99	1.19	1.045	1.19	0.99
	1.13	1.00	1.19	1.045	1.19	0.99
1.5			1.64	1.50	1.67	1.47
			1.64	1.50	1.71	1.51

amount given, they are chromogenic, and, a further complication, histidine reduces the color given by the arginine present. In pregnancy urine the concentration of histidine may be as high as 160 mg. per cent (7) and the method is inapplicable as it stands. The method is satisfactory for normal urine, although the possible presence of other chromogens, such as methylguanidine, must be borne in mind.

SUMMARY

A micromethod for the determination of arginine in biological fluids and tissue extracts is described. The advantages of this new method are that a complete separation of arginine from glycocyamine is effected, and that it gives satisfactory results in the presence of common biological substances which interfere in the procedure of previous methods.

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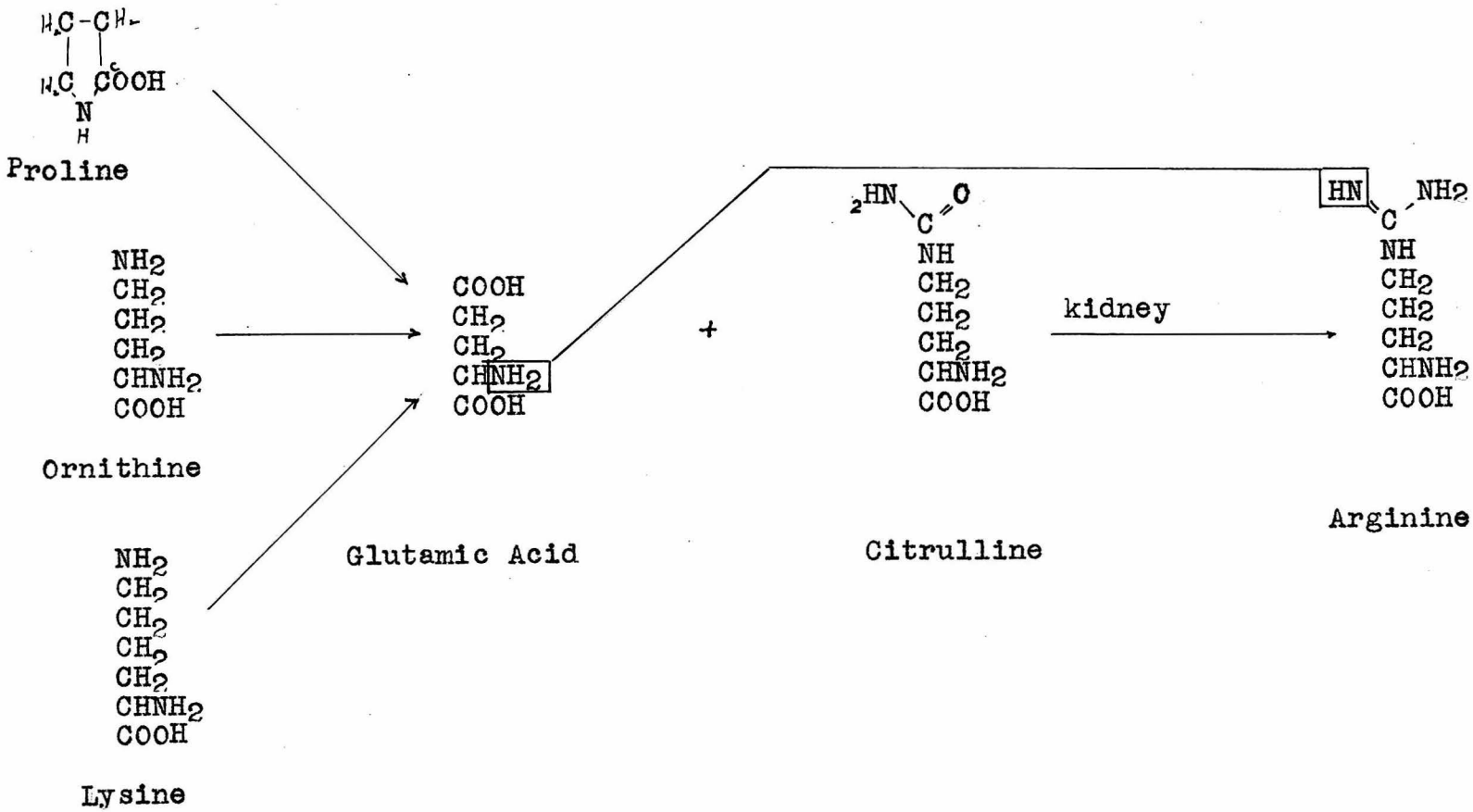

FORMATION OF ARGININE

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THE CONVERSION OF CITRULLINE TO ARGININE IN KIDNEY*

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(Received for publication, August 6, 1941)

Glycocyamine is formed in the kidney by the transfer of the amidine group of arginine to the nitrogen atom of glycine. In the study of this reaction it was observed that glycocyamine was also formed from citrulline and glycine. No other donor or precursor of the amidine group was found (1).

The most probable explanation of this effect of citrulline was that it was first converted to arginine, from which the amidine group was then transferred to glycine. This has now been proved.

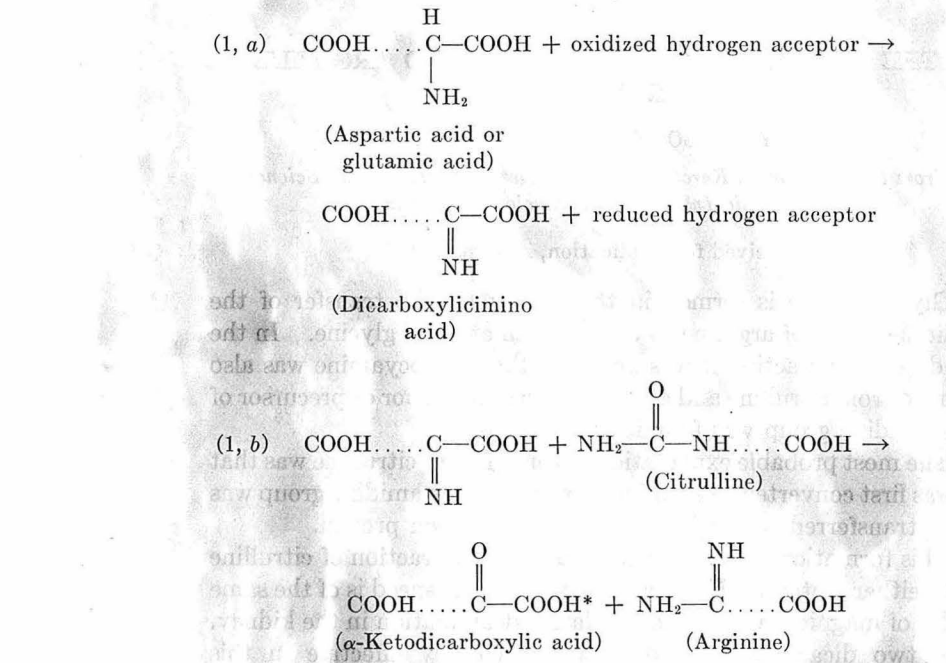
This formation of arginine consists in an interaction of citrulline with either glutamic acid or aspartic acid. Its speed is of the same order of magnitude as that of oxidative deamination in the kidney. The two dicarboxylicamino acids are equally effective in this respect. An oxidation is involved in the reaction; it is nearly completely inhibited by such oxidation inhibitors as KCN, As_2O_3, and As_2O_5 in low concentration. The KCN inhibition is partly relieved by hydrogen acceptors; that of As_2O_3 and As_2O_5 is not.

There are in general three possible types of mechanism for this reaction, the essential differences in them consisting in the point at which the oxidation, *i.e.* the dehydrogenation, occurs: (*a*) at the dicarboxylicamino acid before it reacts with the citrulline, (*b*) at an intermediate compound consisting of the citrulline and the dicarboxylicamino acid, or (*c*), after the cleavage of this hypothetical intermediate compound, at a derivative of the citrulline which is the immediate precursor of the arginine.

* Aided by a grant from Mr. I. Zellerbach.

A summary of this work has appeared (*Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **140**, p. xviii (1941)).

The first of the three possibilities is depicted by the following equations,



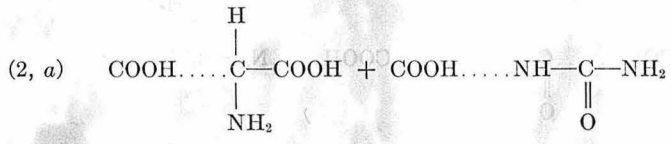
* Here, as in Equations 2 to 4, the dicarboxylic acid is written as being converted to the corresponding keto acid derivative. This is done only for convenience. We have obtained no evidence regarding the nature of the product arising from the deaminized dicarboxylicamino acid. It is possible that it is also decarboxylated in the oxidation; *i.e.*, the reaction mechanism is that suggested by Herbst (2).

This mechanism was eliminated by the finding that arginine formation is inhibited under conditions in which oxidative deamination of the dicarboxylicamino acids proceeds unchecked.

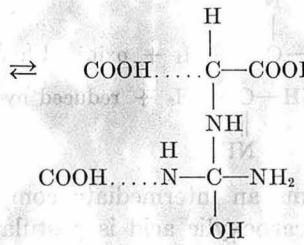
Arginine is also formed from citrulline, ammonia, and α-ketoglutarate or oxalacetate. These reactions are also inhibited by KCN or As_2O_3. The interpretation we have placed on these results is that here also the reaction proper (*i.e.*, arginine formation) is between citrulline and glutamic acid or aspartic acid; that the dicarboxylicamino acids were formed by reductive amination prior to their combination with citrulline. It follows that the imino

acid derivatives of the dicarboxylicamino acids (which are formed spontaneously and are in equilibrium in water with their corresponding α-keto acids and ammonia) do not react with the citrulline. This evidence also therefore excludes the reaction mechanism of Equations 1, *a* and 1, *b*.

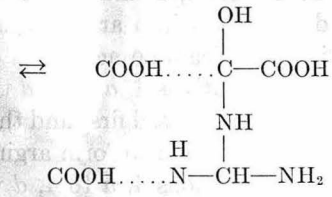
The other two possible mechanisms are given by Equations 2, 3, and 4.



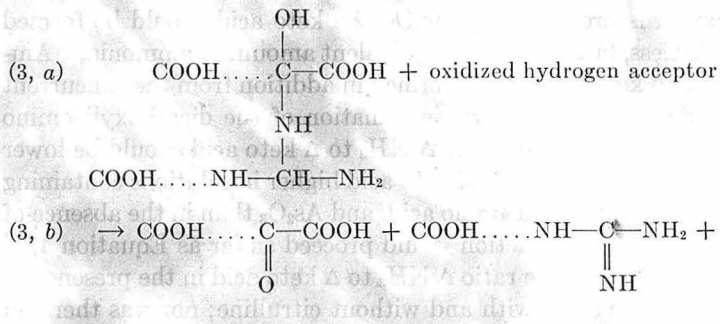
(2, *a*)



(2, *b*)



(2, *c*)

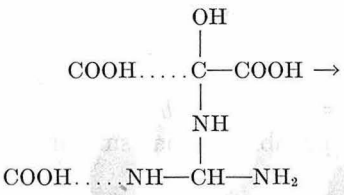


(3, *a*)

(3, *b*)

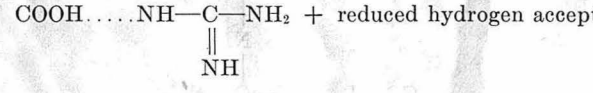
reduced hydrogen acceptor

or

(4, *a*)


(4, *b*)


(4, *c*) $COOH.....NH—CH(NH_2)—NH_2 + \text{oxidized hydrogen acceptor} \rightarrow$

(4, *d*)


In both mechanisms an intermediate compound between the citrulline and the dicarboxylic acid is postulated. According to the mechanism of Equations 3, *a* and 3, *b* this compound is decomposed by dehydrogenation into arginine and the α-keto acid derivative of the dicarboxylicamino acid.

In the mechanism of Equations 4, *a* to 4, *d* the hypothetical intermediate compound is decomposed first and the resulting diamine group undergoes dehydrogenation to form arginine.

If the mechanism of Equations 4, *a* to 4, *d* were correct, when citrulline and the dicarboxylicamino acid are present but arginine formation is prevented by As_2O_3, the keto acid should be formed nevertheless, but without an equivalent amount of ammonia. Ammonia and keto acid will be formed in addition from the concurrent and independent oxidative deamination of the dicarboxylicamino acid. Accordingly the ratio Δ NH_3 to Δ keto acid should be lower and the absolute amount of keto acid higher in solutions containing citrulline, dicarboxylicamino acid, and As_2O_3 than in the absence of citrulline; *i.e.*, the reaction would proceed as far as Equation 4, *b*. It was found that the ratio Δ NH_3 to Δ keto acid in the presence of As_2O_3 was the same with and without citrulline; nor was there an absolute increase in keto acid in the presence of citrulline.

The evidence appears to lead by exclusion to a mechanism of the type of Equations 2 and 3. Braunstein (3) postulated an intermediate compound analogous to that of Equation 2 as a preliminary to transamination. It must be emphasized that in both cases the existence of this hypothetical compound is, as yet, unsupported by any direct evidence. It is an inference only.

Positive results were obtained when proline, hydroxyproline, ornithine, or lysine was used in place of aspartic acid or glutamic acid. All the other amino acids were negative. There is independent evidence that the first three amino acids named are readily convertible to glutamic acid in the rat (4–7). It is reasonable to conclude, therefore, that lysine also is converted to glutamic acid (or aspartic acid). This is the first clue, as far as we are aware, regarding the path of degradation of the carbon skeleton of lysine in the animal body.

In the ornithine cycle by which urea is formed in the liver from ammonia and carbon dioxide, one of the steps proposed is the conversion of citrulline to arginine (8). The mechanism for this conversion which we have found in kidney, *i.e.* the interaction of citrulline with aspartic acid or glutamic acid, does not occur in liver. A study of arginine formation in liver is in progress and will be reported later.

Procedure and Preparations Used

Most of the experiments were carried out with surviving rat kidney slices. In a few experiments guinea pig kidney was used. The details of the technique have been described (9). Two or three small slices were suspended in 4 ml. of bicarbonate-Ringer's solution containing the substances under investigation. The pH of the solution was 7.4 after equilibration with 95 per cent oxygen and 5 per cent CO_2. All the experiments were carried out at 38° for 1 or 2 hours.

At the end of the experimental period the contents of the reaction vessels, acidified to pH 6.0 with a drop of 0.5 N HCl, the slices, and two 2.5 ml. washings with water were transferred to test-tubes graduated at 10 ml. After 10 minutes in a boiling water bath the test-tubes were cooled, and the contents made up to the mark with water, mixed by shaking, and filtered. The tissue slices were dried at 100° and weighed.

Glycocyamine and arginine were then determined in the protein-free filtrates. A 5 ml. aliquot was passed through a column of permutit and the traces of glycocyamine remaining in the column removed with 5 ml. of 0.3 per cent NaCl. The glycocyamine was determined in the combined filtrate by the method we have described (10). The arginine was then eluted from the permutit and determined as described by Dubnoff.[1]

Ammonia and keto acids were also determined in some experiments. These analyses were carried out on the protein-free filtrates which were not passed through permutit. The analytical method used for ammonia has been described (11).

The keto acids were determined as follows: To 2 ml. of protein-free filtrate 0.3 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 1 N HCl was added, followed after standing for a few minutes by 1 ml. of 1 per cent NaOH. An intense, deep red color appears immediately. This quickly fades and after 20 minutes a stable red color remains whose intensity is proportional to the concentration of pyruvic acid, oxalacetic acid, or α-ketoglutaric acid present. A linear relationship is obtained between the concentration of keto acid and the depth of color when it is measured at a wave-length of 0.525 μ, in the concentration range from 0 to 2.0 mg. per cent. The color of higher concentrations is too deep to be measured accurately.

The use of 2,4-dinitrophenylhydrazine in alkaline solution for the colorimetric determination of pyruvic and other keto acids has recently been reviewed by Klein (12). The simple procedure we have employed, without preliminary extraction of the hydrazone, would give positive values with such substances as acetone and acetoacetic acid. Under our experimental conditions the latter two substances are not formed in measurable amounts. The controls (Table VII) showed that for our limited purposes the simplified procedure was satisfactory.

l(+)-Citrulline was used throughout. It was prepared by a combination of the methods described by Kurtz (13) and by Gornall and Hunter (14). The starting material was a commercial preparation of *l*(+)-ornithine. All the other amino acids used were prepared or purified either by Amino Acid Manufactures, University of California at Los Angeles, or in this laboratory. We are in-

[1] Dubnoff, J. W., *J. Biol. Chem.*, **141**, 711 (1941).

debted to Professor H. B. Vickery for a generous donation of glutamine. The naturally occurring isomers of the amino acids were used except when the *dl* form is specified.

Pyruvic acid was prepared by distillation of a commercial product; the distillate was neutralized by NaOH, and the sodium salt crystallized and dried.

α-Ketoglutaric acid was prepared by the method of Neuberg and Ringer (15). We are indebted to Mr. Werner Baumgarten for this preparation.

Oxalacetic acid was prepared by the method of Simon (16).

The α-keto acid derivative of methionine was obtained by oxidation of *dl*-methionine with *d*-amino acid oxidase prepared from hog kidney, ethereal extraction of the acid from the acidified, deproteinized solution, evaporation of the ether, solution of the acid in water, neutralization with NaOH, and recrystallization from water.

The acetaldehyde was a commercial preparation.

Coenzyme I was prepared by the method of Ohlmeyer (17), and coenzyme II and adenosine triphosphate according to the prescription given by Warburg and Christian (18). The coenzyme preparations, as used, were 60 per cent pure. These preparations were made by Dr. Norman H. Horowitz.

Results

Table I is a typical protocol showing the formation of arginine from citrulline and glutamic acid or aspartic acid. There was always a significant increase in arginine over the blank value when citrulline alone was added to the Ringer's solution. This arginine was formed, presumably, from the added citrulline and aspartic acid or glutamic acid (see Table IV) which was present in the kidney slices at the outset or formed in the slices during the experiment. Nearly 3 times as much arginine was formed, however, when either aspartic acid or glutamic acid was added with the citrulline. The two dicarboxylicamino acids were equally effective.

There was a small increase in glycocyamine whenever arginine formation occurred. It may be inferred from previous observations (1) that the amidine group of the arginine was transferred to glycine which was present in the slices initially or formed in the course of the experiment. The quantity of available glycine was

small and also limited because no more glycocyamine was formed when the arginine was increased 3-fold.

It was not feasible, because of the high solubility of arginine and its salts, to attempt to prove by isolating one of them that the substance we were measuring colorimetrically was arginine. This was

TABLE I

Typical Protocol Showing Formation of Arginine by Surviving Rat Kidney Slices from Citrulline and Glutamic Acid or Aspartic Acid

Ringer's solution; 38°; 1 hour.

Solution No.	Tissue weight, dry	Citrulline, 0.0025 M	Glutamic acid, 0.005 M	Aspartic acid, 0.005 M	Arginine found		Glycocyamine found	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	mg.				*mg. per cent*	*mg. per gm. fresh tissue*	*mg. per cent*	*mg. per gm. fresh tissue*
1	3.2	−	−	−	0.02	0.3	0.02	0.3
2	7.3	−	−	−	0.02	0.1	0.04	0.2
3	7.2	+	−	−	0.33	1.8	0.07	0.4
4	6.7	+	−	−	0.31	1.9	0.07	0.4
5	11.7	−	+	−	0.04	0.1	0.04	0.1
6	11.1	−	+	−	0.04	0.1	0.06	0.2
7	8.3	+	+	−	1.07	5.2	0.08	0.4
8	6.2	+	+	−	0.89	5.7	0.07	0.4
9	10.3	−	−	+	0.03	0.1	0.04	0.2
10	11.9	−	−	+	0.04	0.1	0.04	0.2
11	6.1	+	−	+	0.89	5.8	0.06	0.4
12	7.5	+	−	+	1.02	5.4	0.07	0.4

The figures in Columns 6 and 8 give the concentrations of arginine and of glycocyamine in the protein-free solutions after they had passed through the columns of permutit. The figures in Columns 7 and 9 are obtained by multiplying those in Columns 6 and 8 respectively by the factors, $4/100 \times 10/4 \times 2 \times 1000/(5 \times W) = 40/W$, where W is the dry weight of tissue in mg. The basis of these factors is as follows: The volume of the original, undiluted Ringer's solution was 4 ml.; it was diluted to 10 ml. for deproteinization; in the course of separation of the glycocyamine and elution from permutit the aliquot used was diluted with an equal volume of salt solution; the fresh weight of the slices is 5 times the dry weight.

established by three independent pieces of evidence: the specificity of the color reagent under the conditions in which we used it, the disappearance of this chromogenic material when subjected to the action of arginase, and the formation of glycocyamine in the highly specific transamidination reaction with glycine.

The color reagent, the Sakaguchi reagent, gives a positive test with only three common biological substances. These are glycocyamine, histidine, and arginine. Glycocyamine is separated quantitatively from arginine and histidine by the adsorption and elution procedure employed. Histidine, however, remains on the permutit and is removed with arginine by the 3 per cent sodium chloride solution used for eluting the arginine from permutit. Histidine has about 1 per cent the chromogenic power of arginine. Even if all the added citrulline had been converted to histidine, most of the color developed would still remain to be accounted for.

Table II presents direct evidence that the material in question was arginine. Digestion with arginase removed nearly all of the chromogenic material designated as arginine. The details of the experimental procedure are given in the foot-note to Table II.

The transamidination reaction (*i.e.*, formation of glycocyamine from arginine and glycine) is a highly specific test for arginine when an extract of kidney (instead of kidney slices) is used to provide the enzyme. The arginine formed from any citrulline present is negligible for the purposes of this test. The test consists in incubating the material in question with an excess of glycine in the presence of the enzyme. A positive result is indicated by the formation of glycocyamine, and the rate of formation of glycocyamine is proportional to the initial concentration of arginine.

Table III shows that this criterion also afforded clear evidence that we were dealing with arginine. If the chromogenic substance in experiments such as those in Table I was arginine, it was to be expected that there would be (*a*) little or no glycocyamine in Solutions 1, 3, and 4 of Table III, (*b*) large increases in Solutions 2, 5, and 6, with 2 to 3 times as much in Solutions 5 and 6 as in Solution 2, and (*c*) less arginine remaining in this experiment than in the experiment of Table I because of the conversion of some of the arginine to glycocyamine; the "total"[2] arginine formed per hour would, however, be the same in Solutions 5 and 6 of Table III as in Solutions 7 and 8 and 11 and 12 of Table I. The results obtained were in complete accord with these predictions.

Table IV summarizes our findings on the relative effectiveness of

[2] The "total" arginine is the sum of the arginine found and of the amount of glycocyamine multiplied by 1.49 (the ratio of the molecular weight of arginine to that of glycocyamine).

different amino acids and of some other nitrogen compounds as contributors of the =NH group in the conversion of citrulline to arginine. Glutamine was the only compound which was as effective as aspartic acid or glutamic acid. This may be taken to indicate the rapid hydrolysis of the added glutamine rather than that the amide itself is effective. An active glutaminase is present in

TABLE II

Proof from Action of Arginase That Arginine Was Main Chromogen Measured by Colorimetric Method Employed

The arginine values are measured in mg. per gm. of fresh tissue.

Citrulline, 0.0025 M	Aspartic acid, 0.005 M	Glutamic acid, 0.005 M	Arginine present measured colorimetrically	
			Before action of arginase	After action of arginase
−	−	−	1.4	0.2
+	−	−	3.3	0.2
+	+	−	11.0	0.6
+	−	+	8.8	0.5
−	+	−	1.2	0.2
−	−	+	1.0	0.2

Ringer's solution, 38°; 1.5 hours. Each of the six mixtures was carried through in quadruplicate to the end of the period of incubation with kidney slices; pairs of similar solutions were then combined. Each of the values given above therefore is an average of two completely separate but identical experiments run simultaneously from the initial incubation with kidney slices through the digestion with arginase to the final colorimetric determination. The arginine was determined before and after digestion of the protein-free filtrates with arginase. Dry arginase powder was prepared by the method of Hunter and Dauphinee (19). 4 ml. of the protein-free filtrate were digested at pH 8.7 for 1 hour at 38° with 0.2 ml. of a 3 per cent solution of the arginase powder. At the end of the hour the solutions were acidified to pH 6.0, boiled, and filtered. The arginine in the filtrates was then determined in the usual manner after removal of the glycocyamine with permutit.

kidney (20). Asparagine was much less active than aspartic acid (the asparaginase activity of kidney is very low), while acetamide, formamide, and nicotinamide were negative.

The positive results with proline, hydroxyproline, and ornithine were to be expected. Weil-Malherbe and Krebs (4) and Neber (5) have proved that proline and hydroxyproline are converted to

glutamic acid by rat kidney slices. The figures in Table IV show that the formation of arginine from citrulline was approximately twice as fast with proline as with hydroxyproline. This is in accord with the findings of Weil-Malherbe and Krebs that glutamic acid is formed twice as quickly from proline as from hydroxyproline.

The conversion of *l*(+)-ornithine to glutamic acid was first suggested by Krebs (6) on the basis of indirect evidence. *d*(+)-Proline and *d*(−)-ornithine gave the same oxidation product, α-keto-δ-aminovalerianic acid, after treatment with *d*-amino

TABLE III

Glycine Transamidination with Arginine Formed from Citrulline

The results are expressed as mg. per gm. of fresh tissue.

Solution No.	Metabolites used	Glycocyamine	Arginine	"Total" arginine
1		0	0.4	0.4
2	Citrulline	1.2	2.0	3.8
3	Aspartic acid	0.2	0.8	1.1
4	Glutamic "	0.2	0.6	0.9
5	Citrulline + aspartic acid	2.6	5.9	9.8
6	" + glutamic "	2.7	6.6	10.6

The different mixtures of metabolites were first incubated at 38° with rat kidney slices for 2 hours. The citrulline, aspartic acid, and glutamic acid were initially 0.01 M. A 3 ml. aliquot of each mixture was then removed and added to 1 ml. of a cell-free extract of rat kidney to which glycine had been added to a concentration of 0.04 M. These mixtures were then incubated at 38° with shaking for 6 hours. They were then deproteinized and analyzed for glycocyamine and arginine.

acid oxidase. *l*(−)-Proline was proved to be converted to glutamic acid. It was a reasonable surmise then that glutamic acid was formed also from *l*(+)-ornithine via α-keto-δ-aminovalerianic acid in the same manner; *viz.*, oxidation at the δ-carbon atom to form the acid and amination of the α-carbon atom. Direct evidence of this conversion was obtained by Roloff, Ratner, and Schoenheimer (7). These workers fed deutero ornithine to normal adult mice and later found not only deutero arginine but also deutero proline and deutero glutamic acid. Data such as those in Table IV reveal that the conversion of *l*(+)-ornithine to glutamic acid occurs rapidly in rat kidney.

In view of the findings with proline, hydroxyproline, and ornithine it is a reasonable interpretation of the positive result with lysine (Table IV) that this amino acid also is converted to glutamic acid.[3]

TABLE IV

Relative Effectiveness of Different Amino Acids and Other Nitrogen Compounds As Contributors of =NH Group in Conversion of Citrulline to Arginine

Ringer's solution; 38°; 1 hour. The citrulline was initially 0.0025 M; all other compounds 0.005 M. Except where the *dl* form is specified, the naturally occurring isomer was used.

The results are expressed as per cent of "total" arginine formed from citrulline and glutamic acid. "Total" arginine = arginine + glycocyamine × 1.49.

Substance	Relative speed of arginine formation	Substance	Relative speed of arginine formation
Acetamide	−7	*dl*-Isoleucine	−8
Alanine	−8	Leucine	−8
Ammonia	1	Lysine	47
Asparagine	55	*dl*-Methionine	−8
Aspartic acid	108	Nicotinic acid amide	−21
Cysteine	−4	*dl*-Norleucine	−18
Formamide	−13	Ornithine	42
Glutamic acid	100	*dl*-Phenylalanine	−12
Glutamine	101	Proline	51
Glutathione	46	*dl*-Serine	−3
Glycine	1	Threonine	−14
Histidine	0	Tryptophane	−16
Hydroxyproline	23	Tyrosine	−6
		Valine	5

Table V shows the inhibition of the interaction of citrulline and aspartic acid or glutamic acid by low concentrations of KCN, As_2O_3, or As_2O_5. This inhibition is about 95 per cent complete.

[3] It may be expected from their structural similarity that the conversion of lysine to glutamic acid follows a course analogous to that of ornithine. One pathway for which there is some experimental evidence is as follows: oxidative deamination of the α-amino group, followed by oxidative deamination of the ε-amino group, β oxidation at the carbon atom to form α-ketoglutaric acid, which is reduced with the addition of ammonia to glutamic acid.

The cyanide inhibition is relieved to a considerable extent by the α-keto acid derivative of methionine, by oxalacetate, and by

TABLE V

Inhibition of Arginine Formation by Oxidation Inhibitors

Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of =NH donor 0.005 M.

The results are expressed as mg. of "total" arginine per gm. of fresh tissue.

	Uninhibited	KCN 0.001 M	As_2O_3 0.001 M	As_2O_5 0.0036 M
No amino acids added	1.3	0.9	0.9	0.8
Citrulline	2.7	1.1	1.1	1.1
" + aspartic acid	12.2	1.8	1.8	1.7
" + glutamic "	11.2	1.7	1.8	1.7

TABLE VI

Counteraction of Hydrogen Acceptors on Inhibition of Arginine Formation by KCN and As_2O_3

Ringer's solution; 38°; 2 hours. The citrulline was initially 0.0025 M; =NH donor and other metabolites 0.005 M.

=NH donor	Aspartic acid			Glutamic acid		
	Uninhibited	0.001 M KCN	0.001 M As_2O_3	Uninhibited	0.001 M KCN	0.001 M As_2O_3
No amino acids added	1.1	0.9	0.9	1.1	0.9	0.9
Citrulline alone	3.0	1.0	1.1	3.1	1.0	1.0
" + =NH donor	12.2	1.6	1.0	12.0	1.3	1.6
" + " " + acetaldehyde		0.9			0.6	
Citrulline + =NH donor + α-keto acid derivative of methionine		5.5			7.0	
Citrulline + =NH donor + oxalacetate		3.1	1.1		4.3	1.9
Citrulline + =NH donor + pyruvate	7.2	3.1	1.2	6.7	2.2	1.3

pyruvate. These metabolites do not relieve the inhibition by arsenite (Table VI).

Our interpretation of these findings is that both cyanide and

As_2O_3 (and As_2O_5) inhibit the formation of arginine by preventing the necessary dehydrogenation of the citrulline-aspartic acid (or glutamic acid) complex. The effect of cyanide is indirect; it prevents the oxidation of cytochrome. The flow of electron and of hydrogen transfer is thus blocked by the lack of an available acceptor. Arginine formation can be reinstituted, however, by the provision of electron and hydrogen acceptors other than the cytochrome-oxygen system which can function even in the presence of cyanide. They must be provided in sufficient concentration because their reoxidation is also blocked by cyanide and they cannot therefore function in a cyclic manner. The α-keto acid derivative of methionine, oxalacetic acid, and pyruvic acid served this purpose. Acetaldehyde did not relieve the cyanide inhibition. This would exclude complex formation with the cyanide as an explanation of the positive results with the above keto acids.

The arsenite inhibition, on the other hand, is not relieved by these metabolites because this inhibitor interferes with the process of dehydrogenation; *i.e.*, the initial process of donation of electrons and hydrogens is blocked. Hence the provision of additional hydrogen acceptors affords no relief.

In view of the participation of the pyridine coenzymes in the oxidative deamination of *l*(+)-glutamic acid (21) and the evidence that an oxidation of aspartic acid or glutamic acid is involved in this formation of arginine, we tested the effect of coenzymes I and II and of adenylic acid pyrophosphate on the reaction. These substances neither relieved the arsenite inhibition nor increased the rate in the absence of an oxidative inhibitor.

The enzymatic activity of kidney slices is largely lost (about 90 per cent) when the kidney is homogenized into a cell-free suspension. One possible explanation for this loss of activity was that the pyridine coenzymes were dissociated from the protein of the enzyme as a result of the dilution consequent on the preparation of the cell-free suspension. If this explanation were correct, addition of one of the two pyridine coenzymes or of adenylic acid pyrophosphate might be expected to restore some of the lost enzymatic activity. This was not the case. It is possible that some other coenzyme is operative in this reaction.

Another piece of evidence against the above explanation of the loss of enzymatic activity is that kidney tissue homogenized and

tested without any added fluid is no more active than when homogenized and suspended in 4 times its volume of buffer solution.

Table VII shows the separation of oxidative deamination of the dicarboxylicamino acids from the process in which citrulline is converted to arginine. The latter reaction is inhibited by arsenite; the former is not. In fact an inhibitor such as As_2O_3 which retards the disappearance of the products of deamination, the ammonia and keto acids, is necessary for the demonstration of oxidative deamination by tissue slices.

TABLE VII

Separation of Conversion of Citrulline to Arginine from Oxidative Deamination

Guinea pig kidney slices were used; Ringer's solution; 38°; 2 hours. The citrulline was initially 0.0025 M; aspartic acid either 0.005 or 0.05 M as indicated; As_2O_3 0.001 M.

The results are expressed as micromoles per gm. of fresh tissue.

Citrulline	Aspartic acid, initial molality	As_2O_3	Arginine found	Keto acid as pyruvic acid	Ammonia	$\frac{\Delta \text{ ammonia}}{\Delta \text{ pyruvic acid}}$
−		−	0.9	0	4.3	
−		+	1.0	0	10.4	
+		−	1.9	0	7.1	
+		+	0.9	0	10.9	
+	0.005	−	12.1	0	8.7	
+	0.05	−	6.3	1.8	13.6	
+	0.005	+	1.7	13.5	25.5	1.6
+	0.05	+	1.3	36.9	54.2	1.4
−	0.005	−	1.0	0	9.0	
−	0.05	−	1.2	0	20.2	
−	0.005	+	1.0	10.4	23.7	1.9
−	0.05	+	1.0	35.1	54.1	1.4

Another piece of evidence which indicated that the two oxidative processes, arginine formation and deamination, are different, although they involve the same substrate (aspartic acid or glutamic acid), was obtained from a comparison of the effects of two different concentrations of aspartic acid, 0.005 and 0.05 M. The higher concentration of aspartic acid inhibited arginine formation, but the rate of deamination, in an absolute sense, was more than 3 times greater. If the reaction consisted in an exchange of the oxygen on the ϵ-carbon atom of citrulline for the =NH group of

the imino acid derivative of aspartic or glutamic acid, the reaction should proceed with oxalacetic acid and ammonia or ketoglutaric acid and ammonia, even in the presence of cyanide, because the imino acids are formed spontaneously from the latter keto acids and ammonia.

Table VIII shows that arginine is formed from citrulline and the products of deamination of the dicarboxylicamino acids under normal conditions; but the reaction is completely inhibited by cyanide.

TABLE VIII

Aspartic Acid or Glutamic Acid Formation in Rat Kidney Slices; Attested to by Formation of Arginine from Citrulline

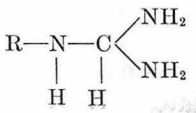
Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of glutamic acid or other metabolites and ammonia 0.005 M.

The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites in addition to citrulline	"Total" arginine found
None	2.4
Citrate + ammonia	2.9
Fumarate + "	4.8
Glucose + "	3.7
Glutamate	10.5
α-Ketoglutarate + ammonia	7.9
Oxalacetate + ammonia	4.8
Pyruvate + ammonia	2.6
Succinate + "	4.5

Three independent lines of evidence concur, therefore, in excluding prior oxidative deamination of the aspartic acid or glutamic acid as part of the reaction mechanism in the formation of arginine from citrulline.

As stated above, another possible reaction mechanism would locate the oxidation on a derivative of the citrulline after it had undergone transamination with the dicarboxylic acid to form a compound of the type



Dehydrogenation of the diamine group would then yield arginine. If this were the reaction mechanism, the addition of citrulline in the presence of As_2O_3 and aspartic acid or glutamic acid would, as discussed above, decrease the ratio Δ ammonia to Δ keto acid, because of an absolute increase in the keto acids without a corresponding increase in ammonia. The data in Table VII eliminate this hypothesis. In the presence of As_2O_3 the absolute amount of keto acids and the ratio of Δ ammonia to Δ keto acid were the same in the presence as in the absence of citrulline.

The evidence, by exclusion, therefore points to the locus of the oxidation on a hypothetical intermediate compound of citrulline and the dicarboxylicamino acid.

The reason that the ratio Δ ammonia to Δ pyruvic acid is greater than 1 is that As_2O_3 does not completely inhibit the oxidation of keto acids. Even in unpoisoned tissue the removal of ammonia is less complete than that of the keto acids. Similar observations were made by Krebs (6).

The formation of arginine from citrulline by surviving kidney slices can be used as a test for potential sources of aspartic acid or glutamic acid. It was shown by this method that certain amino acids are converted in the kidney to one or the other of these two dicarboxylicamino acids (Table IV). The action of tissue slices in this respect parallels closely that of the whole animal. This parallel was also seen in the synthesis of hippuric acid (22) and of creatine (23). The same method can be used as a test for precursors of the carbon skeleton of the dicarboxylicamino acids. The reagents are citrulline, an excess of ammonia, and the metabolite in question. Some examples are shown in Table VIII. Fumarate, glucose, α-ketoglutarate, oxalacetate, and succinate were definitely positive. A dubiously slight positive result was obtained with citrate. Pyruvate was negative.

The mechanisms by which these non-nitrogenous metabolites may be converted to the dicarboxylicamino acids are well known and need not be discussed here. The data in Table VIII are positive evidence that these changes occur readily in the kidney. The citric acid cycle appears to be quantitatively less important than simple oxidation of succinic acid and its derivatives.

The formation of arginine may be used as an indicator to ascertain the "preferred" directions of some of the reversible processes

in the dynamic steady state of metabolic reactions in kidney slices. This test is based on the fact that, in the presence of an excess of citrulline, the formation of arginine is faster the greater the concentration of aspartic acid or glutamic acid (at concentrations below 0.005 M). The following are two examples: The amount of arginine formed from citrulline in the presence of different amino acids and α-ketoglutarate is an indication of the speed of transamination in the direction of glutamic acid formation; the difference in the amount of arginine formed from citrulline, α-ketoglutarate, and ammonia in the presence and in the absence of

TABLE IX

Transamination in Rat Kidney Slices

Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of other amino acids, keto acids, and ammonia 0.005 M. The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites in addition to citrulline	"Total" arginine found
None	2.0
Glutamate	10.9
" + pyruvate	7.8
α-Ketoglutarate	3.1
Ammonia	2.2
α-Ketoglutarate + ammonia	4.6
" + pyruvate + ammonia	1.9
Alanine + α-ketoglutarate	2.8
dl-Alanine + α-ketoglutarate	2.1
Valine + α-ketoglutarate	1.6

pyruvate indicates whether ammonia nitrogen available for amino acid synthesis is bound preferentially as glutamic acid or as alanine.

Table IX is a summary of some experiments along these lines. It is seen that glutamic acid contributes its amino group to pyruvate. On the other hand, amino nitrogen does not pass readily from *l*(+)-alanine, *dl*-alanine, and *l*(+)-valine to α-ketoglutarate. In this respect ammonia is more effective. We may infer then that the formation of glutamic acid from α-ketoglutarate is faster by reductive amination than by transamination.

Similarly, in the presence of ammonia, α-ketoglutarate, and pyruvate there was no evidence of the formation of any glutamic acid available for arginine formation; presumably the nitrogen was bound preferentially as alanine.

The dynamic steady state in kidney slices appears therefore to include a cycle which favors the formation of glutamic acid by reductive amination, followed, if suitable keto acids are available, by transamination to form other amino acids with the regeneration of the α-ketoglutarate.

TABLE X

Inhibition by Pyruvate of Arginine Formation from Citrulline

Ringer's solution; 38°; 1 hour. The initial concentration of citrulline was 0.0025 M; of other amino acids and pyruvate 0.005 M.

The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites added in addition to citrulline	"Total" arginine formed
None	1.3
Aspartate	5.6
" + pyruvate	2.8
Glutamate	4.6
" + pyruvate	2.3
Lysine	3.3
" + pyruvate	1.8
Ornithine	3.7
" + pyruvate	2.4
Proline	2.8
" + pyruvate	1.1

The situation in kidney slices appears to be similar to that in the whole animal. Schoenheimer and his colleagues (24) observed that the most extensive exchange of normal nitrogen for N^{15} occurred in the nitrogen of aspartic acid and glutamic acid whether the N^{15} was introduced as ammonia or as a single amino acid.

The formation of arginine from citrulline was inhibited by pyruvate whether the donor of the =NH group was aspartic acid, glutamic acid, or a precursor of these dicarboxylicamino acids (Table X). This is in accord with the findings in Table IX that pyruvate is converted to alanine at the expense of glutamic acid. It is interesting that pyruvate is inhibitory under the normal cir-

cumstances of the experiment of Table X; yet in the presence of cyanide it permits arginine formation to occur, neutralizing to some extent the cyanide inhibition (Table VI). This latter fact suggests strongly that pyruvate does not inhibit the oxidation necessary for the formation of arginine from citrulline, that it is inhibitory under normal circumstances because it competes effectively with the citrulline for the amino group of aspartic acid or glutamic acid. More direct evidence is required, of course, to determine whether or not this hypothesis is correct.

DISCUSSION

The work of Braunstein and Kritzmann (3, 25), of von Euler *et al.* (21), and of Cohen (26) established by indirect but strong evidence the central position of the dicarboxylicamino acids in the continual and rapid interchange of amino nitrogen which Schoenheimer and his coworkers demonstrated to occur in the body by direct evidence (24).[4] The mechanism of this interchange involves transamination and reductive amination.

Another mechanism is involved in the formation (in the kidney) of arginine from citrulline in which the dicarboxylicamino acids also participate. Instead of transamination, the reaction might be designated as transimination, except that the dehydrogenation of the amino group of the dicarboxylicamino acid does not occur prior to its reaction with the citrulline but while it is in combination with it.

The discovery of an extremely active mechanism for converting citrulline to arginine in the kidney suggests a hitherto unsuspected source of the arginine which the kidney requires for, among other purposes, the transamidination reaction with glycine to form glycocyamine.

It seems improbable that a mechanism which can transform citrulline to arginine so quickly and act upon low concentrations of metabolites, less than 0.001 M, is inoperative *in vivo*, teleological as the argument is. The question then arises, what is the source of the citrulline? The kidney cannot convert ornithine to citrulline.

[4] It is interesting in retrospect that this interchange of amino nitrogen could have been inferred from the fact that ammonia exerts considerable nitrogen sparing action even in man. The continual and extensive synthesis and breakdown of protein was deduced from this and other data (27).

If one accepts the ornithine-urea cycle in the liver as proposed by Krebs and Henseleit (8), the liver can hardly supply the citrulline, because in that cycle the existence of the citrulline is only transitory on its way to arginine.

SUMMARY

1. Citrulline is converted to arginine at a rapid rate by rat and guinea pig surviving kidney slices. This property is almost completely lost when the cell structure is destroyed.

2. Either aspartic acid or glutamic acid is necessary for this reaction (in addition to citrulline).

3. Proline, hydroxyproline, lysine, and ornithine may replace the dicarboxylicamino acids in this reaction. Evidence is adduced that they do so by being converted first to glutamic acid (or, possibly but less likely, to aspartic acid).

4. Arginine is formed from citrulline and α-ketoglutaric acid and ammonia or oxalacetic acid and ammonia. Evidence is presented that these products of oxidative deamination are reduced by kidney slices to form the parent dicarboxylicamino acids.

5. This formation of arginine from citrulline is nearly completely inhibited by oxidative inhibitors, KCN, As_2O_3, and As_2O_5, indicating an oxidative step in the reaction mechanism. The cyanide inhibition is relieved in part by the α-keto acid derivative of methionine, by oxalacetate, and by pyruvate. The inhibition by As_2O_3 and As_2O_5 is not relieved by these metabolites. An interpretation of these findings is presented.

6. The oxidative step is not a dehydrogenation of the amino group of the dicarboxylicamino acid (to form the imino group) prior to its reaction with citrulline. Evidence is presented that this oxidation may be located at a hypothetical intermediate compound of citrulline and the dicarboxylicamino acid.

7. Arginine formation from citrulline can be used as an indicator of the "preferred" direction of some of the reversible processes in the metabolism of the cells. Some examples are presented.

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II CORNEAL VASCULARITY AND ITS RELATION TO ARIBOFLAVINOSIS

It is generally agreed that between the clinically recognizable vitamin deficiency states and the normal, there must exist mild or latent deficiency states. This finds practical expression in the wide difference between the so called minimum protective and optimum level of vitamin intake. According to Kruse (1), the specific pathological process in a deficiency disease is characterized by velocity, intensity, and sequence. An acute process is rapid in its course and also rapid in responding to therapy; a chronic process is slow in its onset, progress, and response to therapy. Both the acute and chronic states may be divided into mild and severe degrees, but with suitable techniques one should be able to show a continuous gradation from normal to markedly pathological states. In ariboflavinosis this gradation has been said to be measured by the extent of corneal vascularization.

A vascularizing keratitis of the cornea in vitamin G deficient animals was first reported in 1931 by Day, Langston, and O'Brien(2). Later in 1939 Eckardt and Johnson (3) noted that all rats on a riboflavin deficient diet which survived to the 48th day showed either keratitis or vascularization of the cornea. In the same year Bessey and Wolbach (4) produced a vascularizing keratitis in animals deprived of riboflavin which could be cured by supplying the vitamin in the diet. This manifestation preceded all other demonstrable lesions due to riboflavin deficiency.

The first report on corneal vascularity in humans receiving insufficient riboflavin was presented in 1940 by Kruse, Sydenstricker, Sebrell, and Cleckley (5).

All nine patients in their study had frank symptoms of riboflavin

deficiency. Nine had cheilosis, (fissures at the angles of the mouth), 8 had a characteristic glossitis, and 3 had seborrheic accumulation at the nasolabial folds. Examination with the biomicroscope revealed that the earliest change was a superficial invasion of the cornea by capillaries from the anterior ciliary vessels at the nasal and temporal sides of the cornea. At a latter stage the capillaries invaded the substantia propria, and superficial and interstitial infiltration appeared. There was no regression on feeding niacin, thiamin, cevitamic acid, cod-liver oil, or crystalline vitamin A. However, riboflavin resulted in a decrease in size and activity of the vessels. Later in the same year this study was supplemented by a report (6) on 47 patients, all of whom were known to be riboflavin deficient. Besides the usual subjective symptoms of itching, burning, roughness, photophobia, and poor vision, 45 patients showed a circumcorneal injection due to congestion and proliferation of the limbic plexus. Thirty-seven patients showed actual invasion of the cornea by the capillaries arising from the limbic plexus. This vascularization developed first just beneath the epithelium, later at varying depths in the substantia propria, and finally just within the endothelium. Increased pigmentation of the iris which disappeared on treatment was seen in 19 cases. In most cases the congestion disappeared within 48 hours after treatment with daily doses of 5 to 15 mg. of riboflavin. If the vascularization was more extensive, 5 to 18 days were required to empty the vessels. The authors stated that "ariboflavinosis is possibly the most prevalent, apparently uncomplicated avitaminosis; it is possible also that it is more easily recognized than others on account of the specific lesions of the eye which occur

early in the period of deficiency." Thus, by means of the slit lamp and biomicroscope all stages of the deficiency from mild to acute could be recognized.

On the basis of this work the Conference on Methods and Procedure for Nutrition Survey (7) recommended the biomicroscopic examination of the cornea for the evaluation of the extent of riboflavin deficiency. This procedure was accordingly employed in the nutrition survey at the Lockheed Aircraft Corporation, Burbank, California. A preliminary report has already appeared (8). This paper is the final report of the application of the biomicroscope to the determination of riboflavin deficiency.

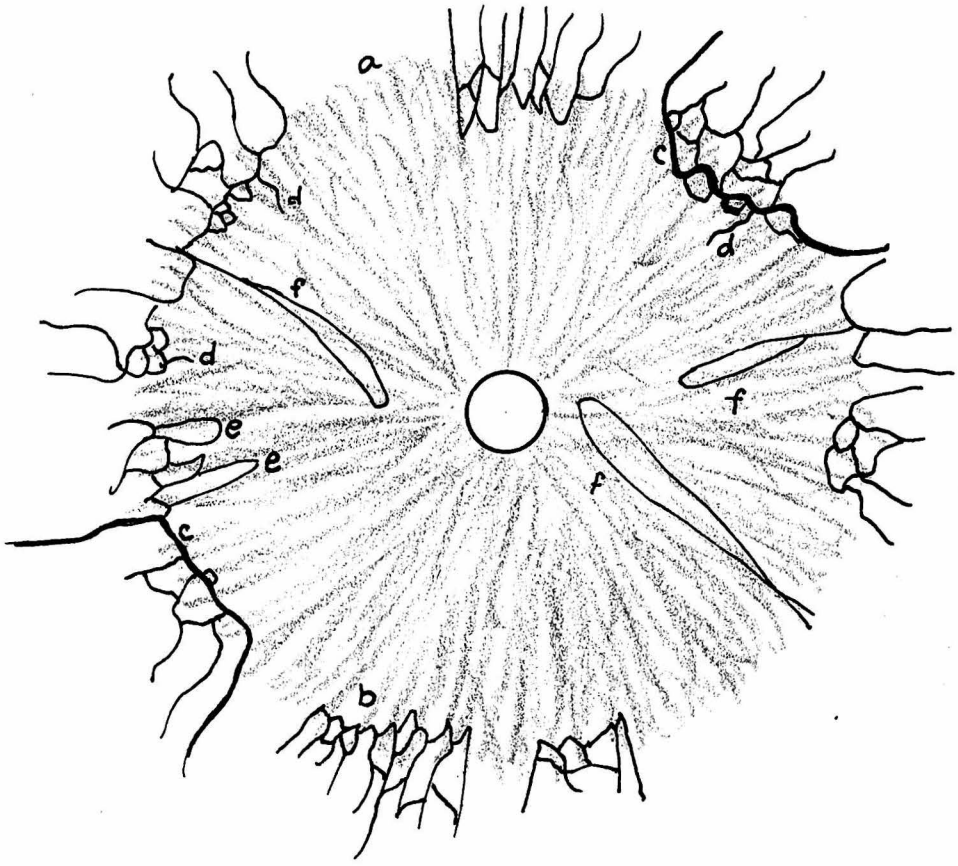
The subjects of this survey were 1,173 white male volunteers from all the major departments of the plant. These were divided at random into two groups: an experimental group which received a vitamin supplement of

Vitamin A (from fish liver oil)	50,000 I.V.
Vitamin D (from fish liver oil)	800 I.V.
Vitamin B_1 (synthetic)	10 Mg.
Riboflavin (synthetic)	10 Mg.
Niacinamid (synthetic)	100 Mg.
Ascorbic Acid (synthetic)	250 Mg.
Calcium ($Ca\ CO_3$)	500 Mg.

and a control group which received the corresponding placebos. The initial examinations began in November, 1941 and ended in February, 1942. The final examinations on the 617 subjects remaining after one year began December, 1942 and ended April, 1943.

The appearance of the limbus under the biomicroscope is shown graphically in figure 1. The fine capillaries which lie just under the

FIGURE 1



a limbus

b two arcades

c trunk (concentric collateral)

d streamer

e streamer type arcade

f white streamer

epithelium arise from the apices of the marginal loops in the conjunctiva and anastamose to form a series of loops extending towards the iris. According to Kruse et al (5) there is no vascularity within the normal limbus.

For the purpose of recording each sign was located according to the hours of the clock. Distances were measured with a micrometer eyepiece.

In the following tables the effect of therapy on a number of signs which have been said to be indicative of ariboflavinosis is recorded. In most instances only enough consecutive cases were analyzed to give a statistically reliable result.

If the extent of capillary invasion of the cornea is measured by totaling the number of hours occupied by capillary loops, the distribution shown in table I is obtained. There is no significant difference in the mean or in the distribution between the experimental and the control group. All subjects had some corneal vascularity, and the extent was marked in most cases.

Table II shows the distribution of the maximum number of arcades in any hour within the cornea. There is a slight decrease in the mean between the first and second examination, but the change is in the same direction in both groups. This may have been due to a difference in grading in the two examinations or to unknown factors other than therapy, but the difference is not statistically significant. The division of the cornea into segments shows no difference between the two groups in the maximum number of arcades (table III) or the extent of invasion to be found in a given sector (table IV). It has been claimed (5, 9) that the streamer type of arcades are particularly indicative of ariboflavin-

osis. These are active capillaries which extend some distance directly towards the iris and then loop back (figure 1). Often the arterial limb is much smaller than the venous limb, and the latter may be mistaken for a free ending capillary. The results recorded in table V show that the mean of the total number of hours of arcades of streamer type of the experimental group is not lower than that of the control, nor is the extension towards the iris less (table VI). If more than one arcade is present, there is no difference in the length of the last arcade between the two groups (table VII). There is no difference between the two groups in the percentage of the total number of hours that are of streamer type (table VIII).

Although we found no evidence for the existence of streamers in the sense of free ending capillaries, those capillaries which were not obviously loops were classified as streamers following the procedure of Kruse et al (5) although closer inspection always showed a fine connection between afferent and efferent vessels. The distribution of these so-called streamers is shown in table IX.

In the course of the second examination a type of vessel was observed which was designated as a white streamer. These streamers, just under the epithelium are sometimes surrounded by a narrow whitish cone whose base is at the limbus and from whose apex the capillary extends as far as the iris in a very fine loop (figure 1). Corpuscles are often seen under high magnification. The cloudy infiltration around the base of the capillary suggested a pathology that might be related to the conjunctival cloudiness of vitamin A deficiency. However, vitamin therapy did not decrease the incidence of these streamers (table X).

About 60% of the subjects were observed to have large trunk vessels (figure 1) for which the term concentric collaterals has been proposed by Vail and Ascher (10). According to these authors "Concentric collaterals are engorged parts of the pre-existent limbal meshwork. Their function is to collect blood from all limbal loops of one entire limbal sector, or a part of them, and to return this blood from the limbus to the larger conjunctival veins". There is no indication in our data (table XI) that the engorged condition leading to the establishment of concentric collaterals is in any way related to vitamin deficiency. This confirms the conclusions of Vail and Ascher. In some subjects a network of pigmented deposits resembling the remains of capillary arcades were observed within the cornea particularly between the five to seven o'clock position. Ten cases were found among the controls and only three in the experimental group out of 617 cases, but the number of cases is too small to be significant.

The finding that corneal vascularity decreased with age (table XII) is just the reverse of what might be expected if corneal vascularity were indicative of vitamin deficiency, for a symptom of a chronic lack would be expected to accumulate with age. This trend has been confirmed in an independent survey conducted among a group of employees of Los Angeles County (table XIII). These results are in direct conflict with the data presented by Scarborough (11) showing that vascularity was greater in patients over 50 years of age. Vail and Ascher (10) have pointed out that there is a normal pericorneal plexus of vascular arcades whose final loops under slit lamp illumination seem to be in the cornea but which are actually in the conjunctivoscleral wedge. This is more clearly

seen in figures 2 and 3. The overlapping of the cornea by the sclera creates a semi-opaque transition zone which makes the location of the true limbus difficult. Vascularization in this corneo-scleral transition zone although actually not within the cornea proper has been considered pathological by Kruse et al. and has been used as a basis for other nutrition surveys and our own. However, the data in this study are still valid, for if a difference in corneal vascularity has existed, a different distribution would have been found between the experimental and the control groups regardless of the point of reference. For example, if the measurements in table IV are decreased by 1 mm., which is the average width of the transition zone, there is still no difference in the distribution of arcades longer than 1 mm. between the two groups. Similarly in table II the number of arcades is decreased if the true limbus is used as the reference point, but the distribution remains the same.

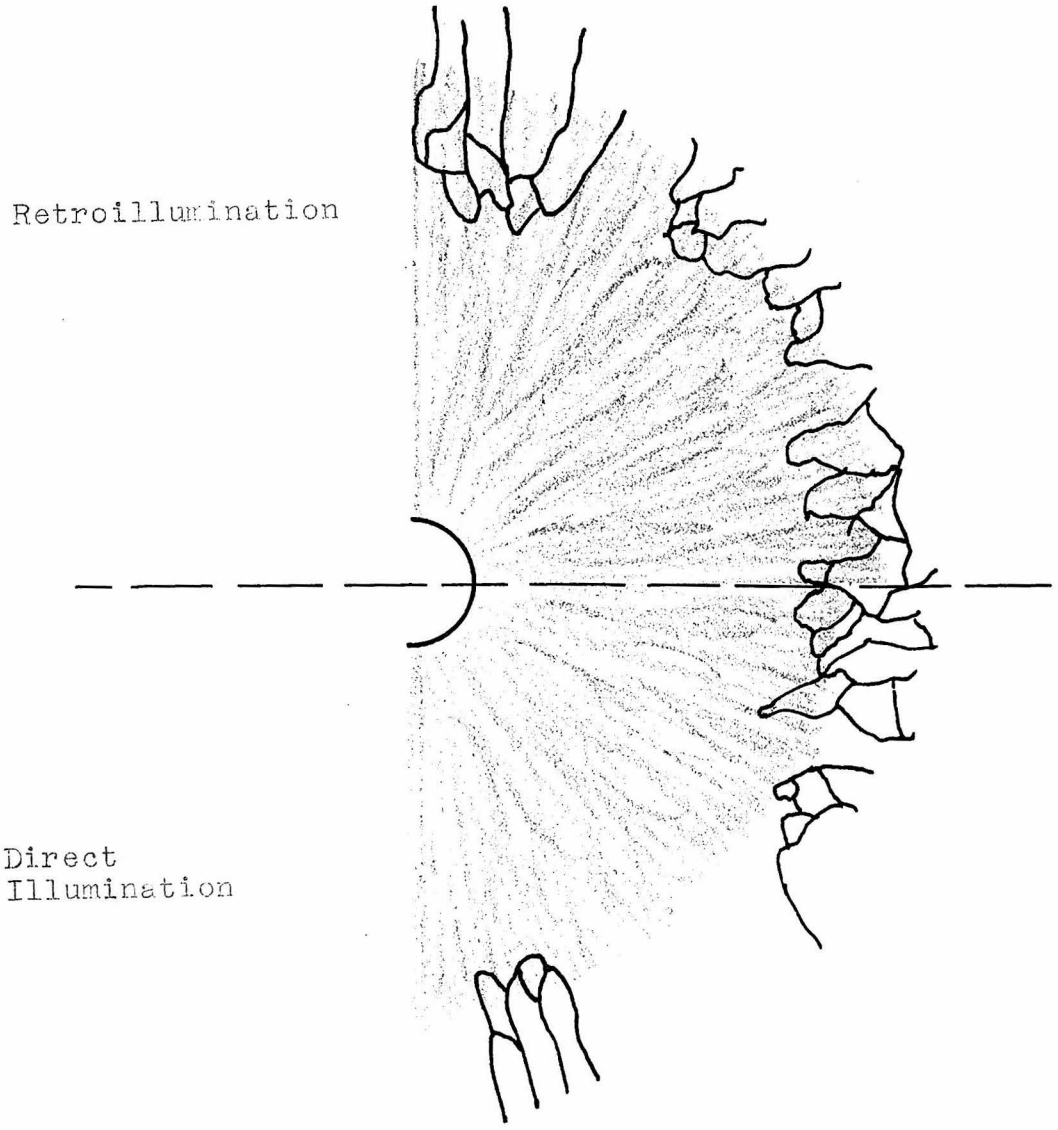


Figure 2

Appearance of the Limbus with Change in Illumination

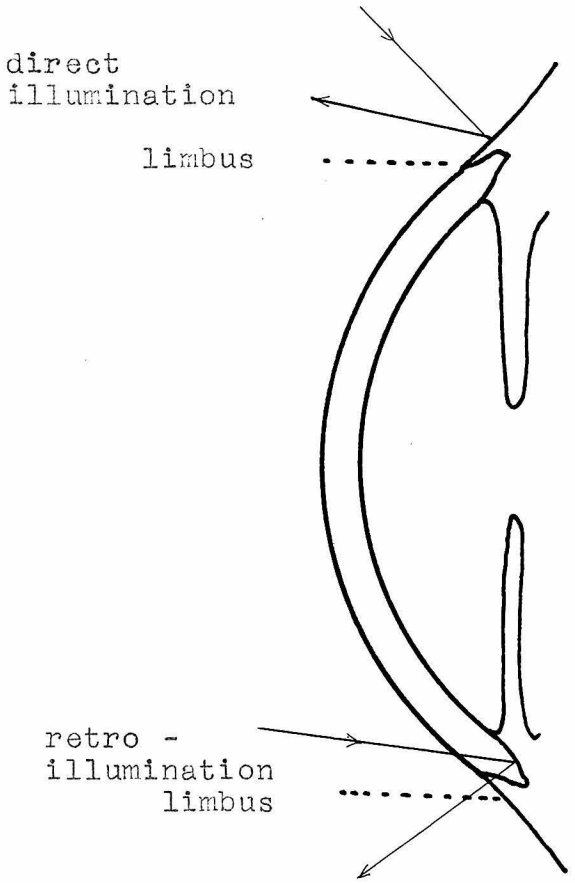


FIGURE 3

Influence of Type of Lighting on the Position of the Limbus

DISCUSSION

In any examination for mild vitamin deficiency the very lack of corroborative symptoms places a greater demand on the specificity of the method employed.

In the original study of Kruse et al (5) all the subjects presented a definite ariboflavinosis syndrome. Since corneal vascularity was found in these subjects and regressed on treatment with riboflavin, it was tacitly assumed that all corneal vascularity was due to riboflavin deficiency. This conclusion was not borne out by subsequent investigation. Many other conditions have been described that give rise to the same sign.

Bessey and Wolback (4) have reported that vitamin A deficient rats show a vascularization of the cornea, and a similar condition has been reported in tryptophane deficient rats by Totter and Day (12) and by Albanese and Buxchke (13). Corneal lesions have been observed in zinc deficiency (14) and in sodium deficiency.(14). They have been observed in poisoning by thallium and on exposure to chemical vapor (9). Sandstead (15) believes that capillary invasion of the cornea may be more commonly the result of exanthenatous diseases as measles. On the other hand, McCreary, Nicholls, and Tisdall (16) believe that ordinary irritants found in every day life - glare, smoke, dust, eyestrain - may be sufficient to maintain a state of vascularity. These authors present photographs which dramatically illustrate the marked engorgment of previously invisible vessels five minutes after irritation by a weak soap solution. Gregory found that the injection of 200 mgs. of nicotinic acid caused a marked circumcorneal injection. The findings of Youmans (17) that vascularity

decreased in winter and increased in summer and that negroes show one tenth the vascularity of whites, point to the possible influence of light on vascularization.

Confirming this lack of specificity is the absence of improvement on therapy which in our present state of knowledge is still the best criterion for determining whether a given sign is typical of a vitamin deficiency. Sandstead found a 80-90% deficiency in 336 persons as judged by the slit lamp technique, but in a controlled experiment on 17 subjects no change was observed on giving 15 mgs. of riboflavin daily for sixty days. 34% of a group of 204 patients studied by Scarborough showed evidence of corneal vascularity. However, 8 patients who were given riboflavin exhibited no change after one month of this therapy. In a study on 41 individuals, McCreary, Nicholls, and Tisdall (16) found no consistent changes in corneal vascularity in either the placebo group or the group given 9.9 mgs. of riboflavin daily for three months. On the other hand a decrease in corneal vascularity on riboflavin therapy has been reported only where subjects showing a definite ariboflavinosis syndrome have been selected for study (5, 6, 11, 18). In these cases regression occurred within a few weeks.

It may be true as Kruse has stated that the reversal of a chronic deficiency state is a very slow process, but if the time required to significantly change the degree of corneal vascularity is beyond the period of one year of therapy employed in this survey, it is doubtful whether that change could be related only to the therapy employed, especially in view of the lack of specificity of this sign. At least a slight trend should have been apparent if there were any relation between

common corneal vascularity and ariboflavinosis, or if only a fraction of the cases were due to ariboflavinosis. The data presented show no change whatever. Even if all corneal vascularity had been brought about by a previous riboflavin deficiency and had remained as scars, this sign would be of no value in the diagnosis of an existing deficiency. In such a case a better relationship might then be established between corneal vascularity and diet, but again no correlation has been found (10, 15, 19, 20).

Some workers (10, 21) believe that there is never any true proliferation of capillaries except in corneal disease, and that the only changes that can be observed are the emptying or filling of preformed vessels with some increase in size as a result of engorgment. Changes of this type can hardly be expected to reflect a specific pathology but rather a non specific irritation or inflamatory condition of which ariboflavinosis is but one case. Conditions that may bring about such a hyperemia have already been presented. The suggestion that everyday irritants as glare, smoke, dust, and eyestrain may maintain a constant state of engorgment of normally present capillaries is more in keeping with the widespread occurrence of this state among normal populations receiving adequate amounts of riboflavin.

Normally the respiratory gases of the normally avascular cornea move by simple diffusion. The vascularization found in riboflavin deficiency has been explained (3, 4) as a response to asphxia brought about by the lack of the oxidative catalyst, riboflavin. That this explanation may not be tenable, even in those cases of vascularity due to ariboflavinosis, is suggested by the work of Philpot and Pirie (22). These workers analyzed the different parts of the eye and found the following average

riboflavin contents in micrograms per gram wet weight of tissue:

corneal epithelium	2.0
substantia propria	0.2
conjunctiva	2.0
aqueous humor	1.0
iris	1.5
lens	0.1
vitreous humor	0.8
meibomian glands	4.0
meibomian secretion	3.5
lachrymal glands	6.5
tears	13.5

These figures indicate that the source of riboflavin in the corneal epithelium is not the pericorneal blood vessels but the meibomian and lachrymal glands. It is suggested that the primary eye lesion is in the eye glands and that secondarily the cornea suffers because of a diminution in the source of flavin. The increased vascularization is assumed to represent an attempt to provide an additional source of riboflavin. More likely, it is merely a response to injury of the epithelial cells.

CONCLUSIONS

Corneal vascularity cannot be considered of any value for the diagnosis of mild riboflavin deficiency since

(1) It is far too extensive in a large population receiving a relatively adequate diet.

(2) There is no correlation between dietary intake and degree of vascularity.

(3) There is no correlation with other symptoms characteristic of riboflavin deficiency.

(4) There is no change on therapy in unselected groups.

(5) The decrease with age is not indicative of a cumulative chronic process.

(6) It may arise from many other causes.

SUMMARY

Six hundred and seventeen white male workers at the Lockheed Aircraft Corporation were examined for corneal vascularity with the biomicroscope and slit lamp. There was no difference in the degree of corneal vascularity between the control subjects and those who had received 10 mg. of riboflavin and other vitamins daily for one year. It was concluded from this and other work that ordinary corneal vascularity is not necessarily due to riboflavin deficiency and is of no value as a diagnostic sign.

TABLE I

TOTAL NUMBER OF HOURS OF ARCADES
(both eyes)

No. of hours	FIRST EXAMINATION				SECOND EXAMINATION			
	Experimental		Control		Experimental		Control	
	No. of cases	per cent of total	No. of cases	per cent of total	No. of cases	per cent of total	No. of cases	per cent of total
0								
1								
2	1	.6						
3								
4					2	1.2	2	1.0
5					1	.6	1	.5
6			1	.5	0		3	1.5
7	1	.6	0	0.	2	1.2	2	1.0
8	1	.6	2	1.0	3	1.7	1	.5
9	2	1.2	5	2.5	2	1.2	3	1.5
10	1	.6	3	1.5	4	2.3	3	1.5
11	2	1.2	4	2.0	5	2.9	5	2.5
12	2	1.2	3	1.5	3	1.7	12	6.0
13	2	1.2	3	1.5	5	2.9	10	5.0
14	4	2.3	10	5.0	9	5.2	3	1.5
15	8	4.7	7	3.5	7	4.0	2	1.0
16	7	4.1	4	2.0	8	4.6	9	4.5
17	11	6.4	10	5.0	14	8.1	14	7.0
18	9	5.2	11	5.5	9	5.2	16	8.0
19	11	6.4	14	7.0	9	5.2	10	5.0
20	13	7.6	12	6.0	13	7.5	15	7.5
21	18	10.5	18	9.5	14	8.1	14	7.0
22	31	18.0	25	12.6	10	5.8	25	12.5
23	28	16.3	35	17.6	26	15.0	21	10.5
24	21	12.2	32	16.1	27	15.6	28	14.1
Total	173	100.3	199	100.5	173	100.0	199	99.6
Mean	19.8		19.6		18.6		18.4	
Sigma	3.5		3.7		4.9		5.0	

TABLE II

MAXIMUM NUMBER OF ARCADES COMMON TO BOTH EYES

No. of Arcades	First Examination				Second Examination			
	number	per cent	number	per cent	number	per cent	number	per cent
	Experimental		Control		Experimental		Control	
0	0	0	0	0	0	0	0	0
1	2	1	11	6	7	4	8	4
2	48	29	58	29	64	39	85	43
3	89	54	91	46	65	39	73	37
4	23	14	36	18	20	12	28	14
5	3	2	2	1	5	3	3	2
6	1	1	0	0	5	3	1	1
Total	166	101	198	100	166	100	198	101
Mean	2.9		2.8		2.8		2.7	
Sigma	0.77		0.93		1.07		0.88	

TABLE III

MAXIMUM NUMBER OF ARCADES
COMMON TO BOTH EYES IN DIFFERENT SECTORS

Number of Arcades	Between the hours of 2-4 and 8-10				Between the hours of 10 - 2				Between the hours of 4 - 8			
	No. of cases		Per cent		No. of cases		Per cent		No. of cases		Per cent	
	Exper-iment	con-trol	exper-iment	con-trol	Exper-iment	con-trol	exper-iment	con-trol	Exper-iment	con-trol	exper-iment	con-trol
0	3	8	3	7	0	0	0	0	0	6	0	5
1	30	37	34	33	2	4	2	4	20	27	23	23
2	35	56	40	49	31	40	36	35	50	61	58	54
3	17	16	20	11	37	44	43	38	16	20	18	18
4	1	0	1	0	7	24	8	21	1	0	1	0
5	1	0	1	0	8	1	9	1	0	0	0	0
6	0	0	0	0	2	0	2	0	0	0	0	0
Total	87	113	99	100	87	113	100	99	87	113	100	100
Mean	1.8	1.6			2.7	2.8			2.0	1.8		

TABLE IV

EXTENSION OF ARCADES FROM LIMBUS

Distance in arbitrary units	Maximum extension between the hours of 2-4 and 8-10				Maximum extension between the hours of 10 - 2				Maximum extension between the hours of 4 - 8			
	Experiment		Control		Experiment		Control		Experiment		Control	
1 mm. =15 units	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
0- 4	5	6	8	7	0	0	0	0	1	1	5	4
5- 9	38	43	62	55	2	2	18	16	28	32	35	31
10-14	41	47	37	33	64	74	68	60	53	61	65	58
15-19	3	3	6	5	20	23	25	22	3	3	8	7
20-24	0	0	0	0	1	1	2	2	1	1	0	0
25-29	0	0	0	0	0	0	0	0	1	1	0	0
Total	87	99	113	100	87	100	113	100	87	99	113	100
Mean	11.5		11.4		14.2		13.5		10.8		10.4	
Sigma	3.3		3.3		2.5		3.3		3.5		3.4	

TABLE V

TOTAL NUMBER OF HOURS OF STREAMER TYPE

(both eyes)

	Experimental		Control	
Hours	Number	Per cent	Number	Per cent
0- 4	45	49	59	55
5- 9	27	29	32	31
10-14	12	13	10	9
15-19	6	7	3	3
20-24	3	3	3	3
Total	93	101	107	101

TABLE VI

TOTAL EXTENSION OF STREAMER TYPE ARCADES

Extension 1 mm. = 15 units	Experimental		Control	
	No. of cases	Per cent of total	No. of cases	Per cent of total
0- 4	44	47	59	55
5- 9	28	30	32	30
10-14	12	13	10	9
15-19	6	7	3	3
20-24	3	3	3	3
Total	93	100	107	100
Mean	6.3		5.4	
Sigma	4.4		4.6	

TABLE VII

EXTENSION OF LAST ARCADE OF STREAMER TYPE

Extension 1 mm.= 15 units	Experimental		Control	
	No. of cases	Per cent of total	No. of cases	Per cent of total
0- 4	29	37	50	58
5- 9	41	53	27	31
10-14	7	9	9	11
15-19	1	1	0	0
Total	78	100	86	100

TABLE VIII

PER CENT OF ARCADES OF STREAMER TYPE

	Experimental		Control	
Percentage Group	No. of cases	Per cent of total	No. of cases	Per cent of total
0-10	8	18	8	15
11-20	10	22	10	18
21-30	2	4	8	15
31-40	8	18	4	7
41-50	4	9	7	13
51-60	2	4	3	5
61-70	6	13	7	13
71-80	1	2	3	5
81-90	1	2	1	2
91-100	3	7	4	7
Total cases	45	99	55	100
Mean	37%		40%	

TABLE IX

TOTAL NUMBER OF STREAMERS
(both eyes)

Number of Streamers	Experimental		Control	
	No. of cases	Per cent of total	No. of cases	Per cent of total
0	56	64	64	62
1	5	6	10	10
2	10	12	8	8
3	5	6	0	0
4	1	1	8	8
5	3	3	7	7
6	5	6	1	1
7	0	0	3	3
8	1	1	1	1
9	1	1		
10				
11			1	
Total	87	100	103	101
Mean	1.2		1.3	

TABLE X

WHITE STREAMERS
(Total of both eyes)

Number of Streamers	Number of cases		Per cent of total	
	Experimental	Control	Experimental	Control
0- 4	58	54	62	50
5- 9	24	33	26	31
10-14	8	8	9	8
15-19	4	6	4	6
20-24		4		4
25-29		1		1
30-34	3	0		0
35-39		1		1
Total	93	107	101	101

TABLE XI

TRUNKS
(Total hours both eyes)

	Number of cases		Per cent of total	
No. of Trunks	Experimental	Control	Experimental	Control
0	355	45	39	41
1	18	19	20	17
2	13	12	14	11
3	4	9	4	8
4	55	9	6	8
5	5	5	6	5
6	6	6	7	5
7	1	3	1	3
8	1	0	1	0
9	1	0	1	0
10	0	0	0	0
11	0	1	0	1
12	1	0	1	0
13	0	0	0	0
14	00	1	0	1
Total	90	110	100	100
Mean	1.9	1.9		

TABLE XII

CHANCE OF VASCULARITY WITH AGE
(Total number of hours both eyes - experimental and control)

LOCKHEED SURVEY

	Age: 20 - 29			Age: 30-39			Age: 40 - 49			Age: 50 - 59			Age: 60 - 69		
No. of hours	Number	per cent	cumulative per cent	Number	per cent	cumulative per cent	Number	per cent	cumulative per cent	Number	per cent	cumulative per cent	Number	per cent	cumulative per cent
1- 3	0	0	0	0	0	0	1	4	4						
4- 6	2	1	1	5	5	. 5	2	7	11						
7- 9	7	3	4	4	4	9	2	7	18						
10-12	13	6	10	14	14	23	5	18	36						
13-15	22	10	20	7	7	30	4	14	50						
16-18	43	19	39	17	17	47	10	36	86						
19-21	52	23	62	23	23	70	2	7	94						
22-24	85	38	100	32	31	10	2	7	100						
Total	224			32			28								
Mean	19			18			14								

TABLE XIII

CHANGE OF VASCULARITY WITH AGE
(Total number of hours both eyes - experimental and control)

LOCKHEED SURVEY

	Age: 20 - 29			Age: 30 - 39			Age: 40 - 49			Age: 50 - 59			Age: 60 - 69		
No. of hours	Number	per cent	cumulative per cent	Number	per cent	cumulative per cent	Number	per cent	cumulative per cent	Number	per cent	cumulative per cent	Number	per cent	cumulative per cent
1- 3							1	3	3	2	6	6	0	0	0
4- 6				5	17	17	5	13	16	2	6	12	2	17	17
7- 9				5	17	34	7	18	34	6	19	31	5	42	59
10-12				3	10	44	5	13	47	5	16	47	0		
13-15				4	13	57	6	16	63	4	13	60	2	17	76
16-18	1	17	17	5	17	74	5	13	76	4	13	73	1	8	84
19-21	4	67	84	5	17	91	6	16	92	6	19	92	2	17	101
22-23	1	17	101	3	10	101	3	8	100	3	9	101	0		
Total	6			30			38			32			12		
Mean	20			15			13			13			11		

TABLE XIV

CHANGE OF VASCULARITY WITH AGE

Ages:	20 - 29	30 - 39	40 - 49	50 - 59	60 - 69
Average both surveys	19	17	13	13	11

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III ROOT BLEEDING

The Candidate wishes to thank Dr. F. W. Went for his direction and guidance in this stimulating problem.

The force within the root which causes an uptake of water from the soil and exudation from the cut stem is known as "root pressure". Many theories have been proposed to explain the mechanism of this uptake of water. The passive forces of osmosis and imbibition are not sufficient to completely account for this phenomenon and there is growing evidence that water uptake is directly controlled by the living cell.

According to Priestly (1920) the nature of the Casparian strip which is formed around the endodermal cells to force the passage of water from the cortical cells to the xylem through protoplasm points to the role of living cells in water transference. The effect of deprivation of oxygen and change of temperature on water uptake led Blackman (1921) to the belief that vital forces were involved. The need for oxygen for bleeding has also been shown by Brewig (1936) and by Grossenbacher (1938). The depression of water uptake by narcotics (Heyl, 1933) and by cyanide (Rosene, 1941 and Van Overbeek, 1941) also emphasizes the relationship of bleeding to respiration. Henderson (1938) has shown that respiration and absorption of water in corn roots run parallel and that energy is expended. The ability to express sap under pressure from a cut stem is decreased when the root is killed. (Kramers, 1932). A definite gradient is established in a living section of Sycamore root with water uptake by the morphologically lower end and exudation at the upper end (James and Baker, 1933). A similar gradient is maintained by the living onion root (Rosene, 1941). These gradients disappear with the death of the root.

Clearly sufficient evidence has been accumulated to indicate that energy provided by respiration is involved in bleeding and that the

rate may be expected to depend on the amount of respiratory metabolite present. It was suggested that the extent of translocation of sucrose from other parts of the plant to the root might be measured by the rate of bleeding. (Went, 1944). This paper is a report of attempts to establish the conditions under which bleeding might be dependent on the sucrose supply.

EXPERIMENTAL

Tomato plants of the San Jose Canner variety which have been extensively studied by Went (1944) were used in this study. These plants were grown in flats in sand, and after three to four weeks were transplanted to four inch pots. They were watered daily with nutrient solution.

The activity of the roots is reflected in the amount of water taken up from the culture solution, in the pressure developed in a manometer attached to the top of the root, or in the rate of exudation of sap from the cut stem. Although these measurements are manifestation of the same phenomena, they are not necessarily proportional. In this study the total amount of exudate in a given period was measured. The stems were cut about two inches above the sand just above the first node, connected to an inverted U tube filled with water by means of a short piece of rubber tubing, and the exudate collected in a vial or graduated tube as shown in figure 1. In all cases the bleeding was conducted at 25° C. and 60-80% humidity.

Sugar was fed to the tomato plants by inserting a leaf top first into sucrose solutions contained in 30 ml. vials supported by small stakes as described by Went and Bonner (1943).

Sucrose was analyzed by the method of Hassid (1938).

The effect of feeding varying amounts of sucrose is recorded in Table 1.

TABLE 1

Plants approximately 24 inches tall; all leaves and stem above second leaf cut off; plants placed in dark room at 18° C. for 24 hours with leaves in sugar as shown in table.

Average exudation of 10 plants in ml.

Treatment	24 hrs.	next 48 hrs.
control	15.8	10.3
1% sucrose 1 leaf	15.1	9.1
1% sucrose 2 leaves	16.6	9.3
2% sucrose 1 leaf	16.1	10.3
2% sucrose 2 leaves	16.1	8.7

There is no significant difference between the groups probably because the sucrose was not limiting. In spite of this the rate of bleeding dropped off rapidly with time.

In the experiments described in table 2, an attempt was made to deplete the amount of stored carbohydrate in the plants by increasing the dark period. Although the rate of bleeding dropped rapidly with the length of the dark period, the increase in bleeding in plants fed sugar over the controls was small.

TABLE 2

Plants 20 inches tall. Average of ten plants in each group. 5% sucrose fed during entire dark period.

Exudation in ml. per 24 hours after varying dark periods

Dark period	control	one leaf in 5% sucrose
0 hrs.	14.8	14.4
24 hrs.	5.1	6.7
48 hrs.	3.2	3.7

In the following experiment (table 3) it was found that removing all but one leaf decreased the bleeding rate over plants that had all leaves during the dark period, but this decrease could not be overcome by feeding sucrose. Lowering the temperature during the dark period apparently decreased respiration of carbohydrate and increased translocation of this material so that plants bled as well with one leaf in this period as the controls at 18° C. with all leaves intact.

TABLE 3

Average of ten plants 15 inches tall. 24 hours dark period.

Effect of temperature and presence of leaves

	Control	one leaf in 5% sucrose
All leaves intact Temperature 18° C.	7.2 ml./24 hrs.	7.4 ml./24 hrs.
1 leaf remaining Temperature 18° C.	4.4	5.5
1 leaf remaining Temperature 7° C.	7.5	8.8

Further experiments were carried out in an attempt to decrease storage material and lower the bleeding of control plants. The most effective method was to expose small plants to high temperature, high humidity, and diffuse light (table 4).

TABLE 4

Plants about 8 inches tall placed in greenhouse at 26.5° C. and 80% humidity for periods shown; then 24 hours in dark room while sucrose was fed. Nine plants in each group.

The effect of light

	Control	5% sucrose
Sunlight 48 hrs.	1.3	2.2
indirect light 48 hrs	0.8	1.7

Analysis for sucrose showed that plants under normal light had adequate amounts of sucrose which could not be greatly increased by the procedure used (table 5).

TABLE 5

Increase of sucrose of roots by feeding leaves

	Control	5% sucrose
Bleeding per root	17.5 ml.	24.2 ml.
Bleeding per gram of root	42.7 ml.	45.1 ml.
Sucrose per gram of root	5.8 mg.	6.6 mg.

In this experiment it was shown that the amount of bleeding was roughly proportional to the weight of the root (figure 3). However, this relationship did not always hold.

Smaller plants in which the stored material was reduced by high temperature and humidity showed little or no sucrose within the limits of the analytical method although the stems of the fed plants did show a slight increase (table 6).

TABLE 6

Tomato plants 7-8 inches tall. 2 days at 26.5° C., 80% humidity

Sucrose content of depleted plants

	Control	5% sucrose
Bleeding per root	2.1 ml./24 hr.	2.4 ml./24 hr.
Bleeding per gram of root	13.5 ml./24 hr.	15.3 ml./24 hr.
Sucrose in roots	0.0 mg/gm	0.0 mg./gm.
Sucrose in stems	0.0 mg/gm	1.5 mg./gm.

Since it was clear that the roots were not getting sufficient sucrose by the procedure employed, an attempt was made to increase the concentration in the roots by infiltrating the leaves in vacuo with the apparatus shown in figure 3. The leaf was placed in a vial in the

usual manner. A split rubber stopper was slipped over the petiole and pushed into the vial. Suction was applied and released. (When properly infiltrated the leaves appeared dark green.) The stopper was then removed and the leaf left in the vial for 24 hours. The data in table 7 show that although the sugar reaching the roots was greatly increased the amount of bleeding was only slightly increased. This infiltration procedure was employed in all subsequent experiments where sucrose was supplied.

It was now evident that sucrose was no longer limiting under these conditions. The finding of Skoog et al (1938) that auxin influences the periodic exudation of Pisum sativum suggested that this substance might now be the limiting factor in these experiments. The data in table 8 show that this is the case. It is seen that the sucrose concentration of the roots is lower when auxin is applied possibly because of increased utilization in the presence of auxin.

TABLE 7

12 inch plants 3 days in darkness. Sucrose treatment during last day. Average of nine plants for bleeding. Average of three roots for sucrose determination.

Increased bleeding by infiltration

	Control	5% sucrose	5% sucrose infiltrated
Average bleeding per root ml./24 hrs.	3.5	3.2	4.3
Average bleeding per gram of root ml./24 hrs.	13	15	18

Increase in sucrose concentration by infiltration

	Control	5% sucrose	5% sucrose infiltrated
Sucrose in stem mg./gm	4	3	40
Sucrose in root mg./gm	2	12	31

TABLE 8

12 to 20 inch plants 4 days at 25° C. and 80% humidity. Leaves infiltrated and auxin applied to roots during a 24-hour dark period.

Effect of auxin on bleeding

	Bleeding first 24 hrs.	second 24 hrs.	Mgs. sucrose per root
Control	1.5	1.2	0.08
Indole acetic acid 10 mgs./ liter	2.1	1.9	0.01
Sucrose 5%	2.8	1.7	0.87
Indole acetic acid plus sucrose	4.2	3.1	0.38

However, the following experiment (table 9) did not show this difference, nor was there any difference in concentration whether auxin was applied during the period that sucrose was fed or just at the time of bleeding.

TABLE 9

10 inch plants 48 hours at 26.5° C. and 80% humidity. In experiment 4 auxin applied to roots while sugar was being absorbed. Bleeding average of 6 plants.

	Bleeding first 24 hrs.	Bleeding next 6 days	Sucrose mg./100 mg. of root
Control	1.7	2.2	0.04
Auxin 10 mg./liter	2.5	5.0	0.12
Sucrose	2.7	2.2	.22
Sucrose plus auxin	4.7	7.4	.24
Sucrose; auxin applied at bleeding time	3.2	6.0	.22
Normal plants; no pretreatment	7.9	5.8	.55

Normal plants of the same lot gave a larger exudate in the first 24 hours than the pretreated plants which had been given auxin and sucrose. This may have been due to the fact that more sucrose was present in the normal plants or to the action of other unknown factors. However, the bleeding of the sucrose plus auxin plants was significantly higher in the second period than the normal plants. From the amounts of bleeding in the second period in both table 8 and 9 it is evident that auxin is more quickly depleted than sucrose and is more likely to become limiting under these conditions. In table 9, for instance, the bleeding of plants given only sucrose was not greater than the controls during the second period, while the bleeding of plants given auxin alone approached the bleeding of plants given both auxin and sucrose.

Van Overbeek (1941) has shown that the active component producing root pressure is reversibly inhibited by cyanide. As determined in this

manner the active component produces about 25 - 50% of the total root pressure. In the present study a more severe poison, sodium arsenite, was employed (table 10) and the inhibition of bleeding was much greater. Since no attempt was made to reverse the inhibition the possibility of destructive changes in the cells or loss of semi-permeability is not ruled out. The fact that small starved plants approximate the low values of exudation in large arsenite treated plants suggest that this low value may be the true measure of the non-active bleeding.

TABLE 10

Tomato plants 12.5 - 15 inches high. 24 hrs. in darkness before bleeding. Average of 12 plants. Sodium arsenite applied to roots.

Effect of M/500 Sodium Arsenite on Bleeding

Pre treatment	Control	Arsenite
None	24.2 ml./24 hrs.	1.9 ml./24 hrs.
8 days 26°C Humidity ~80%	1.3 " "	0.6 " "

DISCUSSION

The problem of the utilization of the rate of exudation for studying translocation has necessitated a study of the factors involved in bleeding. The results obtained so far show that at least two factors are required - a food factor, sucrose, and a hormone factor, auxin. A similar situation holds for the growth of the Avena coleoptile. The region of rapid growth receives auxin from the apical region and the food factor from the basal region (Went, 1928, 1936). Under the conditions of the present study both factors are produced in the leaves under conditions of light and must be translocated to the roots[1].

footnote[1] Van Overbeek (1939) has reported that auxin is produced in isolated tomato roots, but our results indicate that the amounts must be too small to be non-limiting in bleeding experiments.

This depletion may be brought about by placing plants under conditions producing a high rate of respiration, by placing plants in the dark with a resulting decrease in formation of these substances, or finally by topping the plant and thus removing their source. A simultaneous limitation of growth by both auxin and sucrose has also been found by Bonner (1934) and by Schneider (1938) in the Avena coleoptile, and by Fliry (1932) in Helianthus. Normally auxin is not limiting in the growth of tomato plant stems but removal of the tops increases the need for this factor (Went and Bonner, 1943).

It has not yet been possible in our experiments to raise the sucrose concentration of depleted roots to that of untreated controls. This may be the reason that sucrose plus auxin did not increase the rate of bleeding to that of untreated controls. This may also be due to a decrease in other components necessary to bleeding or to irreversible changes brought on by the temporary deficiency. This is again reminiscent of the studies on Avena coeoptiles by Schneider (1938), who showed that a coleoptile section depleted of one factor for any length of time never completely recovered even after the addition of that factor.

The role of salt has not been investigated in this study, but it may be an additional factor involved in bleeding. Its probable importance is suggested in the data presented by Grossenbacher (1938) who showed that the addition of full strength nutrient solution temporarily depressed the pressure developed by Helianthus roots but soon brought about a marked increase in pressure over the low salt control plants. Further studies by Skoog, Broyer, and Grossenbacher (1938) also show a marked influence of salt in the presence of auxin on exudation.

The role of salt in the uptake of water by plant cells is still in dispute. Commoner, Fogel, and Mueller (1943) have shown a marked effect of added potassium in the presence of auxin on the water uptake of potato sections from hypertonic sucrose solutions, and they believe that water uptake may be incidental to auxin directed salt uptake. These experimental results are in agreement with the data of Steward, Stout, and Preston (1940) on potassium induced uptake in potato disks, and Hoagland and Broyer (1942) on water uptake in barley. On the other hand Reinders (1938) and Van Overbeek (1944) have claimed that water is

taken up under the influence of auxin from distilled water alone. The increase in wet weight reported by the latter author was only about 10% and could not be increased by adding salt to the medium. The reason for the discrepency is not clear, but the necessity for salt for any extensive water uptake is not entirely excluded by the distilled water experiments, for it is probable that there was an initial loss of salt from the slices into the distilled water at the beginning of the experiment (Steward, 1932, 1933).

The relationship between salt uptake and water uptake is not clear. It has been stated (Commoner et al, 1943) that the driving force of water uptake is the osmotic pressure of the actively absorbed salt. If this were so, the osmotic pressure of the expressed sap of potato slices should be somewhat greater in the presence of auxin. Van Overbeek (1944) has claimed that this is not the case. The converse view that salt is necessary to maintain the osmotic pressure within the cell after the active uptake of water is also unlikely. The specific role of potassium which stimulates water uptake as compared with calcium which depresses water uptake (Stewart, Stout, and Preston; 1940) argues for a more direct role of potassium than the mere maintainence of osmotic equilibrium. It seems more likely that water absorption and potassium accumulation are to some extent independent of each other but are both reflections of the same respiratory process (Stewart, 1932). The results of Skoog et al. (1938) clearly support this view for the salt concentration in the exudate of auxin treated plants may be greater or smaller than in the controls. It must, nevertheless, be realized that salt concentration in the xylem is not necessarily a measure of the actual amount of

accumulated.(Hoagland and Broyer, 1942).

That auxin activity is influenced by potassium is evidenced in the findings of Kogl, Haagen-Smit, and Erxleben (1933) that activity of auxin decreased sharply on purification and that activity was restored by the addition of KCl. The stimulation of auxin activity by potassium in the growth of the Avena coleoptile has been reported by Alban and Commoner (1941). Still, there remains the possibility that the action of potassium is simply physico-chemical. For instance, it has been shown that potassium shifts the distribution equilibrium of auxin between water and agar (Thimann and Schneider, 1938).

It is interesting to speculate on the mechanism through which a single substance like auxin can play a role in such complex and apparently diverse phenomena as growth, water uptake, protoplasmic streaming, etc. Evidence for the relation between respiration and water uptake has already been presented. A similar relation exists between respiration and growth (Bonner, 1936) and between respiration and protoplasmic streaming (Thimann and Sweeney, 1937). The specific respiratory process affected by auxin in growth (Thimann and Commoner, 1942), protoplasmic streaming (Sweeny, 1941), root number and root length (Sweeney and Thimann, 1942), and salt uptake (Machlis, 1944) is said to be the four carbon acid respiratory system. Recently, Berger and Avery (1943) have presented evidence that it is the alcohol dehydrogenase system that is involved. Further experiments may relate root bleeding to this same respiratory system, and thus further correlate the diverse roles of auxin in plant metabolism with a single energy producing system.

It is very likely that the influence of auxin on growth is through the mechanism of water uptake. Since water can be actively absorbed even by flaccid cells in the presence of auxin (Commoner and Mazia, 1942), no osmotic forces need be postulated for growth. It is not unreasonable to assume that the excess pressure developed by active water (and salt) uptake may extend the cell until the secondary wall is laid down, and by its rigidity prevents further growth. In this connection it has been found that low temperature, by preventing the deposition of secondary walls, allows growth to continue longer than at room temperature (Heyn and Van Overbeek, 1931) (Bonner, 1934). In the light of recent evidence it may be unnecessary to postulate an influence of auxin on wall placticity (Heyn, 1940) or on the lowering of the wall pressure (Van Overbeek, 1944).

Auxin through the control of water movement may act not only in growth but also in non-growing tissue. It has been shown, for example, that auxin applied to the pulvinus of Mimosa will initiate turgor movements (Burkholder and Pratt, 1936).

It has been suggested that root pressure plays a part in refilling the xylem vessels which may become partly blocked with air. If this is so, the value of root pressure to the plant is not in the amount of water moved, which is relatively very small compared to the amount moved by transpiration, but in maintaining a continuous water column which is the very basis for movement by transpiration.

Thus, bleeding is but one example of auxin controlled water relations in the plant.

SUMMARY

The application of root bleeding to the determination of the amount of translocation of sugar has led to a study of the factors limiting this phenomenon.

It has been shown that a respiratory substrate and auxin are involved.

It was possible to deplete tomato plant roots of both of these factors by growing the plants in darkness or at high temperature and humidity. Under these conditions both factors became limiting.

The sucrose concentration of the roots could be raised by infiltrating the leaves, but the values did not reach those of undepleted plants. This may be the reason that roots from treated plants did not bleed as well as roots from normal plants.

The possible relation of salt to water uptake and the role of water uptake to cell elongation is discussed.

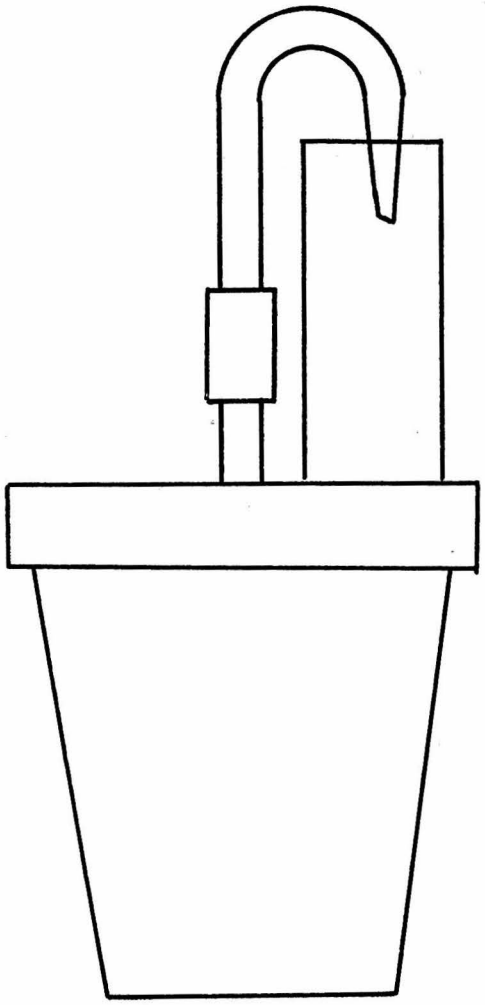


Figure 1

Setup for Measurement of Bleeding

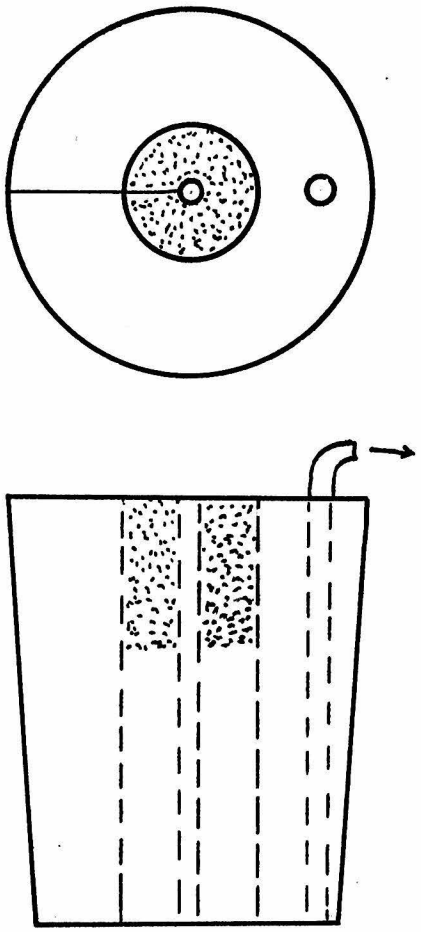
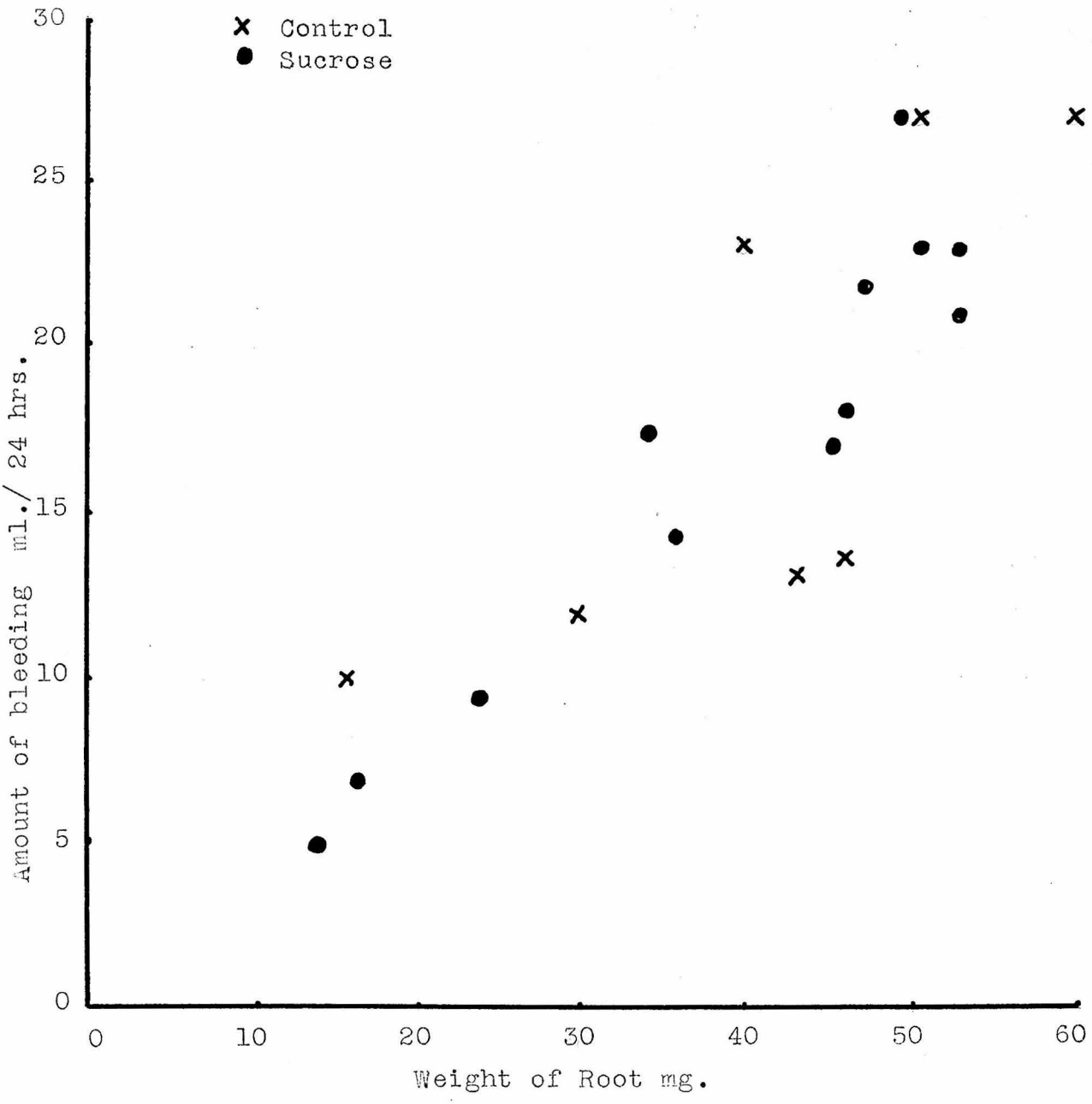


Figure 2

Stopper for Infiltration



RELATIONSHIP BETWEEN BLEEDING AND ROOT WEIGHT

Figure 3

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