XXVIII. THE FORMATION OF CREATINE. EFFECTS ON THE EXCRETION OF CREATINE IN THE BIRD PRODUCED BY PARAFORMALDEHYDE AND HEXAMETHYLENE-TETRAMINE GIVEN SEPARATELY AND COMBINED WITH ARGININE CARBONATE AND OTHER SUBSTANCES.

BY WILLIAM HENRY THOMPSON.

(With an Addendum by EMIL ALPHONSE WERNER.)

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In a series of investigations extending over the past five years, the results of which have been recently published [Thompson, 1917, 1], it has been shown that when arginine is administered to animals a portion of its guanidine nucleus is methylated and either excreted in the urine, or stored in the muscles as creatine, or both. The proportion methylated was greater when the substance was given by hypodermic injection than when added to the food. It was also increased by combining with the arginine (particularly if hypodermically injected) substances containing a methyl group such as methyl citrate and methyl benzoate [Thompson, 1917, 2].

When given with food to dogs on non-meat diet, 2.5 % of methylated guanidine nucleus appeared in the urine: in the case of dogs on meat diet 14.6 %. This latter figure was however admittedly open to question owing to the difficulty of ensuring a constant amount of creatine in the food.

If given hypodermically, dogs on non-meat diet excreted 4.5 % of the guanidine nucleus in the urine, while one dog on a meat diet excreted 4.58 %.

In birds the output of methylated guanidine nucleus when arginine was given with the food, amounted to 1.1 %, if given by hypodermic injection to 2.5 %, of that administered.

Experiments to test the effect of intravenous injection of arginine on the creatine content of rabbit's muscle, showed that there was invariably an increase, which corresponded on the average to a storage in the muscles of

14.5 % of the methylated guanidine nucleus, the variations lying between 8 % and 25 %. At the same time there was an output of the methylated nucleus in the urine secreted in the first three hours after the injection amounting to 0.1 % of the quantity injected.

For the dog the average percentage partition of arginine nitrogen in the urine (a) when given with food, (b) when given by hypodermic injection was as follows:

	Total nitrogen recovered	Urea nitrogen	NH ₃ nitrogen	Amino- acid N	N as total creatinine	N not recovered in the period
(a) Given with food (5 expts.)	56.5 %	34.7 %	13.7 %	2.33 %	3.47 %	43.5 %
(b) Injected	67.87	35.4	4.05	4.7	4.12	32.13

These results amplify and confirm those of previous experiments [Thompson, 1905, 1906].

The main conclusion indicated by these facts is that when a complex substance such as arginine is introduced into the animal body—more especially after reaching the tissues—it is attacked in various ways and dealt with by different agencies.

Many attempts were made to influence the degree of methylation of the guanidine nucleus but in the majority of cases with little obvious effect, so far as the excretion of total creatinine in the urine could be taken as an index. Various circumstances however pointed to the possibility that the presence of formaldehyde in the tissues and the extent to which it is produced, affect the results. Formaldehyde has long been known to be produced by the tissues in plant metabolism. It is also probable that this accounts for the formation of methylated compounds such as betaine, etc., in many vegetables. Betaine has also been shown to be present in the muscle of invertebrate animals whereas creatine is absent. This has been established by Kutscher [1914] for the crab and by D. Wright Wilson [1914, 1, 2] for the scallop and periwinkle. On passing to a stage higher in the animal scale, namely to the lamprey, the latter author was able to isolate both betaine and creatine from the extracts of its muscle. Arginine was also found by these observers to be present in invertebrate muscle. The tissues of invertebrates thus resemble those of plants in the absence of creatine and the presence of betaine. Further the tissues of lower animals contrast with those of the higher in the presence of betaine and the predominance of simple amino-acids in the former, whereas in the latter there is usually an excess of diamino-acids and complex methylated basic extractives such as creatine, carnitine, oblitine, etc.

It seemed therefore probable that formaldehyde, which appears undoubtedly to be produced in the tissues of invertebrates is also formed to some extent in those of higher animals. The presence of formic acid as an excretory product in the urine also lends support to this hypothesis.

Reasoning from these considerations and assuming that under normal conditions the production of formaldehyde in the tissues of higher animals is small, it was inferred that the introduction of formaldehyde combined with arginine would probably result in an increased degree of methylation of its guanidine nucleus, as shown by an increased formation and excretion of creatine.

Preliminary observations were made on two birds to test this hypothesis by noting the effects on the excretion of creatine in the urine. It did not seem advisable however to use formaldehyde as such, owing to its violent action on tissues, either for feeding or hypodermic injection. At the suggestion of my friend Dr Emil Werner, Professor of Applied Chemistry in Trinity College, Dublin (whom I consulted on the possible chemical reactions involved) paraformaldehyde was used instead. This in solution is slowly converted into formaldehyde, and proved very suitable for the experiments. The following is a summary of the results.

TABLE I. *The effects on the excretion of creatine of paraformaldehyde and arginine given with food and injected.*

Animal used	No. of days and quantity given	Creatinine (total) excreted per day in mg.			Effect on weight of animal in g.
		(a) before	(b) during	(c) after	
Duck 8	2 days, arg. 1 g., paraformald. 0.3 g., with food	6.72	15.09	8.21	+ 7
„ 8	1 day, same quantities, with food	8.21	8.00	8.16	+ 40
„ 9	2 days, same	21.61	34.12	31.10	+ 20
„ 9	1 day, same quantities, injected	8.99	50.13 1st day 70.92 2nd „ 21.90 3rd „	8.27	+ 16

Only in two of the observations in which paraformaldehyde was given with food were effects seen on the excretion of total creatinine in the urine. It is to be remarked however that the third case was a “repeat” observation and in such cases as previously pointed out there is usually a decline or actual reversal of the effect on the excretion in the urine. In the two cases where an effect was seen, the increase of total creatinine corresponded to a methylation of 2.2 % of the guanidine nucleus given, being double the amount

observed as stated in previous experiments with birds when arginine alone was given with the food.

Turning now to the experiment in which paraformaldehyde and arginine were given by subcutaneous injection it will be seen that the effects were very pronounced, much more so than in any of the previous experiments with birds. Only one injection was made, the effects of which lasted for three days. During this period there was an average daily excretion of 47.65 mg. as compared with the normal daily average of 8.99 mg. That is to say in the three days there was an excess of total creatinine in the urine amounting to 115.98 mg. over the normal, which represents an excretion in methylated condition of 24.2 % of the guanidine nucleus given. It may be recalled that the injection of arginine alone in previous experiments led to a corresponding excretion of 2.5 % of its guanidine nucleus. It cannot however be taken as definitely proven that the whole of the methylated guanidine nucleus excreted in the recent experiments in excess of the normal, was actually derived from the arginine injected. The results given in the following table seem to indicate the contrary since the injection of paraformaldehyde alone was followed also by a great increase in the excretion of total creatinine.

TABLE II. *The effects on the excretion of creatine of paraformaldehyde alone, given with the food and injected.*

Animal used	No. of days and quantity given	Creatinine (total) excreted per day in mg.			Effect on weight of animal in g.
		(a) before	(b) during	(c) after	
Duck 8	2 days, 0.3 g., with food	8.03	9.92	6.72	+ 25
„ 9	2 days, same	13.74	12.55	15.75	- 50
„ 8	1 day, 0.3 g., injected	11.79	79.36 1st day } 63.42 2nd „ }	10.31	nil

It will be seen that in the two observations in which paraformaldehyde was given with the food there was no appreciable effect produced on the excretion of creatinine, while in the third in which the substance was injected there was a marked increase which lasted for two days. During this period the excess excretion of total creatinine over the normal, amounted to 109.2 mg. This excess cannot however be strictly compared with the corresponding excess seen after the injection of paraformaldehyde and arginine combined, since the two observations were made on different birds. Further experiments are needed to settle beyond doubt whether or not the simultaneous injection of arginine with paraformaldehyde produces a decidedly greater effect on output of total creatinine than the latter alone.

One thing however seems unquestionably to be established, namely that formaldehyde is in part at all events disposed of in the tissues by combination with a guanidine nucleus to form creatine. This is confirmed also by results obtained from the injection of urotropine (hexamethylene-tetramine), a substance which is known to liberate formaldehyde in the tissues. Thus

Three observations were made on the same bird, one in which urotropine and arginine were given with the food, a second in which urotropine alone was similarly given, and a third in which the last-named substance was given by subcutaneous injection. The results are shown in Table III. On examining them it will be seen that in the observations in which the substances were added to the food, urotropine alone produced no appreciable effect; whereas when arginine was given along with it the output of creatinine was more than doubled. When the same quantity of urotropine was *injected* but without arginine, the effect which followed lasted three days and even then had not

TABLE III. *The effects on the excretion of creatine of urotropine (hexamethylene-tetramine) and arginine given with food, and of urotropine alone given with food and injected.*

Animal used	No. of days and quantities given	Creatinine (total) excreted per day in mg.			Effect on weight of animal in g.
		(a) before	(b) during	(c) after	
Duck 9	1 day, urotropine 0.3 g., with food	8.27	10.07	—	- 5
„ 9	1 day, arg. 1 g., urotropine 0.3 g., with food	8.27	18.99	9.83	+ 10
„ 9	1 day, urotropine 0.3 g., injected ...	9.83	13.11 1st day 24.13 2nd „ 20.68 3rd „	12.27	- 20

fully subsided. During this period an excess of 28.43 mg. of creatine over and above the normal (9.83 mg. per day) was excreted. That is to say on the average the output was doubled for three days following a single injection. If the whole of the formaldehyde theoretically liberated from the quantity of hexamethylene-tetramine injected were disposed of by combination with a guanidine nucleus and forthwith excreted, the output of creatine should have been much greater, that is approximately equal to the output after a corresponding injection of paraformaldehyde. Further experiments are needed before an explanation of the difference in the two cases can be assigned.

Three observations were also made on the effects of combining paraformaldehyde with substances other than arginine. The substances used

were glycine, sarcosine and guanidine carbonate, and in all cases they were given with the food. The results are shown in Table IV. On examining them it will be seen that in all cases there was an increased output of total creatinine during the feeding period. The effects are small in comparison with some of those considered in this paper but by no means negligible, the increase in each case being approximately 60 % over the normal.

TABLE IV. *The effects on the excretion of creatine of paraformaldehyde given in the food with other substances than arginine.*

Animal used	No. of days and quantities given	Creatinine (total) excreted per day in mg.			Effect on weight of animal in g.
		(a) before	(b) during	(c) after	
Duck 8	2 days, glycine 1 g., paraformald. 0.3 g. ...	6.94	11.40	—	+20
„ 8	1 day, sarcosine 1.3 g., paraformald. 0.3 g....	6.94	11.79	—	+30
„ 8	2 days, guanidine carb. 0.25 and 0.3 g., paraformald. 0.25 g.	4.67	7.06	6.94	-20

Observations were also made in each case on the output of preformed creatinine but in no case was the effect appreciable.

DISCUSSION.

It remains to suggest a chemical hypothesis by which the foregoing facts might be explained.

It has been pointed out by Neubauer [1910], Dakin [1912, p. 62] and others that in accordance with typical oxidation processes known to occur in the animal body guanidine-acetic acid (glycocylamine) can readily be derived from arginine, the stages being as follows:

It has also been conclusively proved [Jaffé, 1906; Dorner, 1907; Thompson, 1917, 1] that the last-named body is methylated in the tissues with the production of creatine, which is partly stored in the muscles and partly excreted in the urine.

No suggestion has been offered as to how this methylation occurs, but it is not improbable that in the production of creatine from guanidine-acetic acid the methylation arises from the action of formaldehyde. The reaction may provisionally be represented in the following way:

By migration of the CH_3 group which is more likely to occur in the animal body than in vitro, the isomer of creatine,

would become

This hypothesis has the merit that the results obtained from the injection of arginine and paraformaldehyde both singly and combined could be explained on similar lines. It is improbable however that the methylation of the arginine would be deferred till, by the process of oxidation, it had reached the stage of glycoeyamine. It is more likely that methylation and oxidation go hand in hand, the end results being the production of creatine, ammonia, carbon dioxide and water, the major part of the NH_3 and CO_2 appearing in the urine as urea.

Before considering this suggestion further it is necessary to refer to Sørensen's results [1908] on the interaction of formaldehyde and amino-compounds. With neither arginine (free base) nor with guanidine was acidity immediately developed when titration with neutral formaldehyde was carried out. Acidity was however obtained when he used arginine hydrochloride. Sørensen concluded from these results that the amino-group of the arginine reacts as usual with formaldehyde but that the guanidine group on the contrary is unable to fix the aldehyde.

I am indebted to my colleague, Prof. E. Werner, for a critical discussion of Sørensen's results and conclusions in which he states that the results are exactly what he would have predicted but that the inferences are not what he would have drawn. Nor would the acidity in the case of arginine hydrochloride be entirely due to a liberation of HCl but to the production also of formic acid. Werner holds, and I think rightly, that when CH_2O is added

to any compound containing NH_2 in such a complex as occurs in all the amino-acids the result may be expressed in general terms as follows:

In the case of guanidine a methylguanidine¹ is formed which neutralises the formic acid as fast as it is produced, hence the neutrality observed by Sørensen. In the case of arginine free base the guanido-group is methylated and neutral methylated arginine formate is produced. If the arginine be present as a stable salt, this salt is formed by the acid (say HCl) being attached to the guanido-group

while on the "cyclic" or "internal ammonium salt" theory of the structure of amino-acids there would be mutual neutralisation of the NH_2 and COOH end groups as here indicated:

Hence the formic acid produced would remain free, the guanido-group being neutralised by the HCl. This explanation implies that in all cases where acidity is developed by titrating a neutral amino-acid compound with neutral formaldehyde, formic acid is set free, at all events, to some extent. (See Addendum, p. 318.)

Applying these considerations to the interaction of arginine and formaldehyde in the tissues the stages would conceivably be those already suggested (p. 312).

The arginine would thus by the processes of methylation and oxidation pass through the stages of (1) methylene-guanido-butyric acid, (2) methylene-guanido-acetic acid, (3) to an isomer of creatine which by migration of the methyl group becomes ordinary creatine.

The scheme at all events provides a working hypothesis, which will I believe, in the main if not in all its details, prove to be correct. I had hoped to find time before publishing this preliminary account to test on the animal two of the points which could readily be confirmed or the reverse, namely (1) whether the combination of formaldehyde with glycocyamine would increase the methylation of the latter, (2) whether formic acid appears or is increased in the urine as a result of the injections. Unfortunately, however, although I have

¹ A methylene-guanidine formed in accordance with equation (1) would no doubt be itself a strong base and hence no acidity would be developed. In a lesser degree this would also hold for arginine (free base).

waited a year in the hope of finding the necessary time this has not proved possible and the prospect is so distant that I am constrained to publish the investigation in its present incomplete condition. Obviously much has yet to be done for which it is hoped that opportunity will ultimately present itself.

In conclusion I have to express my indebtedness to Dr Werner for his kind criticism and valuable suggestions.

SUMMARY.

1. The administration of arginine and paraformaldehyde with food to ducks was followed by an increase in the excretion of creatine which corresponded to a methylation of 2.2 % of the guanidine nucleus contained in the arginine. Previous experiments with arginine alone, given in the same way led to a corresponding excretion of 1.1 % of the guanidine nucleus. Paraformaldehyde alone produced no effect.

2. Arginine and paraformaldehyde given to the same birds by subcutaneous injection led to an excess excretion of creatine which represented a methylation of 24.2 % of the guanidine nucleus of the arginine. In previous experiments with arginine alone the corresponding amount methylated and excreted was 2.5 % of the guanidine nucleus. Paraformaldehyde injected alone also caused a large excess in the excretion of creatine. It is concluded therefore that formaldehyde is disposed of in animal tissues, in part at least, by combination with a guanidine nucleus to form creatine.

3. Arginine and hexamethylene-tetramine similarly given with food to one of the ducks increased the output of creatine in the urine to double the normal. Hexamethylene-tetramine alone had no effect.

4. Hexamethylene-tetramine given by subcutaneous injection to the same bird, doubled the output of creatine for three days after a single injection. The whole of the formaldehyde theoretically obtainable and presumably liberated in the tissues from the quantity of hexamethylene-tetramine used, cannot have been disposed of by combination with a guanidine nucleus or if so, the creatine formed was not forthwith excreted in the urine.

5. Paraformaldehyde given in the food along with glycine, sarcosine, and guanidine carbonate respectively increased the excretion of creatine in the urine by about 60 % in each case.

6. No appreciable effect was produced by any of the substances on the excretion of preformed creatinine.

7. A chemical explanation is offered to account for the rôle played by formaldehyde in the methylation of the part of the guanidine nucleus of arginine converted into creatine in the animal body.

PROTOCOLS.

Duck VIII. In each case the days marked with letters are those on which the effects appeared, that is to say, the administration of the substance was begun a day earlier than that marked. Lines of space across the page are introduced to mark off the periods.

Date 1916	Preformed creatinine per day in mg.	Total creatinine per day in mg.	Total nitrogen per day in mg.	Weight kilos
June 16	4.24	8.17	0.698	1.495
„ 17	4.11	5.85	0.669	1.495
„ 18	4.16	10.06	0.734	1.540
„ 19 (a)	4.17	12.47	0.718	1.560
„ 20 (a)	2.81	7.37	0.561	1.565
„ 21	2.41	7.31	0.581	1.540
„ 22	2.01	6.14	0.664	1.550
„ 23 (b)	2.91	15.33	0.485	1.560
„ 24 (b)	2.66	14.87	0.595	1.545
„ 25	3.85	9.14	0.598	1.520
„ 26	2.82	7.28	0.660	1.540
„ 27 (c)	2.62	8.00	0.559	1.580
„ 28	3.36	8.16	0.428	1.560
„ 29	2.73	4.67	0.625	1.565
„ 30 (d)	3.13	7.77	0.556	1.550
July 1 (d)	2.79	6.35	0.599	1.545
„ 2	2.42	6.94	0.609	1.580
„ 3 (e)	4.33	10.82	0.819	1.590
„ 4 (e)	3.98	11.98	0.859	1.600
„ 5 (f)	4.06	11.79	0.777	1.630
„ 6 (g)	4.01	79.36	0.985	1.630
„ 7	4.06	63.42	0.708	1.630
„ 8	3.71	13.12	0.736	1.625
„ 9	3.66	7.50	0.681	1.630

(a) Paraformaldehyde 0.3 g. with food.

(b) Paraformaldehyde 0.3 g., arginine carb. 1 g. with food.

(c) Paraformaldehyde 0.5 g., arginine carb. 1 g. with food.

(d) Paraformaldehyde and guanidine carbonate with food, first day 0.25 g. of each, second day 0.25 g. of paraformaldehyde and 0.3 g. guanidine carbonate.

(e) Paraformaldehyde 0.3 g., glycine 1 g. with food.

(f) Paraformaldehyde 0.3 g., sarcosine 1.3 g. with food.

(g) Paraformaldehyde 0.3 g., injected.

Duck IX.

Date 1916	Preformed creatinine per day in mg.	Total creatinine per day in mg.	Total nitrogen per day in g.	Weight kilos
June 10	2.53	13.33	0.387	1.770
„ 11	3.14	14.16	0.386	1.750
„ 12 (a)	3.57	12.35	0.422	1.770
„ 13 (a)	3.49	12.74	0.444	1.740
„ 14	2.97	13.16	0.374	1.720
„ 15	4.03	17.38	0.355	1.725
„ 16	4.08	18.75	0.402	1.725
„ 17	4.25	20.83	0.315	1.720
„ 18	3.82	22.39	0.300	1.700
„ 19 (b)	3.83	28.43	0.567	1.710
„ 20 (b)	2.71	39.80	0.453	1.720
„ 21	4.49	40.78	0.517	1.690
„ 22	4.44	21.43	0.471	1.720
„ 23	2.30	7.78	0.429	1.730
„ 24	3.61	8.05	0.297	1.725
„ 25	3.37	9.93	0.318	1.705
„ 26 (c)	4.16	50.13	0.644	1.750
„ 27	3.75	70.92	0.506	1.725
„ 28	3.25	21.90	0.446	1.740
„ 29	3.26	8.11	0.402	1.750
„ 30	3.97	8.43	0.346	1.760
July 1 (d)	3.45	10.58	0.475	1.750
„ 2 (d)	4.19	9.55	0.470	1.750
„ 3 (e)	5.70	15.69	0.678	1.700
„ 4 (e)	4.39	22.30	0.625	1.760
„ 5	3.76	9.83	0.315	1.750
„ 6 (f)	5.13	13.11	0.567	1.730
„ 7	4.69	24.13	0.645	1.700
„ 8	4.12	20.68	0.465	1.710
„ 9	—	12.06	0.449	1.710
„ 10	4.29	12.48	0.649	1.710

(a) Paraformaldehyde 0.3 g. with food.

(b) Paraformaldehyde 0.3 g., arginine carb. 1 g. with food.

(c) Paraformaldehyde 0.3 g., arginine carb. 1 g. injected.

(d) Hexamethylene-tetramine 0.3 g. with food.

(e) Hexamethylene-tetramine 0.3 g., arginine carb. 1 g. with food.

(f) Hexamethylene-tetramine 0.3 g. injected.

ADDENDUM.

By EMIL ALPHONSE WERNER.

It may be well to point out the following facts, in connexion with equations (1) and (2) given by Prof. Thompson (p. 313), which briefly represent the mechanism of the methylation of amino-compounds by formaldehyde in accordance with the theory recently put forward by the writer [Werner, 1917].

Whilst equation (1) expresses the main change upon which Sørensen's titration method is based; three conditions are necessary for the promotion and completion of equation (2). These are, (a) rise of temperature, (b) the presence of formaldehyde in excess, and (c) the production of a methylene-amino-compound of the type $:C.N:CH_2$, more or less prone to reduction. It is obvious that in Sørensen's method, condition (b) is always fulfilled, whilst (c) will depend upon the nature of the amino-compound under investigation.

It will be seen therefore, that it is not suggested by the theory in question, that equation (2) is completed under the conditions employed by Sørensen, but that it may proceed simultaneously with equation (1) in certain cases seems highly probable.

So far as the interaction of formaldehyde and arginine with the ultimate formation of creatine is concerned, it would appear from a purely chemical point of view, that the scheme put forward by Prof. Thompson gives a very plausible explanation of the series of changes.

It may be fairly assumed that in an animal body the conditions would be favourable for the promotion of equation (2).

In conclusion, it may be pointed out that the velocity of reaction (2) is enhanced by another condition, namely the removal of formic acid as it is produced, either by neutralisation, or by its oxidation to carbon dioxide, and water.

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