XXIII. ON THE PRECURSOR OF CREATINE IN CHICK MUSCLE.

BY DAVID BURNS.

From the Department of Physiology, Glasgow University.

(Received May 16th, 1916.)

In the search for the precursor of creatine and creatinine workers naturally turned to arginine and its disintegration products. According to the theories of Knoop [1910] and Neubauer [1911], arginine is oxidised in the animal organism to guanidine-butyric acid, then to glycocyamine. Glycocyamine is then methylated to form creatine. There is no doubt that from the oxidation of arginine-containing proteins, guanidine-butyric and guanidine-acetic acids and even guanidine itself are obtained [Kutscher, 1901]. The methylation of a compound, *in vivo*, has been carried out successfully by many investigators. It is, therefore, reasonable to presume that the administration of compounds containing a guanidine or methylamino radicle will produce alterations in the creatine-creatinine metabolism.

Glycocyamine. The earliest researches were made by Czernecki [1905] who added quantities of glycocyamine to the diet of rabbits, and thereafter estimated the urinary creatinine. Using the zinc chloride method he obtained, in some instances, an increased excretion. He, however, considered that his results were indecisive.

Jaffé [1906] and his pupil Dorner [1907] observed a transformation to creatinine of from 4.5 to 12.7 % of the glycocyamine given to rabbits by the mouth. They also obtained a considerable increase in the creatine content of muscle. Dorner, too, after a study of the effect of adding glycocyamine to autolysing muscle came to the conclusion that methylation of guanidine-acetic acid to creatine takes place.

Mellanby [1908] arrived at the opposite result. He added glycocyamine in suspension to the diet of chicks on each of five days, and then determined the creatine content of their muscle and also of the muscle of the same number of controls. He found less creatine in the glycocyamine-fed chicks than in the normal.

Recently, Palladin and Wallenburger [1914, 1915] have repeated Dorner's experiments on the addition of guanidine-acetic acid to autolysing muscle. They used rabbit and eel muscle and found a decided production of creatine. In injected rabbits methylation occurred invariably, producing an increase in creatine content of from 21.5 to 35.6 %.

It is unfair to dismiss the question of the possibility of the methylation of glycocyamine or its anhydride, glycocyamidine, in the organism, on the ground that these bases do not occur naturally [Barger, 1914] and that, therefore, the formation of creatine or creatinine from them is not a physiological process. A substance may occupy a very important position in intermediary metabolism and yet be totally undetectable. It may be an absolutely necessary link in a chain of chemical changes but because of the rapidity with which it is transformed into the next substance in series, it may only momentarily exist in a free state.

Arginine. Arginine is the only known constituent of protein which contains the guanidine grouping. It has, therefore, been suggested as a possible parent of creatine and creatinine.

Negative or indecisive results were obtained by Van Hoogenhuyze and Verploegh [1905], Jaffé [1906] and Thompson [1906]. The latter worker found that 73-96 % of the arginine given was excreted as urea. More recently, however, he [1913] was able to demonstrate an increase in the creatine content of the urine after the administration of this amino-acid to dogs and birds.

Positive results are recorded by Inouye [1912], Myers and Fine [1915] and Kutscher [1914]. On the contrary, Baumann and Marker [1915] could detect no increase in total creatinine when arginine was added to hashed muscle.

Kutscher [1914] thinks that the formation of arginine is an intermediate step in the formation of creatine. It may be so rapidly transformed into the latter that detection is impossible, but in the lower organisms, for example, crustacea, the chain of chemical changes may not go so far as creatine and so arginine be found. In mammalian muscle creatine is a constant constituent and arginine unknown, while in the crayfish Kutscher demonstrated the presence of arginine and the absence of creatine.

Other possible precursors. Sarcosine may yield creatine *in vitro* [Volhard 1868, cited by Barger 1914], and Riesser [1913] administered this amino-acid in conjunction with urea, *per os* and subcutaneously, and in half of his experiments got evidence of creatine formation.

Recently [1914] he put forward the hypothesis that creatine was formed as one of the by-products of lipid metabolism. To confirm his theory he studied the influence of injections of choline and of betaine on the creatine content of rabbit muscle, and on the creatinine elimination in urine. Choline produced positive results in four out of seven experiments while betaine gave small increases every time. Methylureido-acetic acid was perfused through isolated limbs by Baumann and Marker [1915] but without result.

Baumann, Hines and Marker [1916] perfused arginine, betaine, methylureido-acetic acid, sarcosine plus urea, and choline plus urea through muscle tissue. Only choline plus urea consistently led to an increase in muscle creatine.

It seems natural to suppose that guanidine itself might be the precursor of creatine or creatinine. It could combine with acetic acid to form glyco-cyammine which could add a methyl group to form creatine, or guanidine might first be methylated and then combine with acetic acid. Guanidine (or methylguanidine) may exist in muscle in two states. It may either form part of the arginine molecule or, as suggested by Jaffé and Otori, it may be the chemical nucleus to which the various peptide chains are attached.

Evidence of the formation of creatine from guanidine or methylguanidine has not as yet been found. Success did not attend the efforts of Jaffé, Dorner, Pommerenig [1901] or Achelis [1906] who administered methylguanidine. Neither did Riesser [1914] who injected both guanidine and methylguanidine obtain any increase in the quantity of urinary creatine.

From a review of the literature on the subject of the origin of muscle creatine and its relationship to urinary creatinine, one must come to the conclusion that adequate facts are wanting on which to base a satisfactory theory of creatine metabolism.

Muscle of chick embryo contains no creatine up to the twelfth day of incubation. Does it contain guanidine?

In 1911, Dr Cathcart suggested to me that a study of the total guanidine content of fertilised eggs at various stages of development might throw some light on the formation of creatine. Mellanby [1908] has shown that fertilised hen's eggs contain no extractable creatine till the twelfth day of incubation, and then a trace only, which, however, increases in amount till the egg has hatched.

Kutscher [1901] suggested that possibly arginine may be the precursor of

creatine. Arginine may be oxidised *in vitro* to γ -guanidine-butyric acid. Through β -oxidation this would yield guanidine-acetic acid. Jaffé, Dorner, and others have demonstrated the methylation of guanidine-acetic acid to creatine.

Arginine	Guanidine- butyric acid	Guanidine- acetic acid	Creatine
NH ₂	NH ₂	NH ₂	NH ₂
$\dot{C} = NH$	$\dot{C} = NH$	$\dot{C} = NH$	$\dot{C} = NH$
$\dot{N}H$	$\dot{N}H$	$\dot{N}H$	$\dot{N} - CH_3$
$\dot{C}H_2$	$\dot{C}H_2$	$\dot{C}H_2$	$\dot{C}H_2$
$\dot{C}H_2$	$\dot{C}H_2$	$\dot{C}OOH$	$\dot{C}OOH$
$\dot{C}H_2$	$\dot{C}H_2$		
$\dot{C}HNH_2$	$\dot{C}OOH$		
$\dot{C}OOH$			

The attempts that have been made to cause a formation of creatine in the body through the administration of arginine, guanidine and methylguanidine have already been considered above.

Method.

The guanidine molecule is very resistant to oxidation. It was noted by Kutscher and his co-workers that a soluble protein, treated with successive small quantities of a boiling solution of a permanganate, yielded a complex, from which most of the products of oxidation had been driven off as volatile aldehydes, ammonia, carbon dioxide, etc. By the use of a suitable precipitant, an insoluble salt of guanidine could be obtained.

Choice of Precipitant. Guanidine is a strong monovalent base forming a series of crystalline salts with acids. Of the salts formed with mineral acids, the nitrate, $CH_5N_3 \cdot HNO_3$, is among the least soluble. It forms large plates melting at 214°.

The picrate, $CH_5N_3 \cdot C_6H_3O_7N_3$, and the picrolonate, $CH_5N_3 \cdot C_{10}H_8O_5N_4$, generally come down as amorphous precipitates, often needing repeated recrystallisation to obtain the characteristic crystals.

Guanidine picrate when pure forms irregular clusters of leaflets (Fig. 4, Plate IV). On slow heating they melt at 311–315°—on rapid heating up to 320°. Emich [1891] states that one part of the guanidine salt is soluble in 2630 parts of water at 9°. It is more soluble in hot water, slightly soluble in alcohol and very slightly soluble in ether. Dilute soda and other alkalis have apparently no effect on it while concentrated acid and alkali dissolve

Fig. 1. Ammonium Picrate. $\times 50$

Fig. 2. Ammonium Picrate from Egg. $\times 50$

Fig. 3. Methylguanidine Picrate. $\times 50$

Fig. 4. Guanidine Picrate. $\times 50$

Fig. 5. Methylguanidine Picrate
from Albumin. $\times 24$

Fig. 6. Sodium Picrate. $\times 24$

it rapidly. On heating on platinum foil it burns with a clear hissing flame leaving a coaly ash. It does not explode on heating or detonate when struck.

To precipitate guanidine from solution as picrate, the solution must not be too concentrated nor must it contain arginine [Kutscher and Otori, 1904].

Choice of Oxidising Agent. Calcium permanganate was chosen as oxidising agent, since the calcium can readily be removed as carbonate by the addition of the necessary quantity of sodium carbonate. After a trial of one or two "makes" of calcium permanganate, Merck's preparation was chosen as being most active and as being practically dry. In all the oxidations recorded below, a 10 % solution of Merck's calcium permanganate was the oxidising agent used.

Tests. Preliminary investigations were made on the guanidine content of gelatin, caseinogen, egg albumin and goose muscle.

(1) *Gelatin.*

Ten grams of commercial sheet gelatin (which gave a slight but distinct xanthoproteic reaction) were dissolved in 250 cc. water, and placed in a long-necked, round-bottomed Jena flask of one litre capacity, and the solution was brought to boiling point over an open flame. The solution of calcium permanganate was added, drop by drop, to this boiling fluid. The first drops produced a violent effervescence, and they were rapidly decolorised. As more permanganate solution was added, the effervescence became less marked, and the surface tension of the boiling fluid became greater, producing large bubbles. The boiling point of the semi-oxidised fluid was also raised. Finally, the surface tension returned to about its normal value, but each drop of the oxidising agent took a much longer time to become reduced.

The process of oxidation was stopped, either (a) when a certain quantity of calcium permanganate had been added, namely, 50 g.: or (b) when the last drop of permanganate was reduced only after prolonged boiling.

It will be seen that the oxidation process is divided into three periods.

(1) *Early stage.* This part is easily carried out and requires only occasional attention, for the purpose of regulating the rate of dropping of permanganate. There is a steady evolution of the aldehydes of the higher fatty acids, ammonia and carbon dioxide. Hydrocyanic acid was detected, and its presence confirmed by chemical means.

(2) *Intermediate stage.* Constant attention was necessary at this stage. Oxidative by-products were formed which raised the surface tension of the

fluid, and these had to be boiled off before another drop of the oxidising medium could be added. There was less smell of aldehyde and more of ammonia among the volatile by-products.

(3) Final stage. This stage presented no great difficulty. Its inception was sudden.

After cooling, the black manganese precipitate was filtered off on a Buchner funnel. The precipitate was transferred to the oxidising flask and lixiviated with about 250–300 cc. of hot water for six to eight hours, and was then cooled and filtered as before. This was repeated a second time. The filtrate and washings were united, evaporated on the steam bath to about 30–50 cc. and allowed to stand for twenty-four hours. The precipitate formed was then filtered off and washed with a small quantity of cold water. The filtrate was completely freed from calcium by the careful addition of a saturated solution of sodium carbonate, excess being avoided. The mixture was gently warmed, allowed to stand for about an hour, and then the calcium carbonate was filtered off. The filtrate which should be about 100 cc. was made up to that volume (approximately), and treated with a saturated solution of pure recrystallised sodium picrate. The guanidine picrate was allowed to stand in the mother liquor for twenty-four hours, filtered off, washed with a small quantity of cold water and then with ether. It was dried at 60° and weighed.

Results.

(a) Complete oxidation of 10 g. gelatin ...	1. 0.777 grams picrate
	2. 0.772 „ „
	3. 0.773 „ „
(b) Oxidation with 50 g. $\text{Ca}(\text{MnO}_4)_2$...	4. 0.401 „ „

Two estimations of nitrogen were made of the picrate obtained from gelatin (2) and (3) (combustion process).

N of (2) 29.3; of (3) 30 %.

The picrate from gelatin (4) yielded 29.09 % of N.

Guanidine picrate contains 29.16 % nitrogen.

The substance did not melt below 280°. It was identified microscopically as guanidine picrate. (See later under “eggs.”)

From the work of Kossel and Kutscher, we know that pure gelatin contains 9 % of arginine. Kutscher and Zickgraf [1903] consider that 10 g. of commercial gelatin correspond to 8 g. of the absolutely dry pure substance. Theoretically, 8 g. of pure gelatin should yield 1.19 g. of guanidine picrate.

The above workers actually got 0.75 g. from 10 g. of commercial sheet gelatin, that is 63 % of the theoretical yield. Our figures are sufficient to show that, under standard conditions, the method used is a comparative one for gelatin.

(2) *Application of the method to substances which are insoluble in water.*

Two methods are available for getting the insoluble protein material into solution, viz.:

I. Digestion with a proteoclastic enzyme.

II. Hydrolysis with a hot mineral acid.

Tests were carried out on commercial gelatin by both methods.

I. (a) 10 g. of gelatin were digested with 250 cc. of a 0.4 % solution of sulphuric acid, to which a small quantity of pepsin was added. The digestion lasted five days at 40°.

I. (b) The same quantity of gelatin was digested for the same time with 250 cc. of a 0.2 % solution of sodium carbonate, to which some pancreatin had been added.

II. The same quantity of gelatin was hydrolysed for nineteen hours with 250 cc. of 25 % sulphuric acid.

I (a) and II were then almost neutralised by the addition of less than the calculated quantity of barium carbonate, and were then oxidised as above, without the removal of the barium sulphate. I (b) was oxidised as it was.

Results.

A. *Complete oxidation.*

Peptic digest ...	0.6348 g. guanidine picrate
Tryptic digest ...	0.23 „ „
Acid hydrolysis	0.834 „ „

B. *Oxidised with 50 g. permanganate.*

Peptic digest ...	0.4446 g. guanidine picrate
Acid hydrolysis	0.4459 „ „

It will be seen that oxidation after acid hydrolysis is productive of a greater yield of guanidine than after the substance has been brought into simple solution with water, or by means of a peptic digest. This is in accordance with the results of Kunze [1910] and Henzerling [1910] who found that an alkylated creatinine gave (on oxidation) a better yield of a substituted guanidine if it had previously been hydrolysed with acid than if it had been digested with pepsin or trypsin or both. Apparently, the enzymic splitting of the molecule differs from that produced by acid hydrolysis.

(3) *Caseinogen.*

Several attempts were made to isolate guanidine from the oxidation products of commercial caseinogen.

Two experiments using a peptic digest, and two using a pancreatin solution, were made. In all cases a paranuclein was formed. Twice, caseinogen (10 g.) was hydrolysed for twenty hours with sulphuric acid and then, after being almost neutralised, was oxidised with calcium permanganate. In no case was any guanidine found.

30 g. of caseinogen were shaken up with 300 cc. of a dilute acetic acid solution and then oxidised direct. No guanidine was isolated.

According to Hart [1901] pure caseinogen contains 4·8 % of arginine and therefore should yield about 1 % guanidine. The commercial commodity probably contains less. It follows that the method detailed above is not suitable for the isolation of small quantities of guanidine from large amounts of material.

(4) *Muscle.*

An attempt was made to determine the guanidine content of goose muscle.

I. 10 g. of minced goose muscle (moist) were digested for seven days at 40° with pepsin and hydrochloric acid, as above.

II. The same quantity of goose muscle was similarly treated with pancreatin solution.

They were both completely oxidised, as above. The peptic digest required almost 20 g. and the pancreatic digest 16 g. of permanganate, for complete oxidation.

Results.

I. Peptic digest of goose muscle gave 1·72 g. of guanidine picrate

II. Tryptic ,, ,, ,, 1·94¹ ,, ,,

That is, moist goose muscle yields from 0·34–0·39 % guanidine.

Nitrogen in the picrate by combustion. I = 29·1 %. II = 24·7 %.

Dr Cathcart took samples of the above muscle, and, by the method described by Brown and Cathcart [1909], estimated the creatine content.

I. Leg muscle, 0·387 % creatine

II. ,, 0·393 ,,

Creatine contains 45·7 % guanidine. That is 0·17 and 0·18 respectively should be subtracted from the guanidine percentage given for muscle in order to obtain a figure for the non-creatine guanidine. Muscle, therefore, contains

¹ This was almost entirely methylguanidine picrate contaminated with a small amount of sodium picrate.

some other source of guanidine than creatine as it yields from 0.17–0.2 % non-creatine guanidine.

(5) *Deaminised protein.*

In the process of auto-deamination albumins are altered beyond the stage of amino-acids. Kutscher [1901] failed to obtain arginine from auto-digested thymus.

Reaction with either nitrous acid, or with an alkali nitrite and acetic acid, is supposed to destroy completely the $\text{CO} \cdot \text{NH}_2$ -group of the protein while it does not alter the protein in such a way as to prevent the biuret reaction. It has been shown that a deaminised protein may be hydrolysed into products containing NH_2 -groups.

In order to ascertain whether deaminised gelatin or caseinogen contained guanidine, 50 g. commercial gelatin were thoroughly shaken on an electric shaker with 500 cc. of 10 % acetic acid. The resultant mixture was transferred to a large basin, and was warmed to less than 40° on a water bath. A 10 % solution of sodium nitrite was added drop by drop to the well stirred liquid, till a drop no longer produced foaming. The precipitate was filtered off, washed with water and then with alcohol, and finally with ether, and dried *in vacuo*.

10 g. at a time of this deaminised gelatin were hydrolysed, and completely oxidised, as above. No guanidine picrate was obtained (two expts.). In the same way, deaminised caseinogen was prepared and oxidised. Guanidine was not obtained.

Therefore, from the gelatin experiments, one may conclude that nitrous acid destroys the guanidine radicle and also the arginine molecule. Gergens and Baumann [1876] and Pommerenig [1901] demonstrated that both guanidine and methylguanidine underwent little or no deamination in the animal body. It is, therefore, probable that guanidine, as such, does not exist in muscle, except perhaps in the merest traces.

(6) *Pure Egg Albumin.*

4 g. of Merck's Egg Albumin (powder) were hydrolysed and oxidised as above.

Results.

- (1) 0.254 g. of the picrate. (Nitrogen 28.7 %.)
- (2) 0.288 " " (" 27.8 %.)
- (3) 0.238 " " (" 27.9 %.)

Microscopic examination showed that the bulk of the yield was methylguanidine picrate. No. (2) contained a very small quantity of sodium picrate.

Guanidine hydrochloride ($\text{CH}_5\text{N}_3\text{HCl} \cdot \text{H}_2\text{O}$) should yield 253 % of the picrate.

0.2 g. of the hydrochloride was boiled up with acid and permanganate as above. 0.512 g. of the picrate was obtained (nitrogen 27.5 %); this contained traces of the precipitant.

0.2 g. of guanidine hydrochloride + 4 g. of egg albumin powder were hydrolysed, neutralised and oxidised as before. Yield, 0.875 g. of the mixed picrates of guanidine and methylguanidine.

(7) *Eggs.*

The fertilised eggs, on arrival, were carefully repacked, and allowed to stand for twenty-four hours. They were then placed in the laboratory incubator kept at 40° , the air being saturated with moisture at that temperature. Each day, at the same hour, the eggs were turned by hand. At certain periods, indicated below, two eggs (Set I only) were removed from the incubator and their weight and the weight of their shell taken. The embryo was carefully examined as to development, and if its age, thus determined, did not agree with that calculated from the number of days spent in the incubator, it was at once rejected. (I am much indebted to Dr W. Abel—Mrs Shaw Dunn—for her kind help in adjudicating ages.) The embryo was then transferred to the oxidising flask and roughly extracted with ether. This removed most of the fatty matter of the yolk (triolein and tristearin), the colouring oil (chromatin) as well as cholesterol and ovine. The resultant matter was hydrolysed with 250 cc. of 25 % sulphuric acid for twenty hours, almost neutralised by the addition of 140 g. of barium carbonate, and oxidised with 50 g. of calcium permanganate, as above. That the results of this tedious process are at least comparative is shown by the similarity of the figure obtained for eggs of the same age. For example, the amount of guanidine in an egg incubated for fourteen days is given (Set I) as 0.47 and 0.44 g. of the picrate. If this is calculated as the amount of the base per 100 g. of egg substance, the figures are even closer, viz.: 0.19 % and 0.188 %. For eggs which had been incubated for about two and a half days, one gets (Set II) 0.24 % and 0.236 % of guanidine. Eggs of different sets are also comparable age for age. From Sets II, III and IV, results may be obtained for eggs of three days' incubation, namely, 0.298 %, 0.3 % and 0.3 % guanidine. The results are tabulated below, and are also presented graphically, Set I in Graph 1, and the others in Graph 2. The first series is not strictly comparable with the later sets, as the process was slightly improved in detail.

It was found to be useless to carry out estimations of the N content

of the picrates as the yields consisted of mixtures of guanidine and methylguanidine. Microscopic analyses had to be taken as the sole criteria of composition, and photomicrographs of various substances met with are given in Plate IV.

Results.

Set I. May, 1911.

Days incubated	Weight of egg g.	Weight of shell g.	Weight of material taken g.	Guanidine picrate g.	% Guanidine picrate	% Guanidine (base)
0	68.7	8.3	60.4	0.1828	0.30	0.06
4	56.6	5.84	50.76	0.231	0.45	0.09
6+	60.9	6.9	54	0.5328	0.98	0.196
12	60.45	6.75	53.7	0.779	1.45	0.29
14	56.9	7.7	49.2	0.4707	0.95	0.19
14	54.35	7.15	47.2	0.44	0.94	0.188
16	59.52	7.75	51.77	0.4295	0.82	0.164

Graph 1. Eggs—Set I

In Sets II, III and IV slight alterations were made in the method of analysis. For instance, the barium sulphate, formed on neutralising the products of acid hydrolysis, was filtered off, and was extracted three times by boiling with water. Further, oxidation was carried out more slowly. Care was taken that as much as possible of the volatile products of oxidation was driven off by boiling before any additional permanganate was dropped in. The same quantity of permanganate was used—50 g. The final liquid was reduced further in bulk, before the precipitation of guanidine by sodium picrate.

Set II. Incubation started March 11, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
20/3/12	48 hrs.	56.6	6.3	50.3	0.6126	1.2	0.24
23/3/12	56 hrs.	59.0	6.95	52.05	0.636	1.2	0.24
"	"	57.4	7.47	49.93	0.5835	1.18	0.236
22/3/12	3 days	57.25	6.05	51.2	0.7656	1.40	0.28
"	"	55.45	6.62	48.83	0.7759	1.5	0.3
21/3/12	4 days	56.27	6.7	49.57	0.9005	1.82	0.364

Set III. Incubation started May 31, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
June 3	16 hrs.	60.05	5.9	54.15	0.4315	0.79	0.16
„ 4	3 days	54.95	5.85	49.1	0.76	1.5	0.3
„ 5	4 „	52.3	6.25	46.05	0.586	1.3	0.26
„ 7	6 „	56.09	6.59	50.05	1.2727	2.5	0.5
„ 10	9 „	53.61	6.4	47.21	1.3593	2.8	0.56
„ 10 ¹	8 „	47.69	5.96	41.73	1.3076	3.1	0.62

¹ June 10, 1912. Age 8, had died the day before removal. Guanidine picrate contaminated with larger reddish crystals of ammonium picrate (Fig. 2, Plate IV).

Set IV. Incubation started June 25, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
July 4	9 days	53.3	6	47.3	1.1603	2.6	0.52
„ 5	3 or 4 days (dead)	53.6	6	47.6	0.6951	1.5	0.3

Graph 2. Sets II, III, IV and V

The incubation of the eggs in the laboratory incubation chamber had to be abandoned as it was difficult to keep the eggs alive more than nine days.

The following was the death rate in the earlier sets.

Set I. 6 died before the ninth day.

„ II. 8 „ „ „

„ III. 7 „ „ „

„ IV. 11 „ „ „

That is, over 66 % of the eggs had to be rejected as undeveloped.

Arrangements were then made with Mr A. B. Templeton, the proprietor of the "Sussex Hatcheries," to forward eggs to the laboratory as required. The eggs were removed from his incubator at 8.30 a.m., placed in a receptacle

designed to retain warmth, and sent by special messenger to the laboratory where they were at once weighed, examined and submitted to acid hydrolysis.

In this series the removal of ether-soluble bodies was omitted to save time. The actual result, however, of the omission was to cause additional trouble. The presence of oily matter should, of course, have kept the surface tension of the fluid low. The oxidation products of fats seem to have the opposite effect. The second stage of the oxidation process was marked by a particularly high surface tension which produced intense frothing. This necessitated careful heating often over several days and so the process was

Set V. Incubated at Sussex Hatcheries.

Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
5 days	58.8	5.915	52.885	1.0128	1.91	0.38
8 "	52.6	4.85	47.75	1.1666	2.44	0.49
9 "	53.3	5.9	47.4	1.1062	2.33	0.466
9 "	49.7	0.0	43.7	1.0123	2.3	0.46
10 "	52.67	6.5	46.17	1.3068	2.83	0.566
10 "	51.5	6.3	45.2	1.1881	2.70	0.54
11 "	53.03	6.01	47.02	1.1744	2.50 (error)	0.50
12 "	49.92	6.32 ¹	43.6	1.2956	2.94	0.59
13 "	49.95	5.6	44.35	1.3013	2.93	0.586
14 "	56.1	6.4	49.7	0.9975	2.01 ¹	0.42
15 "	55.22	5.99	49.23	1.3297	2.70	0.54
16 "	53.25	5.78	47.47	1.3756	2.89	0.58
16 "	45.5	5.25	40.25	0.7832	1.94 ¹	0.39
17 "	50.95	5.99	44.96	1.0692	2.37	0.474
18 "	52.65	6.9	45.75	1.166	2.5	0.5
19.75 "	52.0	6.65	45.35	0.961	2.11	0.42
20 "	49.6	5.7	43.9	1.0691	2.43	0.486
22 "	—	—	39.1	0.9754	2.49	0.498
23.5 "	—	—	34.8	0.8886	2.55	0.51

¹ Accidentally allowed to evaporate to dryness after filtration from Mn. ppt.

considerably lengthened. The volatile products of oxidation were particularly odorous. In addition to hydrocyanic acid and the other bodies already mentioned, butyric aldehyde and acid were noticed.

Great differences were noted in the difficulty of oxidising different eggs. Those at both ends of the series were fairly easy to work with—i.e. up to eight days and after sixteen days—while it was with great difficulty that the eggs in the middle of the series were oxidised.

Egg No. 18—age ten days—was particularly refractory. About half way through the oxidation, that is when about 25 g. of permanganate had been added, a very high surface tension was developed. The last 10 g. of

permanganate were added with great difficulty. Each drop caused a persistent frothing which subsided after prolonged boiling. At times, the oxidising agent was being added at the rate of one drop in thirty minutes.

Before adding sodium picrate, precautions were taken to remove any ammonia present in solution. After the removal of the guanidine picrate formed, the mother liquor was evaporated down and allowed to stand. In most cases, a crop of crystals of the picrates of the mixed methylguanidines, was obtained. On further reduction of volume, ammonium picrate crystallised out in small amount.

The results of Sets II to V are plotted in Graph 2.

Discussion of Results.

The seven eggs in Set I show an increase in total guanidine content from 0.06 % of the unincubated egg to 0.29 % of the twelfth day of incubation. Thereafter, the percentage amount decreases rapidly to the fourteenth day and more slowly to the sixteenth.

The thirty-one eggs forming the other series represent ages from a one day egg to a two and a half day old chick (unfed). The results are plotted on the second graph, and it will be seen that both curves reach their maximum at the same point, namely the twelfth day. There is, as before, a steady increase in the amount of guanidine present from 0.16 % to 0.59 %. The decline after the twelfth day is not as regular as the incline before it. Just before the maximum is reached, there is a slight oscillation in value. On the ninth day, for instance, a slight dip occurs on the curve. If the curve were to remain smooth, there should be 0.56 % guanidine in the egg on that day. The actual figures are 0.5 and 0.46 %. The eleventh day embryo contains much less guanidine than one would expect from a study of the content of the tenth and twelfth day embryos. The filtrate from this guanidine precipitate contained a larger quantity of ammonium picrate than usual. Similarly, the 14 and 16.5 day old eggs are poorer in guanidine than one would naturally expect.

The first question that arises is, what is the cause of the twelfth day maximum?

(1) Mellanby [1908] has shown that, at that stage of development, creatine makes its first appearance as an extractable constituent of egg bioplasm. One might, therefore, consider that guanidine or its methyl derivative is continuously formed till its concentration is about 0.3 % of the

total weight of the egg. At that point, a rupture of the guanidine-containing molecule takes place with the formation of methylguanidine-acetic acid. This formation of creatine, however, does not seem to me to account for the subsequent decrease in the total guanidine content of the egg. The presence of creatine, if synthetic, might increase but in no way could decrease the amount of guanidine. It has been demonstrated that both creatine and its anhydride are readily oxidised, by the process used above, to guanidine and methylguanidine.

(2) Neither can it be that an equilibrium has been established between guanidine and creatine, for then after the twelfth day till hatching, the total guanidine would remain constant.

(3) This would also be the result if the complex containing guanidine ceased to be formed.

Two other possible explanations present themselves.

(4) The configuration of the protein complex may have been altered so that the new complex is more resistant to oxidation.

(5) It has been shown above that enzymes may split the protein complex through the guanidine molecule, so that the product gives a lower yield of guanidine on subsequent oxidation (page 269). From the work detailed above (page 271) on the action of nitrous acid on the guanidine content of gelatin, one concludes that the guanidine group (in arginine) remains free in the linking together of amino-acids to form the protein complex. This is shown in the diagram of the protein molecule given by Plimmer [1913, p. 10]. The position of arginine renders it open to the attack of enzymes, especially trypsin. Something of this nature may take place with the result that the precursor of guanidine which has not been converted into creatine is partially destroyed.

I fail to understand Mellanby's reasoning when he correlates the amount of creatine present in chick embryos of different ages with the development of the liver. There seems to be no striking alteration in either the weight or the activity of this organ on the twelfth day of incubation.

Further Noël Paton has clearly demonstrated the absence of any direct connection between the liver and creatine metabolism in the bird.

Important changes preparatory to hatching take place on the seventeenth to the nineteenth days. This is shown embryologically by the total absorption of the amniotic fluid, the retraction of the intestine and of the yolk-sac into the body cavity and the position of the head. Mellanby's curves—body weight, liver, and creatine—all show a flattening at this stage indicating an arrest in development during the period of rearrangement. The amount of

guanidine also varies irregularly during those days. There is primarily a rise followed by a slight drop. On the twentieth day a steady increase begins, lasting, at least, until two and a half days after hatching.

What importance may be attached to this arrested development and oscillation of the total guanidine content just before hatching can not yet be discussed.

Very little can be said at present of the relative proportions of guanidine and the various methylguanidines obtained from developing eggs. The ease with which methylation takes place has already been alluded to, and one cannot but fear that much of the methyl derivative obtained is artefact.

Before any very decisive opinion could be expressed on the relation of guanidine to creatine formation in the developing egg, further work is necessary. When opportunity offers, a study of the free (extractable) guanidine as apart from that combined in protein or in creatine is to be carried out.

SUMMARY.

1. In the developing chick embryo there is a regular increase of the guanidine content till the twelfth day of incubation.
2. Thereafter there is a marked decrease, followed by a period of oscillation and finally a slight but steady rise.

This research was begun under the tenure of a Carnegie Scholarship, and continued by the aid of an annual grant from the Carnegie Trustees to whom I am much indebted. I am also under a debt of gratitude to Professors Noël Paton and E. P. Cathcart for their helpful guidance and kindly criticism.

REFERENCES.

- Achelis (1906). *Zeitsch. physiol. Chem.*, **50**, 10.
Barger (1914). *The Simpler Natural Bases*, p. 78.
Baumann and Marker (1915). *J. Biol. Chem.*, **22**, 49.
Baumann, Hines and Marker (1916). *J. Biol. Chem.*, **24**, Proc. xxiii.
Brown and Cathcart (1909). *Biochem. J.*, **4**, 420.
Czernecki (1905). *Zeitsch. physiol. Chem.*, **44**, 294.
Dorner (1907). *Zeitsch. physiol. Chem.*, **52**, 225.
Emich (1891). *Monatsh.*, **12**, 23.
Gergens and Baumann (1876). *Pflüger's Arch.*, **12**, 205.
Hart (1901). *Zeitsch. physiol. Chem.*, **33**, 347.
Henzerling (1910). *Arch. Pharm.*, **248**, 604.
van Hoogenhuyze and Verploegh (1905). *Zeitsch. physiol. Chem.*, **16**, 169.
Inouye (1912). *Zeitsch. physiol. Chem.*, **81**, 71.
Jaffé (1906). *Zeitsch. physiol. Chem.*, **48**, 430.
Knoop (1910). *Zeitsch. physiol. Chem.*, **67**, 495.

- Kunze (1910). *Arch. Pharm.*, **248**, 578.
 Kutscher (1901). *Zeitsch. physiol. Chem.*, **32**, 415.
 — (1914). *Zeitsch. Biol.*, **64**, 240.
 Kutscher and Benech (1901). *Zeitsch. physiol. Chem.*, **32**, 278.
 Kutscher and Otori (1904). *Zeitsch. physiol. Chem.*, **43**, 92.
 Kutscher and Zickgraf (1903). *Sitzber. Akad. Berlin*, 1.
 Mellanby (1908). *J. Physiol.*, **36**, 447.
 Myers and Fine (1915). *J. Biol. Chem.*, **21**, 389.
 Neubauer (1911). *Abderhalden's Handlexicon d. Biochemie*, **4**, 386.
 Palladin and Wallenburger (1914). *Bull. Acad. Imp. Sciences Petrograd*, 1427.
 — (1915). *Compt. Rend. Soc. Biol.*, **78**, 111.
 Plimmer (1913). *The Chemical Constitution of the Proteins*. Part II.
 Pommerenig (1901). *Beiträge*, **1**, 561.
 Riesser (1913). *Zeitsch. physiol. Chem.*, **86**, 415.
 — (1914). *Zeitsch. physiol. Chem.*, **90**, 221.
 Thompson (1906). *J. Physiol.*, **33**, 106.
 — (1913). *Inter. Congress of Med.* (Physiol. Section).