Creatin and creatinin / by Edward Mellanby.

[*Reprinted from the Journal of Physiology.*
Vol. XXXVI. No. 6, *February* 28, 1908.]

CREATIN AND CREATININ.

By EDWARD MELLANBY, B.A. *Research Student of Emmanuel College, Cambridge.*

(*From the Physiological Laboratory, Cambridge.*)

CONTENTS.

1. Historical.

The chemical investigation of muscle was started by Liebig[1] in 1847 when he published work describing the effect of fatigue on muscle. He found that muscle work increased its contents of creatin so that those muscles which performed most work contained the most creatin. At this time he performed the classical experiment of the hunted fox, in muscles of which he found ten times the quantity of creatin present in the muscles of the resting animal.

Since Liebig's time many physiologists have worked on creatin in muscle and their results have been widely different. Sarokow[2] found that resting muscle contained twice as much creatin as creatinin and that the result of work was to increase the creatin and at the same time to change some of it to creatinin. It was stated by Sczelkow[3] that tetanus produced an increase, rest a decrease of creatin in muscle,

also that different groups of muscles contained different quantities of creatin. Nawrocki [4] found on the contrary that different muscles of dogs contained the same quantities of creatin, also that in dogs and frogs there was no difference in the resting and tetanised muscle. The results of Voit [5] were again different. He decided that tetanus produced a decrease of creatin in frog's muscle, while the total quantity of creatin and creatinin was not increased. He thought that the creatin was changed during muscular contraction to another substance not identical with creatinin.

Thus far, the work mentioned was performed in the sixties of last century. Of more recent workers on the direct muscle, Monari [6] stands out, and his results have up to the present been regarded with greatest favour. His results will be dealt with in greater detail later. Suffice it to say now that in fatigued dogs he got a large increase of total creatin and creatinin and at the same time a most marked conversion of creatin into creatinin.

Many experimenters have attempted to elucidate the life history of creatin and creatinin in an indirect manner by finding the effect of different bodily conditions on the creatinin output in the urine. Here again results are very variable. In the urine of a dog that had run for eight hours, Voit found no increase of the creatinin. In men, however, the performance of work produced an increased creatinin excretion. Meissner [7] examined the urine of a dog which had run for five hours, and found a decrease in creatinin on the day of exercise, with a corresponding increase the next day. Taken together the exercise produced no increase of creatinin. Working on men, K. B. Hofmann [8] only got an unsteady creatinin excretion having no evident relation with the work performed. P. Grocco [9] found that the creatinin output in men was greater on the day of exercise than on the following day. Moitessier [10] found that the creatinin excreted was always larger on a day of work than on a resting day. Living on a creatinin-free diet Gregor [11] decided that muscular exercise increased the creatinin output. These experimenters and many others used the Neubauer method of estimating creatinin.

Making use of Jaffé's colour test for creatinin, Folin [12] in 1901 introduced a colorimetric method for estimating this substance. The creatinin excreted he regards as of two different modes of production—one part exogenous, which is taken in with the food, and a second portion endogenous produced in the metabolic changes in the life of the cell. He regards the endogenous creatinin as being a measure of true meta-

bolic changes in the tissues, since it is constant in each person and varies almost proportionately as the weight of the individual.

Folin's method of estimation has been widely adopted for creatinin estimation in urine.

Van Hoogenhuyze and Verploegh [13] carried out a large series of experiments to investigate the effect of exercise on nitrogenous metabolism particularly in regard to creatinin. Whereas they found a close relation between the excreted urea and the intaken proteid, the excreted creatinin showed no such agreement. When eating a sufficient diet—creatinin free—strenuous work did not affect the creatinin elimination. In the case of a subject who was doing a fast for 14 days as a public exhibition, on the 10th day performance of muscular work produced a small rise of creatinin excreted. Van Hoogenhuyze and Verploegh therefore came to the conclusion that muscular work only produces an increased elimination of creatinin when "the body is forced to live at the cost of its own tissues." The metabolism of the starving subject seems certainly very much modified from the normal condition, the uric acid and creatinin excreted being very low compared to a person on a creatinin-free diet. Van Hoogenhuyze and Verploegh suggest that the intensity of living is in this case very low, being accompanied at the same time by a low creatinin production.

Working on a dog which was fed on oatmeal and milk Noël Paton [24] found a relation between the creatinin excreted and the intaken proteid. Dorner experimenting on a dog found the addition of fibrin to the food raised the output of creatinin, although flesh freed from creatin did not do so. It is questionable however whether these results of Van Hoogenhuyze and Verploegh, Noël Paton and Dorner indicate that the increased creatinin comes directly from proteid. It no doubt simply implies an increased metabolism, affecting all the organs of the body and therefore that organ responsible for the formation of creatin or creatinin.

Another experimenter, Koch [15], has suggested the hypothesis that creatinin is a measure of metabolic change of the methyl group and that the methyl groups of lecithin and cephalin can all be accounted for by the methyl groups of creatinin excreted. This hypothesis would have to be put to much greater proof than it has up to the present experienced before being accepted, but it is valuable in that it suggests a method of investigating the precursor of creatinin in the body. The methyl group of creatin has received attention from Czernecki, Jaffé and Dorner. The increased excretion of creatin after glyco-

cyamin feeding, obtained by these observers, may indicate that creatin is normally formed by the methylation of glycocyamin.

Recently, work has been done on the urinary creatinin by several workers, among whom may be mentioned Klercker[16] and Closson[17]. Both these men confirm Folin's conclusion that the endogenous creatinin elimination is constant despite varying nitrogenous intake of creatin-free food. Also that the quantity excreted appears to be quantitatively dependent on the body weight.

Considering the amount of research being carried out on creatinin eliminated under various conditions of diet intake and energy output, it was thought well after having tested the colorimetric method of estimating creatin as creatinin, to go to the root of the matter like the earliest researchers and to experiment with the muscle direct.

2. Method of estimation.

At the beginning of this research, the idea was to confirm Folin's colorimetric method of estimation by isolating creatinin from urine and comparing the weight of the isolated substance with that calculated from the colorimetric reading. The necessity of this kind of confirmation was felt because of the belief that a colorimetric estimation of pure creatinin would be quite different from the result of an estimation of urinary creatinin.

An endeavour was made to estimate creatinin in urine by the Neubauer[20] method. This consists of the addition of $Ca(OH)_2$ to urine, evaporating the filtrate and making it up to an 80 per cent. alcoholic solution. To this solution alcoholic zinc chloride is added and the precipitated creatinin-zinc chloride compound is weighed. This method was quickly found to be impossible. The alcoholic solution after standing in a cold place for several days was generally found to contain a great deal of creatinin. In one case as much as ·26 gr. of creatinin-zinc chloride was further precipitated from the filtrates after the application of the Neubauer method. Many other criticisms of this method of estimation might be made but as it now stands generally condemned, even if only because of the amount of controversy which it has brought about, it will be sufficient to say that I feel no confidence whatever in the results of all the older workers who used this method. As for those experimenters who deduced the quantitative relation of creatin to creatinin in muscle using this method of estimation, their results must be quite wrong. In the first place the solutions used are always made

alkaline and secondly, as will be seen later, even where acid and alkali never approach the extractives, mere boiling converts creatin to creatinin.

An attempt was made to improve this method by making the final alcoholic solution as nearly absolute as possible. Creatinin itself is almost insoluble in this strength of alcohol but creatinin hydrochloride is very soluble. A few drops of hydrochloric acid added to the alcohol quite easily dissolve a large quantity of creatinin. The difficulty then was to get creatinin precipitated from such an acid solution as the zinc chloride compound. For the crystallisation of this compound, the liquid must be perfectly neutral and the base added to neutralise the liquid must not have an insoluble chloride. Lithium carbonate was tried, and although the creatinin could then be precipitated by zinc chloride, the solubility of the creatinin-zinc chloride in alcohol was increased by the presence of lithium chloride.

Another effort was made with similar results. The creatinin-hydrochloride-zinc chloride compound is precipitated from strong alcoholic solutions by the addition of sodium acetate as creatinin-zinc chloride. In this case also it was found that the solubility of the creatinin-zinc chloride compound in alcohol was increased by sodium acetate to such an extent that the method would be useless to test the colorimetric method.

Experiments were made to test the reliability of the creatinin estimation on the principle that a double salt is formed by creatinin with mercuric chloride. This salt is obtained by adding to the urine $\frac{1}{20}$ its volume of sodium acetate and $\frac{1}{4}$ its volume of mercuric chloride. The first precipitate formed is composed of organic bodies and urates. This is quickly filtered off and on standing another precipitate containing the creatinin compound develops in the filtrate. This method was also found to be impracticable because (1) the first precipitate contains also creatinin, (2) the final filtrate contains much creatinin.

After the failure of the efforts to get any method of estimating creatinin with sufficient accuracy to be used as a standard, it was decided to test the colorimetric method directly by altering all the conditions to which it was subjected. Such conditions as the presence of other bodies, organic and inorganic, temperature, time relations, quantities of picric acid, caustic soda and creatinin, and the volume of these bodies were all tested.

Organic and Inorganic salts. Colorimetric estimations of creatinin solutions were made to which were added bodies which might be found

in the urine. Such substances as urea, dextrose, sodium chloride, calcium chloride, sodium phosphate acid and normal, were added singly and together to pure solutions of creatinin. The colorimetric readings were never affected when reasonable quantities of these bodies were present. Sodium acid phosphate has a marked action on the developed colour but the quantity required to produce this action is not likely to be found in urine. In the curves of Fig. 1, where the relation of the colour produced to creatinin present in solutions of *A* pure creatinin, *B* muscle extracts, *C* urine, is shown, it will be seen that in the case of concentrated urine there is a little departure from the curve obtained with pure creatinin. This is no doubt due to the other bodies present in the urine.

Temperature. Folin stated that it was necessary to have the water which is added to the reagents at a constant temperature and Van Hoogenhuyze and Verploegh also took this precaution. It is of great importance that the reacting solutions—the creatinin solution, picric acid and caustic soda—should always be used at the same temperature. A difference of two or three degrees temperature in these reacting fluids is sufficient to spoil any result in the experiments on muscle.

Time relations. Although five minutes is the regulation time for allowing the reacting fluids to stand before dilution, a certain amount of latitude is not unsafe. For instance, in one experiment there was no difference in the colorimetric readings when the reacting fluids were allowed to stand three, five and nine minutes. The final colour after dilution fades away in time, but a reading taken within a quarter of an hour after dilution can be made with safety.

Quantities of picric acid and caustic soda. The solutions used are saturated solution of picric acid, of which 15 c.cs. are added, and 5 c.cs. of a 10 per cent. solution of caustic soda. Here again no great accuracy is necessary. The colorimetric reading was the same when 15 as when 12 c.cs. of saturated picric acid were added. In the case of caustic soda, similar readings were obtained when 10 and 3 c.cs. were added to the reacting fluids.

Varying quantities of creatinin. Other experimenters have worked at Folin's recommendation between depths of solution in the colorimeter of 5 to 12 mms. and between these points have regarded the depth of colour as varying directly as the amount of creatinin. It was found cumbrous to have constantly to alter solutions of meat extract until they gave the readings between these numbers. Consequently it was decided to draw curves (cp. Fig. 1) representing the variation of depth

of colour with varying quantities of creatinin so that the amount of creatinin in solution can be immediately read off as soon as the depth of colour has been determined.

The estimation of creatin as creatinin in muscle. It was thought at first that much of the differences in the results of previous workers on creatin in muscle was probably due to the same cause as the differences found[1] in the amount of lactic acid, viz. the improper methods

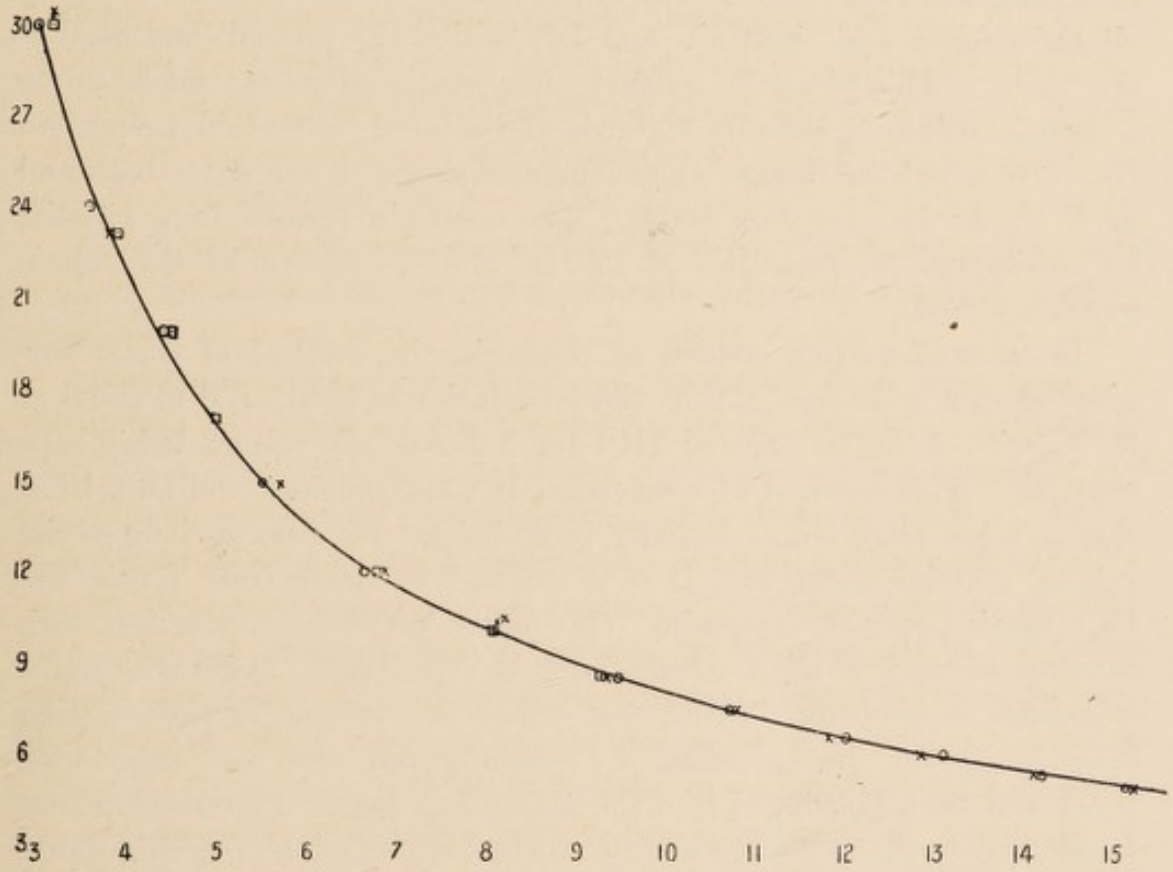

Fig. 1. Curve showing the variation of depth of colorimetric reading with differing quantities of creatinin, in

A. Pure watery solutions. ⊙ points.

B. Meat extract as used throughout the research. ⊡ points.

C. Urine. × points.

Abscissæ represents colorimetric readings in millimetres.

Ordinates represent weight of creatinin in milligrams.

employed in killing the muscle before extracting it. Consequently in all the earlier experiments the muscle was quickly killed after severance from the bone by grinding finely in alcohol. It was however afterwards found that there was no difference in the creatin contents if the muscle were straightway extracted with water. After grinding in alcohol, the

[1] *Vide* Fletcher and Hopkins, *Journ. of Physiol.* xxxv. p. 251. 1907.

alcohol was poured off and a watery extract of the muscle made by adding four lots of water to the muscle and filtering the last two extracts through muslin. The alcoholic and watery extracts were then added together and evaporated down to dryness. To get rid of proteids previous experimenters have boiled the watery extract, but this method was found cumbrous, especially when other colloidal matter in the solution prevented rapid filtration, and it appeared better to evaporate the extracts to dryness and then extract with 75 per cent. alcohol. This dissolved up all the creatin and creatinin and left behind the proteids and most of the salts. This alcoholic extract after evaporation was made up to a certain volume generally 150 c.cs. and filtered; a certain proportion (*i.e.* 100 c.cs.) of the filtrate was evaporated down on the water bath and made up to 50 c.cs. with water. The creatinin, formed from creatin, was estimated in quantities of 10 c.cs. of the solution by the colorimetric method.

In order to convert creatin to creatinin the method of Folin was adopted, that is, 10 c.cs. of the original solution were allowed to stand at 90° C. with 5 c.cs. of normal HCl for 5 hours, the volume being kept constant. This method of conversion is quite efficient and no further change takes place if the heating is prolonged for days so long as the volume remains constant. If it is allowed to concentrate much, the HCl breaks up the creatinin. One other point in connection with colorimetric work on meat extracts must be mentioned. After conversion of creatin to creatinin in the test tubes the volume of the reacting fluids must not be increased by washing out the test tube with water and then pouring it into the measuring flask. The development of colour depends to a large extent on the volume of the reacting fluids, that is, of meat extract, picric acid and caustic soda. It is also necessary to neutralise exactly the acid which has been used to effect the change of creatin to creatinin.

Work was done for the estimation of creatinin on the residues of frog's muscle used by Dr Hopkins and Mr Fletcher after the extraction of lactic acids. The results obtained were very perplexing and after much labour it was eventually discovered that boiling with animal charcoal—a very necessary step in the estimation of lactic acid—in some way removed creatinin. For instance 10 c.cs. of solution containing 14 mgrs. of creatinin after heating on water bath for half an hour with 2 grs. of charcoal contain only 3 mgrs. of creatinin. Whether the charcoal adsorbs the creatinin or not is not known but an effort to recover it by boiling the charcoal with 3 per cent. acid was unsuccessful.

Finally it may be said that great reliance may be placed on the colorimetric method of estimating creatinin so long as the conditions are kept rigidly constant. The results of experiments detailed later in the paper will certify to the efficiency of the method. At the same time great practice is needed in matching the colours accurately, and even when this accuracy is attained there is still great opportunity for the personal equation to assert itself.

3. The stability of creatin in muscle.

(*a*) *The alleged presence of creatinin in muscle.*

The presence of creatinin and its relative proportion to creatin in muscle has been a source of considerable discussion since the time of Liebig.

Heintz [18] regarded creatinin as produced from creatin in the manipulation of the muscle by the action of the acids. Liebig did not agree with Heintz's conclusion since (he stated) a mineral acid equal in concentration to the organic acids of meat could not convert creatin to creatinin even when heated. Sarokow [2] found that resting muscle contained twice as much creatin as creatinin, and that muscular work caused a conversion of creatin to creatinin. Voit [5] found ·0666 per cent. creatinin in frog's muscle, ·0197 per cent. in calf's muscle, and ·0384 per cent. in heart muscle. Contrary to previous observers, Borszezow [19] and Johnstone said that only creatinin was present in muscle but that some is changed to creatin in the manipulation required for isolation of the substance. Monari [6] found ·066 per cent. creatinin in the resting muscle of a dog.

The result of research on this point by the author is that creatinin is quite absent from resting muscle of all animals examined.

As has been already stated the method employed throughout the early work of this research in dealing with the muscle has been first to grind it up finely in 95 per cent. alcohol, and then after having poured off the alcohol a watery extract of the residue has been made; the two extracts being then added together and evaporated down to dryness. Using the colorimetric method in the residue there are found equal quantities of creatin and creatinin. If, however, the alcoholic extract alone be evaporated down and taken up with water no trace of creatinin can be found by the Jaffé colour reaction. If, now, the

requisite quantity of HCl be added to this solution and heated, quite a considerable amount of creatinin can be found. For instance, the alcoholic extract of 45·4 grs. of resting frog's muscle were evaporated down and made up to 30 c.cs. with water. This solution gave no evidence of the presence of creatinin, but when heated with HCl for some hours 60 mgrms. were found to be present.

Neubauer and Nawrocki assert that merely boiling in water changes creatin to creatinin. Monari[6] found that by boiling together concentrated solutions of creatin and potassium acid phosphate, both of which he prepared from muscle, then on neutralising with NH_3, adding $ZnCl_2$ and filtering off the zinc phosphate, he could recognise microscopically the zinc chloride creatinin compound. That is, he found that potassium phosphate converted creatin to creatinin.

In consequence of this, an effort was made to find in muscle a substance soluble in water and insoluble in alcohol capable of converting creatin to creatinin with ease. No such substance could be found, and the whole explanation of the presence of creatinin in watery extracts of muscle, and its absence in alcoholic extracts, depends on the lower temperature and the shorter time required by alcoholic solutions in evaporation.

One other possible explanation, viz. that alcohol might destroy creatinin in muscle, was found not to be the case. For instance, rabbit's muscle allowed to stand a week under alcohol had as much creatin (estimated as creatinin) as muscle estimated direct. Also after dissolving pure creatinin in alcohol, allowing to stand for a week does not affect the colorimetric reading.

From the above considerations it is seen that the absence of creatinin in alcoholic extracts of resting muscle signifies the complete absence of this substance in such muscle.

The presence of creatinin in fatigued muscle must be considered separately because of the agreement of previous workers, particularly Monari, that there is a conversion of creatin to creatinin when muscle does work.

Monari's[6] results confirmed those of Sarokow and Ranke, and his figures are so striking that little doubt was felt as to the truth of the fact that abundant creatinin is present in fatigued muscle. As regards creatin, Monari's experiments give inconsistent results but they always show a considerable increase of creatinin after fatigue.

In order to test Monari's results, I have estimated the amounts of

creatin and creatinin in fatigued rabbit's muscle[1] by the colorimetric method:

(*a*) By directly extracting the muscle with alcohol and water as previously described.

(*b*) After isolating the creatin according to Monari's method and then estimating the creatinin in the filtrate.

Both sciatic nerves of an anæsthetised rabbit were dissected out near the cord and stimulated for two hours, after which the rabbit was bled to death. As regards (*b*) treatment, 175 grs. of muscle were extracted with water and the extract boiled to coagulate proteids. To the filtrate basic lead acetate was added in small excess to precipitate phosphates and the excess of lead got rid of by H_2S. The final solution was evaporated down to a thick brown solution, allowed to stand three days in a cool place, and the precipitate collected on a weighed filter paper. The weight of the precipitate was ·605 gr. This creatin precipitate was dissolved in water, converted to creatinin and estimated colorimetrically and only ·306 gr. creatinin was found. It is evident, therefore, that about half of the first precipitate, supposed by Monari to be almost pure creatin, was composed of bodies other than creatin.

Monari estimated creatinin, after the separation of creatin, by the Neubauer method, but this has been already shown to be unreliable, and it was not proceeded with. Examined colorimetrically the mother liquors contained ·350 gr. creatinin. This creatinin was no doubt formed from the creatin, for in addition to the large amount of boiling necessary for evaporation, the muscle extracts could not be kept neutral owing to their amphoteric reaction.

In the examination of the rabbit's fatigued muscle according to treatment (*a*), no creatinin was found in the alcoholic extract while the total creatin (estimated as creatinin) present was ·43 per cent.

Further, if water extracts of muscles be evaporated down under a vacuum below 40° C., no trace of creatinin can be found in muscle under any circumstances.

From the above consideration, I conclude

(1) That Monari's results are unreliable owing to the opportunity afforded in his technique for the conversion of creatin to creatinin, as well as the impurity of his precipitates.

[1] This experiment was kindly done by Dr H. K. Anderson.

(2) That creatinin is never present in muscle in quantities capable of detection, and that creatin is not converted to creatinin as the result of prolonged work.

(*b*) *The influence of survival and the contraction of muscle on creatin.*

The next set of experiments were performed in order to determine whether isolated survival and prolonged work cause an increase of creatin in muscle. The previous experiments on this point have already been referred to in the historical introduction, when it was seen that, whereas the original workers worked with muscle, more recent attempts have been made to solve the problem indirectly by finding the influence of exercise on urinary creatinin, on the assumption that creatinin excretion is the result of excessive creatin formation in the muscles. The following experiments were made on frogs and rabbits.

The technique followed was that of Hopkins and Fletcher[38]. After being pithed and skinned the frogs are divided in the lower part of the abdomen so that the legs are separated from the body. The legs of the frogs to be stimulated are weighed in tens. The legs are then hooked on to each other, the hooks passing through each leg below the gastrocnemius, and the long strings of the legs of ten frogs are allowed to hang from a bar. A tetanising current is passed through the strings of legs which are made to lift weights of 100 grs. By means of an automatic arrangement the current is allowed to pass through the muscle for five minutes and then ceases for ten minutes and so on. When one lot of legs has been sufficiently stimulated, the muscles are carefully dissected off and the bones weighed, thus giving the exact amount of muscle ground up in alcohol. The estimation of creatin as creatinin is then proceeded with as previously described.

Exp. 1.	Weights of muscle	Total creatinin	
A	45·4 grs.	·255 p.c.	Control.
B	32·9	·260	Stimulated in O_2 for 3 hours.
C	39·0	·256	Stimulated in O_2 for 11 hours.
D	45·9	·257	Stimulated in N_2 for 5 hours.
E	35·6	·255	Stimulated in N_2 for 5½ hours.

In the following experiment 40 frogs were used. It being evident that whatever changes creatin underwent on stimulation were very small, it was thought well to have two control lots of muscle.

Exp. 2.	Weights of muscle	Total creatinin	
A	36 grs.	·250 p.c.	Control.
B	30·6	·240	Control.
C	30	·258	Stimulated in O_2 for 6 hrs. 40 mins.
D	29·1	·255	Stimulated in O_2 for 11 hrs. 45 mins.

Temp. 18° C.

At the same time as above an experiment was made to see if any development of creatinin could be observed when the frog's muscles simply survived in oxygen.

Exp. 3.				
	E	34·1 grs.	·256 p.c.	In oxygen for 7 hours. Oxygen bath at 18° C.
	F	26·1	·266	In oxygen for 28 hours.

Three lots of ten muscles were stimulated for an hour—three mins. stimulus and two mins. rest. Two of these three lots were then placed in oxygen for some time. Temperature throughout experiment 18°.

Exp. 4.	Muscle weights	Creatinin estimated	
A	36·2 grs.	·257 p.c.	Control.
B	30·2	·267	Stimulus 1 hour and then ground up.
C	34·0	·264	Stimulus 1 hour. 9 hours 40 mins. in oxygen at 18°.
D	26·6	·261	Stimulus 1 hour. In oxygen for 23 hours at 18°. The gastrocnemii only irritable when taken out.

Exp. 5. Two lots of muscles were again allowed to remain in oxygen.

	Weights of muscle	Creatinin estimated	
A	36·2 grs.	·257 p.c.	Control.
B	32·2	·258	In oxygen at 18° for 11 hours.
C	34·1	·254	In oxygen 25½ hours. Muscles quite fresh at end of Exp.

In the above experiments the creatin production may have been too small to be detected. In order, therefore, to afford every opportunity for the detection of an increase of creatin in muscle, the following experiment, with frog's muscle, was performed later in the year during cold weather.

Exp. 6. Dec. 5, 1906. The legs of 50 frogs were allowed to survive three days at 9° C. in tubes through which oxygen was passed. 20 frogs' legs were used as a control and estimated direct. The legs were divided into batches of 10, and the amount of total creatinin obtained as follows:

	Weight of muscle ground up	Creatinin estimated (from creatin)	
A	37·5 grs.	·235 p.c.	Three days survival in oxygen.
B	34·6	·239	
C	47·8	·220	
D	42·3	·222	
E	45·2	·217	
F	43·0	·222	Control muscles.
G	37·3	·235	

The muscles were quite irritable after the three days survival.

The amounts of creatinin estimated in these frogs are low. Possibly the time of year influences the creatin in the muscle to some extent.

The experiments on rabbits differ from the experiments on frogs, in that here the blood supply to the muscles was intact during the time of stimulation, while with the frogs the legs were isolated.

Exp. 7[1]. Rabbit. The sciatic nerve on one side was dissected out as near the cord as possible, cut and the peripheral end stimulated discontinuously for 1½ hours: the first hour the nerve was stimulated for 10 minutes and allowed to rest 5 minutes. After this, stimulation last 3 minutes and rest 1 minute. The creatin contents of the resting and stimulated limbs were compared.

	Weights of muscle	Creatinin found	
A	95·9 grs.	·448 p.c.	Control (leg muscle).
B	26·5	·435	Control (back muscle).
C	109·7	·436	Stimulated muscle.

Exp. 8[1]. Rabbit. Both sciatic nerves were cut and the peripheral ends stimulated for two hours[2].

The creatinin (from creatin) estimated in a portion directly by the colorimetric method = ·430 per cent. Control resting muscle = ·440 per cent.

It is clear from these two experiments on the intact muscle of rabbits that great muscular contraction does not influence the creatin content of mammalian muscle.

From a general consideration of all the above results it is seen:

(1) That the performance of muscular work leaves creatin unaffected.

(2) That the survival of frog's isolated muscle, even for three days, does not increase the creatin content of muscle.

[1] These experiments were kindly done by Mr V. J. Woolley of King's College, Cambridge, and Dr H. K. Anderson, respectively.

[2] The observations on the absence of creatinin in fatigued muscle given on page 457 were made on this rabbit.

(*c*) *The influence of autolysing tissues on creatin and creatinin.*

Schmidt-Nielsen [27] found an increase of xanthin bases and amino-acids in the autolysis of the muscle of fish, but it is only quite recently that any work has been done to discover how creatin and creatinin behave during autolysis.

Seemann [33] endeavoured to prove that the autolytic breakdown of muscle results in an increase in the creatinin content. Not only so, but if gelatine is added to the autolysing muscle, he found a still further increase of creatinin. Seemann's figures, however, are both scanty and unsatisfactory. He reverted to the older unsatisfactory methods of estimation. It may be stated here that the colorimetric method of estimation Seemann describes as unworkable, owing to the rapid disappearance of colour, but, so far as I know, this is the first time the colorimetric method has been quite condemned by one working with it. Whatever value Seemann's theory of the origin of creatinin may have, no great weight can be attached to his experimental facts. Before the publication of his results, work had already been done by the author on autolysing muscles with quite negative results. In order to prevent the adverse effects of antiseptic bodies, such as chloroform and toluol, efforts were made to cut out muscle aseptically. In many cases the experiments failed, for the muscle became septic in a few days after standing at a temperature of 37° C. Wherever the muscle became thoroughly septic, all the creatin in the muscle entirely disappeared.

Exp. 1. 48·3 grs. of the leg muscle of a full grown rabbit were cut off aseptically and allowed to stand two days at 21° C. and two days at 28° C. On the third day a slight mould was evident and on grinding up the muscle did not smell quite fresh.

Total creatinin estimated	·383 p.c.
Control muscle	·430

The decrease on autolysis was no doubt due to the small amount of bacterial action which had gone on.

Exp. 2. In this experiment the muscle stood five days at 37° C. and remained absolutely aseptic until the end.

64·5 grs. of the back muscle of a rabbit were autolysed and the creatinin estimated colorimetrically was ·403 per cent. Control muscle contained ·390 per cent.

In an alcoholic extract of the autolysed muscle no creatinin could be detected. It will be seen from the above experiments, that in autolytic experiments where every opportunity for complete breakdown has been offered, no change in the creatin content of the muscle can be detected. In both experiments the breakdown of the tissue was very

complete. The slightly unsatisfactory difference in the results is explained by the fact that it is quite impossible to deprive the muscle of fibrous tissue in aseptic autolytic experiments.

Antiseptic autolysis. Autolytic action in the presence of antiseptic bodies must of necessity be different from aseptic changes. It is doubtful, however, whether this difference extends beyond time relations. It has been abundantly proved that changes which take place in aseptic tissues after a few days will take weeks in the presence of antiseptic substances. The effect of bacterial action, however, is so great in the case of creatin, that the following experiments were done in the presence of chloroform water.

Exp. 3. Leg muscle of a rabbit was allowed to autolyse for three weeks at 30° C. The muscle at the end of this time was well broken up. Creatinin estimated in autolysed muscle ·438 per cent. Control (1) ·441 per cent., (2) ·437 per cent.

Exp. 4. 53·8 grs. of rabbit's muscle were allowed to stand under Ringer's solution at 37° C. for 21 days. The tissue at the end of this time was well broken up, there being a great deal of crystalline substances resulting from the change. Creatinin estimated in autolysed muscle ·380 per cent. Control ·382 per cent.

In order to see whether creatin had been changed to creatinin the first extract was evaporated down below 50° C. No creatinin could be detected either by the Jaffé or Weyl reaction.

These experiments were regarded as conclusive that autolytic action (antiseptic and aseptic) left creatin in muscle untouched, and consequently the subject was not proceeded with until Gottlieb and Stangassinger[29] published the results of numerous experiments which seemed to prove conclusively that there are throughout the animal kingdom many ferments which have a marked action on creatin and creatinin. The following facts were claimed as established by these workers: (1) By the autolysis of muscles and other organs, creatin is produced. (2) Creatin is changed in part to creatinin in autolysis by a ferment. (3) Creatin and creatinin in advanced autolysis are destroyed by ferments (creatase and creatininase). (4) That the very complex curves representing the amounts of creatin and creatinin in autolysing experiments can be explained by the action of the various ferments resulting in creatin formation, and change to creatinin and the destruction of both bodies. (5) The above ferment actions also take place in the urine.

The number of ferments required by Gottlieb and Stangassinger to explain their results are almost bewildering, and it was thought necessary to repeat some of their experiments, especially as previous

experiments on the autolysis of muscle had failed to demonstrate any ferment capable of affecting creatin.

Before describing further experiments it may be pointed out that there is one source of error which may at least explain some of Gottlieb's and Stangassinger's results. It has already been shown how very easily creatin is changed to creatinin on raising the temperature to 100° C. These experimenters seemed to have been aware of this fact, and yet in their experiments they evaporate to dryness on the water bath. Such a procedure is fatal where the object is to estimate preformed creatinin. However, it must be admitted that it would require much more criticism than this to explain away all their results.

I have made some observations to determine whether the kidney and liver contain ferments capable of influencing creatin or creatinin.

Exp. 1. The liver of a guinea-pig which had been killed by chloroform was ground up with sand, extracted with Ringer's solution, allowed to stand half an hour and centrifuged. Weight of liver = 20 grs.

	Liver extract	Creatin and creatinin solution	Ringer solution
A	50 c.cs.	20 c.cs.	0
B	50	20	0
C	—	20	50 c.cs.

A little toluol was added to each.

The flasks A, B and C were allowed to stand 66 hours at 37° C. Proteids were precipitated by 60 per cent. alcohol. Liquids filtered and evaporated on water bath at 37° C. The three residues were taken up with 100 c.cs. of water and the preformed and total creatinin estimated.

	Preformed creatinin	Total creatinin (*i.e.* creatin and creatinin)
A	135 mgrs.	270 mgrs.
B	135	270
C	132	264

It will be seen that there has been no change whatever either to the creatin or creatinin, under the influence of extract of guinea-pig's liver.

Exp. 2. Rabbit eight weeks old. The liver was ground up with Ringer's solution immediately on killing, and then allowed to stand half an hour, and the liquid poured off and roughly filtered.

	Creatin	Creatinin	Ringer	Muscle	Liver extract
A	25 c.cs.	0	25 c.cs.	0	0
B	25	0	25	0	30 c.cs.
C	0	10 c.cs.	50	0	0
D	0	10	50	about 10 grs.	0
E	0	10	50	0	30
F	0	0	50	12·1	0

Toluol was added to each.

The above were allowed to stand with toluol, in water bath at 37° C. for 48 hours. Proteids as before were got rid of by 60 per cent. alcohol and in evaporation the temperature was kept below 50° C. The following quantities of creatin and creatinin were estimated :

	Creatin (estimated as creatinin)	Creatinin
A	82 mgrs.	—
B	81	—
C	—	31·5 mgrs.
D	—	31·25
E	—	31·25
F	—	no creatinin

From these results it is apparent that the creatin and creatinin are unaffected by extracts of rabbit's liver and muscle.

It seemed possible that the absence of results confirmatory of Gottlieb and Stangassinger might be due to the fact that most of their experiments were done on cats and dogs. It has been shown by various experimenters how very variable is the susceptibility of uric acid to the tissues of different animals. For instance Wiener [35] has shown that extracts of the livers of dogs and pigs can destroy uric acid while the livers of herbivorous animals increase the quantity rather than destroy it. In order to avoid this possible source of difference the following experiment was done.

Exp. 3. Cat. The liver was cut out immediately after death and ground up with ice-cold Ringer solution. After standing for half an hour, the liquid extract was added to solutions of creatin as follows :

	Pure creatin solution	Ringer	Extract of liver
A	10 c.cs.	50	0
B	10	25	25
C	10	25	25

The above solutions were allowed to stand for one week at 37° C. with toluol. The following amounts of creatinin were estimated after conversion of the creatin :

A. 35·5 mgrs. B. 35 mgrs. C. 34 mgrs.

No creatinin could be found before conversion of the creatin, *i.e.* in the extract after evaporation below 50° C.

Creatin is therefore uninfluenced by an extract of cat's liver.

Exp. 4. Hedgehog. The liver was ground up with Ringer's solution and added to solutions of creatinin as below :

	Creatinin	Liver extract
A	5 c.cs.	50 c.cs.
B	5	50
C	5	50

C was first boiled. A little toluol was added to each and they were then allowed to stand at 37° C. for three days. At the end of this time the proteids were precipitated by

60 per cent. alcohol, filtered, and the filtrate evaporated down and made up to 50 c.cs. in water. The following average readings on colorimeter for 10 c.cs. of solution were obtained:

A	B	C
7·6 mms.	7·6 mms.	7·5 mms.

It will be seen that creatinin has undergone no change.

Further experiments of a qualitative nature were performed, in which mixtures of muscle and liver, also muscle and kidney of cats, were allowed to autolyse under antiseptic conditions. Such experiments were chosen because it was thought possible that if creatin is normally changed to creatinin before excretion, then this change might be regulated in a similar manner to the glycolytic action obtained with mixtures of muscle and pancreas (Cohnheim). No creatinin could ever be found in these experiments after the mixed tissues had undergone considerable autolytic action.

From the results of the above experiments on the autolysing tissues of rabbits, cat, guinea-pig and hedgehog, it is seen that Gottlieb and Stangassinger's statement that tissues contain numerous ferments affecting creatin and creatinin can be in no single respect confirmed. In fact in all autolysis experiments kept rigorously clear of bacterial action and where precautions were taken to prevent the conversion of creatin to creatinin by heating, creatin and creatinin have remained unaffected.

The outstanding fact of the foregoing examination of muscle is the stability of muscle creatin. This stability has remained unaffected by any activity such as muscular contraction, isolated survival and autolysis (aseptic and antiseptic).

4. The effect of glycocyamin feeding on the creatin of chickens' muscle.

Czernecki[34] found an increased excretion of creatinin in the urine of a rabbit after feeding it with glycocyamin. Jaffé[30] and Dorner[28] have also found considerable amounts of creatin excreted by rabbits fed with the salts of glycocyamin, although normally little or no creatin is present in the urine. From these observations, it has been assumed that glycocyamin is methylated in the body to creatin in the same manner as are tellurous acid, xanthine, caffein and pyridine. Against this assumption the following criticism is offered.

1. In addition to a modified Neubauer method for estimating creatin, Jaffé decolourised his solutions with animal charcoal. This has been

shown to remove creatinin from solutions in a manner impossible to control.

2. As Dorner has shown, the excretion of creatin in rabbits is easily produced by abnormal conditions. When starving they excrete considerable quantities. The ill effects produced on rabbits by the glycocyamin feeding taken in conjunction with the creatin excretion in cancerous patients (*vide* infra) make it possible that the creatin excreted after glycocyamin feeding is the result of a direct muscle breakdown following on a toxic condition of the blood, rather than due to methylation of glycocyamin.

Dorner also got increased quantities of creatin by adding glycocyamin to autolysing muscle. His results are inconstant and not convincing. Jaffé found an increase of creatin in the muscles of the rabbits that had been fed on glycocyamin. This is an important point, for whereas the methylation of glycocyamin and its direct excretion as creatin might be regarded as physiological only in so far as it is a normal method of rendering poisonous substances innocuous, any increase of creatin in muscle that it produces must be regarded as of greater significance, for then the question whether it is the normal precursor of creatin in the body must be considered. Jaffé does not mention whether in these later experiments he decolourised with charcoal. If he did so, the additional errors attached to the estimation of creatin by crystallisation from muscle extracts raise considerable doubt as to the trustworthiness of his results.

It seemed to me that if glycocyamin increases the amount of creatin in muscle, this would be more marked in young than in adult animals. The following experiments were accordingly performed.

Of 18 chickens (born July 5th), 12 were given four grams of glycocyamin in addition to their normal diet during five days, *i.e.* July 14th to 18th. They were all killed July 19th and the creatin in their muscle estimated in the usual way previously described. The 12 glycocyamin fed chicks were divided into two batches, A and B.

The following results were obtained:

	Weight of muscle ground up	Percentage creatinin estimated (from creatin)
A. 6 Glycocyamin-fed chicks	25·0 grs.	·314 p.c.
B. 6 „ „	29·8	·284
C. 6 control chicks	33·1	·285

The result of batch A is certainly high, but considering individual differences in chickens at this age, it is almost certain that glycocyamin

feeding has no effect on muscle[1]. Experiments to be mentioned later show, however, that the muscles of chicks at this age have almost attained their maximal percentage of creatin, so that it is possible that glycocyamin might have an effect at an earlier age before the maximum is reached.

5. The effect of feeding with creatin and creatinin on the percentage of creatin in muscle.

As a result of feeding experiments with creatin Folin [26] has shown that under certain conditions creatin appears to be stored in the body. On a diet of high nitrogen value, nearly all the creatin was excreted as such, while with deficient food nitrogen little or none appeared in the urine, and at the same time the unchanged nitrogen excretion pointed to its being stored. These observations make it probable that the creatin under these conditions is stored as such.

It has been previously seen in this paper that a hedgehog's muscle contains only ·2 per cent. of creatin (calculated as creatinin), a quantity much lower than any other animal examined. When this was first estimated, being in the winter, it was thought to prove that the muscle creatin had disappeared to some extent in the hedgehog as a result of starvation, and that here was a good opportunity of seeing whether creatin taken with food can be stored in the muscle. A hedgehog was roused from its state of torpor by bringing into the warmer laboratory and given as much meat as it felt inclined to eat. This quantity was not inconsiderable, for it ate 1½ lbs. in the first two days. At the end of four days it was killed and the creatin estimated in the muscle. The amount of creatinin by the conversion of creatin was found to be ·197 per cent. It has already been seen that normally the percentage is about ·2. It is evident therefore that food creatin is not stored up in hedgehog's muscle after hibernation.

[1] *Notes on glycocyamin experiment.* Normal diet for first week consisted of Spratt's Chikko and water and a little boiled rice. After the first week, a mixture of boiled egg and bread was occasionally given.

About 4 grs. of glycocyamin were given altogether, in small doses several times a day. It was prepared by the method of Nencki and Sieber, by heating together guanidin carbonate and glycocoll in definite proportions. A further criticism of the above experiment is that the glycocyamin was given in the form of a suspension and not as a soluble salt. This probably explains why the chickens thrived and showed no poisonous symptoms as did the rabbits of Jaffé and Dorner. On the other hand the absence of deleterious effects may point to the glycocyamin having passed through the intestinal canal unabsorbed.

When dealing with the question of the methylation of glycocyamin it was seen that whereas Jaffé got an increase of creatin in the muscle of full grown rabbits after feeding with this substance, I could obtain no increase when young chickens with their muscles unsaturated by creatin were fed with glycocyamin. The method of experiment described has been proceeded with and there will now be set out some results obtained by feeding young chickens on creatin. The chief reason that chickens were chosen in these experiments was because they are easily fed when young.

Exp. 1. 19[1] chickens born July 13th to 14th. 13 were fed on one gram creatin from July 21st to July 25th, in addition to their ordinary food, which was quite free from creatin. Six control chickens received only the creatin free food. All the chickens were killed July 26th, and the muscle dissected off. Great trouble was taken to free the muscle of all visible fat. The 13 creatin fed chicks were divided into two lots X and Y, and their creatin estimated by the previously described method.

Results	Muscle ground up	Total weight of chicks	Percentage creatinin (from creatin) to muscle
6 control chickens	28·1 grs.	283·5 grs.	·30 p.c.
6 creatin fed chicks X	33·5	330·2	·295
7 creatin fed chicks Y	36·2	372	·285

Exp. 2 15 chickens born August 12th and 13th.

A. 5 fed on creatin free diet.
B. 5 fed on creatinin and ordinary diet.
C. 5 fed on meat extract, and ordinary diet.

The creatinin and meat extract[2] were given to the chicks from August 20th to 25th. About 1½ grs. creatinin in solution were given to B chicks. The chickens were killed August 26th, *i.e.* a fortnight old. The meat extract contained mostly creatin but also contained a little creatinin. The creatin was not estimated but the extract represented about 400 grs. of rabbit's muscle.

Results	Weight of chicks	Average weight	Percentage creatinin obtained from muscle
A. Control	257 grs.	51·4 grs.	·287 p.c.
B. Creatinin fed	282·5	56·5	·297
C. Meat extract (*i.e.* mostly creatin)	264	53	·293

It is seen that in the above experiments feeding with creatin or creatinin produce no apparent increase in the percentage creatin content of muscle. If creatin feeding in young animals has no such effect on chickens at this age, it seems more improbable that glycocyamin given in the food will be methylated and stored in muscle as creatin.

[1] Most of the chickens used in this research were supplied by Mr W. Bateson, to whom my thanks are due.

[2] The creatin, creatinin, meat extract and glycocyamin, according to the experiment, were always introduced directly into the chicken's stomach by means of thin indiarubber tubing.

It was thought probable that the above negative results might be due to the fact that the muscle of any animal can hold only a definite amount of creatin, which cannot be increased and that in the time of living, *i.e.* a fortnight, the chickens' muscle had nearly reached this limiting point. The muscle of a hen was examined and found to contain ·31 per cent. creatinin (from creatin).

In the following experiments the chickens were only allowed to live a few days.

Exp. 3. 12 chickens hatched out September 6th and 7th. Killed September 12th, *i.e.* 5 days old.

On September 10th and 11th, one gram of pure creatin was given them by mouth. They were divided into two lots and the creatin estimated in the muscle of each.

	Results	Weight of chicks	Average weight	Muscle ground up	Percentage creatinin from creatin in muscle	
A.	6 chicks	243 grs.	40·5 grs.	20·48 grs.	·275 p.c.	1 gram creatin.
B.	6 chicks	262·5	43·7	21·18	·264	
	6 control chicks	256·3	42·7	22·07	·238	creatin free diet.

These latter control chicks were born September 17th and killed September 22nd, *i.e.* 5 days.

The result of this latter experiment would certainly point to an increase in the creatin of muscle as a result of giving this substance by the mouth.

Exp. 4. 16 chickens born Sept. 17th. Killed Sept. 21st, *i.e.* 4 days old.

On Sept. 19th and 20th, 6 were given ½ gr. pure creatin in 50 c.cs. water. A.
10 ,, 1·2 grs. creatinin ,, 75 ,, ,, B and C.

These substances were given by the mouth, a little at a time, about five times a day. The 10 creatinin fed chicks were divided into two lots, B and C. Immediately on killing the chickens, the creatin was estimated in the muscles, the latter being carefully deprived of visible fat.

	Results	Total weight	Age	Average weight	Muscle ground up	Percentage creatinin estimated	
A.	6 chicks	262 grs.	4 days	43·7 grs.	20·6 grs.	·257 p.c.	(creatin fed).
B.	5 chicks	222	4	44·4	16·72	·246	(creatinin fed).
C.	5 chicks	231	4	46·2	18·1	·257	,, ,,

In a control set of 6 chicks, which were killed Sept. 22nd, and were thus one day older than above chickens, the estimated creatinin was ·238 per cent.

Exp. 5. 10 chickens born Friday, Sept. 27th. Killed Sunday, Sept. 29th, *i.e.* 2 days old.

On Sept. 28th, 5 chickens A were given 300 mgrs. of creatinin in 20 c.cs. water, in addition to other food.

The other 5 chicks B were fed on creatin free diet.

	Results	Weight of muscle ground up	Percentage creatinin estimated	Age
A.	5 creatinin-fed chicks	18·78 grs.	·205 p.c.	2 days
B.	5 control	17·15	·187	2

In this experiment a 9 per cent. increase of estimated creatinin results from creatinin feeding. It is impossible to say that such a result is positive evidence of storing, for at this early age, when creatin is normally being stored at a great rate, there must be considerable individual differences, which can only be eliminated by a large number of experiments. It might be suggested that if creatinin in the food were at all stored in muscle, then since it has been previously shown that creatinin is normally absent from muscle, this storing should easily be detected by an examination for preformed creatinin. This possibility was followed up. Three chickens of six days old were given about 250 mgrs. of creatinin during two days. Some hours after the last injection of creatinin they were killed, their alimentary canal dissected out, and their bodies macerated and extracted with water. On evaporating the extract below 50° C., no trace of creatinin could be found. From this, it is evident that creatinin in food is not stored as such in the muscle.

From the above experiments it is seen:

(1) That creatin and creatinin feeding has no effect upon the creatin content of muscle after the muscle has reached a certain saturation point. The small increase with glycocyamin feeding in one lot of chickens recorded previously must be therefore regarded as of little significance, while the increase obtained by Jaffé in muscle creatin on feeding rabbits with glycocyamin must be also considered doubtful.

(2) There is some evidence of an increase in muscle creatin at an early stage of life on feeding with creatin and possibly also with creatinin. For these facts to be established more feeding experiments would be required in order to eliminate the differences in chicks, particularly at an early age.

(3) That the normal absence of creatinin in muscle is not influenced by creatinin feeding.

The first conclusion makes Folin's observations difficult to interpret. When the men were on a low nitrogen diet and the creatin retained, it would not appear from the above results that it was stored up in the muscle as such. The creatin content of muscle after the first few weeks of life is a constant value for any animal, and every attempt to increase it in this research has been unsuccessful.

The very great susceptibility of creatin to bacterial action[1], which has been already pointed out, must be taken into account in all feeding experiments. This factor must have been smaller than usual in the experiments carried out on very young chickens, particularly as they were all hatched out by an incubator. Bacterial action in the alimentary tract might possibly explain the non-appearance of the creatin nitrogen in the urine in some of Folin's experiments, particularly as the product of bacterial action on creatin is of a colloidal nature, which in solution is difficult to deal with in the laboratory. At all events the above results on chicken feeding with creatin make it doubtful whether Folin's interpretation of his results, viz. that creatin is of great importance as a food-stuff, is correct. This doubt is further increased by the fact that in starvation the excretion of creatin is evidently a normal characteristic.

6. The ontogeny and phylogeny of creatin.

It is a very significant fact that, as stated by Krugenberg[23], creatin seems to be entirely confined to the vertebrate kingdom. It is but right to mention, however, that this point has caused controversy and the importance of the fact demanded further investigation. The earthworm and the lobster failed to give evidence of the presence of creatin in their muscles. Having in mind Gaskell's theory of the origin of vertebrates, efforts were made to find creatin in limulus, as representing the invertebrate most nearly allied to the vertebrate animal, and ammocœtes, the larval form of the lamprey, as the lowest form of vertebrate. An alcoholic extract of limulus after heating with hydrochloric acid gave with sodium nitro-prusside and caustic soda a colour reaction somewhat similar to Weyl's reaction for creatinin, except that it did not fade on standing, as does the latter. The Jaffé colour reaction was negative. It would appear probable that creatin is not present in limulus muscle. Ammocœtes, on the other hand, contains a considerable amount of creatin, but it was found impossible to estimate the percentage of creatin in these animals.

[1] In a sample of Liebig's extract which had stood in the laboratory some months, no creatin could be found. A 1 per cent. solution of creatin in water, which had been left for a few days, developed a basic reaction and contained no creatin. An effort was made to identify the base by distilling the liquid into hydrochloric acid. The colloidal nature of the solution caused some difficulty, and the experiment was not completed. The gas evolved was almost certainly NH_3 as the characteristic smell of methylamine was absent. This observation would tend to show that the methyl guanidin in Liebig's meat extract discovered by Kutscher is really due to bacterial action on creatin.

The presence of creatin and its relative proportion to the muscle in the adult lamprey was considered interesting because it was thought that the developmental history of the substance might throw some light on the evolution of vertebrate animals. It was found, however, that the muscle of lamprey contained ·25 per cent. (creatin estimated as creatinin), that is, as much as the skate (·24 per cent.), which is an elasmobranch fish. As a typical teleostean fish, the muscle of cod was found to contain ·304 per cent.

The amounts of creatin (estimated as creatinin) in various animals have been found as follows:

Lamprey	·25 p.c.	Hedgehog (winter)	·2 p.c.
Skate	·24	,, (summer) ...	·2
Cod	·3	Rats (2 months old) ...	·300
Frogs	·260	Bullock	·30
Fowl	·31	Pig	·330
Guinea-pig	·320	Rabbits	·440

From the above figures it will be seen that generally speaking there is a greater quantity of creatin in muscle accompanying the development from cold blooded to warm blooded animals.

It is a very striking fact that the hedgehog contains a less percentage creatin than any other animal. The smallness of the quantity was thought to be due to its disappearance during inanition, as the first estimation was made in February.

As previously pointed out, an effort made to increase the muscle content of creatin in this animal was without success. This quantity remained constant throughout the year as is seen in the following figures:

February	·21 p.c.	April	·20 p.c.
July	·230	October	·197

A consideration of the great differences seen in hedgehog metabolism at different periods in the year makes this constancy very remarkable, and it would appear to remove creatin right away from the ordinary products of metabolic change. It has been shown by Dorner that when a rabbit is starved, a very considerable amount of creatin is excreted, while at the same time the rapidly diminishing weight and the large nitrogen excretion point to great tissue breakdown. The hedgehog, on the other hand, loses little or no creatin during hibernation, and its muscles at the end of this period are almost as well developed as in the autumn. Correlated with these facts, it is interesting to find that a hedgehog contains in its muscle less creatin and a rabbit more creatin

than any other vertebrate examined. It is evident from the above table of the comparative amount of creatin in muscle that the animals examined which can exist for a long time without food, *e.g.* frogs, fish, and hedgehog, have a markedly smaller proportion of this substance. It would be interesting to know the proportion of creatin in other hibernating animals, viz. marmot, bat and bear, if only to see whether it is a generalisation that the percentage creatin in muscle is an inverse measure of its stability during starvation.

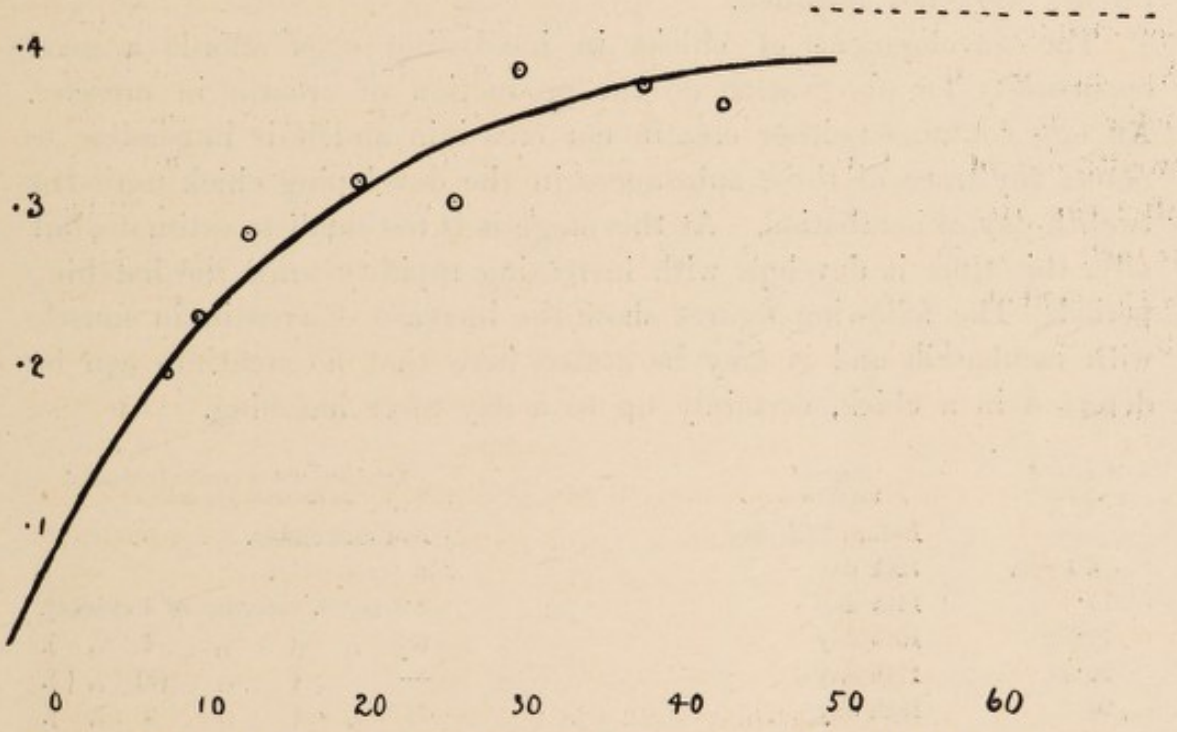

Fig. 2. The development of creatin (estimated as creatinin) in rabbits with age.
Abscissæ represent age in days.
Ordinates represent percentage creatin.
Dotted line indicates percentage creatinin obtained from adult rabbits' muscle.

It was now necessary to see whether creatin has an ontogenetic history in accordance with its phylogeny, and a preliminary experiment on fœtal rabbits, in which only traces of creatin were found, led to further work on this question. Working on animals of different ages, the following amounts of creatinin (from creatin) were found in the muscles:

Rabbits (fœtal 21 days)	...	a trace only	
Kittens (2 days old) ...	...	·153 p.c.	
3 rabbits (7 days old) ...	...	·191	
2 ,, (9 ,,) ...	...	·228	
1 rabbit (12 ,,) ...	...	·283	
1 ,, (19 ,,) ...	...	·316	
1 rabbit (25 days old)	...	·300 p.c.	
1 ,, (39 ,,)	...	·390	
1 ,, (46 ,,)	...	·373	
1 adult rabbit	...	·435	
1 ,, ,,	...	·430	

The rate of development of creatin is seen in Fig. 2.

The development of creatin in the first week of life is very remarkable, while at a later stage, when the muscle is approaching either a saturation or an equilibrium point, the development is very slow. The unsteady results obtained in animals of two or three weeks old are rather unsatisfactory, but it is not so surprising when one considers the differences of metabolic changes experienced by different animals, as indicated by their different rates of growth. Unfortunately the weights of the rabbits were not recorded.

The development of chicks in incubating eggs affords a good opportunity for observation on the production of creatin in muscle[1]. An egg contains neither creatin nor creatinin and it is impossible to detect any trace of these substances in the developing chick until the twelfth day of incubation. At this stage it is too small to estimate, but after this time it develops with increasing rapidity until the hatching period. The following figures show the increase of creatin in muscle with incubation and it may be stated here that no creatinin can be detected in a chick, certainly up to a day after hatching.

Weight of chick	Stage of incubation	Total amount of creatin (estimated as creatinin) in chicks
—	Before 12th day	Not detectable.
6·1 grs.	12th day	A trace only.
11·3	14th day	3·8 mgrs. (average of 4 chicks).
17·8	16th day	6·6 ,, (,, 4 ,,).
22·5	17th day	— (,, (2) ,,).
26	18th day	11·8 ,, (,, 2 ,,).
30·25	20th day	13·25 ,, (,, 2 ,,).
36	1 day after hatching (*i.e.* 21st day) (no food taken)	23 ,, (,, 3 ,,).

These numbers are represented in Fig. 3.

It can be seen from these figures:

(1) That although there is considerable muscle formed by the 12th day of incubation, there is no creatin in this muscle.

(2) That from the 12th day of incubation until hatching the growth of muscle, as represented by the body weight, and the formation of creatin are synchronous.

(3) That at hatching the rates of development of muscle and creatin

[1] This work was started with the idea of deciding whether the development of creatin had any apparent connection with the lecithin of the egg, since these substances both possess the methyl-imido group. The small amount of creatin in the new born chick, viz. 23 mgrs., prevented this experiment from being completed.

are widely different, the former remaining little altered[1], while the latter is nearly doubled from the 20th to the 21st day.

It is evident then that the formation of creatin in muscle is independent of the growth of that tissue. Further evidence of this

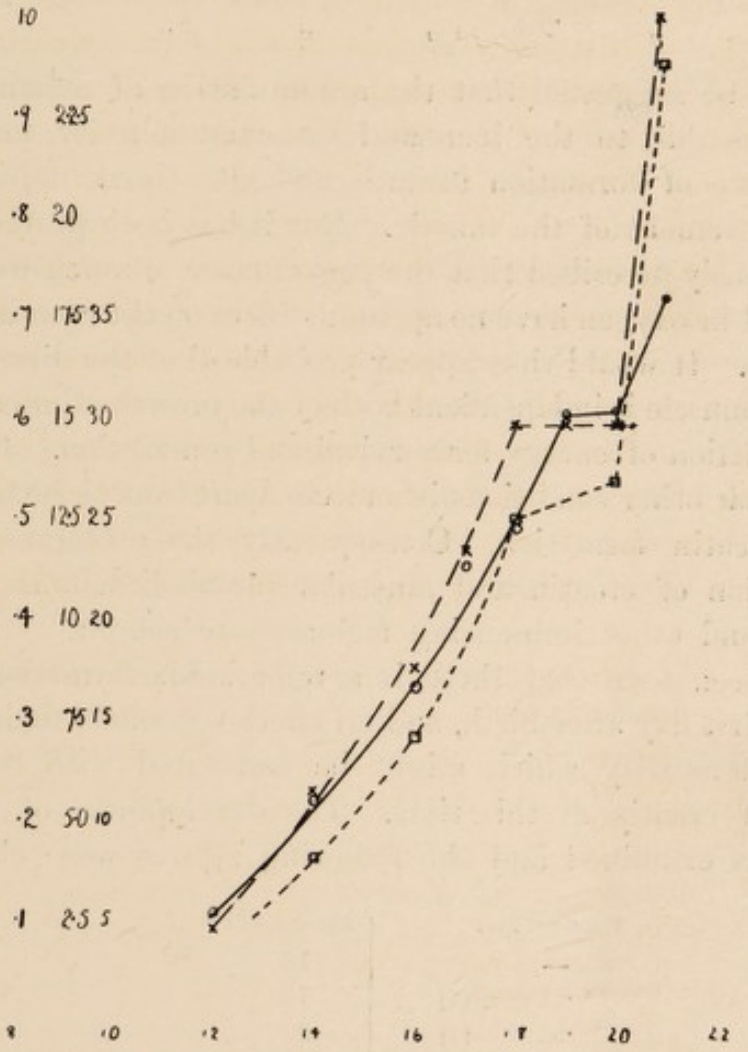

Fig. 3. The development of body weight, liver and creatin in chickens during incubation.
——— and ⊙ indicate body weight.
– – – – – – and ⊡ indicate creatin.
—— —— and × indicate liver.
Abscissæ represent periods of incubation in days.
Ordinates represent weights of liver, body and creatin respectively.
Outside figures weights of liver in grams.
Middle figures weights of body in grams.
Inside figures weights of creatin in milligrams.

[1] The increase of weight from 30·5 to 36 grams in the 20th day does not mean that the muscle weight has so increased. It is remarkable how quickly at hatching chickens receive a complete coating of egg-substance under their skins and consequently it is impossible to get the correct weight at this time. Even this apparent increase in weight, however, is not comparable to the increase in creatin.

independence is seen in the varying quantities of creatin in muscle after birth. For instance chickens' muscle

3 days old contains ·20 per cent. creatin estimated as creatinin,
5 „ „ ·24 „ „ „ „
14 „ „ ·29 „ „ „ „

It might be suggested that the accumulation of creatin in muscle after birth is due to the increased muscular activity, and that the increasing rate of formation towards and after birth depends on the increased movement of the muscle. But it has been proved in experiments previously described that the performance of much work and also long survival in oxygen have no apparent effect on the creatin content of frog's muscle. It would thus appear probable that the direct formation of creatin in muscle is independent both of the growth of muscle and also of the production of energy for survival and contraction. It is difficult to realise what other conditions in muscle apart from these factors would influence creatin formation. Consequently the attempt to correlate the production of creatin and muscular metabolism was temporarily abandoned and other influencing factors were sought.

It has been seen that there is a remarkable formation of creatin during the first day after birth, and an effort was made to discover some physiological activity which might be correlated with the increased formation of creatin at this time. The development of the liver in chickens was examined and the following figures were obtained.

Age of chicks	Weight of liver		Age of chicks	Weight of liver	
9	—		18	·59 grs.	(2)
12	·11 grs.	(2)	19	·58	(2)
14	·25	(4)	20	·57	(2)
16	·42	(2)	21 (1 day after hatching)	1·04	(3)
17	·46	(2)	24 (4 days after hatching)	1·7	(6)

The development of the liver of chickens is also seen in Fig. 3. Comparing the development of the liver with the formation of muscle and creatin it is evident:

(1) That during the incubation period the development of liver is synchronous with that of the muscle as represented by the body weight and from the 12th day to hatching with the formation of creatin.

(2) That after birth a rapid growth of the liver accompanies the accelerated production of creatin.

After birth the growth of muscle is apparently very slow. The real weights of the chickens are impossible to obtain at this time, because of

the yolk which they draw into themselves at hatching and a large portion of which appears to be quickly deposited over their body underneath the skin. The decreased weight with age, seen in the following figures, is no doubt due to assimilated yolk, although the chicks began to eat vigorously soon after birth.

Number of chicks, average weight taken	Age	Average weight[1]
1	1 day old	48 grs.
16	4 days old	45
18	5	43
19	14	53

The diminution of total weight of chickens from 48 to 43 grs. in five days after birth is at least evidence that any increase of muscle weight there is at this time is out of all proportion to the increase in liver, viz. from 1 gram to 1·7 grams.

Although the absolute amount of creatin in chickens was not obtained during this period, the short time required for muscle saturation, taken in conjunction with the results obtained with rabbit's muscle, would point to a further rapid formation of this substance after birth, well in keeping with the awakened liver activity.

It is evident then that while the growth of muscle seems to be independent of creatin formation, the growth of the liver shows a remarkable relation to the development of this substance in chicks both during incubation and after hatching.

The increase in rate of growth of the liver at hatching is no doubt due to the closing of the ductus venosus so that a much greater blood supply reaches this organ. The fact that the growth of the liver goes on at the same rate as that of the rest of the chicken during most of the incubation period, would seem to show that during this time its own special functions are not particularly called upon. Were this not the case, the ductus venosus would, no doubt, close sooner. The first function of any organ is to grow, and the increased blood supply to the liver at hatching causes the corresponding increase in rate of growth of this organ. This closing of the ductus venosus is a very special change, and it is not likely that there are many similar changes in the body at this time, which do not depend on the increased blood supply to the liver. It is therefore reasonable to put forward the synchronous rates of growth of the liver and creatin both during incubation and after hatching as strong evidence of an intimate connection between the blood supply of the organ and the development of creatin. The many

[1] All these chicks were intact and contained yolk.

functions of the liver would make one hesitate to conclude that this connection was direct—that, in fact, creatin was made by the liver and stored as such in muscle. At the same time it is of great importance to recognise that there is some evidence of another organ beside muscle being intimately related with the production of creatin.

Having now seen there is ontogenetic evidence of creatin formation being connected with the liver, it is necessary to consider if there is any supporting evidence to be derived from the phylogeny of this substance. It was seen that creatin only appears with the introduction of the vertebrate. It is absent in the cross striated muscle of the lobster, and present in that of ammocœtes. Is there any difference in the developmental story of the cross striated muscle of these animals between the invertebrata and vertebrata to account for the creatin in the latter? Zoologists are agreed that there is no difference. Here again there is some evidence, although of a negative nature, that creatin does not depend solely on muscle metabolism.

On the other hand there is ample evidence that the "gland of the mesenteron" of the invertebrate is both physiologically and morphologically different from the liver of the vertebrate.

There is no doubt then, that with the introduction of the vertebrate a new organ of the utmost metabolic importance appears, and at the same time there is a corresponding development of creatin. Thus the phylogenetic history of creatin as well as its ontogeny provide evidence of the intimate relation between the liver and creatin.

To sum up, the main facts of the ontogenetic and phylogenetic development of creatin, which show its formation does not depend on the muscle alone but probably also on the liver, are:

(1) In the developing chick there is muscle (before the 12th day of incubation) when creatin is absent.

(2) That although for a considerable time during incubation the body weight, liver and creatin develop synchronously as shown by the foregoing curves, yet towards and after hatching the rapid development of the liver, due to the closing of the ductus venosus, is accompanied by a corresponding increase in creatin formation, while at the same time muscular growth almost stops.

(3) That although creatin is absent from invertebrate muscle, morphologically and physiologically the cross striated muscle of these animals is identical with that of the vertebrates.

(4) That the "gland of the mid-gut" of invertebrates has no morphological or physiological connection with the liver of vertebrates, and therefore the newly introduced liver might account for the development of creatin in vertebrates.

7. The excretion of creatin and creatinin in pathological conditions.

The phylogenetic and ontogenetic history of creatin suggests that the liver plays a considerable part in its formation. Therefore the excretion of creatinin in people affected with pathological livers was determined to see if any further evidence of this connection could be obtained. It may be stated at this point that in my opinion creatin in muscle and urinary creatinin are intimately related, and the observations that injected creatin and creatinin are excreted unchanged, also that these substances cannot be transformed, the one into the other, under autolytic conditions, are not regarded as strong evidence of their independence.

Below are recorded the creatin and creatinin excretions of patients suffering from different disorders influencing the liver[1]:

	Sex	Age	Total creatinin excreted per diem	Total creatin excreted	Volume of urine	Remarks
A. *Cirrhosis of Liver.*						
(1)	♂	43	·756 grs.	0	305 c.cs.	Ascites. Urine collected immediately after paracentesis.
(2)	♀	43	·521	0	285	Oedema in legs. No enlargement of liver.
			·521	—	305	
(3)	♂	46	·575	0	710	No enlargement of liver. Albuminuria. Ascites.
			·68	0	885	
(4)	♀	53	·25	0	350	Alcoholic Cirrhosis. Ascites.
			·44	0	650	
(5)	♂	52	·89	·070 grs.	865	Enlarged liver. Ascites and oedema of legs.
(6)	♀	49	·325	0	350	Ascites.

[1] Most of these figures have appeared in a preliminary communication. *Proc. Physiol. Soc.* p. xxiii (This *Journal*, xxxvi. 1907).

I take this opportunity of thanking Dr Garrod and Mr T. S. Hele, of St Bartholomew's Hospital, for assistance in the procuring of material.

	Sex	Age	Total creatinin excreted per diem	Total creatin excreted	Volume of urine	Remarks
B. *Venous engorgement of liver due to mitral stenosis.*						
(1)	♂	28	·113	·074	190	Double murmur, endocarditis. Ascites.
			·114	·041	180	
(2)	♂	50	·317	0	250	Double murmur, heart enlarged, cyanosis, enlarged liver. Ascites.
			·345	—	230	
C. *Carcinoma of Liver.*						
(1)[1]	♂	40	·7	1·17	685	Died of 2nd carcinoma of liver, primary growth fundus of stomach. Some ascites. Marked cachexia.
			·38	·91	530	
			·39	1·16	530	
(2)	♂	60	·46	1·5	1775	No ascites. Jaundice. From 9 st. 11 lbs. to 7 st. 11 lbs.

The daily urine of a normal person on a creatin free diet contains from 1 to 2 grams of creatinin and no creatin. The above results therefore prove that:

(1) The excretion of creatinin in people suffering from liver affections is markedly subnormal.

(2) Patients suffering from cancer of the liver excrete a large amount of creatin, while in cases of cirrhosis and engorged livers the creatin excretion is not affected.

In most of the above cases the patients had ascites. The diminution of the excreted creatinin might have caused an accumulation of this substance in the ascitic fluid. Therefore a large quantity of this fluid was evaporated down to small bulk after first precipitating the proteids by alcohol. No creatin or creatinin could be found in it.

The diminished excretion of creatinin might have been due to:

(1) Disturbances of the general circulation.

(2) Depressed activity of the liver.

It is difficult to decide which is the more important of these factors, but in cases of ascites, where there is no oedema, the vascular disturbances are largely limited to the portal and hepatic systems. It is probable (although the question is of such importance that more work will be required to be done on it) that the creatin excreted in cancer represents a muscle breakdown. If as in case C 1, where there is ascites, the general circulation is such that it can carry more than a gram of creatin from the muscles to the kidney, then if creatinin production depended on muscular metabolism, there is no cogent reason

[1] My thanks are due to Dr J. A. Wright of Addenbrooke's Hospital, Cambridge, for assistance with this case.

why it should not be adequately excreted as in normal people. Then again in case C 2, there was no ascites, yet this patient, suffering from carcinoma of the liver, excreted only ·46 grs. of creatinin.

One other sign of liver disturbance was seen in nearly all the above cases—the acidity of the urine. It might be thought that depression of liver activity resulting in the failure to convert ammonia to urea would make the urine alkaline. However, although the ammonia may be increased, the acids, particularly lactic acid, are much more increased, so that actually the urine becomes acidic. Lest it might be thought that the large creatin output in the carcinoma cases was due to a conversion from creatinin to creatin, it may be stated that in case C 1 the urine of each day was markedly acid.

To sum up, the diminished creatinin excretion is regarded as due to depressed liver activity rather than to any change in the general circulation, for the following reasons:

(1) That with no ascites, a patient with a pathological liver (C 2) may excrete little creatinin.

(2) Although having ascites a large amount of creatin may be excreted (C 1).

(3) The urine is markedly acidic in most cases.

The excretion of creatin in the above cases of carcinoma of the liver[1] must also be considered.

It is suggested that this creatin may be due to

Either (1) a muscle breakdown caused by a general (or special) toxæmia due to the new growth resulting in the freeing of creatin,

Or (2) a failure of the tissues to convert creatin into creatinin before excretion,

Or (3) a direct production from the new growth.

If the third suggestion is true, great importance must be attached to it. In this connection it has been suggested that since in experiments, recorded earlier in this paper, it has been found that embryonic growth is accompanied by the production of creatin, and since cancerous growths are probably a reversion to the embryonic type, it might be expected that the growth itself is directly responsible for the creatin.

The origin of creatin from a direct muscle breakdown resulting

[1] The excretion of creatin is probably not *peculiar* to carcinoma hepatis. I have lately found it in the urine of patients suffering from cancer of the uterus, of the breast and of diffuse melanotic sarcomata. In these cases it may be that the livers are affected by metastatic growths.

in the freeing of this substance is indicated by the following evidence:

(1) It has been seen, earlier in the paper, that there is only creatin in muscle and under no circumstances has creatinin been found.

(2) That creatin taken by mouth is excreted as such and is not changed to creatinin. Thus creatin is not changed to creatinin in the blood stream or kidneys.

(3) In both cases of carcinoma examined there was a marked loss in weight. At the post-mortem examination of C 1, he was found to have very little muscle. The weight of C 2 had dropped from 9 st. 11 lbs. to 7 st. 11 lbs.

(4) In the case of a starving rabbit a considerable output of creatin in the urine accompanies a rapid diminution in weight. A rabbit's weight diminished from 2530 grams to 1578 grams in 9 days when starving. In this time there was excreted nearly 2 grams of creatin (Dorner).

One fact might be supposed to seriously affect the above assumption, and that is, the total nitrogen excretion of patient C 1 on 2 days was only 8·68 and 6·97 grams when his total creatin excretion was 1·06 and 1·36 grams respectively. Supposing human muscle to contain ·3 per cent. creatin, then an excretion of 1·36 grams would represent the destruction of 450 grams of muscle. Muscle is said to contain 18 per cent. total proteids including sarcolemma and connective tissue, so that the total proteid breakdown supposing all the muscle to be involved would be 81 grams, and the nitrogen of this is about 12 grams, while it was seen that the total nitrogen excreted was only 6·97 grams on this day.

This diminished nitrogen excretion may be accounted for thus:

(*a*) An incomplete muscle breakdown involving only the muscle cell containing the creatin and not the sarcolemma and connective tissue.

(*b*) The nitrogen of the broken down muscle may be carried to other tissues, particularly the new growth, and there stored.

If this explanation of the origin of the excreted creatin is correct, then it may be capable of proof, for the muscles of a patient deceased of carcinoma hepatis ought to contain a less percentage of creatin than normal muscle. The creatin content of the muscle of such a subject has not yet been estimated. Assuming a similar origin of the excreted creatin in starving rabbits, it may be noted (although the fact was not

commented on) that the percentages obtained by Dorner in autolytic experiments in which he used the muscle of such animals, are much below the normal. For instance, in two estimations of such muscle he only found ·356 p.c. and ·357 p.c., whilst with adult normal rabbits his results were ·456 p.c., ·428 p.c., ·428 p.c., and ·435 p.c. It is unlikely that there would be such a large difference in individual adult rabbits under normal conditions. Thus we have direct evidence that a muscle breakdown can release creatin and excrete it unchanged.

The question whether the carcinomatous patients excreted creatin because they were really being starved as the rabbits above mentioned, must be considered. This is clearly not the case, for experiments by Cathcart [36], Benedict and Diefendorf [37] on starving people, show that although there is some creatin excreted during starvation, yet the quantity is very much smaller than in the above cases of carcinoma. It has been suggested above that the muscle breakdown in carcinoma is due to a general or special toxæmia[1] due to the new growth. It must be admitted, however, that recent research on cancer in mice appears to be against the presence of toxins in this disease. Even in the starvation of rabbits, however, it is difficult to think that muscle breakdown results simply because of its inherent instability. It is much more likely that this breakdown is the result of a stimulus from an organ involved in general metabolism, and this stimulus would no doubt be in the form of a chemical body. Here again then, analogy would point to the presence of some chemical stimulus going from the new malignant growth to the muscles, causing them to break down and supply their nitrogen stores for this abnormal development.

The second hypothesis to account for the production of creatin in cases of carcinoma hepatis, viz. that the tissues fail to convert creatin to creatinin before excretion, must now be briefly considered. It has been advocated lately by Cathcart [36] that the excretion of creatin during starvation points to the normal production of creatinin as depending upon a change from creatin under food influence. There is no doubt also, that creatinin excretion is to some extent dependent on ingested food. It may be noted that the phrase "food influence" does not imply that the change takes place in the alimentary canal,

[1] As case C 1 had a primary growth in the stomach, and probably in C 2 the carcinoma hepatis was secondary to some intestinal lesion, the toxæmia may have been due in these cases to septic absorption. However, as previously stated in a note, creatin has been found excreted by patients suffering from cancer with no evident intestinal affection or cachexia.

but in the individual tissues. This hypothesis is vague and the following observations indicate its probable inaccuracy.

(1) So far as ingested creatin and creatinin are concerned, they are independent.

(2) In the above cases of pathological livers, *e.g.* cirrhosis and venous engorgement, the creatinin excretion is diminished with no corresponding increase in creatin.

(3) For some days after the hatching of chickens no creatinin is excreted, although a considerable quantity of food is ingested.

From this consideration of the production of creatin in the urine of patients suffering from carcinoma hepatis, it is probable that the first hypothesis is correct, viz. that there is a large muscle breakdown due to either a general or special cancer toxæmia, or more generally to some chemical stimulus, resulting in the excretion of the liberated creatin.

To summarise, it would appear from the above that

(1) The small amount of creatinin excreted in hepatic disease offers additional support to the suggestion that the liver is responsible for the formation of creatinin.

(2) The excretion of creatin in carcinoma of the liver accompanied by a decrease in body weight makes it probable that when muscle cells break down the creatin is liberated and suffers no change to creatinin before excretion.

8. Have creatin and creatinin a function?

When this research was started and the generally accepted belief that creatin is changed to creatinin was held, it was thought possible that creatin performed some function in connection with the contraction of voluntary muscle. Against such a belief no creatin was found in the cross striated muscle of a lobster, and so far as electrical stimulation is concerned, a lobster's muscle is apparently identical with vertebrate muscle. Experiments were performed to see whether creatin had any obvious effect either directly on the muscle or on the nerve endings. The sartorius muscles of frogs were carefully dissected out and placed in solutions of creatin, the strength of which varied from ·1 p.c. to ·3 p.c. When the solvent used was Ringer's solution, the muscles were quite unaffected by the creatin and remained motionless.

In a second series of experiments, a cannula was placed in the aorta of a frog and the blood vessels were washed out with Ringer's

solution. The femoral artery of one leg was then tied, and a ·25 p.c. solution of creatin allowed to flow through the frog. The sciatic nerves were dissected out high up near the cord and the minimal current found which caused the gastrocnemius on each side to contract. There was never any real difference of stimulus required in the case of the leg which had been washed out with creatin, and in the other, which had not been so treated. These experiments seem conclusive that creatin has no marked influence on contraction, or the passage of the nervous impulse in muscle. The lack of positive evidence to demonstrate the function of creatin in muscle cannot have much weight in a consideration of this body and certainly cannot be held to support the absence of such a function. However, other substances which are innocuous and probably useless are known to be stored by tissues. For instance a considerable amount of urea is found in the muscle of elasmobranch fishes, while in invertebrate muscle taurin is found.

The only observations pointing to the presence of creatin in muscle as having any significance is in the comparative estimations in various animals. It was seen that the percentage creatin had some connection with general metabolism. This was especially indicated in the rabbit and hedgehog, the former of which has a large creatin percentage, the latter a small percentage. Correlated with this the rabbit's muscle breaks down on starvation, while hedgehog's muscle is very stable. Whether such observations point to creatin playing an active part in the transference of nutrition from some such central metabolic organ as the liver to the muscles, or whether it is simply an accompanying factor in the different relations of liver to muscle, seen in various animals, cannot be decided.

All the evidence points to creatinin being entirely of an excretory nature after the muscles are saturated with creatin. Thus (1) all ingested creatinin is excreted as such, and (2) creatinin has never been found in the tissue of an animal after any treatment.

To sum up

(1) Creatin has apparently no influence on muscular contraction or the passage of a nervous impulse into muscle.

(2) Creatinin, after the earlier period of life, is an excretory product of metabolism.

9. General conclusions.

A summary of the chief results of this paper has been given at the end of each section, and it is unnecessary to repeat them here. The broad general conclusions to which I have come are

(1) That in the formation of creatinin muscle plays a small part.

(2) That the liver is intimately connected with the production of creatin and the excretion of creatinin.

As regards the action of the liver I suggest that it is continuously forming creatinin from substances carried to it by the blood stream from other organs, and that in the developing muscle this creatinin is changed to creatin and stored, while after the muscle has reached a saturation point, creatinin is continuously excreted.

I advocate the origin of muscle creatin from creatinin for the following reasons:

(1) The feeding experiments on young chickens yield some slight evidence that food creatinin can be changed to creatin and stored.

(2) In no physiological experiment in this research has creatin ever been changed to creatinin.

(3) Creatinin is not excreted by chickens until about a week after hatching, *i.e.* not until the muscles are saturated with creatin. The almost complete absence of creatinin from the urine of young children has been confirmed by Rietschel [25], and by Van Hoogenhuyze and Verploegh [13], and in the case of puppies by Closson [17]. This shows the intimate relation of creatin and creatinin, for it is unlikely that an organ would commence making an entirely new substance a week after birth. It is more probable that the power of making creatinin innocuous and storing it in the muscles as creatin has been reached at this age.

(4) The change from creatinin to creatin is from every point of view more likely than the change from creatin to creatinin. From a chemical consideration it is more probable that the ring formation of creatinin comes direct from a tissue breakdown, and that this ring is then hydrated to a creatin chain by muscle, rather than that the creatin chain is dehydrated to form the creatinin ring.

From a physiological point of view it cannot be thought that tissues would make an innocuous neutral substance like creatin into a strongly basic substance like creatinin. Such a change would be

contrary to all that is known of the changes undergone by chemical substances in the organism.

Part of the creatin used was purchased from a grant allotted to Dr Hopkins by the Government Grant Committee of the Royal Society.

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This work was undertaken at the suggestion of Mr F. G. Hopkins, F.R.S., to whom the Author is also indebted for constant advice.