CLXXXV. STUDIES ON INOSITOL.

III. THE METABOLIC BEHAVIOUR OF *i*-INOSITOL IN THE DEVELOPING AVIAN EGG.

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IN the previous communications of this series [Needham, 1923, 1, 2, 1924], experiments were described which were planned to throw some light on the physiological significance of *i*-inositol. In the first paper an estimation method for this substance was described, and in the second it was shown that the animal body appeared to possess the powers of effecting its synthesis. The present paper describes some work which has been done to determine what changes inositol may undergo during embryogenesis. In view of the correlation of the cycloses with the growth process by Starkenstein [1911] such an undertaking was the more desirable.

The importance of inositol in the egg was partially realised by two of the earlier observers. Rosenberger [1908] was unable to find any inositol in the fertilised unincubated hen's egg, but reported that he had been able to isolate a small quantity from it after it had been suspended in saline for some hours. Klein [1909] was also unable to isolate any from the fresh egg, but succeeded in isolating "eine reichliche Menge der Inosit" from eggs which had been allowed to incubate twenty days. His conclusion was that the chick had either made it by synthesis or that it had arisen from some precursor. Lastly, Starkenstein [1911] isolated 20 mg. % from the yolk of a fresh egg. No other researches have been carried out on the cyclose of the egg.

Methods.

In any one experiment the eggs were all of the same breed. They were brought to the laboratory packed in sawdust and after having been aired for half-an-hour, they were placed in electric incubators kept at a temperature of 101° F. and an atmosphere saturated with water vapour. The estimation method was not found to need any great modification, but had to be adapted to the new material in certain ways. The eggs to be estimated were broken into evaporating basins, and carefully examined as to age: if the size of the embryo did not correspond with its calculated age, it was rejected. The

amniotic membrane and fluid were counted as chick, the vitelline membrane (when it could be separated) as yolk, and the shell-membrane as shell, with which it was thrown away.

The embryos were pounded up with clean sand and the different fractions then extracted with acetone in the way described in the first paper of this series. After standing overnight, the tissue was removed by straining through muslin and the acetone distilled off. The aqueous residue was next boiled to coagulate the protein which had escaped precipitation with the acetone, and the mixture was then poured into a large separating funnel. It was diluted to about twice its bulk, so that after an hour or so the protein had settled to the bottom and the oily matter had come to the top. The underlying liquid and the precipitate were drawn off into a beaker and the oil washed once or twice with distilled water. Then the combined aqueous fractions were precipitated with neutral lead acetate and the estimation proceeded with as usual. These were the only modifications necessary.

EXPERIMENTS TO DETERMINE THE CHANGES IN THE CONCENTRATION OF CYCLOSE AS EMBRYOGENESIS PROCEEDED.

The first experiment was done on eggs from Black Leghorn hens. At first the white and the yolk were analysed separately, but before long the vitelline membrane becomes too fragile to allow of this, and the partition is then into embryo and remainder. The results of this are recorded in Table I, and a graphic presentation of them given in Fig. 1.

Fig. 1. Inositol-content of *Black Leghorn* eggs.

Table I. *Black Leghorn eggs. Concentration of i-inositol during development.*

Day of Incubation	Fraction	No. of eggs taken	Weight of tissue	cc. of CO ₂ per cc. of final solution	Inositol			Total mg. %
					Mg. % of final solution	Mg. % of tissue	Mg. per unit of tissue	
1	White	9	289.5	0.0971	12.6	4.35	1.4	6.792
1	Yolk	9	173.9	0.0351	4.25	2.4	0.47	
4	White and yolk	4	197.2	0.0596	14.24	7.1	2.95	7.221
10	Embryo	12	29.12	0.0338	4.51	15.49	0.37	33.67
10	Remainder	2	93.88	0.1279	17.07	18.18	8.53	
13	Embryo	10	57.7	0.0551	7.35	12.74	0.735	23.97
13	Remainder	1	42.3	0.0356	4.75	11.23	4.75	
16	Embryo	4	63.5	0.0199	2.66	4.19	0.66	12.21
16	Remainder	2	66.8	0.0610	5.20	8.02	3.90	
19	Embryo	2	53.8	0.0722	9.63	17.89	4.81	33.16
19	Remainder	3	53.3	0.0610	8.14	15.27	2.71	
20	Embryo	2	58.0	0.0413	5.51	19.5	2.75	44.84
20	Remainder	3	53.2	0.1009	13.46	25.34	4.48	
21	Embryo	2	70.1	0.1182	15.78	22.54	7.89	51.50
21	Remainder	3	25.0	0.0543	7.24	28.96	2.41	
Hatched	Embryo	2	89.0	0.1416	18.89	21.22	9.44	60.16
„	Remainder	2	16.0	0.0467	6.23	38.94	3.11	

From these data it will be seen that the total inositol in the egg rises from about 7 mg. % at the beginning to over 60 at the end. The general shape of the curve is doubly peaked, the first maximum occurring about the tenth day and the second one being coincident with hatching. Between the two maxima there is a depression in which the total inositol descends to a level not greatly above that which it occupied at the beginning. After the depression, which seems to occur about the sixteenth day, there is a marked rise, and now the amounts in embryo and remainder, which before kept very nearly parallel, begin to differ. The inositol in the remainder increases at the very end of incubation much more rapidly than does that in the embryo.

The significance of the two maxima is a subject which will be returned to later. But attention should be paid at this point to the light which these data throw on the problem of the synthesis of inositol. *A priori* the possibility exists that inositol is contained in the unincubated egg solely in combination with phosphorus in the form of some phytin-like compound. This is, indeed, the supposition which would follow naturally from the general view of Starkenstein [1911]. During development the phytin would break down and free inositol would accumulate. We possess, however, some very reliable figures for the phosphorus-partition of eggs at all stages of their development though, unfortunately, the breed of hen is not given [Plimmer and Scott, 1909], and by their aid we can calculate what amount of water-soluble organic phosphorus would have to be present at the beginning in order to account for all the inositol set free during development. In every molecule of phytic acid there are six atoms of phosphorus: giving a molecular weight of 813 for the phytic acid molecule. It thus appears that the 60 mg. % of free inositol present at the end of incubation would require 62 mg. % of water-soluble

organic P at the beginning, whereas Plimmer and Scott found that the total water-soluble phosphorus in the fertilised but unincubated egg amounted only to 26.64 mg. % P. It is, therefore, clear that if the inositol produced at the end arose from phytin present at the beginning: about three times as much organic water-soluble phosphorus as is actually present would be needed to account for it. Of course the precursor might be a di- or tri-phosphate of inositol; but there is no reason at present to suppose that such compounds occur in the animal body.

Before proceeding to the next experiment, it is interesting to note that the findings of Rosenberger and Klein that no inositol could be found in the undeveloped egg, could not be confirmed. On the contrary, the single figure of Starkenstein is seen to be nearer the real state of affairs.

The second series of experiments was done on White Leghorn eggs, of genuine ancestry, and was in all respects comparable with the first. The results which were obtained are given in Table II, and plotted in the form of a curve in Fig. 2.

Table II. *White Leghorn eggs. Concentration of i-inositol during development.*

Day of incubation	Fraction	No. of eggs taken	Weight of tissue	cc. of CO ₂ per cc. of final solution	Inositol			Total mg. %
					Mg. % of final solution	Mg. % of tissue	Mg. per unit of tissue	
0	Yolk	8	171.0	0.3872	51.66	30.21	6.45	33.633
0	White	8	252.75	0.0648	8.65	3.42	1.08	
4	Yolk	3	86.0	0.1728	23.05	26.8	7.68	33.80
4	White	3	59.0	—	—	7.0?	2.50	
6	Embryo	21	23.0	0.4342	57.94	252.0	2.76	302.06
6	Remainder	3	149.0	0.5590	74.59	50.06	24.86	
7	Embryo	14	28.0	0.4566	60.94	217.60	—	250.00?
9	Embryo	8	40.0	0.3982	53.14	132.80	6.64	160.8
9	Remainder	5	250.0	0.5400	70.00	28.00	14.0	
12	Embryo	6	50.0	0.2116	28.33	56.46	4.7	74.12
12	Remainder	4	250.0	0.3808	44.14	17.66	11.03	
15	Embryo	2	60.0	0.4980	66.45	110.80	33.22	257.2
15	Remainder	2	55.0	0.6036	80.54	146.40	40.27	
18	Embryo	3	74.0	0.6968	92.98	125.50	30.99	393.7
18	Remainder	3	57.0	1.1454	152.80	268.00	101.86	
21	Embryo	3	60.0	0.8400	112.1	186.8	37.30	450.0?

In the shape of the curves a striking resemblance is seen. There are two maxima as before; the only difference is that the time-relations are slightly different. The same relations between embryo and remainder at the end of development hold, however, in the second breed, for the inositol in the remainder increases much more rapidly than it does in the embryo in the final stages of incubation. But quite the most remarkable point about the two sets of experiments is that in the White Leghorn eggs, the quantities concerned are two or three times as great as in the Blacks, while yet the shape of the curve remains the same. Thus, the inositol in the whole Black Leghorn egg is 33 mg. % on the tenth day, in the White it is 74 mg. % on the twelfth day. Other points on the curve exhibit even greater difference. Accordingly the

probability that the inositol is not formed from phytin becomes even greater.

This state of affairs is very difficult to account for. That the breed of hen is a highly important matter in dealing with developmental work, is shown by the analyses of Langworthy [1906] and the experiments of Moran [1924]. The former, in an extended series of researches on the chemical constitution of eggs, found considerable differences in their composition. Moran later found that eggs varied greatly in their resistance to cold, and the temperature of their optimum viability.

Fig. 2. Inositol-content of *White* Leghorn eggs.

The fact that the difference here is between a coloured and an uncoloured breed brings up at once the possibility that the phenomenon may have something to do with that factor. Unfortunately there is no indication anywhere in our knowledge of inositol that it might have some part to play in this connection. White Leghorns are a breed in which the white character is dominant, and the writer is proposing to repeat these estimations on eggs from White Minorca hens, in which the colour factor is recessive.

EXPERIMENTS TO DETERMINE THE PRECURSOR OF THE CYCLOSE OF THE EGG.

From the results which have already been described in this paper, it seems certain that whatever may be the source of the *i*-inositol in the egg, that source cannot be phytin. In order to gain further information as to what its precursor might be, it was decided to make use of the injection-method devised by Tomita [1921], who injected glucose into hen's eggs and found by

subsequent analyses that the lactic acid content had risen considerably above the controls. This did not occur when he injected alanine. The following experiment was therefore done.

Two hundred eggs of known White Leghorn breed were divided into five equal groups. One group was injected with phytic acid prepared from commercial phytin by the method of Plimmer and Page [1913], each egg receiving 200 mg. The second group was injected with the same quantity of hexamethylenetetramine. The third and fourth groups were injected with glucose, each egg receiving in the first case 250 mg. of glucose and in the second case 125 mg. These groups will be referred to hereafter as Groups P, B, G, and D. The fifth group, C, was used as a control. Hexamethylenetetramine was injected to test the hypothesis of Posternak [1900], who suggested that inositol might arise in the body by the condensation of six molecules of formaldehyde. That hexamethylene does indeed break down into formaldehyde when introduced into the egg was proved as follows. One of the unincubated eggs which had been injected with hexamethylenetetramine was broken into a distilling flask, diluted to 600 cc. with distilled water, and the mixture steam distilled for four hours. The distillate was then tested for formaldehyde and found to contain it in a concentration certainly exceeding 1 in 50,000. On the solution of hexamethylenetetramine that had been injected, the tests were also positive but exceedingly feeble.

The injection of the eggs was accomplished in the following manner. The pointed end of each egg was painted over with an alcoholic solution of iodine, and a small round hole drilled in the shell with a dentist's burr. The liquid to be injected, having previously been sterilised, was then introduced into the egg with the aid of a sterile hypodermic syringe, and the albumin which exuded wiped off. Finally a small square piece of adhesive plaster was stuck over the hole, and the egg placed in the electric incubator to develop. The whole technique was done under precautions to ensure sterility, the drill, for example, being well boiled first. It is noteworthy that Tomita, using this method, was unable to get his chicks to develop for more than three days, but in the present experiments, injected eggs developed as far as the fifteenth day. The mortality is always greater among injected than among control eggs, but it is likely that with care in sterilisation and an avoidance of shocks during the drilling, this could be still more reduced, and development proceed as far as hatching.

The results obtained in this way are recorded in Table III and Fig. 3. The figures were not all obtained in the first experiment; they are the summarised results of all. The eggs of Group P and Group H did not develop at all. On the seventh day the eggs of Group P possessed an unpleasant smell, and showed traces of decomposition. The white was coloured brown and there were distinct evidences of coagulation around the point of injection. In some cases there had been development for one or two days. The eggs of Group H were also unfertile. The yolk was seen to be wrinkled in appearance by the seventh day, and this wrinkling became progressively more pronounced. It was accom-

panied by an increasing viscosity of the yolk, so it was clear that the normal current of water yolkwards [Riddle, 1916; Aggazzotti, 1923] had been reversed. There was, however, no sign of decomposition or coagulation. Groups G and D developed excellently.

Table III. *Effect of injection of glucose.*

Day of incubation	Description		No. of eggs taken	Weight of tissue	cc. of CO ₂ per cc. of final solution	Inositol			Total mg. %
						Mg. % of final solution	Mg. % of tissue	Mg. per unit of tissue	
7	G	Embryo	17	18.0	0.5854	78.22	434.5	25.57	476.5
7	G	Remainder	4	190.0	0.5986	79.87	42.04	19.97	
7	G	Embryo	6	12.0	0.4973	66.37	553.1	92.17	582.9
7	G	Remainder	3	146.0	0.3261	43.51	29.8	10.0	
7	C	Embryo	14	28.0	0.4566	60.94	217.6	15.54	—
7	D	Embryo	14	25.0	0.5336	71.22	284.9	5.088	313.09
7	D	Remainder	5	240.0	0.5070	67.66	28.19	13.53	
14	D	Embryo	3	40.0	0.5184	69.16	172.9	23.05	260.36
14	D	Remainder	3	100.0	0.6554	87.46	87.46	29.15	
14	H	Infertile whole egg	3	130.0	0.4798	64.01	49.25	21.33	—

Fig. 3. *Effect of injection of glucose.*

It is clear from Fig. 3 that the injection of glucose into the egg at the beginning of incubation has a great effect upon the first inositol maximum. In Group D, indeed, this is not very marked at the seventh day, though considerable at the fourteenth. In Group G, on the other hand, in which 250 mg. were injected into each egg, the effect is to double the first inositol maximum,

so that the condition at the seventh day approximates to that found normally at the end of incubation. It is to be noted that in all cases the change is seen only in the embryo. From this fact, it would seem likely that the synthesis of inositol from glucose only takes place in the embryo. This has a bearing on the shape of the curves at the end of incubation, where it was seen that inositol in the remainder rises more rapidly than in the embryo. Perhaps then the inositol is being formed in the embryo more rapidly than the needs of the growing organism demand.

DISCUSSION.

The conclusion that inositol has been synthesised from glucose by the developing embryo can admit of no doubt, but whether this is the normal process, and with what other happenings the shape of the inositol curves should be correlated, is a much more difficult matter. There is evidence, however, that the glucose in the hen's egg sinks continuously from the beginning of development until the tenth day. It must be remembered in the discussion of this subject that no glycogen or other polysaccharide has been found in the avian egg. The free glucose [see Bywaters, 1913; Satô, 1916; Tomita, 1921], which at the beginning of incubation amounts to between 400 and 500 mg. % sinks until at the tenth day there is barely 200 mg. %. The maximum amount of lactic acid formed is about 100 mg. % according to Tomita's figures, and putting the figure for the inositol maximum at 200 mg. %, it is evident that much of the free glucose is accounted for. As a matter of fact, such a calculation fails to take into account the considerable stores of glucose contained in ovomucoid, and it is possible that the second maximum of the inositol may be due to the glucose from that source. Tomita found that a maximum of lactic acid occurred on the fifth day, so perhaps the glucose first forms lactic acid and afterwards inositol. But as to what function the inositol may perform when it has been synthesised we have as yet no clue.

Not a few attempts have been made by earlier workers to demonstrate a relationship between the cycloses and the carbohydrates, a possibility which, from a study of the formula of inositol, had a certain *a priori* likelihood. Greenwald and Weiss [1917] tried to show that glucose was formed when inositol was fed, but their results were not very conclusive, and Anderson [1916] could find no rise in the Respiratory Quotient of dogs after the feeding of inositol. Long previously it had been shown by Külz [1876] and von Mering [1876] that inositol was incapable of producing glycogen in the livers of phloridzinised dogs. Then Oppenheim [1912], Griesbach [1913] and Embden [1914] all obtained entirely negative results when inositol was perfused through various tissues, and glucose or lactic acid looked for. Lactic acid had, indeed, been obtained as a product of bacterial action by Hilger [1871] and Vohl [1876] but, as later workers have shown [Hewitt and Steabben, 1922], it occurs only along with a host of other products, and can have little significance

in this connection. The experiments of Mayer [1907] were also inconclusive, though mainly negative.

All this work failed, then, to relate inositol metabolism to that of the carbohydrates in any way. The noticeable characteristic of it is, however, that all these workers tried to show that inositol could be the precursor of glucose, and none of them that glucose could be the precursor of inositol. But there is no reason why the reaction, $\text{glucose} \rightleftharpoons \text{inositol}$, although theoretically reversible, in the body might be so controlled that inositol could be produced from glucose but little or no glucose from inositol. This hypothesis receives support from the effect of insulin convulsions on the free inositol of the rat [Needham, Smith and Winter, 1923]. Insulin convulsions, it was found, raised the free inositol in the tissues of rats by 50 %, although this difference was small compared with the amount of glucose which had disappeared. It seems likely that here also, a change in the carbohydrate side of the equation has resulted in a change on the cyclose side.

In spite of the superficial similarity between the formulae of *i*-inositol and glucose, to picture how the transfer takes place is not easy. That the formation of rings in the body is possible, is of course well known; the case of kynurenic acid need only be referred to. Other cases are also known [see Kikkoji and Neuberg, 1909; Stolte, 1908; Spiro, 1907].

In the last paper of this series [Needham, 1924] it was shown that the body probably possessed the power of synthesising inositol, and in view of the data reported in this paper, it seems probable that it may do so from its supplies of glucose. But it is too early as yet to say so definitely, and there may well be more than one manner in which the cycloses can be produced in animal metabolism.

SUMMARY.

1. The changes in concentration of *i*-inositol during the development of the avian embryo have been studied in detail. Although the absolute quantities concerned differ for Black and White Leghorn eggs, yet the shape of the curve is the same.

2. The injection of glucose into the fertilised unincubated egg causes a very large rise in the inositol content of the egg during its subsequent development. Accordingly it may be considered likely that glucose is the normal precursor of inositol.

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