CCLXXIX. THE ORIGIN OF THE PHOSPHORUS COMPOUNDS IN THE EMBRYO OF THE CHICKEN

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Several of the numerous compounds containing phosphorus present in the embryo of the chicken[1] occur in the yolk and the white of the egg. Those which do are chiefly phosphatides and nucleoproteins but, as Table I shows, other phosphorus compounds also occur in those parts of the egg.

Table I [Plimmer & Scott, 1909]. *Percentage of the total P (94 mg.) at the beginning and the end of incubation of a hen's egg*

	Beginning	End
Inorganic P	Trace	60
Water-soluble P	6·2	8·6
Ether-soluble P	64·8	19·3
Vitellin-P	27·1	0
Nucleoprotein-P	1·9	12

Kugler [1936] has lately found that, on the twentieth day of incubation, i.e. the last day but one, only 25 mg. of the 65 mg. of lipoid P originally present in the yolk remained there; 8 mg. were found in the embryo, and the remainder had been hydrolysed yielding inorganic P. About two-thirds of the phosphatides present were found to be lecithin and one-third kephalin. In view of the large store of phosphatides present in the yolk even shortly before the egg is hatched, we should expect the embryo to avail itself of this store when it needs phosphatides to build up its nervous system and other organs containing these substances. We can test this point by introducing labelled (radioactive) sodium phosphate into the egg before incubation and investigating if and to what extent the phosphatide of the yolk and of the embryo become labelled. If none becomes labelled, we can conclude that the phosphatide molecules in the embryo are not newly synthesized from inorganic phosphate present there; if, however, the yolk phosphatide remains unlabelled while that of embryo becomes radioactive, we can conclude that the phosphatide molecules present in the embryo have not come from the yolk but have been built up in the embryo with the participation of labelled inorganic P. Similar considerations apply to certain other compounds occurring in the embryo.

Methods

The phosphorus content of a series of solutions is usually determined colorimetrically. For example, the inorganic P present in one sample of an acid-soluble fraction can be determined in this way, and then in another sample the

[1] A detailed investigation of the acid-soluble phosphorus compounds present in the embryo of the chicken was recently carried out by Needham *et al.* [1937].

phosphagen-P present can be converted into inorganic P, so that colorimetric determination now supplies the value for the inorganic P+phosphagen-P. In our experiments this was inadequate. We had to measure not only the P content but also the activity of the various fractions, so we had to obtain precipitates in each case. To obtain sufficient precipitate when dealing with eggs only incubated for a few days, it was necessary to work with several eggs simultaneously.

We precipitated the phosphorus, after bringing it into the inorganic state, as ammonium magnesium phosphate. The precipitate was then dissolved in 0·1 *N* HCl and an aliquot part was sucked into a glass cuvette. This was placed below the Geiger counter used to determine the activity of the preparations, while another aliquot part was utilized for the colorimetric determination of the phosphorus content. The glass cuvettes were covered with a thin mica window (5–6 mg. per cm.^{2}) which only absorbed to a negligible extent the β-rays emitted by the radioactive phosphorus; the area of the mica window was 1·1 cm.^{2} and the liquid content of the cuvette amounted to about 0·5 ml. If we attempt to precipitate ammonium magnesium phosphate from the same solution several times, both before and after hydrolysis, large amounts of salts accumulate in the solution and hinder quantitative precipitation.

We were interested in the determination of the activity of 1 mg. P prepared from different phosphorus compounds present in the embryo or in the remains. Accordingly we were not concerned with the quantitative amounts of the P compounds present and so concentrated our efforts on obtaining the various fractions in a pure state—to avoid, for example, traces of inorganic phosphate remaining in the phosphatides extracted from the yolk. As the phosphatides of the yolk were found to be but slightly active, while the inorganic P was strongly active, even a small contamination of the former by the latter was to be avoided. The white, the yolk, the embryo and, in some cases, the amniotic and allantoic liquids were worked up simultaneously.

As regards the white we were only interested in the total activity present after incubation. The white was ignited (reduced to ash) and the phosphorus in it precipitated as ammonium magnesium phosphate.

The yolk was dried with acetone and the phosphatides extracted three times from the dry product with a 3 : 1 alcohol-ether mixture. The alcohol and ether were then evaporated off at about 50° *in vacuo* and the residue was taken up with light petroleum and filtered. The filtrate was evaporated *in vacuo*, the residue ignited, and the phosphorus precipitated as ammonium magnesium phosphate.

Another part of the yolk was treated as follows. The acid-soluble compounds were extracted, then the phosphatides were removed as described above, and the residual part containing mainly vitellin-P and nucleoprotein-P was ignited; the P content of this last part was determined as ammonium magnesium phosphate.

The embryos were dropped, immediately after being removed from their eggs, into liquid air and were subsequently pulverized. The embryo powder was then extracted several times with cold trichloroacetic acid—in the first two extractions a 10 % solution was used, and later one of 5 %. The extract was filtered into cold concentrated NaOH solution and divided into three parts, (*a*), (*b*) and (*c*). From (*a*) a sample of the average acid-soluble P of the embryo was secured, (*b*) was precipitated with 25 % barium acetate solution at *p*H 6·5. The cold precipitate was washed with a dilute barium acetate solution, centrifuged and dissolved in a few drops of cold HNO_3. The inorganic P present was then precipitated by adding Fiske's reagent. The remaining filtrate was hydrolysed with *N* HCl at 100° for 7 min. to split the two labile phosphate radicals of

adenosinetriphosphoric acid. The phosphorus set free was finally precipitated as ammonium magnesium phosphate. Barium hydroxide was added to the filtrate from the barium precipitation to remove any inorganic P, the precipitate was separated by centrifuging and ethyl alcohol was added to the remaining liquid until an alcohol concentration of nearly 60% was reached. The precipitate obtained after addition of alcohol [Ostern *et al.*, 1936] contained the hexosemonophosphate. Its P content was determined in the usual way. The third part, (*c*), was hydrolysed with *N* HCl and 0·1 *M* ammonium molybdate for 30 min. at 40°. In the course of 30 min. most of the phosphagen present decomposed, so that the inorganic P originally present as such, and that obtained by the decomposition of the phosphagen,[1] were secured together in this fraction.

After removal of the acid-soluble P the embryo was thoroughly treated with an alcohol-ether mixture, as described above, to remove the phosphatides. The residue, containing mainly nucleoprotein-P, was ignited with concentrated sulphuric and nitric acids and the P precipitated in the usual way.

Results

Eggs incubated for 6–18 days. The results of the determination of the specific activities (activities per mg. P) of the different fractions extracted from seven embryos and from the remaining parts of eggs incubated for 11 days are shown in Table II, while Tables III–V give the results obtained with eggs incubated for 18, 16 and 6 days. In addition to the specific activity (activity per mg. P, with that of the P extracted from the white of the egg taken as 100), we have also recorded in Tables II and III the activity (in kicks per minute or in % of amount injected) and the P content of the fraction—this last quantity being determined, in all cases, by the method of Fiske & Subbarow.

Table II. *Specific activity of P extracted from different fractions of an egg incubated for 11 days. (Specific activity of P extracted from the white taken as 100)*

	Fraction	mg. P*	Kicks per min.	Specific activity
Embryo:	Average acid-soluble P	0·074	3·5	59
	Inorganic P	0·077	3·1	51
	Adenosine-P + inorganic P	0·121	6·0	63
	Creatine-P	0·171	8·1	60
	Phosphatide-P	0·561	29·6	67
	Residual ("nucleoprotein") P	1·49	85·6	72
Yolk:	Phosphatide-P	10·4	0·55	0·067

* Indicator mg. P in the sample measured.

Table III. *Specific activity of P extracted from different fractions of an egg incubated for 18 days. (Specific activity of P extracted from the white taken as 100)*

	Fraction	mg. P	% of amount injected	Specific activity
Embryo:	Average acid-soluble P	19·7	53·5	19
	Inorganic (without skeleton) P	10·91	27·2	17
	Tibia and femur-P	4·50	7·6	11
	Adenosine-P	0·048	0·14	20
	Phosphatide-P	1·08	1·7	11
	Residual ("nucleoprotein") P	0·204	0·3	10
Yolk:	Acid-soluble P	0·828	1·3	11
	Phosphatide-P	17·50	0·28	0·11
	Residual P	2·16	0·12	0·40

[1] On the phosphagen content of the embryo of the chicken, cp. Lehmann & Needham [1937].

The figures for the specific activities (activities per mg. P) of different fractions extracted from an embryo and from the remaining parts of an egg incubated for 18 days are shown in Table III. The P content in mg., the percentage of the injected activity present in the fraction and the relative specific activity are recorded; the specific activity of the P extracted from the white of the egg is taken as 100.

The specific activities obtained when the eggs were incubated for 16 and 6 days respectively are seen in Tables IV and V.

Table IV. *Specific activity of P extracted from different fractions of an egg incubated for 16 days. (The specific activity of P extracted from the white taken as 100)*

	Fraction	Specific activity
Embryo:	Average acid-soluble P	14
	Inorganic (without skeleton) P	14
	Tibia and femur-P	15
	Creatine-P	14
	Hexosemonophosphate-P	19
	Phosphatide-P	12
	Residual ("nucleoprotein") P	16
Yolk:	Acid-soluble P	12
	Phosphatide-P	0·14
	Residual P	1·22

Table V. *Specific activity of P extracted from different fractions of 10 eggs incubated for 6 days. (Specific activity of embryo phosphatide P taken as 100)*

	Fraction	Specific activity
Embryo:	Phosphatide P	100
	Average (phosphatide) P	113
Yolk:	Inorganic P	60
	Acid-soluble minus inorganic P	34
	Phosphatide P	0·032
	Residual P	1·3

As the figures show, the phosphatides extracted from the yolk are only slightly active, while those extracted from the embryo show strong activity; 1 mg. of embryo phosphatide-P is at least 100 times as active as 1 mg. yolk phosphatide P. Furthermore, the specific activity of the embryo phosphatide-P is about as high as that of the embryo inorganic P, showing that an inorganic P atom reaching the embryo has about the same chance of entering the skeleton as of being incorporated in a phosphatide molecule by an enzymic process—which of the two systems it enters is governed solely by probability considerations. From this it follows that the phosphatide molecules in the embryo are not identical with those derived from the yolk, but are synthesized in the embryo.

The formation of labelled phosphatides in growing eggs was investigated by Hevesy & Hahn [1938]. It was found that the phosphatides present in the yolk are taken up from the plasma by the ovary and incorporated into the latter; as soon as the yolk leaves the ovary no more change occurs in the content or composition of its phosphatides. When labelled phosphate is administered to a hen after the yolk has left the ovary and is located in the oviduct, the egg takes up active phosphate but no active phosphatide is formed. In experiments *in vitro* as well, eggs placed in radioactive sodium phosphate solution take up active phosphate but no active phosphatides are formed. The slight activity of the phosphatides present in the yolk of incubated eggs is presumably due to the

influx into the yolk of small amounts of active phosphatides synthesized in the embryo. This view is supported by the fact that the ratio of the specific activities of the embryo phosphatide-P and yolk phosphatide-P was much larger (3000) in the 6 days experiment than in the 18 days experiment (100). The activity of the residual P of the yolk, which is mainly composed of vitellin and nucleoprotein, was larger than that of the phosphatides; this can be understood if we admit the possibility that the extraction of the strongly active, non-protein constituent of the yolk is not quantitative, for in this case the specific activity of the residual P would be increased.

The embryonic residue obtained after extraction of the acid-soluble and ether-soluble constituents is composed chiefly of nucleoproteins. That the specific activity of the nucleoprotein-P is the same as that of the inorganic P extracted from the embryo is not surprising, because much less nucleoprotein is present in the yolk than in the embryo (Table I). The greater part of the nucleoproteins present in the embryo must therefore have been built up in the course of incubation; during this process labelled phosphate has an opportunity of entering the nucleoprotein molecules.

The radioactive sodium phosphate of negligible weight injected into the white of the egg labels the inorganic P present in the latter. The labelled inorganic P is transported from place to place, along with the other water-soluble phosphorus compounds present in the white and the yolk, and becomes partly incorporated in the embryo. The inorganic P so removed is replaced by some formed by hydrolysis, mainly of phosphatides and vitellin. In this way the radioactive inorganic P injected into the egg becomes more and more diluted with inactive inorganic P and its specific activity (activity per mg. P) diminishes accordingly. Whereas at the start of the experiment only traces of inorganic P are present in the egg, at the end of the incubation about 60 mg. are found, mostly in the skeletal part of the embryo. As the inorganic P used in the synthesis of the different P compounds present in the embryo becomes less and less active in the course of incubation, we should expect to find the phosphorus compounds synthesized at a later date much less active than those built up at an early stage in the incubation. This does not, however, seem to be the case. Actually, we have not yet compared the activity of, for example, the phosphatides extracted from the nervous system, which is built up at an early stage, with that of the phosphatides extracted from the skeleton, which is synthesized at a late stage of development. But the fact that the specific activities of the inorganic P, the phosphatide-P and the nucleoprotein-P were found to be equal to within the errors of the experiment suggests that no large differences in the specific activities of phosphorus compounds formed at different dates can be expected, though minor differences could possibly be found. This must be interpreted as being due to the ceaseless breaking-up and the rebuilding of the molecules in the embryo under enzymic action, a process which leads to equipartition of the activity between the different phosphorus molecules.

We should most expect to find phosphate layers of different specific activity in the skeleton. The rate of phosphorus exchange in the bone tissue is a comparatively slow process, though the atoms of the embryonic bone tissue may be comparatively easily replaceable. A difference in the specific activities of inorganic P extracted from bone tissue (tibia + femur) and from the other organs of the embryo is shown in Tables III and IV, which is, however, to be interpreted cautiously.

Distribution of radioactive phosphate in the egg

The greater part of the sodium phosphate injected into the white is still found at the end of the experiment in that part of the egg. The distribution of the activity between white, yolk, connecting fluids (which were not, however, free from white and yolk) and embryo is seen in Table VI.

Table VI. *Distribution of injected active phosphate between different parts of the egg*

Time of incubation	Fraction	% activity
6 days	White	61·6
	Yolk	10·3
	Liquids	26·0
	Embryo	1·7
18 days	White	14·9
	Yolk	1·7
	Liquids	19·8
	Embryo	63·0

The low activity of the yolk might possibly be due to a slow rate of penetration of the vitellin membrane by the phosphate ions; this point is under investigation. Another possible explanation is that the inorganic P content of the yolk is lower than that of the white. If a distribution equilibrium is reached, the activity should be proportional to the amount of inorganic phosphate present in the phase in question, since the inorganic P, among all the P compounds present in the yolk and white, is practically the only source of activity; in the 6 days experiment, for example, 10% of the 10·3% activity found in the yolk was present as inorganic P. Finally we have to envisage the possibility that a part of the inorganic phosphate injected is not freely movable in the white—it might be precipitated as calcium phosphate or attached to proteins, its mobility being lowered thereby.

We have also carried out experiments in which 0·1 ml. physiological NaCl solution containing a negligible amount of labelled sodium phosphate was injected into eggs which were not incubated. After the lapse of 5 days the distribution of the activity in different parts of the egg was determined; 97% was found in the white and 3% in the yolk. As was of course to be expected, a still greater preference for the white was shown by the active phosphorus in this experiment; the duration of the experiment was shorter than that of those discussed above, and transport of phosphorus from the white to the embryo was absent.

To test whether the water injected encountered any hindrance in its propagation through the egg, we injected 0·2 ml. heavy water into the white of the egg; after the lapse of 5 days water was distilled separately from the white and from the yolk and the densities determined. We are much indebted to Mr O. Jacobson for carrying out the density determinations using Linderström-Lang's float method. He found that the water prepared from the white had a density exceeding that of normal water by 484 parts per million, while the corresponding figure for the water obtained from the yolk was 437. The deuterium content of the water distilled off from the yolk was thus found to be only about 10% lower than that of the water from the white, showing that in the course of 5 days the water injected was very nearly evenly distributed throughout the egg, in contrast to the injected active phosphate. The anomalous behaviour of the latter, while of interest in the study of the circulation of phosphate ions in white and yolk, in

no way influences the investigation of the main problem discussed in this paper—namely, if and to what extent the molecules of the different phosphorus compounds present in the embryo are built up there or drawn, ready made, from the yolk.

Introduction of labelled hexosemonophosphate into the egg to be incubated

In one set of experiments, instead of following up the fate of labelled inorganic P in incubated eggs, we introduced radioactive hexosemonophosphate. Prof. Parnas very kindly presented us with this radical (prepared by Dr Ostern) in the form of barium hexosemonophosphate, from which, by treatment with sodium sulphate in the cold, the sodium compound of the ester was obtained. 0·2 ml., containing about 0·2 mg. P as hexosemonophosphate salt and about 3 mg. sodium sulphate, was injected into the white of each of the eggs to be incubated; to avoid decomposition of the ester, the solution was kept ice-cooled until it was injected into the egg. Of the 10 eggs receiving this treatment, only two supplied living embryos. After a lapse of 14 days, 7·7 % of the activity injected was found to have been incorporated in the embryo (5·8 % in the yolk) and a large fraction was also to be found in the white and in the connecting liquids. If, of the various fractions extracted from the embryo, we had only found activity in the fraction containing hexosemonophosphate, we should have had to conclude that the hexosemonophosphate does not decompose in the egg but enters the embryo as such. In view of the results obtained in the experiments carried out with labelled inorganic phosphate, however, such behaviour was hardly to be expected. Furthermore, Kay [1926] found that in the embryo the phosphatase activity of the developing bone was extremely high, the phosphatase decomposing the hexosemonophosphate. We isolated the hexosemonophosphate from the embryo, as described on p. 2148, and compared the specific activity of this fraction with that of the inorganic phosphate (+ creatine-P). We also isolated the phosphatide fraction and the residual phosphorus fraction containing mainly nucleoprotein-P. As Table VII shows, no conspicuous difference can be seen between the specific activities of the different fractions of the embryo, with the possible exception of the residual P. In these experiments small activities had to be measured and the differences found between the first three fractions lie within the errors of the experiment. The results obtained suggest the explanation that active inorganic P splits off from the labelled hexosemonophosphate injected and is incorporated in the different phosphorus compounds of the embryo, while the hexosemonophosphate molecules extracted from the embryo are not those synthesized by Dr Ostern but are molecules built up by the chicken's embryo.

Table VII. *Specific activity of P from different fractions extracted from two eggs incubated for 14 days after the injection of radioactive hexosemonophosphate. (Specific activity of P extracted from the white taken as 100)*

	Fraction	Specific activity
Embryo:	Inorganic P	24
	Hexosemonophosphate-P	26
	Phosphatide-P	20
	Residual ("nucleoprotein") P	11
Yolk:	Inorganic P	36
	Hexosemonophosphate-P	18
	Phosphatide + residual P	0

The low value found for the residual P of the embryo may possibly be due to the building up of a part of the nucleoprotein fraction at an early date before much of the active hexosemonophosphate introduced has decomposed. The phosphatide-P and residual P extracted from the yolk were found to be inactive. These fractions were found to be only slightly active even after the injection of strongly active inorganic P, and the absence of activity after the injection into the egg of the much weaker hexosemonophosphate was only to be expected. The hexosemonophosphate fraction isolated from the yolk had a specific activity of 18; the inorganic P, 36. The larger value found for the specific activity of the inorganic P is possibly to be explained in the following way. Some active hexosemonophosphate diffuses into the yolk and partly decomposes into active inorganic P; this is the source of most of the active inorganic P which we isolated from the yolk. The hexosemonophosphate, isolated by the method outlined on p. 2149, contains, besides the active hexosemonophosphate, some non-active hexosemonophosphate and possibly also some other acid-soluble P compound separated simultaneously, which diminishes the specific activity of the "hexosemonophosphate" fraction isolated from the yolk. In the embryo, on account of the strong enzymic action prevailing there, all phosphorus compounds become activated; on the other hand, in the yolk, as we have just mentioned, no such activation takes place.

On the phosphatide synthesis in the embryo of the chicken

We saw that the phosphatide molecules present in the chicken's embryo are not identical with those formerly located in the yolk, but that they were synthesized in the embryo. The work of Schönheimer & Rittenberg [1936] gives us important information about the units which are utilized in the synthesis. They found, by making use of deuterium as an indicator, that the developing hen's egg forms no new fatty acids and their result excluded also the possibility that unsaturated fatty acids present in the egg had been hydrogenated during development. Needham [1931], on the other hand, found that a marked desaturation occurs in an aqueous emulsion of embryonic tissues mixed with the corresponding yolk and vigorously shaken. The embryo must thus make use of the fatty acids present in the yolk to build up its phosphatides; in doing this it possibly gives some preference to the less saturated fatty acids. The fatty acid components of the phosphatides extracted from the embryo are found to be less saturated than those extracted from the yolk residue. The at first sight puzzling fact that the embryo, instead of using the phosphatide molecules found in great abundance in the yolk, synthesizes its own phosphatide molecules, becomes less puzzling when we envisage the likely possibility that the synthesis of phosphatide molecules is also a step in other chemical processes, which occur simultaneously in the growing embryo.

Summary

Radioactive sodium phosphate was injected into hen's eggs which were then incubated in some experiments for 6, and in others for 11, 16 and 18 days. While the phosphatide-phosphorus extracted from the embryo always showed a high specific activity (activity per mg. P), that extracted from the yolk was hardly active at all. The phosphatide molecules present in the embryo could not therefore have been taken from the yolk only, but must have been synthesized in the embryo. The investigation of the "acid-soluble" and residual (mainly nucleoprotein) phosphorus extracted from the embryo led to a similar

result—namely, that the ratio in which the labelled inorganic phosphorus atoms are incorporated into the different phosphorus compounds present in the embryo is governed solely by probability considerations. Practically all the phosphorus atoms present in the various compounds of the embryo must pass through the stage of inorganic P; only the inorganic phosphorus present in the embryo is taken as such from the yolk or the white.

In some experiments, instead of radioactive sodium phosphate, labelled hexosemonophosphate was injected into the egg before incubation. The hexosemonophosphate-phosphorus extracted from the embryo had about the same specific activity as the inorganic and the phosphatide phosphorus extracted. This result suggests that inorganic phosphate radicals which have split off from the hexosemonophosphate and from other compounds present in the yolk and the white, rather than the hexosemonophosphate molecules introduced into the latter, are utilized to build up the phosphorus compounds of the chicken's embryo.

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