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A STUDY OF BIOLOGICAL VARIABILITY: THE
DISTRIBUTION OF P³² IN THE PHOSPHORUS
COMPOUNDS OF THE 8-DAY CHICK EMBRYO

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A Study of Biological Variability: the Distribution of P^{32} in the Phosphorus Compounds of the 8-Day Chick Embryo¹

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IN A series of experiments on phosphorus metabolism in the developing chick embryo including the effects of various physical agents (*e.g.* high electric field) and chemical agents (*e.g.* vitamins), we have been faced with the difficult problem of deciding just how great should a quantitative difference between two determinations be, in order for us to attribute the difference to the agent. What we have is essentially a problem in statistics—the same statistical problem encountered in biological assays. The specific investigation we have undertaken in this paper, the metabolism of phosphorus in the 8-day chick embryo, has two aspects. In the first place, when using the method of following the turnover of radioactive phosphorus in the phosphorus compounds of the embryo what variation is to be expected from the combination of normal variability from group to group and the

inherent experimental errors. The data are subjected to elementary statistical analysis to find the mean, variance, and some characteristics of the population of groups from which our sample was taken by using Student's *t*-test. The second aspect is that the information gained from the study gives us insight into the functioning of the embryo as a biological system.

The general outline of the experiment was as follows. An aliquot of radioactive phosphorus was injected into a group of weighed eggs. The eggs were incubated for eight days. At the end of the incubation period, the viable embryos were frozen immediately in liquid air. After weighing the embryos were ground to a powder and the chemical analysis performed to determine the specific activities—radioactivity per microgram of phosphorus corrected for decay—of the phosphatide, acid soluble, inorganic and adenosine, inorganic and inorganic from phosphocreatine, residual, and inorganic phosphorus fractions. Intercomparison of

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these data give an indication of biological variability for each phosphorus fraction.

PROCEDURE

Sixteen dozen eggs were obtained from a local hatchery. From this sample, thirty-six eggs were selected at random. They were then weighed, sterilized with Lugol's solution and injected into the white through the air sac with 0.05 ml. of P³² solution with a 1 ml. calibrated tuberculin syringe having a No. 27 hypodermic needle. The needle was inserted to the same depth in each egg. The P³² solution was in the form of Na₂HPO₄ having an activity 51 μ c./ml. The activity of the P³² had been determined by using the Tracerlab simulated P³² reference source. The activities obtained by this technique never differed from the designated Oak Ridge values by as much as 5%. The eggs were then sealed with sterile wax and placed in an incubator. After eight days incubation, the embryos were removed, cleared of adhering membranes, and immediately frozen in liquid air. The fact that all of the embryos survived gave us increased confidence in our injection procedure. Immediately following freezing, the embryos were weighed and then powdered in preparation for chemical analysis.

The chemical procedure employed for the fractionation of the acid-soluble phosphorus compounds and the ether-soluble phosphorus compounds of the chick embryo for use in our tracer studies is similar to that of Hevesy, Levi and Rebbe (1938). It is based principally on the solubilities of the barium salts of the compounds and their susceptibility to hydrolysis. The spectro-photometric method of analysis is fashioned after that of Kitson and Mellon (1944) which does not require any very cumbersome gravimetric determination. The counting of

the sample is done very conveniently by merely taking an aliquot portion of the final solution used for chemical analysis, evaporating it to dryness, and counting with an end-window Geiger Müller tube, window thickness 3 to 4 mgm./cm.² A single 8-day embryo was too small for reliable chemical analysis. We found that a group of three could be handled easily. In all of our discussion, the single values given refer then to the means for three embryos pooled prior to extraction. The "population" of our statistical analysis is not, therefore, the population of individual eggs but that of groups each consisting of three eggs selected at random from the original thirty-six eggs. The variation in the original population would be expected to exceed the variation in our "population."

The fractions that have been obtained and analyzed for phosphorus are: acid soluble phosphorus, inorganic phosphorus, inorganic phosphorus plus adenosine phosphorus, inorganic phosphorus plus inorganic phosphorus from phosphocreatine, residual phosphorus (mainly nuclear protein phosphorus), and phosphatide or phospholipid phosphorus. A description of the chemical procedure is given in the following flow sheet.

RESULTS

Table 1 gives the data determined from the experiment. The second and third columns display the relation between the specific activity and weight of egg and between specific activity and weight of embryo. These figures show no correlation between these factors. There is no advantage, therefore, in expressing the specific activity as counts per microgram or phosphorus per gram of egg or per gram of embryo.

Table 1 reveals that the maximum deviation from the mean varied from

FLOW SHEET FOR PHOSPHORUS FRACTIONS

Drop embryo into liquid air; pulverize frozen embryo; extract 3 times (twice with 10% trichloroacetic acid and once with 5% TCA); filter into cold NaOH (conc.)

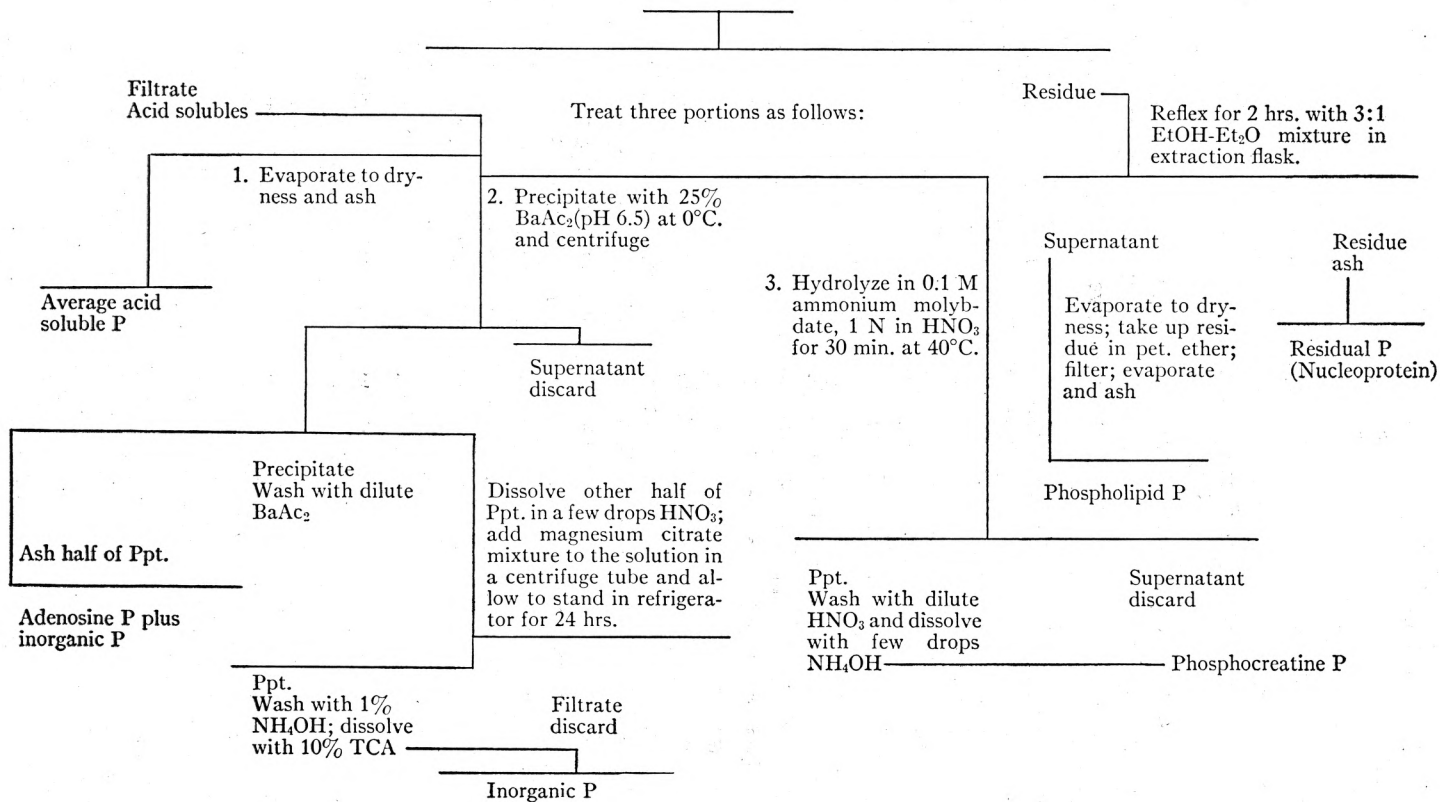


TABLE 1.—Data on specific activities of phosphorus fractions

Sample	Average weight of embryo	Average weight of egg	Specific activity of phosphorus fractions (Counts per sec. per microgram of phosphorus)					
			Phosphatide P	Acid soluble P	Inorganic & adenosine P	Inorganic P plus inorganic P from phosphocreatine	Residual P	Inorganic P
1	4.09 gm.	62.8 gm.	.1013	.0533	.0864	.0930	.0600	.0209
2	5.07 gm.	60.5 gm.	.0982	.0613	.0815	.0956	.0624	.0353
3	5.24 gm.	60.6 gm.	.0927	.0518	.0948	.0813	.0517	.0248
4	5.12 gm.	60.0 gm.	.0991	.0523	.0882	.0959	.0534	.0304
5	5.05 gm.	59.5 gm.	.0892	.0629	.0922	.0896	.0572	.0268
6	5.52 gm.	61.3 gm.	.0846	.0611	.1000	.1189	.0624	.0349
7	5.24 gm.	62.3 gm.	.0883	.0537	.0855	.1006	.0672	.0196
8	5.50 gm.	58.7 gm.	.0943	.0547	.1084	.0893	.0491	.0258
9	5.03 gm.	61.2 gm.	.0837	.0555	.0954	.0872	.0574	.0248
10	5.11 gm.	61.4 gm.	.0994	.0615	.0891	.1017	.0700	.0329
11	4.83 gm.	59.0 gm.	.0974	.0506	.0793	.0851	.0687	.0240
12	4.73 gm.	58.5 gm.	.0744	.0603	.0815	.0973	.0647	.0302
Mean			.0919	.0565	.0901	.0946	.0603	.0275
Maximum Deviation from Mean			+11% -19%	+11% -11%	+20% -12%	+25% -14%	+16% -18%	+28% -29%
Coefficient of Variation			8.4%	7.6%	9.0%	9.9%	10.73%	18.14%

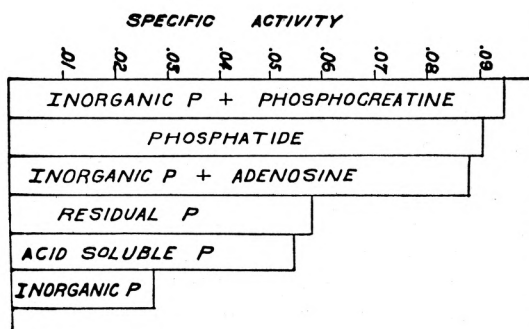
±11% for the acid soluble phosphorus fraction to +28% and -29% for the inorganic phosphorus fraction. The coefficient of variations are respectively 7.6% and 18.1%.

The average of the specific activities of the six fractions are plotted in Graph 1. They range from a high of 0.0946 for

the phosphocreatine fraction to 0.0271 for the inorganic phosphorus fraction. In most of our subsequent discussion based on Table 1, we shall express values in percentage of the mean rather than the numerical values.

DISCUSSION

In computing the coefficients of variation for the phosphorus fractions in Table 1, we have tacitly assumed that the distribution is normal, that is, it follows the familiar bell-shaped curve. This assumption seems reasonable for the original population from which the individual eggs were drawn. Fortunately, it is true that even if a population deviates from normality, the distribution of means of samples tends to follow a normal distribution (Hoel, 1947). In this work we have used the mean of the three embryos as our individual values, hence we would expect such values to follow a normal



GRAPH 1. Relation among the specific activities of the phosphorus fractions. Specific activity is defined as counts per second per microgram of phosphorus.

curve even though the parent population might be abnormal.

With the coefficients of variation we may set up the usual limits for deviations from the means. Our primary concern, however, is with how reliable are the determinations of the means for the phosphorus fractions in permitting us to estimate the means for the population consisting of groups of three eggs from the same flock which we might have procured for the experiment. This problem seems to fit all conditions necessary for an application of Student's *t*-distribution for the determination of confidence limits for the mean (Hoel, 1947). We may state the problem as follows: Let the specific activities for each phosphorus fraction for all such groups of these eggs in the manner of this experiment be normally distributed with mean m and variance σ^2 ; let \bar{x} and s^2 be their sample values determined from a random sample of size, n , i.e., n groups consisting of three embryos each, then the new variables

$$U = \frac{(\bar{x} - m)}{\sigma/\sqrt{n}}$$

and

$$V^2 = \frac{ns^2}{\sigma^2}$$

satisfy the conditions for the *t*-distribution; then

$$t = \frac{(\bar{x} - m)\sqrt{n}}{s}$$

has a *t*-distribution with n degrees of freedom.

From the tabulated values of *t* (Hoel, 1947, p. 248), we find for a 99.0% and a 95.0% confidence level for m , the values $\bar{x} \pm 0.82\sigma$ and $\bar{x} \pm 0.54\sigma$ respectively. Such values are shown in Table 2. The first column states that the probability is

TABLE 2.—Confidence levels for the means

	95.0%	99.0%
Phosphatide P	4.54%	6.88%
Acid Soluble	4.1 %	6.22%
Inorganic P and adenosine P	4.86%	7.38%
Phosphocreatine plus inorganic P	5.35%	8.16%
Residual P	5.78%	8.78%
Inorganic P	9.78%	14.85%

95.0% that the mean determined for the phosphatide P fraction is within $\pm 4.54\%$ of the mean of this phosphorus fraction for the population of groups of eggs from which our sample is drawn. The other values are interpreted similarly.

Two different types of errors are recognized in such statistical problems as we are treating: Type 1, the hypothesis (that the agent influences the effect) is correct but the experiment rejects the hypothesis; Type 2, the hypothesis is incorrect but the experiment accepts the hypothesis. It is our belief that in experiments such as we are concerned Type 2 errors are more serious than Type 1 since Type 1 errors are not likely to be published. We feel then that a fairly high probability should be set for accepting a result as showing influence of the agent. In this discussion we have set 95.0% as the lower limit with the 99.0% level as being preferable. It is our belief, too, that experiments of this type should be designed to keep the Type 2 error within some such bound. The phosphatide phosphorus fraction will be used to illustrate the testing for Type 2 errors.

Let us suppose that an experiment has been performed where the effect of the agent is to decrease the specific activities. Where \bar{x} is the value found from our experiment, m and σ are the mean and coefficient of variation of the population for the trait under investigation—in this instance, they are mean specific activity

(0.0919) and coefficient of variation—(0.0077) of the phosphatide phosphorus fraction. The 0.05 range for a Type 1 error is then, $\bar{x} > (m - 2\sigma)$ which equals 0.0765 for the phosphatide fraction.

The probability of a Type 2 error is that \bar{x} will not fall within this initial region ($\bar{x} < m - 2\sigma$) when the hypothesis is false. We may treat the problem as 1 minus the probability that \bar{x} will fall in the region when the hypothesis is false. Thus in order to make the Type 2 error 0.05, it is necessary that \bar{x} satisfy the inequality when the experiment yields a value z . We assume that the experimentally determined mean would have the same coefficient of variation as before, then the 0.95 requirement may be written

$$\frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^{m-2\sigma} e^{-\frac{1}{2}(\bar{x}-z/\sigma)^2} d\bar{x} = 0.59.$$

This integral states that as long as the true experimental mean, \bar{x} , is less than $(m - 2\sigma)$ we will reject the hypothesis with the experimentally determined value z in 95.0% of the cases. From this we may determine the limits on z which will ensure the rejections.

From the table for the error integral, we get

$$\frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^{11.15-129z} e^{-u^2/2} du = 0.95$$

which requires

$$11.15 - 129z = 1.64$$

$$z = \frac{9.51}{129} = 0.0737$$

Thus if a single value of the specific activity of the phosphatide phosphorus fraction is less than 0.0737 the probability is 95.0% that we do not commit the Type 2 error, that is, the experiment accepts an incorrect hypothesis.

Graph 1 shows that the specific activities of the phosphorus fractions fall into three groups: (1) inorganic P from phosphocreatine, phosphatide, inorganic P plus adenosine P; (2) residual P, acid soluble P; (3) inorganic P. The differences within each group amount to only a few percent and are less than the coefficient of variation for each fraction. These findings are consistent with the interpretation of Hevesy *et al.* (1938) that the organic phosphorus compounds of the embryo are synthesized from the inorganic phosphorus from the yolk. The findings of Hevesy *et al.* (1938) that the specific activities of all the phosphorus fractions including inorganic phosphorus were the same in the 11 and the 18-day embryo would require that the differences we have observed would be equilibrated by that time. Unfortunately, Hevesy *et al.* (1938) gave no value for the specific activity of the inorganic phosphorus for the six-day embryo.

The results in Graph 1 are consistent with the view of phosphorus metabolism in the chick embryo reported by Branson, Brooks and Piper (1950) who interpreted their data to imply that the embryo first used the inorganic P in the egg for building of the phosphorus compounds. Their proposed sequence of reactions is presented in Figure 1. The organic phosphorus compounds are hydrolyzed outside the embryo by a phosphatase activated or released by the embryo. The resulting inorganic phosphorus enters the embryo where it is used to build the organic phosphorus compounds.

According to this view, the inorganic phosphorus which enters the embryo in the early days of development should have a higher specific activity than that which enters later since the latter has been produced by hydrolysis from inactive phosphorus in an organic com-

pound. We may interpret our results as indicating that the high specific activity group (1) was formed from inorganic phosphorus which entered the embryo earlier in its development while the lower activity group (2) was formed from later inorganic phosphorus. As time goes on the phosphorus in the embryo would be

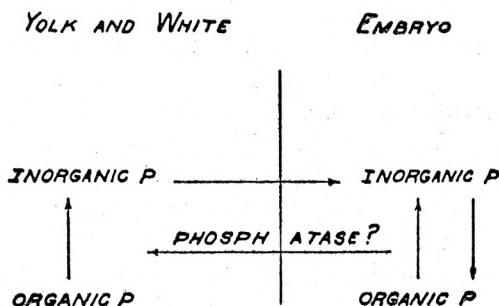


FIG. 1. The proposed path of phosphorus between the yolk and white and the embryo. The vertical line represents the membranes separating the embryo from the remainder of the egg.

expected to travel around the metabolic cycle and become thoroughly mixed, hence one would expect the specific activities of all fractions in older embryos to tend to the same value. An alternative to the preceding interpretation would be that the group (2) compounds, residual phosphorus (mainly nucleoprotein) and acid soluble phosphorus compounds, exchange more rapidly with the inorganic phosphorus and are thus more reactive in the embryo than they are in the adult.

Popjak and Beeckmans (1950) found that the foetal placenta of the rabbit does not transmit unhydrolyzed phospholipin (phosphatide) molecules to the foetus. Their findings and these reported here are additional evidence that as a general rule in living organisms phosphorylated organic molecules are not transported across foetal membranes but are hydrolyzed into

simpler compounds or components and the components transported.

SUMMARY

1. In experiments on phosphorus metabolism studied with P^{32} in the 8-day chick embryo, analyzed in groups of three, the specific activities of the phosphatide, acid soluble, inorganic plus adenosine, inorganic plus phosphocreatine, residual and inorganic phosphorus fractions varied from a low of $\pm 11\%$ of the mean for the acid solubles to a maximum of $+28\%$ and -29% of the mean for the inorganic fractions.

2. In experiments on the effects of chemical or physical agents, by working with fairly large samples (*e.g.* 12 values) for each fraction we may greatly increase the sensitivity of the experiment in revealing differences between the experimental and control values. From Table 2, with such a sample, we can conclude that if the means of the inorganic P fractions differ by only 35.0% (the smaller taken as 100%) the probability is 99.0% that the difference is attributable to the agent.

3. The specific activities of the phosphorus fractions fall into three groups with the inorganic P from phosphocreatine, phosphatide, and inorganic P plus adenosine P having essentially the same value (0.09), the residual P and acid soluble P form the second group (0.065) and the inorganic P is lowest (0.027). These relations are shown in Graph 1. These findings are consistent with the view that the embryo uses the inorganic phosphorus in the yolk to build its organic phosphorus compounds. This inorganic phosphorus is produced by the hydrolysis of organic phosphorus compounds in the yolk.

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