CLV. INTERMEDIARY CARBOHYDRATE METABOLISM IN EMBRYONIC LIFE

II. THE FORMATION AND REMOVAL OF PYRUVIC ACID

III. THE PASTEUR EFFECT AND THE MEYERHOF CYCLE

IV. THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS

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II. THE FORMATION AND REMOVAL OF PYRUVIC ACID

INTRODUCTION

In the preceding paper of this series, grounds have been given for suspecting the existence of a non-phosphorylating glycolysis in the tissues of the chick embryo. Since, owing to the work of Utewski [1929], Hahn & Haarmann [1930],

Case & Cook [1931], Case [1932] and Meyerhof & McEachern [1933], we now know that pyruvic acid is an important intermediary in the phosphorylating glycolysis of muscle, it was of much interest to examine its accumulation during the glycolysis of the embryo.

ISOLATION OF PYRUVIC ACID AND METHYLGLYOXAL HYDRAZONES

Following the method of Neuberg & Kobel [1930] as developed by Case & Cook [1931] we obtained the 2:4-dinitrophenylhydrazones of these compounds from embryo tissue after a period of glycolysis. To the trichloroacetic acid filtrates or centrifugates from the manometric cups were added sufficient amounts of a saturated solution of 2:4-dinitrophenylhydrazine in 2*N* HCl, and the mixture was warmed for some time on a boiling water-bath. After it had evaporated to something more than half its original volume, it was transferred to the ice-chest and left overnight. Some experiments carried out thus are the following:

Exp. 33. Forty-five 4-day embryos were incubated at 38° for 2 hr. in Ringer-bicarbonate anaerobically in the presence of *M*/50 sodium bisulphite. After the above procedure, the small amount of precipitate was filtered off on a small Jena hard-glass filter and washed with water. 1 ml. of warm 25 % Na₂CO₃ solution was then passed through the filter, giving a reddish coloration with the precipitate, the main part of which dissolved and passed into the filtrate. To the filtrate strong HCl was added; when enough had been added the solution became turbid, indicating the probable presence of pyruvic (or an acid) hydrazone. The residue on the filter gave a deep blue colour with alcoholic potash, indicating the probable presence of methylglyoxal.

Exp. 193. Thirty 6-day embryos were made into brei under ice-cold conditions, then dialysed in a collodion sac for 16 hr. against tap water at 0°. A portion was kept an equal length of time at 0° without dialysis. Then side by side with manometric experiments, samples of the dialysed and undialysed material (3 ml.) were incubated at 38° for 3 hr. with 0.4 ml. 2.5 % glucose solution and 0.4 ml. *M* sodium bisulphite. The results were as follows:

Relative glycolytic rates of the two samples:

Undialysed: 515 μ l. gas output in 1½ hr.

Dialysed: 36 μ l. gas output in 1½ hr.

	Total hydrazone precipitate	Alcoholic potash test on residue
Undialysed 0 min.	+	+
Undialysed 3 hr.	+	+
Dialysed 0 min.	+	—
Dialysed 3 hr.	+	+

This indicates that parallel with loss of glycolysing power through dialysis, there has taken place a loss of power to form methylglyoxal, limited though this may be in any case. This disposes of the other alternative, namely, that in conditions where lactic acid formation was interfered with, as here, a large methylglyoxal formation would be found.

Exp. 211. A freshly made ice-cold brei of 7-day embryos was divided into two portions as follows:

	A ml.	B ml.
Brei	16	16
Glucose	—	2.4
Water	2.4	—
Bicarbonate	3.65	3.65
<i>M</i> Bisulphite	1.0	1.0

There was thus glucose present in B to the extent of 60 mg., and from Exps. 29 and 30 (described later) we should expect a utilization of between 36 and 37 mg. of this glucose. Yet the

amounts of methylglyoxal and pyruvic acid which it was possible to isolate as hydrazones and weigh were very small:

	A mg. found	B mg. found
Methylglyoxal	4.7	2.6
Pyruvic acid	1.1	2.1
Total	5.8	4.7

From these experiments we conclude that the amounts of methylglyoxal and pyruvic acid formed by embryo tissue are relatively small. In view of the difficulties of collecting a large amount of starting material we did not carry out any further attempts at isolation but turned our attention to the quantitative experiments now to be described.

FORMATION OF PYRUVIC ACID IN THE PRESENCE OF VARIOUS SUBSTRATES

The pyruvic acid was estimated according to the method of Clift & Cook [1932]. Bisulphite was added either at the beginning or at the end of the glycolysis period and the trichloroacetic filtrate divided into two portions. One was titrated directly, giving the total content of carbonyl compounds, the other was neutralized and treated with NaOH in an open flask on a boiling water-bath to destroy all alkali-labile carbonyl compounds of the type of methylglyoxal, then acidified and titrated. The titration method consists in removing the excess bisulphite from the solution by adding the required amount of iodine, then liberating the carbonyl compounds from bisulphite combination by the action of sodium bicarbonate and titrating the bisulphite so freed with a dilute standard iodine solution. Thus 1 ml. of *N*/500 iodine is equivalent to 0.088 mg. pyruvic acid. The titration after alkali treatment should thus always be lower than before, but it was not so in all cases, a fact which may be explained by the formation under conditions unknown of parapyruvate. Parapyruvate (a polymeric form) is converted into pyruvate by the alkali treatment.

In our experiments with embryos, the titration values before and after alkali treatment were often not very different, and there seemed to be no regular relation between the effect of the treatment and the substrate which had previously been glycolysed. Thus when glucose was the substrate, rises were obtained in four cases and falls in three. Typical figures were the following: in Exp. 44 (glucose) the titration figure before the alkali treatment was 1.5 ml. of *N*/500 iodine, and after, 2.68. On the other hand, in Exp. 42 the figure fell from 1.89 to 0.4 ml. Since we were primarily interested in studying the formation of pyruvate, we did not seek to elucidate the conditions under which methylglyoxal was formed. It may be mentioned, however, that the results with mannose as substrate were particularly striking, for here (Exp. 42) the values prior to alkali treatment were 5.3 and 5.9 ml. respectively, falling after it to 0.53 and 0.82.

We first established that the formation of pyruvic acid occurs to a greater extent in the presence of glucose than in its absence. The data assembled in Table I show that rather over twice as much pyruvic acid is formed in the presence of glucose as in its absence. The average amount is not inconsiderable, 83 mg./100 g. wet wt./hr., or just twice as much as was reported by Mendel *et al.* [1931] for Jensen rat sarcoma, and four times as much as the highest values (for liver and kidney of polyneuritic chicks, 15–20 mg./100 g. wet wt./hr.) given by Sherman & Elvehjem [1936], using the Clift-Cook method. Their value for muscle was as low as 2–3 mg./100 g. wet wt./hr. In normal avian brain pyruvic acid does not accumulate at all. The figures of Meyerhof & McEachern [1933] for frog muscle approach ours more nearly.

Although it is sure that more pyruvic acid is formed in the presence of glucose than in its absence, it was at once obvious that the increase was in no

Table I. *Formation of pyruvic acid in autoglycolysis and glucose glycolysis, anaerobically*

Exp.	Autoglycolysis				Glucose				
	Wet wt. mg.	Time hr.	Pyruvic acid mg.	mg./hr./ g. wet wt.	Wet wt. mg.	Time hr.	Pyruvic acid mg.	mg./hr./ g. wet wt.	
29	127	2	0.035	0.189	99	2	0.07	0.354	
30	332	1.4	0.08	0.172	80	1.5	0.062	0.518	
	94	1.5	0.035	0.249	79	1.4	0.062	0.561	
	87	1.5	0.062	0.475	—	—	—	—	
35	363	1.5	0.187	0.343	314	1.5	0.214	0.454	
38	—	—	—	—	12	2	0.044	1.84	
39	87	2	0.098	0.562	25	2	0.085	1.70	
42	67	2	0.068	0.507	124	2	0.076	0.307	
43	137	1.6	0.102	0.465	—	—	—	—	
44	168	2	0.091	0.270	125	2.1	0.236	0.900	
45	44	2	0.026	0.290	—	—	—	—	
				Av. 0.352					0.830

way comparable with the difference between the manometric acid productions in the two cases. Embryo glycolysis is increased ten times or more when glucose is given, pyruvic acid formation only doubled. It was found, on exploring further this relationship, that in every case when the glycolytic quotient was high, indicating utilization of substrate, the pyruvic acid formed in percentage of the carbohydrate broken down (calculated from the gas-output) was very low, while when no substrate was given or the substrate was not utilized the pyruvic acid percentage was quite high. This uniform relationship is shown in Table II. Autoglycolysis gives a pyruvic percentage of 55, that is to say, at the end of a 2 hr. glycolysis period, 55 % of the carbohydrate (or other substances) broken down are to be accounted for as pyruvic acid. Glucose, on the other hand, gives a pyruvic percentage of only 8. Glucolysis is not interfered with by the presence of hexosemonophosphate, and the pyruvic percentage is correspondingly of the same order of magnitude, 10. Nor is it affected by the presence of considerable amounts of inorganic phosphate [see Needham & Lehmann, 1937], so that the quotient remains high, and the pyruvic percentage is only 6. With mannose or with glucose and mannose in competition, the same result is found. But the condition of affairs where non-utilizable substrates are present is in striking contrast. Pyruvic percentages are: for glycogen 36, hexosemonophosphate 41, mannosemonophosphate 55, glucosamine 89 and glucosamine with inorganic phosphate 35. The effect of the presence of bisulphite during the glycolysis is also interesting. It very markedly increases the autoglycolysis (Q_L^{Na} rises from +2.2 to +7.7) and correspondingly diminishes the pyruvic percentage. Conversely, it inhibits the glucolysis (from +17.9 to +2.1) and raises the pyruvic percentage.

There can be only one simple explanation for this state of affairs, namely, that upon a large and regular pyruvic acid formation in autoglycolysis is superimposed a small and regular pyruvic acid formation in glucolysis. The following calculation elucidates the relative intensities of formation:

	Autoglycolysis	Glucolysis
Pyruvic acid formed mg./g. dry wt./hr.	3.5	4.8
Gas output, μ l./g. dry wt./hr.	2160	17,900
Calculated corresponding mg. glucose broken down/g. dry wt./hr.	8.63	71.7
Percentage of carbohydrate broken down normally appearing as pyruvic acid	40.5	6.7

Table II. Pyruvic acid percentages with various substrates in glycolysis, 4-5 day embryos (see text)

Exp.	Nil	Nil + B	Gluc.	Gluc. + B	Glyc.	Glyc. + B	HmP	Q_{11}^*						Mann.	MannmP	Glucos-amine	Glucos-amine + P
								HmP + Gluc.	Nil + P	Gluc. + P × 4	Gluc. + Mann.	+ 17-76	+ 0-0				
		+ 2-16	+ 7-74	+ 17-9	+ 2-06	+ 0-03	+ 0-03	+ 0-3	+ 9-81	+ 5-56	+ 16-01	+ 18-15	+ 17-76	+ 0-0	+ 0-27	+ 0-30	
26	—	—	—	5	20	36	13	—	—	—	—	—	—	—	—	—	
29	33	8	4	11	10	—	—	—	—	—	—	—	—	—	—	—	
—	—	5	—	10	—	—	—	—	—	—	—	—	—	—	—	—	
30	28	29	5	78	—	—	—	—	—	—	—	—	—	—	—	—	
—	60	7	7	15	—	—	—	—	—	—	—	—	—	—	—	—	
—	24	8	—	24	—	—	—	—	—	—	—	—	—	—	—	—	
35	37	—	9	—	—	—	—	41	10	—	—	—	—	—	—	—	
38	157	—	12	—	—	—	—	—	54	7	—	—	—	—	—	—	
39	76	—	18	—	—	—	—	—	38	5	—	—	—	—	—	—	
42	59	—	4	—	—	—	—	—	—	—	9	6	—	—	—	—	
43	43	—	—	—	—	—	—	—	—	—	—	11	47	64	—	—	
44	70	—	11	—	—	—	—	—	—	—	—	—	—	—	88	—	
45	18	—	—	—	—	—	—	—	—	—	—	—	—	—	136	—	
Aver.	55	11	8	26	36	36	13	41	10	36	6	9	8	55	89	35	35

Abbreviations. Nil, autoglycolysis; B, bisulphite *M*/10 to *M*/50; Glyc., glycogen; HmP, hexosemonophosphate; P, inorganic phosphate to make equivalent to phosphate Ringer; P × 4, four times this amount of inorganic phosphate; Mann., mannose; MannmP, mannosemonophosphate.

As has already been pointed out, there is, of course, no proof that the autoglycolysis is a real carbohydrate breakdown, and the pyruvic acid formed in it may therefore have another, e.g. a protein, origin. On the other hand, it is clear that at the end of an experiment lasting from 1.4 to 2.1 hr. some 7% of the glucose glucolysed has been transformed into pyruvate. We next investigated the change in this quota with time.

PYRUVIC ACID PRODUCTION AT DIFFERENT STAGES OF GLYCOLYSIS

Suspecting that in the earlier stages of glycolysis the pyruvic acid production might be more intense, we withdrew samples after successive periods and estimated the pyruvic acid in them. The experiments to be described (Exps. 114 and 116) were carried out in the following way. Large round-bottomed flasks with glass stoppers through which passed inlet and outlet tubes (with taps) were arranged so that they could be gently shaken in the bath at 38°, the usual manometric proportions as regards tissue, Ringer-bicarbonate, gas mixture and gas volume being maintained. About 10 or 12 4½-day embryos were used in each flask, giving a total wet weight of about 1 g. Flasks with glucose received 60 mg. each. The results, shown in Table III and Fig. 1*a, b*,

Table III. *Pyruvic accumulation; mg./g. wet wt./hr.*

	Time hr.	Anaerobic		Aerobic	
		Auto- glycolysis	Glucolysis	Auto- glycolysis	Glucolysis
Exp. 114	1	0.117	0.610	0.147	1.00
	2	0.082	0.277	0.124	0.306
	3	0.052	0.195	0.093	0.192
Exp. 116	½	0.262	1.04	0.208	0.752
	1½	0.087	0.250	0.109	0.240
	2½	0.061	0.176	0.070	0.144
	3½	0.043	0.105	0.050	0.078

demonstrated that our expectation had been correct and that the intensity of formation of pyruvic acid reached a maximum after about half an hour's glycolysis, falling off progressively afterwards.

The subject was further pursued by studying in parallel the pyruvic accumulation, the anaerobic glycolysis quotient and the pyruvic percentage. For this purpose we reverted to the usual Warburg manometers, starting with as many as possible and withdrawing them one by one after various successive intervals. The results, summarized in Table IV and Fig. 2*a, b, c, d*, give a fairly complete picture of the pyruvic acid changes during anaerobic glycolysis. There is a quick rise from a low initial value to a maximum rate of production of pyruvic acid, followed by a steady fall till the end of the period. In each successive period Q_L^N also falls, as expected, while the consumption of glucose continually rises and the percentage of it remaining in the form of pyruvic acid steadily declines. It is important to note that the pyruvic acid falls not only relatively but also absolutely. If we might assume that it is converted into lactic acid by reduction, we could summarize the facts by saying that in the early stages of glycolysis it is formed faster than it is reduced, but later the speed of the formation process falls below that of the reduction. There seems also to be a relationship between the pyruvic acid and the glycolytic intensity; for so long as the former is accumulating the latter remains constant, but when the former

Fig. 1 a. + Aerobic. x Anaerobic.

Fig. 1 b. o Aerobic glucose. • Anaerobic glucose.

Fig. 1 a and b. Formation and disappearance of pyruvic acid in the absence of glucose (1a) and in its presence (1b).

Table IV. *Pyruvic accumulation compared with pyruvic percentage, and anaerobic glycolysis quotient*

	Time hr.	Initial mg. pyruvic/g. wet wt.	mg. pyruvic/g. wet wt./hr.	$Q_L^{N^1}$	Calculated glucose breakdown mg.	Pyruvic percentage
Exp. 118	0	0.041	—	—	—	—
	$\frac{1}{4}$	—	0.406	+13.8	0.58	7.35
	$\frac{1}{2}$	—	0.300	+14.5	1.32	5.17
	$\frac{3}{4}$	—	0.146	+14.6	1.85	2.52
	1	—	0.068	+11.0	2.48	1.55
	$1\frac{1}{4}$	—	0.048	+5.3	2.44	2.28
	$1\frac{1}{2}$	—	0.022	+6.8	3.69	0.81
	$2\frac{1}{2}$	—	—	Total possible	5.00	—
Exp. 120	0	0.070	—	—	—	—
	$\frac{1}{4}$	—	0.464	+10.9	0.81	10.6
	$\frac{1}{2}$	—	0.208	+10.4	1.32	5.7
	1	—	0.056	+7.0	1.88	2.2
	$1\frac{1}{2}$	—	0.044	+6.1	2.66	1.0
	2	—	0.035	+4.8	2.62	1.8
	3	—	0.090	+4.1	3.34	0.4
				Total possible	5.00

Fig. 2. Comparison of pyruvic acid accumulation and glycolysis: *a*, anaerobic glycolysis, $Q_L^{N^1}$; *b*, mg. pyruvic acid formed or removed/g. wet weight embryo; *c*, percentage of glucose glycolysed appearing as pyruvic acid; *d*, mg. pyruvic acid/g. wet wt./hr.

ceases to accumulate the latter quickly falls off. This would be in agreement with the suggestion that pyruvic acid is an intermediate in the formation of lactic acid from glucose.

PYRUVIC ACID PRODUCTION BY OLDER EMBRYOS

Since the suggestion has been made by Edson *et al.* [1936] that pyruvic acid may be involved in the synthesis of uric acid by avian tissues from the ammonia of protein breakdown, it was thought worth while to enquire whether at later stages of development the same level of pyruvic acid production exists, or whether it is appreciably lowered. At the ages hitherto under examination

(2½–6 days) the embryo does not form uric acid, but by the tenth day its maximum speed of uric acid production has already been attained [Needham, 1926]; the tenth day was therefore chosen for this purpose. Embryos of this age are extremely difficult to cut into slices by the usual method with safety-razor blades, and the resulting state of the tissue was something intermediate between slices and brei.

Table V

Accumulation of pyruvic acid during glycolysis of tenth-day embryo "slices", in absence of ammonia

	Initial	Anaerobic				Aerobic	
		Nil	Glucose	Mannose	Sorbose	Nil	Glucose
Exp. 88. Time 2 hr.							
$Q_L^{N_2}$	...	+1.98	+9.02	+8.82	+0.14	—	—
Pyruvic %	—	20	3	7	46	—	—
Pyruvic acid:							
mg./g. wet wt.	0.048	0.257	0.180	0.470	0.650	0.234	0.277
mg./g. wet wt./hr.	—	0.105	0.067	0.212	0.301	0.094	0.116
Pyruvic acid from 4-day embryos (Table III)	—	0.082	0.277	—	—	0.124	0.306

Accumulation of pyruvic acid in tenth-day embryo "slices", aerobically, in presence of ammonia

(Ammonium chloride, 3.2 mg./ml.)

	mg./100 g. NH ₃	Pyruvic acid, mg./g. wet wt./hr.	
		Nil	Glucose
Exp. 93. Time 3 hr.	6.36	—	0.279
	12.72	—	0.315
	25.44	0.052	0.308
	50.88	—	0.474
			Av. 0.344

Table V, which gives the results, shows that pyruvic acid accumulates at this stage of development just as at the earlier stages, and the pyruvic percentages in relation to the hexoses used agree with those previously found. The presence of ammonia, inviting the formation of uric acid, does not reduce the pyruvic acid accumulation.

THE LACTATE $\rightleftharpoons$ PYRUVATE REACTION

In experiments to be reported in Section III (p. 1196) it was found that when the aerobic disappearance of lactate was compared with the oxygen consumption in presence of lactate, a certain discrepancy was sometimes seen. Thus in Exps. 59 and 72 $Q_L^{O_2}$ was -4.7 while Q_{O_2} was -9.4 . Yet if all the lactate disappearing was being oxidized, $Q_L^{O_2}$ should be equal to $\frac{Q_{O_2}}{3}$, since $C_3H_6O_3 + 3O_2 = 3CO_2 + 3H_2O$. Some lactate therefore disappears without being oxidized. To find whether this portion is converted into pyruvate, the following experiments were made:

	Time hr.	mg. pyruvic/g. wet wt./hr.			
		Anaerobic		Aerobic	
		Nil	Lactate	Nil	Lactate
Exp. 76	2	—	0.108	0.053	0.173
Exp. 78	2½	—	—	—	0.200
From Table III		0.082	—	—	—

It appears, therefore, that a proportion of lactate may be oxidized to, and remain as, pyruvate, the magnitude of which varies according to as yet unknown conditions.

It will later be shown [Needham & Lehmann, 1937] that the reverse reaction does not proceed anaerobically.

RELATION OF VITAMIN B₁ TO PYRUVIC ACID ACCUMULATION

The work of Peters and his collaborators on avian brain metabolism in relation to avitaminosis-B [Peters & Thompson, 1934; Gavrilescu *et al.* 1932] led us to investigate whether the pyruvic acid formations observed by us in embryonic glycolysis could be lowered or abolished by addition of vitamin B₁.

Table VI, containing several experiments on this point, shows that crystalline vitamin B₁ has no effect whatever on the aerobic or anaerobic pyruvic acid accumulation of embryos.

Table VI. *Vitamin B₁ and accumulation of pyruvic acid in embryo glycolysis*

1 ml. crystalline vitamin B₁ solution contained 5γ; 3 ml. Ringer-bicarbonate were adjusted to contain 1.65γ; in the experiments of Peters, 3 ml. contained 2γ.

Exp.	mg. pyruvic acid/g. wet wt./hr.							
	Anaerobic				Aerobic			
	Nil	Nil + vitamin	Glucose	Glucose + vitamin	Nil	Nil + vitamin	Glucose	Glucose + vitamin
98	—	0.047	—	0.041	—	0.040	—	0.103
116	0.043	—	0.105	—	0.050	—	0.078	—
106	0.050	0.040	0.045	0.055	0.032	0.030	0.070	0.072
112	0.033	0.029	0.107	0.092	0.050	0.052	—	—

It is unlikely that a state of physiological avitaminosis-B exists normally in the chick embryo, since, as we know from the work of Chick *et al.* [1931] and others, vitamin B₁ is contained in appreciable amounts in the yolk, though not in the white. It is interesting to compare the present results with those of Sherman & Elvehjem [1936], who report that the anaerobic glycolysis rate of the tissues of polyneuritic chicks is quite normal and is unaffected by addition of vitamin B₁. The anaerobic accumulation of pyruvic acid, moreover, is unaffected by the addition of vitamin B₁. The general conclusion must therefore be that the effect of avitaminosis is seen only in aerobic conditions and that the chick embryo is not in a state of physiological avitaminosis.

PYRUVIC ACID AND THE INDUCTION PERIOD IN GLUCOLYSIS

In a number of experiments throughout the present work, it was noticed that, when glucose was tipped into brei or intact embryos previously maintained anaerobically without substrate for some 20 min., the onset of the acid production was preceded by an initial "induction period" lasting from 10 to 15 min. This had already been observed for some adult tissues and tumours by Rosenthal [1930; 1932] and by Dickens & Greville [1932; 1933], who believed it to be connected with the oxidation-reduction potential level of the system, since methylene blue and other dyes would abolish it as effectively as pyruvate. The accompanying figures (Figs. 3, 4, 5) show that this interpretation is probable also for embryonic glucolysis. Fig. 3 gives a few typical induction-period curves

Fig. 3. Curves to show induction period in glucolysis.

Fig. 4. Abolition of induction period by sodium pyruvate (Exp. 244).

Fig. 5. Abolition of induction period by methylene blue (Exp. 249).

(Exps. 168, 170, 172, 216 *a* and *b*); Fig. 4 illustrates the abolition of the delay by sodium pyruvate (Exp. 244), while Fig. 5 illustrates the same for methylene blue ($6.5 \times 10^{-4} M$) (Exp. 249).

III. THE PASTEUR EFFECT AND THE MEYERHOF CYCLE

INTRODUCTION

It has been shown by K. C. Dixon [1935] that the rate of disappearance of lactic acid from brain cortex is not nearly sufficient to account for the Pasteur effect on the Meyerhof theory. The argument employed was as follows. The rate of accumulation of lactic acid from glucose in nitrogen ($Q_L^{N_2}$) is much greater than that in oxygen ($Q_L^{O_2}$). If Meyerhof's theory provided the correct explanation for this fact, the rate of disappearance of added lactate (in the absence of glycolysable substrate) should be equal to the observed difference between the values of $Q_L^{N_2}$ and $Q_L^{O_2}$ in the presence of glucose. In brain this was shown not to be the case. In fact the rate of disappearance of added lactic acid is only equal to about one-eighth of the difference between the anaerobic and aerobic glycolyses. The disappearance of lactic acid by oxidation and resynthesis to carbohydrate cannot therefore explain the effect of oxygen in reducing the accumulation of lactic acid.

In order to test the applicability of Meyerhof's theory to the metabolism of embryonic tissue (in which the Pasteur effect is extremely marked) we have performed similar experiments using $4\frac{1}{2}$ -day chick embryos. The rate of disappearance of added lactic acid was compared with the difference between the anaerobic and aerobic glycolyses.

METHODS

The rate of accumulation of lactic acid in nitrogen and in oxygen and the rate of disappearance of lactic acid in oxygen were measured by manometric methods. We first employed the two-vessel method of Warburg [1924] to determine these entities. Similar calculations to those employed by K. C. Dixon [1935] were followed. This method assumes a respiratory quotient of unity. Accordingly, later the bicarbonate method of Dickens & Šimer [1930] was employed. From the results obtained by the use of this method the rates of aerobic acid production and disappearance were determined absolutely. Further, since the R.Q. obtained by the method of Dickens & Šimer both in the presence of glucose and of lactate are equal to unity, the arguments based on results obtained by the two-vessel method of Warburg are also fully justified.

Chick embryos (4–5 days) were used throughout. These were immersed in mammalian bicarbonate-Ringer to which the appropriate substrates were added. The manometric vessels containing these fluids were then filled with oxygen or nitrogen containing 5% CO_2 .

RESULTS AND DISCUSSION

The main results are shown in Table VII. It is seen that the aerobic glycolysis, though measurable, is usually very low in the chick embryo, as has been shown by Warburg *et al.* [1924]. Further, the difference between the rates of anaerobic and aerobic glycolysis ($Q_L^{N_2} - Q_L^{O_2}$) in the presence of glucose is much greater in

Table VII

	Sodium <i>dl</i> -lactate containing 0.2% <i>dl</i> -lactic acid			Glucose 0.2%			
	Q_{O_2}	$Q_L^{O_2}$	R.Q.	Q_{O_2}	$Q_L^{O_2}$	R.Q.	$Q_L^{N_2}$
W* 72 A†	- 6.8	-4.8	—	- 9.1	+1.3	—	+10.8
W 72 B	- 7.7	-3.7	—	—	—	—	—
W 59 A	-11.9	-5.8	—	-12.1	+0.7	—	+ 9.3
W 59 B	-11.3	-4.6	—	—	—	—	—
W 110 C	—	—	—	- 8.3	+0.6	—	+ 7.8
W 110 D	—	—	—	—	—	—	+ 8.0
DS* 105	—	—	—	- 6.35	+1.6	1.03	+14.2
DS 107	—	—	—	- 5.8	+1.7	1.18	+17.4
DS 108	—	—	—	-24.3	+2.8	—	+ 9.7
DS 109 A	-10.2	-3.9	1.08	—	—	—	—
DS 109 B	-10.4	-4.2	1.11	—	—	—	—
DS 110 A	- 8.05	-1.7	1.3	—	—	—	—
DS 110 B	-12.2	-0.47	1.05	—	—	—	—
DS 111 A	- 8.6	-1.1	0.91	—	—	—	—
DS 111 B	-10.4	-3.05	0.93	—	—	—	—
DS 113 A	- 6.25	-2.7	1.12	—	—	—	—
DS 113 B	- 6.25	-2.5	1.05	—	—	—	—

* W indicates 2-vessel method of Warburg; DS indicates method of Dickens & Šimer.

† Experiments A, B, C, etc., with same preceding numerals were performed on same batch of embryos.

value than the rate of disappearance of lactic acid ($Q_L^{O_2}$ here negative in value) where lactate is the substrate used. It is thus clear that the oxidative disappearance of lactic acid is not large enough to account for the effect of oxygen in reducing glycolysis. It thus appears that Meyerhof's theory for the mechanism of the Pasteur effect does not apply to the embryo.

Two possible criticisms of the above arguments present themselves.

(1) It might be supposed that in embryo to which lactic acid is added the oxidative removal mechanism is already saturated by lactic acid formed from some intracellular precursor. Thus the rate of disappearance of added lactate would be small. There is, however, hardly any intracellular precursor of lactic acid present in embryonic tissue, since we find that lactic acid formation in nitrogen when no substrate is present is almost negligible ($Q_L^{N_2}$ usually below 0.5). Embryo in this regard is very similar to brain [see K. C. Dixon, 1935; Holmes & Holmes, 1925; 1927].

(2) It has been shown (p. 1193) that lactic acid is oxidized in part to pyruvic acid in embryo. Thus the actual disappearance of lactic acid may be larger than that recorded in the above experiments which only measure the disappearance of total acid. However, some of the acid formed from glucose is also pyruvic acid. If Meyerhof's theory were correct there should be a disappearance of total acid equal to the difference between that formed from glucose in nitrogen and that formed from glucose in oxygen. No matter whether pyruvic acid is accumulating or not from the oxidation of lactic acid, the disappearance of lactic acid or pyruvic acid which this theory supposes to be built back to carbohydrate should cause an equivalent disappearance of acid. The total acid disappearance is, however, not nearly great enough to account for the diminution in the production of acid caused by the presence of oxygen. These criticisms are thus shown not to invalidate the arguments presented.

We may conclude that Meyerhof's theory for the mechanism of the Pasteur effect is not applicable to embryonic tissue. In embryo, as in brain, it seems

likely that oxygen reduces the gross rate of carbohydrate destruction either by reducing the amount of glycolytic enzyme accessible to the substrate [Dixon, K. C. & Holmes, 1935] or by inactivating the glycolytic enzyme [Lipmann, 1933, 1, 2].

The fact that lactic acid is not resynthesized according to the Meyerhof quotient may explain the accumulation of lactic acid in the intact egg during normal development (reaching a maximum on the fifth day) which has been observed by Tomita [1921] and many subsequent workers. Contrary to what is often stated, the chick embryo has quite a measurable aerobic glycolysis, approximately $Q_L^{O_2} + 2.0$.

Two more points have been investigated. First it was of interest to ascertain if embryo exhibits on immersion in Ringer containing $M/10$ potassium chloride the same increase in respiration and aerobic glycolysis as observed in brain cortex by Ashford & K. C. Dixon [1935]. The following results eliminate this possibility (see Table VIII). The addition of potassium chloride causes no

Table VIII. *Exp. 85*

Glucose + normal Ringer		Glucose + normal Ringer + $M/10$ KCl	
Q_{O_2}	$Q_L^{O_2}$	Q_{O_2}	$Q_L^{O_2}$
-9.9	+3.4	-7.7	+2.9

increase in the metabolism of chick embryo, unlike that of brain. Dickens & Greville [1935] showed the potassium effect to be absent from mammalian yolk sac, kidney etc. Similarly, Lasnitzki [1933, 1, 2; 1934] found that the degree to which respiration depends on the cation balance of the medium varied with age, embryonic tissues being less sensitive than adult tissues. Removal of potassium, or of calcium, or of both from the medium resulted in much greater inhibition of the respiration of adult liver and kidney than of foetal liver and kidney (rat). Lasnitzki also found that changes in the cations had a much greater effect upon brain glycolysis than on that of other organs, and that embryonic liver glycolysis was very little affected.

Secondly, we have investigated the aerobic and anaerobic metabolism of mannose in the chick embryo. It is clear from the following results that oxygen reduces mannolysis as much as glucolysis. The Pasteur effect is thus seen in both cases.

Table IX. *Exp. 115. (Mannose)*

Q_{O_2}	Q_L^O	R.Q.	Q_L^N	Meyerhof quotient
-6.7	+2.0	0.97	+14.2	1.8
-7.5	+1.7	0.98	—	—

The results given in this paper might appear to be in contradiction with those of the first paper of the series, since it was there shown that the autorespiration is very little affected by added substrates. Hence the present assumption that the removal of lactate is oxidative would be unjustified. But there is really no contradiction. It is true that in the former paper we showed that the respiration of the embryo in the presence of hexose substrates is not much increased above that of the autorespiration, but we also showed—not for the first time—that the R.Q. of the autorespiration is only 0.75 whilst that of the substrate respiration is unity. This must mean that the autorespiration is

abolished or very greatly decreased in the presence of substrate. We are therefore justified here in saying that the removal of lactate is oxidative, especially as we have shown that the R.Q. in its presence is unity and not 0.75. Without going further into the nature of the autorespiration, we may mention that we regard it as probably protein in character, ending in ammonia.

IV. THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS

INTRODUCTION

Facts reported in the preceding papers of this series have suggested that the glycolysis of the chick embryo does not proceed through a mechanism of phosphorylation. It was therefore of interest to make a systematic examination of the distribution of phosphorus between the various compounds to be found in a trichloroacetic acid extract of the embryo itself. Hitherto there has been no systematic attempt to find hexosephosphates in the embryonic tissue, for, apart from earlier work reviewed by Needham [1931], that of Kugler [1936] was specially concerned with the lipin phosphorus and its changes in the embryo during development. After the conclusion of the present experiments it was found that Takahashi [1935] had made some progress in the desired direction; his results will be discussed below.

METHODS

The description of a typical experiment will be the simplest way of summarizing the methods used, since all the later experiments followed this as a basis. Estimation of the inorganic phosphorus was carried out colorimetrically according to the method of Fiske & Subbarow [1925].

Forty-three $5\frac{1}{2}$ -day embryos, of a total wet weight of 8.685 g., were dropped, immediately on being taken from their eggs, on to ice-cold sand, and when all were collected, ground with 43.5 ml. of 5% trichloroacetic acid. After filtration through an asbestos mat at the pump, the total volume of extract was 45 ml. Duplicates of 2 ml. each were then removed for incineration to estimate directly the total phosphorus of the extract. The remaining 36 ml. were precipitated with 4 ml. of 25% barium acetate solution, and made just alkaline to phenolphthalein with strong NaOH. After standing some 20 min. in ice, the flocculent precipitate was centrifuged down and washed twice on the centrifuge with 8 ml. of 1% barium acetate. The precipitate was then dissolved in a drop or two of *N* HCl, diluted to 6 ml., and the barium removed by saturated sodium sulphate. The centrifugate was then divided into as many aliquot parts as necessary, some for determination of total barium-precipitable phosphorus, some for inorganic phosphorus, and others for fractional hydrolysis in the boiling water-bath with *N* HCl, the 7 min. value giving the adenylypyrophosphate phosphorus, and the difference between the 7 and 100 min. values giving the "hexosediphosphate" phosphorus.¹ Meanwhile the centrifugate from the barium precipitation was also divided into aliquot parts, some for determinations of total phosphorus,

¹ We put hexosediphosphate in inverted commas because in 100 min. only one of the phosphoric acid groups is split off, and the more difficultly hydrolysable hexosemonophosphate is left. There remains the possibility that this fraction contains esters other than hexosediphosphate.

others for phosphagen phosphorus, that fraction spontaneously hydrolysing on standing with the molybdate reagent for half an hour at room temperature. "Hexosemonophosphate" phosphorus would appear in this portion as the difference between the phosphagen phosphorus and the total barium-non-precipitable phosphorus.

THE DISTRIBUTION OF PHOSPHORUS

The figures of Table X show the results obtained on three normal batches of 5½-day embryos. As comparisons are more conveniently made on the basis of percentages of the total acid-soluble phosphorus, these are shown in Table XI.

Table X. *Distribution of acid-soluble phosphorus in 5½-day chick embryos*

	mg./g. wet wt.		
	Exp. 124	Exp. 125	Exp. 126
g. wet wt. embryos taken	8.68	4.9	14.25
Total P (direct)	0.401	0.380	0.304
Ba-precipitable:			
Total P	—	0.158	0.122
Inorganic P	0.056	0.033	0.029
"Adenylpyrophosphate" P (7 min.)	0.051	0.070	0.060
"HdP" (100 - 7 min.) values	0.019	0.010	0.016
Unhydrolysed after 100 min. (<i>t</i> - 100)	—	0.045	0.018
Ba-non-precipitable:			
Total P	0.256	0.260	0.125
Phosphagen P	Trace	0.019	0.014
"HmP" P	<0.256	0.241	0.111
Total P, sum of fractions	0.382	0.418	0.249

Table XI. *Phosphorus-distribution in 5½-day chick embryos; percentages of the total acid-soluble phosphorus*

	Exp. 124	Exp. 125	Exp. 126	Aver.
Inorganic P	—	8.0	11.6	9.8
Phosphagen P	—	4.6	5.7	5.2
"Adenylpyrophosphate" P	13.4	16.7	24.3	18.1
Ba-precipitable, hydrolysed in 100 min., "Hexosediphosphate" P	4.7	2.7	6.4	4.6
Ba-precipitable, not hydrolysed in 100 min. P	—	10.6	7.3	8.9
Ba-non-precipitable, "hexosemonophosphate" P	67.2	57.4	45.0	56.5

The amounts of inorganic phosphate and phosphagen found are in good agreement with the figures of Baldwin & Needham [1933, 1]. The "hexosediphosphate" fractions are very small, almost on the limit of detectability with assurance, but on the other hand the enormous amount of phosphorus in the "hexosemonophosphate" fraction is extremely striking. No less than half the total phosphorus extractable with trichloroacetic acid is to be found in a fraction, unprecipitable with barium, accompanying the phosphagen.

Comparison with the data in the literature for the tissues of adult animals is therefore invited. When we place side by side in one table (Table XII) the figures obtained in this way from a number of sources, we note the surprising fact that only in tumour, heart muscle, and possibly blood, is a similar state of affairs found.

Table XII. *Phosphorus distribution in a variety of tissues, for comparison with the chick embryo*

		Percentages of the phosphorus in the trichloroacetic acid extract					
		Barium-precipitable					Barium-non-precipitable esters "HmP" P
		Inorganic P	Phosphagen P	Adenylpyrophosphate P	"HdP" P	Not hydrolysable in 100 min.	
Chick embryo	(Present work)	9.8	5.2	18.1	4.6	8.9	56.5
		15.0					
Muscle	Frog [Cori & Cori, 1932]	13.5	49.0	22.6	11.3		3.7
		62.5					
	Rat [Hill & Kupalov, 1930]		62.1	22.2	11.1		4.6
	Fly [Baldwin & Needham, 1933, 2]	25.6	12.8	30.0	14.5		17.1
		38.4					
	Moth [Heller, 1936]	53.4	7.1	16.0			
Brain	Rabbit* [Ashford & Holmes, 1931]		62.5	4.2		33.3	
Nerve	Rabbit [Gerard & Wallen, 1929]	38.0	18.0		44.0†		
	Frog [Gerard & Wallen, 1929]	51.0	22.0		27.0†		
Liver	Rabbit [Takahisa, 1936]	15.0	4.0	6.3	31.7‡		
Heart muscle	Frog [Clark <i>et al.</i> 1931]	13.8	8.6	13.2		13.2	51.0
Blood	Human [Kerr & Daoud, 1935]§	13.2	0.0	23.0			
Tumours	[Outhouse, 1935]						
	Benign		59.0				30.0
	Malignant		61.0				29.0
	[Boydland, 1932]	48.0	4.0	22.0	1.0		15.0

* Holmes [1933] and Kerr [1935] both find small amounts of phosphagen in brain.

† Includes all fractions other than inorganic and phosphagen.

‡ Includes all fractions other than inorganic, phosphagen and adenylpyrophosphate.

§ These authors also found 52% undetermined; perhaps diphosphoglyceric acid.

|| Not hexosemonophosphate; perhaps hexaminephosphate or aminoethylalcoholphosphate.

As mentioned above, Takahashi [1935] investigated the water-soluble phosphorus compounds of the chick embryo. His methods differed from ours, in that he made no barium precipitations, relying entirely upon fractional hydrolyses. Nevertheless the amounts of difficultly hydrolysable material found by him were exceedingly large in proportion to the total water-soluble phosphorus and bear out well the findings now reported by us. In the following summary of his figures, which we reproduce since his paper is accessible only with difficulty, it will be seen that his "glycerophosphate P" (total phosphorus—phosphorus hydrolysable in *N* HCl in 180 min.) corresponds to our "hexosemonophosphate" phosphorus+our barium-precipitable phosphorus not hydrolysable in 100 min.

Percentage of the total water-soluble phosphorus

	Days of development...	5	10	15	20
Inorganic P		4.5	11.0	31.3	34.4
Phosphagen P		7.1	5.0	3.9	4.2
"Adenylpyrophosphate" P (7 min.)		3.3	3.5	1.6	5.5
Hexosediphosphate P (180 - 7 min.)		4.1	3.4	3.4	2.6
"Glycerophosphate" P (<i>T</i> - 180 min.)		80.9	77.2	65.2	53.3

The chief divergence from our work lies in the smaller amounts of adenylpyrophosphate found by Takahashi. We cannot, of course, accept without further evidence his designation of "glycerophosphate" for the difficultly hydrolysable material. According to Takahashi, it accounts for 84 % of the water-soluble phosphorus of the yolk.

THE NATURE OF THE "HEXOSEMONOPHOSPHATE" FRACTION

The phosphoric esters of tumours have been investigated principally by Outhouse [1935], who found, as is recorded in Table III, very little barium-precipitable material, but a great deal of phosphorus in the "hexosemonophosphate" fraction. This could not be hexosemonophosphate for very little reducing power (Hagedorn-Jensen) appeared on acid hydrolysis, and the Molisch test was not given. Part of it was thought to be hexosaminephosphate and part (unprecipitable by lead) probably aminoethylalcoholphosphate; a supposition subsequently confirmed by the same author [Outhouse, 1936].

Our examination of the nature of this barium-soluble ester, contained in chick embryo in such large amount, was directed in the first instance to deciding whether or not it was fermentable, and therefore whether or not it was likely to play an important part in carbohydrate metabolism. Only secondarily did we attempt to discover the chemical nature of the ester, reserving this in the main for a subsequent communication. In passing, however, we may give a few details regarding its general properties. On the unhydrolysed ester, the tests of Benedict, Fehling, Cole and Molisch were all found to be negative (Exp. 126). On the hydrolysed ester, the quantitative method of Elson & Morgan [1934] for glucosamine was quite negative, indicating strongly that the substance is not an aminohexose of any kind (Exp. 131). The reducing power (Hagedorn-Jensen) in Exp. 131 corresponded to 1.05 mg. of hexosemonophosphate where 8.5 mg. of the ester were calculated from the phosphorus estimations to be present. This reducing power of only 12 % is reminiscent of the figure given by Outhouse [1935] who could obtain a Hagedorn-Jensen figure only 9 % of that expected on the phosphorus determinations, in the case of the barium-non-precipitable esters of tumours. The material is hydrolysed with great difficulty. Thus in Exp. 133 where 49 % of the total acid-soluble phosphorus was in the "hexose-

monophosphate" form, only 6% was set free after 3 hr. hydrolysis in *N* HCl at 100°, and only 10% after 6 hr., or one-fifth of the fraction in question. The same results were obtained in Exp. 135, where out of 52.7% of the total phosphorus in this fraction, only 9.7%, or under one-fifth, had been set free after 7 hr. hydrolysis. Again, in Exp. 142 only 21.7% of the fraction was hydrolysed in 7 hr., and in Exp. 143 only 17.6%.

The point of most importance to us, however, was the question of the fermentability of the fraction. In experiments of which the following (131) is typical, we found that it is not fermentable by yeast. Fifty-nine 5-day embryos, weighing 12.48 g. wet weight, were worked up in the usual way, and the 37 ml. of centrifugate from the barium-precipitable esters were precipitated with 151 ml. of alcohol, as it has been shown by Cori & Cori [1932] that 80% alcohol is the most suitable concentration for precipitating barium esters in solution. The alcoholic solution, just alkaline to phenolphthalein, was allowed to stand at 0° for 3 hr., the precipitate centrifuged and dried in an air-stream, then taken up in a few drops of *N* HCl and brought to pH 6.0 in about 10 ml. water. The barium was next removed by precipitation with strong sodium sulphate; a control incineration showed that practically no phosphorus remained adsorbed on the barium sulphate. The final volume of unknown ester solution was just under 13 ml. Manometric experiments with Lebedev-extract¹ then showed that the ester could not be fermented:

	μ l./ml. Lebedev-extract/ hr. in air
Yeast alone	- 5
	- 12
Yeast + unknown substance	- 39
	- 25
Yeast + glucose	+ 506
Yeast + glucose + unknown substance	+ 305
	+ 374
Yeast + unknown substance + hexosemonophosphate	+ 56

The substance, then, could not itself be glycolysed. In this experiment the unknown barium-soluble fraction was 58.5% of the total acid-soluble phosphorus.

Another experiment (134) may be given in its entirety. Here the inorganic and phosphagen fractions totalled 15.9% of the whole water-soluble phosphorus, the adenylypyrophosphate fraction 15.3% and the barium-non-precipitable fraction 45.6%. On being precipitated with alcohol, the barium-non-precipitable fraction was made up to 1 mg. per ml. of aqueous solution (calculated as hexosemonophosphate) and tested manometrically as before. The results were as follows:

	μ l./ml. Lebedev-extract in 2 hr. in air
Yeast alone	- 1
Yeast + unknown substance	- 8
	+ 12
	- 16
	+ 4
Yeast + glucose (same concentration)	+ 528
Yeast + glucose + unknown substance	+ 736
Yeast + hexosemonophosphate (same concentration)	+ 348
Yeast + hexosemonophosphate + unknown substance	+ 173
Yeast + glucosamine	+ 9

The previous conclusion was therefore confirmed.

¹ The Lebedev-extract was prepared as follows. 5 g. dried yeast powder were shaken with 15 ml. distilled water and incubated at 37° for 3 hr. Solid material was centrifuged down and the supernatant liquid taken for use.

In examining the persistence of the fraction into the later stages of embryonic development, a quicker method was adopted, that of progressive hydrolysis, not barium precipitation, since we know that most of the barium-precipitable part (some 72 % of it) is hydrolysed in *N* HCl at 100° in 100 min., while in that period not more than 5 % of the barium-non-precipitable part will be hydrolysed. The following figures, then, were obtained:

	Days age	P in % total water-soluble P		
		Inorganic and phosphagen	Easily hydrolysable	Difficultly hydrolysable
Exps. already quoted	5½	15	22	65
Exp. 310	11	47	21	32
Exp. 313	15	70	7	23
Exp. 188	19	66	8	26

From this it appears that the relative amount of the unknown ester decreases as development proceeds.

From fourteenth and nineteenth day materials two samples of the substance were prepared in the usual way as dry white powders. From 29 g. fourteenth-day embryo, 0.284 g. was isolated, and from 271 g. nineteenth-day embryos 1.255 g. (samples 14 L and 19 N). From the older embryos a less pure sample was obtained, accompanied by some substance not containing phosphorus. The elementary analyses were found not to correspond with any of the likely possibilities. The phosphorus content (2.63 %) is too low for hexosemonophosphate (7.85 %) and much too low for diphosphoglyceric acid (12.98 %) or aminoethylalcoholphosphate (11.2 %), although both these latter compounds, like the present fraction, are extremely resistant to acid hydrolysis [Greenwald, 1925; Outhouse, 1935]. Absence of hexosamine (Elson-Morgan method) rules out glucosamine phosphate. Total carbohydrate (orcinol method) was very low, 3.6–5.3 %. The nature of the fraction will form the subject of a later communication in this series.

EFFECTS OF *IN VITRO* GLUCOLYSIS AND FLUORIDE ON THE PHOSPHORUS DISTRIBUTION

It seemed of interest to study the effect of *in vitro* glucolysis upon the phosphorus distribution in the presence and absence of fluoride. Our information as to the expected results of such an investigation in the classical phosphorylating case of muscle could serve as a background against which to compare what happens in the embryo.

The treatment which might be expected to cause the greatest deviation from the normal phosphorus distribution if embryo behaved like muscle would be a period of glucolysis inhibited by fluoride. From the work of the schools of Embden & Meyerhof it would follow that an accumulation of hexosediphosphate and of phosphoglyceric and glycerophosphoric acids should occur.¹ The former would greatly increase the amount of phosphorus hydrolysable in 100 min. under the conditions already specified, while the two latter, being hydrolysable only with great difficulty and being likely to be distributed between the barium-precipitable and barium-non-precipitable fractions, would increase the amount of residual phosphorus in the former and the non-phosphagen phosphorus in the latter.

¹ This, of course, assumes that the hypothetical esterification of glucose is relatively unaffected by fluoride. Enough pyruvic acid would be present in the embryos to play the part of acetaldehyde in fluoride-poisoned yeast.

The results obtained in such experiments, summarized in Tables XIII and XIV, show, however, that nothing of the kind took place. The embryos were placed in Ringer-bicarbonate-glucose solution in large stoppered flasks with

Table XIII. *Distribution of phosphorus in the chick embryo after periods of glycolysis etc.*

Conditions	mg./g. wet wt.				
	Exp. 133 Fluoride and phosphate 3 hr.	Exp. 135 Fluoride and phosphate 3 hr.	Exp. 142 Ringer Ca 3 hr.	Exp. 143 Ringer normal 3 hr.	Exp. 153 Fluoride and phosphate $\frac{1}{2}$ hr.
g. wet wt. embryos taken	9.19	7.05	12.58	11.16	4.30
Age in days	6	6	$5\frac{1}{2}$	6	6
Total P (direct)	0.398	0.328	0.310	0.414	0.555
Ba-precipitable:					
Total P	0.172	0.147	0.206	0.194	0.144
Inorganic P	0.140	0.108	0.172	0.180	0.108
Adenylpyrophosphate P (7 min.)	0.006	0.018	0.017	0.004	0.007
"HdP" P (100 - 7 min. values)	0.037	0.009	0.010	0.018	0.007
Unhydrolysed after 100 min. ($t - 100$)	0.0	0.011	0.006	0.0	0.021
Ba-non-precipitable:					
Total P	0.165	0.164	0.186	0.146	0.274
Phosphagen P	Trace	Trace	0.012	Trace	Trace
"HmP" P	<0.165	<0.164	0.174	<0.146	<0.274
Total P, sum of fractions	0.337	0.311	0.392	0.340	0.418

Table XIV. *Distribution of phosphorus in the chick embryo after periods of glycolysis etc.*

	% of the acid soluble P					
	Before glycolysis Average of Exps. 124, 125 and 126	After glucolysis 3 hr.			After glucolysis inhibited by fluoride	
		Normal Ringer- bic. Ca			3 hr. Average of Exps. 133 and 135	
		Ringer- bic. Exp. 143	Ca Exp. 142	Average	$\frac{1}{2}$ hr. Exp. 153	
Inorganic P	9.8	53.0	44.1	48.5	34.7	25.9
Phosphagen P	5.2	0	0	0	0	0
Adenylpyrophosphate P	18.1	1.3	4.3	2.8	5.8	1.6
Ba-precipitable, hydrolysed in 100 min., "hexosediphosphate" P	4.6	5.5	2.6	4.0	3.2	1.7
Ba-precipitable, not hydrolysed in 100 min. P	8.9	0	1.4	0.7	3.6	5.2
Ba-non-precipitable, "hexose- monophosphate" P	56.5	43.0	47.6	45.3	52.7	65.6

(N.B. Percentages are calculated on the sum of the fractions)

inlet and outlet tubes permitting of a flow of the gas mixture, in such proportions as to imitate manometric conditions, and shaken gently for most of the time (3 hr.) during which they were in the water-bath at 38°. Fluoride was added to make a concentration of 20 mMol. per litre ($M/50$), which, as was previously shown [Needham, 1933], is sufficient to inhibit the glucolysis of embryo about 90%. In order that there should be no lack of inorganic phosphate, an addition of this to the Ringer-bicarbonate was made, giving a concentration of 0.88 mg.

per ml. or 0.2 mg. inorganic phosphorus. At the end of the period of inhibited glucolysis, the embryos were carefully washed free from the strong phosphate by ordinary Ringer-bicarbonate and the phosphorus partition of the trichloro-acetic extract worked out as before. It was at once noticeable that no accumulation of the "hexosediphosphate" or the barium-non-precipitable fractions had taken place, but that on the contrary there had been a piling up of inorganic phosphate at the expense of the phosphagen phosphorus, the "hexosediphosphate" phosphorus, the adenylypyrophosphate phosphorus and the residual phosphorus, i.e. that barium-precipitable but not hydrolysable in 100 min. This effect could not possibly be ascribed to the use of inorganic phosphate in the Ringer, since the total phosphorus was of just the same order in these embryos as in those worked up directly without any glucolysis period (cf. Table X).

Nor could it be due to any indirect effect of the phosphate, since in a special experiment (129) glucolysis was allowed to proceed side by side with glucolysis inhibited by fluoride in the absence of added inorganic phosphate, with exactly the same results (see Table XV).

Table XV

Exp. 129				
42 6-day embryos 8.35 g. wet wt. Ringer-bic. 52.8 ml. 2.2 ml. 2.5% glucose 2½ hr.			42 6-day embryos 8.36 g. wet wt. Ringer-bic. and 20 mMol./l. NaF 52.8 ml. 2.2 ml. 2.5% glucose 2½ hr.	
	mg./g. wet wt.	% of water soluble P	mg./g. wet wt.	% of water soluble P
Total P (direct)	0.400	—	0.280	—
Ba-precipitable:				
Total P	0.160	—	0.112	—
Inorganic P	0.157	39.3	0.076	27.0
Adenylypyrophosphate P	0.047	11.7	0.009	3.2
HdP P	0.0	0	0.055	19.8
Residual P	0.0	0	0.0	0
Ba-soluble:				
Total P	0.245	—	0.167	—
Phosphagen P	Trace	—	Trace	—
"HmP" P	0.245	49.0	0.167	50.0

This experiment suggested that the disappearance of most of the barium-precipitable phosphorus except the inorganic phosphorus, and the steady level of the barium-non-precipitable phosphorus, might be due, not to the action of the fluoride, but to the mere incubation of the embryos in Ringer-bicarbonate whether glucolysing or not. Experiments 142 and 143 were accordingly made to test this. Embryos were allowed to glucolyse for 3 hr. in Ringer-bicarbonate containing added phosphate as before. The only difference between these two experiments was that in the first of them (142) the Ringer contained no calcium (it had been omitted from the Ringer used in the fluoride experiments), while in the second one calcium was included to make the conditions as nearly normal as possible. This minor adjustment had no effect; in both cases the distribution of phosphorus at the end of the experiment was just as in the experiments with fluoride; i.e. an accumulation of inorganic phosphate at the expense of every other fraction except the stable non-fermentable barium-non-precipitable "hexosemonophosphate" fraction.

The great difference between these experiments, with and without fluoride, is worthy of note. In Exp. 142, for example, where glucolysis was allowed to

proceed normally, fifty-four 6-day embryos were present, a wet weight of 12.58 g., and they were given 55 mg. glucose. Taking the Q_L^N at +10.0 [Needham & Nowinski, 1937, p. 1171] this would mean between 20,000 and 38,000 μ l. in 3 hr., so that all the glucose given was probably exhausted before the end of the period. On the other hand, when fluoride was present (Exps. 133 and 135) the glucolysis would have been hardly more than 100 μ l. Yet in spite of these great differences, the distribution of phosphorus between the various water-soluble fractions at the end of the period was much the same in both cases, and in neither case what would have been expected on the assumption that embryo follows the phosphorylation scheme accepted for muscle.

It could have been suggested that the accumulation of hexosediphosphate, residual phosphate, and barium-non-precipitable phosphate would be seen if the distribution had been measured after a relatively short period of fluoride-inhibited glucolysis. Later on, the activity of phosphatases of all kinds would monopolize the picture. But in Tables XIII and XIV we give an experiment (153) which shows that even after only half an hour the inorganic phosphate has nearly trebled its original proportion, while the adenylypyrophosphate and the residual phosphate have sunk a great deal below their normal level. The barium-non-precipitable fraction has, it is true, risen slightly, but figures of 60 % had already been obtained for this fraction on perfectly fresh embryos (cf. Table X) so that no significance can be attached to it.

Summing up, then, it may be said that when embryos are allowed to glucolyse *in vitro* the phosphagen phosphorus, the adenylypyrophosphate phosphorus, the "hexosediphosphate" phosphorus, and the residual barium-precipitable phosphorus all fall to minimum levels. On the contrary the inorganic phosphorus correspondingly rises, while the large barium-non-precipitable "hexosemonophosphate" fraction remains constant. These changes are already far advanced after only half an hour. They are not affected by the presence or absence of calcium or added phosphate in the medium. Most important of all, they are not affected by the almost complete suppression of the glucolysis by fluoride.

SUMMARY

1. During glucolysis methylglyoxal accumulates in small amounts. When glucolytic activity is much diminished, as by the dialysis of brei, there is no larger accumulation of methylglyoxal.

2. Pyruvic acid accumulates during both autoglycolysis and glucolysis. Although in absolute amount it increases more during glucolysis, its amount relative to the total acid production is much less (thus in mg./hr./g. wet wt. 0.35 in autoglycolysis, 0.83 in glucolysis; yet in percentage of the total acid production, 55 % in autoglycolysis, 8 % in glucolysis). This must mean that to a large pyruvic acid formation in autoglycolysis, a small pyruvic acid formation in glucose breakdown is added.

3. The intensity of formation of pyruvic acid reaches a maximum after about half an hour's glucolysis, falling off progressively afterwards. There is a coincidence between this maximum and the falling off of the anaerobic glycolytic rate, which begins as soon as the maximum is passed.

4. At later stages (tenth day) the rate of pyruvic acid formation is about the same, although by this time uric acid excretion (in which pyruvic acid may be involved) has established itself.

5. Aerobically, a certain proportion of lactate may be oxidized to pyruvate.

6. Pyruvic acid formation during glycolysis was not affected in any way by addition of vitamin B₁.

7. In the onset of glycolysis after substrate deprivation there is an induction period. This can be abolished by the addition of sodium pyruvate or of methylene blue.

8. The rates of aerobic and anaerobic glycolysis and the rate of oxidative disappearance of lactic acid in the chick embryo were measured.

9. The rate of oxidative disappearance of lactic acid is insufficient to account for the effect of oxygen in reducing glycolysis. In this respect the metabolism of embryo resembles that of cerebral cortex.

10. The potassium effect is not seen in the embryo; in this respect the embryo differs from brain.

11. The Pasteur effect is exhibited in the metabolism of mannose as well as in that of glucose in the chick embryo.

12. A survey has been made of the distribution of phosphorus among the acid-soluble phosphorus compounds of the chick embryo, at the fifth day of development.

13. Small amounts of inorganic phosphorus, phosphagen phosphorus, "adenylpyrophosphate" phosphorus, "hexosediphosphate" phosphorus and residual barium-precipitable phosphorus are present.

14. The main peculiarity in the phosphorus distribution is the extremely high proportion of phosphorus not precipitable by barium. This fraction, which is very resistant to acid hydrolysis, is not fermentable by yeast and probably plays no part in the mechanism of carbohydrate breakdown. During later development it declines relatively in amount.

15. The phosphorus distribution was also measured after varying periods of *in vitro* glucolysis. After a period of glucolysis inhibited by fluoride there was no accumulation of hexosediphosphate and of phosphoglyceric and glycerophosphoric acids, as happens in muscle under similar conditions. On the contrary, the only change was an accumulation of inorganic phosphorus at the expense of all the other fractions save that not precipitable with barium. This change was found to occur also in the absence of fluoride during uninhibited glucolysis and to be well advanced half an hour from the beginning of the experiment. It may be concluded that the various phosphorus fractions in embryo have little to do with carbohydrate breakdown.

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