CLVI. INTERMEDIARY CARBOHYDRATE METABOLISM IN EMBRYONIC LIFE

V. THE PHOSPHORYLATION CYCLES

VI. GLUCOLYSIS WITHOUT PHOSPHORYLATION

VII. EXPERIMENTS ON THE NATURE OF NON-PHOSPHORYLATING GLUCOLYSIS

BY JOSEPH NEEDHAM AND HERMANN LEHMANN

From the Biochemical Laboratory, Cambridge

(*Received 16 March 1937*)

CONTENTS

V. THE PHOSPHORYLATION CYCLES

INTRODUCTION

THE problem approached in the work here to be described arose from the marked substrate preference shown by the chick embryo, glucose and mannose being glycolysed, glycogen and hexosediphosphate not. Laying on one side the

question of the mechanism of the glucolysis proper, for what reason, it may be asked, are the phosphorylated hexoses unattacked? If they penetrate the cell-wall, is it because the whole array of phosphorylation enzymes is lacking, or are certain essential steps, for one reason or another, impossible for the embryo? Recent work has shown [Ostern *et al.* 1935; Needham & v. Heyningen, 1935; Lehmann, 1935, 2] how adenylpyrophosphate is an essential phosphorus transporter in the phosphorylating cycles of muscle. It was therefore necessary to observe the effect of adding it to the embryo in the presence of substrates such as glycogen and hexosediphosphate. Since cozymase preparations may also function in this way, experiments with cozymase were also necessary [Vestin, 1936].

We examined these problems in the following order: (1) the presence of adenylpyrophosphate and cozymase in the embryo, (2) the effects on glycogen and hexosediphosphate breakdown of added coenzymes, (3) the presence of each one of the enzymic mechanisms in the phosphorylation cycles.

Presence of dehydrogenase coenzymes

In an earlier paper of this series [Needham *et al.* 1937] it was shown that the chick embryo during the first week of its development contains a fraction of barium-precipitable phosphorus which is hydrolysed by HCl in the boiling water-bath in 7 min. This fraction, which amounts to as much as 18% of the total phosphorus of the trichloroacetic extract, must be partly adenylpyrophosphate, if we may judge from work which has been done on other material. Another argument which makes it likely that adenylic acid is present, is that phosphagen is present from the 70th hour onwards [Baldwin & Needham, 1933], phosphagen-forming enzyme from the 40th hour [Lehmann & Needham, 1937], and up to now we know of no mechanism whereby phosphagen may be synthesized other than from phosphate carried by adenylic acid, even if disguised in cozymase [Vestin, 1936].

For a decision on the presence of cozymase in the early embryo the literature is not very helpful. The work of Waterman [1925] and of Kraut & Bumm [1928] may be disregarded, for (*a*) they only studied the effect of saline extracts of tissues on the glycolytic rate of kidney slices (unspecific stimulus), and (*b*) they give no particulars of the embryos used. Sym *et al.* [1930], on the other hand, worked with apozymase preparations and found a value of 1·6 for the rat embryo (age not stated, but probably near term), contrasting with 8·5 for skeletal muscle and less than 0·5 for a number of adult organs such as thymus and ovary. By the same method, it was found to be present in human placenta [v. Euler & Johansson, 1928]. Of embryos in early stages, nothing is to be found.

In the present investigation the methods of the Lwoffs [1937] and Green [1936] were employed. The former is a test for either the Harden-v. Euler cozymase or the Warburg-Christian hexosemonophosphate codehydrogenase [Warburg & Christian, 1931]; i.e. coenzyme I and coenzyme II in Green's nomenclature. It uses the glucose dehydrogenase of *Bacillus influenzae*, or, at a more dilute range, the growth rate of this organism, which requires the coenzymes as an accessory growth factor [see Lwoff & Lwoff, 1936]. The latter is a specific test for cozymase and employs the malic dehydrogenase of mammalian tissues.

With the glucose dehydrogenase of *B. influenzae* trichloroacetic extracts of 5- and 2½-day chick embryos were strongly positive. Extracts were then made from embryos of 30 hr. incubation, about the time of the closure of the neural

folds. These were tested by the growth-promotion method on cultures of the bacillus, side by side with diluted yolk and white from the unincubated, but fertilized, egg. The results were as follows:

	Growth-promotion test
30-hr. embryo	+ + + +
Yolk	+ +
White	+

Details of the preparation of extracts for coenzyme tests are given in the paper of Lwoff already quoted. The general conclusion was that either coenzyme I or coenzyme II, or both, exist in appreciable amounts right back to the stage of the open neural folds and to a smaller extent in the yolk (Exp. 186).

We were next able to go far towards excluding coenzyme I (cozymase), by the method of Green. A number of 8-day embryos were ground with sand in 30 ml. of trichloroacetic acid and the extract was concentrated *in vacuo* in a bath at 55° to 2 ml. and neutralized. The test with malic dehydrogenase of heart muscle then proved absolutely negative, though given by mammalian tissue brei without any concentration at all. Fearing, however, some destruction during the concentration, we performed the following experiment:

Fourteen 7-day old embryos, with hearts still beating, were placed in 50 ml. 2% trichloroacetic acid and ground with sand. Their wet weight was 12·6 g. The protein precipitate was removed by centrifuging and the centrifugate treated with saturated normal lead acetate solution till no further precipitate came down. To the centrifugate from this 2 ml. more of the lead solution were added. Weak alkali was then added till the solution was blue to thymol blue (*p*H 9), then the precipitate was quickly centrifuged and suspended in $N/10$ H_2SO_4. The lead sulphate resulting was centrifuged away. The solution was then brought to *p*H 5 with cold baryta and the barium sulphate removed. Concentration *in vacuo* at about 35° to 4 ml., followed by the addition of 60 ml. 97% alcohol gave a fairly voluminous white precipitate. This was washed with alcohol and ether and dried. On testing a solution of it by the malic dehydrogenase method, the result was completely negative, and a parallel control demonstrated the absence of any inhibitor.

We may therefore conclude that coenzyme I (cozymase) in appreciable amounts is absent from the chick embryo at this stage, and therefore probably from all earlier stages (Exp. 187). It would be of much interest to follow the matter up by investigating later stages and the chick after hatching.

At the stages used by us in this series of papers, then, adenylpyrophosphate and coenzyme II are probably present, but coenzyme I is probably absent.

Effects of addition and removal of adenylpyrophosphate, adenylic acid and cozymase

A partial explanation of the inability of the embryo to attack glycogen and hexosediphosphate might lie in the insufficiency or unavailability of the phosphorus transporters present in the embryo at the stage in question. We therefore added these substances in various combinations to intact embryos, embryo brei and acetone powders of embryo prepared according to v. Euler *et al.* [1936]. As extracts of embryo are invariably inactive, we shall not describe work with them. The general results may be summarized by saying that the effect of adding phosphorus transporters is always small and only sometimes demonstrable. When it appears it falls off after a comparatively short time and may be renewed

by the addition of more phosphate transporter. Sample experiments are the following:[1]

Exp. 77. Effects of adenylpyrophosphate on hexosemonophosphate and hexosediphosphate breakdown of intact 4-day embryos; $Q_L^{N_2}$ HmP +0·78; HmP + APP +3·02; HdP +1·14; HdP + APP +2·00.

Exp. 79. The same, HmP +0·0; HmP + APP +0·84; HdP +0·0; HdP + APP +0·38.

Exp. 166. Effect of cozymase on glycogen glycolysis of intact 4-day embryos; glycogen + 0·0; glycogen + cozymase +0·11.

Exp. 157. Effect of adenylpyrophosphate and magnesium on hexosediphosphate glycolysis of 5½-day embryos; $Q_L^{N_2}$ glucose +14·9; HdP +0·53; HdP + APP + Mg +1·92, +2·15.

Exp. 177. Effect of adenylpyrophosphate on HdP and glycogen glycolysis, when tipped in during the experiment (see Fig. 1); 5¾-day embryos. μl. after tipping; autoglycolysis 5; HdP 20; HdP + APP 97; HdP + AA 90; glycogen + APP 85; glucose 190. Control on solutions alone 10–15.

Exp. 177*a*. Effects of adenylpyrophosphate and adenylic acid on HdP and glycogen glycolysis, when tipped twice during the experiment (see Fig. 2); 6½-day embryos.

	μl. after tipping	
	First	Second
Autoglycolysis	15	15
HdP + AA	43	48
HdP + inorganic P + AA	60	—
Glycogen + AA	65	80
Glycogen + APP	50	115

Exp. 180. Adenylpyrophosphate and adenylic acid tipped into HdP; no rise at all (4-day embryos, intact). μl. after tipping, glycogen 2; glycogen + APP 102 (see Fig. 3).

Exp. 182. Hexosediphosphate and adenylic acid tipped into 4-day intact embryos; no rise in any case.

Exp. 185. Glucose tipped into 4-day intact embryos after autoglycolysis; great rise after induction period. Glycogen and APP present in another cup, also glycogen followed by APP tipped. μl.: glycogen + APP 63; glycogen + autoglycolysis 31; glycogen, tip APP 31; glucose 256 (see Fig. 4).

Exp. 160. Effect of adenylic acid on glycogen breakdown by 5-day embryo brei; μl. in 2 hr.; glycogen + inorganic P + Mg 37; glycogen + AA 19; glycogen + inorganic P + Mg + AA 21; no activation.

Exp. 162. Effect of cozymase on hexosediphosphate breakdown by 6½-day embryo brei; μl. in 2 hr.; HdP 18; HdP + cozymase 41.

---

[1] In the following experiments, except when otherwise stated, the following conventions hold good:

Material: 2·0 ml. of embryo brei, made by grinding in a mortar under ice-cold conditions, but not with sand; undiluted but buffered with stock bicarbonate in the usual proportions as if normal Ringer; kept, if necessary, in ice; or 1–3 weighed intact embryos.

Glucose: 0·2 ml. of 2·5% to make 5 mg. per cup or just over 0·01 *M*.

Adenylpyrophosphate: 0·2 ml. containing 0·2 mg. of P hydrolysable in 7 min.

Adenylic acid: 0·2 ml. containing 0·1 mg. organic P.

Cozymase: prepared by the method of Myrbäck [1933] slightly modified; 0·2 ml. containing 0·2 mg. organic P.

Magnesium: 0·1–0·3 ml. containing 0·7 mg. Mg as magnesium chloride in 0·3 ml.

Hexosediphosphate: 0·2 ml. of 0·08 *M*, giving 5·2 mg. in the cup.

Hexosemonophosphate: 0·2 ml. of 0·11 *M*, giving 6 mg. in the cup.

Glycogen: 0·2 ml. of 2·5%.

Inorganic phosphate: 0·2 ml. of 0·075 *M*.

Abbreviations: AA, adenylic acid; APP, adenylpyrophosphate; HdP, hexosediphosphate; HmP, hexosemonophosphate.

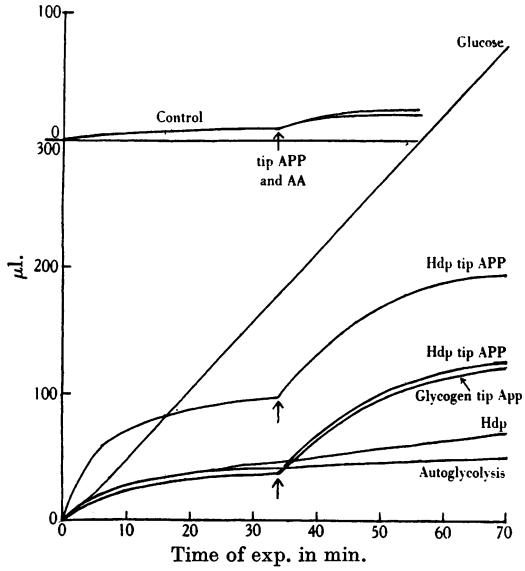

Fig. 1. Effects of adenylpyrophosphate and adenylic acid on hexosediphosphate and glycogen glycolysis of embryo brei; a positive experiment (Exp. 177). 100 μl.$\equiv$0·4 mg. lactic acid. Control on the solutions alone shown above, and a glucolytic rate on the same brei for comparison. HdP = hexosediphosphate, APP = adenylpyrophosphate, AA = adenylic acid, P = extra inorganic phosphate.

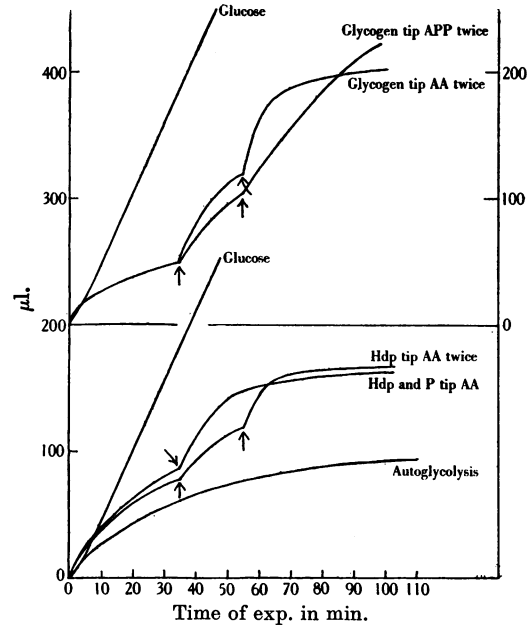

Fig. 2. Another positive experiment (177a); successive amounts of coenzymes tipped during the glycolysis. For abbreviations and other particulars see Fig. 1.

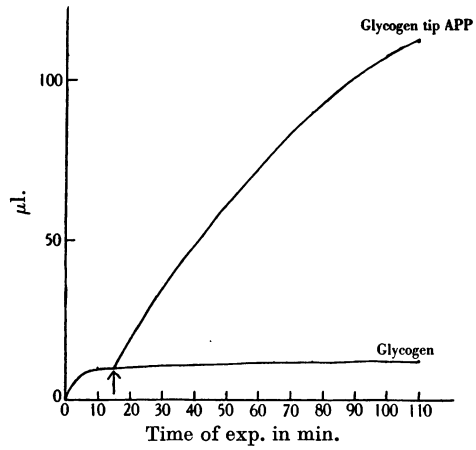

Fig. 3. Another positive experiment (180); utilization of glycogen only in the presence of added adenylpyrophosphate. For abbreviations and other particulars see Fig. 1.

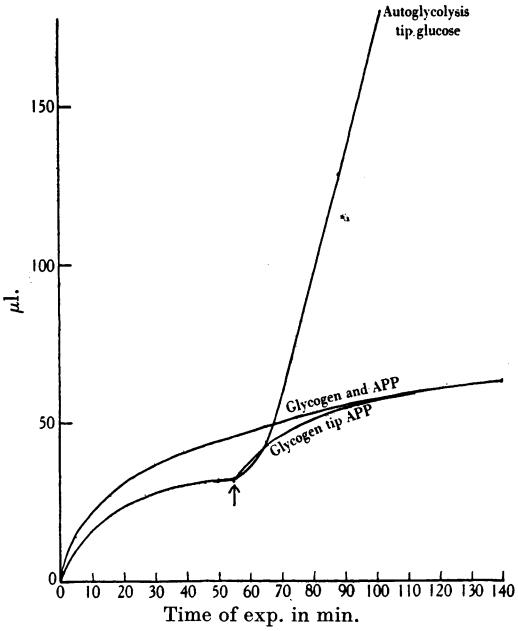

Fig. 4. Another positive experiment (185); explanation in text. For abbreviations and other particulars see Fig. 1.

Exp. 178. Addition of adenylpyrophosphate, adenylic acid and cozymase to 8-day embryo brei at the conclusion of the autoglycolysis, by tipping. μl. after tipping: autoglycolysis 6; HdP 10; HdP + AA 66; HdP + APP 64; HdP + cozymase 46; glycogen 10; glycogen + AA 61; glycogen + APP 64; glycogen + cozymase 34.

Exp. 163. Effect of cozymase on glycogen breakdown by acetone powder of 6½-day embryos; μl. in 2 hr.; glycogen 6; glycogen + cozymase 30.

Table I. *Test of lactic acid production from pyruvate, phosphopyruvate, phosphoglycerate and glycerophosphate*

$6\frac{1}{2}$-day embryo brei, made under ice-cold conditions. Exps. 155, 156

All the cups were shaken in the bath at 38° for the time indicated, and to each except the initial, the nil and the glucose were added 0·2 mg. adenylpyrophosphate P, and 1·1 mg. Mg in the form of magnesium chloride. Of sodium pyruvate (Py) 0·2 ml. of a 2·5% solution was used; of sodium α-glycerophosphate (GP) 0·2 ml. of a 0·1 *M* solution; of sodium phosphopyruvate (PP) 0·1 ml. of a 0·37 *M* solution; of sodium 3-phosphoglycerate 0·1 ml. of a 0·43 *M* solution.

Note. As has already been mentioned, no acid formation is obtained from hexosediphosphate + pyruvate (manometric).

	Contents of medium	ml. brei	Corresponding dry wt. embryo mg.	mg. glucose added or equiv.	Av. $Q_L^{N_2}$	Total μl. CO_2	mg. lactic acid found	mg. lactic acid formed during exp found	mg. lactic acid formed during exp calc.	mg. lactic acid per g. dry wt.	mg. lactic acid formed from added substrate	Turnover % of substrate added
Initial	—	1·5	155	—	—	—	0·49	—	—	3·1	—	—
2 hr.	Nil	1·5	155	—	+0·48	149	—	—	0·598	3·9	—	—
2 hr.	Glucose	1·0	103	20	+6·58	1409	6·14	5·65	5·65	61·4	5·05	**25·2**
2 hr.	Py + GP	1·5	155	30	+0·59	185	1·19	0·70	0·745	7·7	0·10	**0·3**
2 hr.	PP + GP	1·5	155	30	+0·53	167	1·55	1·06	0·671	10·0	0·46	**1·5**
2 hr.	PG + GP	1·5	155	30	+0·63	196	1·26	0·77	0·788	8·1	0·17	**0·6**

From all this it is clear that the effect of adding phosphate transporters is variable, slight and not long-continuing. As we shall see in section VI of this series (p. 1229), the effect of phosphate transporters on glucose breakdown is definitely nil. Exp. 147 was of especial interest in view of experiments described in the next section. A brei of 5-day embryos showed a very active glucolysis while at the same time its glycogen breakdown was only slightly increased by adding adenylpyrophosphate, inorganic phosphorus and magnesium, and in the presence of glycogen no esterification of inorganic phosphate was found. The figures were as follows: μl. in 2 hr. from 0·5 ml. brei: autoglycolysis 30; glucose 354; glycogen 46; and glycogen + 0·3 ml. $M/10$ disodium hydrogen phosphate + 0·15 ml. adenylpyrophosphate (containing 0·075 mg. 7 min. P) + 0·15 ml. $MgCl_2$ solution (equivalent to 0·15 mg. Mg) 64.

Addition of large amounts of adenylpyrophosphate to 9-day embryo brei maintained for a period at room temperature in order to inactivate coenzymes already present was no more successful (Exp. 183). Addition of 0·1–0·3 ml. 2·5 % sodium pyruvate to hexosediphosphate glycolyses gave values in no case significantly larger than the autoglycolysis (Exps. 141, 144).

Addition of phosphorylated intermediates

The action of embryo brei upon the intermediates pyruvate, phosphopyruvate, glycerophosphate and phosphoglycerate, not open to study by the manometric method, was checked by lactic acid estimations, according to Friedemann *et al.* [1927], (Exps. 155, 156). The results, shown in Table I, indicate that no appreciable lactic acid production occurs from these intermediates. The extra lactic acid production above the autoglycolysis value (about 0·73 mg.) is some 4·7 mg./g. dry wt. or one-twelfth that of the glucolysis value after subtraction of the autoglycolysis, namely, 57·5 mg./g. dry wt. It will be noticed that, owing to the acid nature of the substrates, the calculated lactic acid productions are only a certain percentage of the estimated lactic acid productions, and were it not for the considerable autoglycolysis, these percentages would be much lower still.

The enzymes of the phosphorylation cycles and the entry of phosphoric ester into the cell

We now examined step by step the question of the existence of the separate constituent enzymes of phosphorylating glycolysis (phosphorus estimations in trichloroacetic acid (2 %) filtrates according to Fiske & Subbarow [1925]; hydrolyses according to Lohmann [1928]; phosphoric esters all carefully freed from barium, neutralized, and expressed in terms of mg. phosphorus).

Aldolase (zymohexase), which converts hexosediphosphate into triosephosphate, was first investigated.

Exp. 340. Four 4-day intact embryos gently shaken at 37° for 2 hr. in Ringer-bicarbonate 2·6 ml. each, containing each 0·9 mg. hexosediphosphate P, in 5 % CO_2/N_2 gas mixture. From the previous experiments [Needham & Nowiński, 1937] we know that under these conditions the embryos freely form lactic acid from glucose, but not from hexosediphosphate. Phosphate estimations showed that the hexosediphosphate had not been dephosphorylated, but that triosephosphate had been formed. This, which, as Meyerhof & Lohmann [1934] showed, can be estimated as alkali-labile phosphate, stands in equilibrium with hexosediphosphate. In our experiments, the triosephosphate appeared already in 10 min. and rose to a maximum during a period of 2 hr.

1 embryo in 2·6 ml. Ringer-bicarbonate containing 0·9 mg. HdP P

Time in min.	Inorganic P mg.	P after alkali hydrolysis mg.	Alkali-labile P mg.	Decrease of HdP P mg.
0	0·12	0·12	—	—
10	0·13	0·15	0·02	0·03
45	0·16	0·22	0·06	0·10
120	0·20	0·40	0·20	0·28

Thus after 2 hr. only 69 % of the original amount of hexosediphosphate is still present; 22 % has been changed into triosephosphate and 9 % has been broken down to inorganic phosphate.

From this experiment, it is important to note, it follows that the hexosediphosphate passes into the intact embryos, yet, apart from the 9 % dephosphorylated, is not broken down under precisely the same conditions as allow of a vigorous formation of lactic acid from glucose. The relatively slow production of triosephosphate probably results from the fact that the equilibrium

$$\frac{\text{Mol. conc. HdP P}}{(\text{Mol. conc. triosephosphate P})^2} = K$$

establishes itself not in the total volume of 2·6 ml., but only in the interior of the embryo, where the hexosediphosphate comes into contact with the aldolase. The prolonged osmotic return of the triosephosphate through the embryo's surface into the solution and the corresponding entry of more hexosediphosphate eventually lead to a reflection in the whole system of the state of affairs existing within the embryo.

Investigation of the aldolase was continued under such conditions as to exclude the presence of coenzymes.

Exp. 312. Twenty-three 6-day embryos ground to a brei and maintained 2 hr. at 37°, so that all easily hydrolysable phosphorus should be broken down [Needham *et al.* 1937]. The brei then kept 24 hr. at 0°. 1 ml. of this brei, corresponding to 2 embryos, added to 1 ml. solution containing 4·34 mg. HdP P and 0·62 mg. inorganic P with 1·0 mg. Mg as magnesium chloride.

As a control, and for comparison, the same solution was added to 1 ml. of a 3-weeks-old 15-hr. dialysed rabbit muscle extract.

	Inorganic P mg.	P after alkali hydrolysis mg.	Triose-phosphate P formed mg.
Rabbit muscle extract			
0 min.	0·64	0·63	—
30 min. at 37°	0·85	1·05	0·20
Embryo brei			
0 min.	0·73	0·73	—
30 min. at 37°	1·08	1·35	0·27

The fact that in muscle extract freed from coenzymes, as also in embryo brei freed from coenzymes, the same amount of triosephosphate is formed, makes it very probable that the same equilibrium and the same enzyme, aldolase, occur. The comparatively low amount of triosephosphate formed may be explained by the exceptionally large addition of magnesium, which is known to shift the equilibrium in favour of hexosediphosphate [Meyerhof, 1935; Takahashi, 1935].

The proof of the penetration of hexosediphosphate into the embryonic tissues seemed to us of such importance that we made further efforts to establish it by testing for aldolase in the medium in which embryos had been for some time immersed. As the following experiment (400) shows, this test was quite negative,

i.e. no appreciable amount of the enzyme leaks out from the intact embryos under the conditions of our experiments.

Exp. 400. 6-day-old embryos were carefully washed three times in Ringer solution and then distributed, in groups of three, to five manometric cups, each containing the usual 2·6 ml. Ringer-bicarbonate solution. After various treatments the alkali-labile phosphorus was estimated. Cups 1 and 2 contained sodium hexosediphosphate (1 mg. P); the former was precipitated with trichloroacetic acid at 0 min.; the latter was shaken anaerobically for 2 hr. in the bath at 37°, the gas phase containing the usual mixture of 5% CO_2. Cup 3 was like cup 2, but without hexosediphosphate. Cups 4 and 5 also had no hexosediphosphate; at the end of 2 hr. the embryos were carefully filtered off from the medium, and the former (6 embryos) made into 4·5 ml. of brei with distilled water. Four tubes (6, 7, 8, 9) were then put up, two with 2 ml. embryo brei, and two with 2 ml. of the medium. Hexosediphosphate was added to each, as above, and while one of each was estimated at 0 min., the other was held at room temperature for 30 min. The results follow:

		Inorganic P mg.	Alkali-labile phosphate + inorganic P mg.	Difference mg.
Cup				
1	0 min. intact embryos + HdP	0·11	0·14	0·03
2	120 min. intact embryos + HdP	0·54	0·76	0·22
3	120 min. embryos alone	0·23	0·23	0·00
Tube				
6	0 min. embryo brei + HdP	0·21	0·21	0·00
7	30 min. embryo brei + HdP	0·28	0·38	0·10
8	0 min. medium + HdP	0·03	0·07	0·04
9	30 min. medium + HdP	0·03	0·07	0·04

Calculation of turnover

	Time min.	Inorganic P formed from HdP mg.	Alkali-labile (triose-phosphate) P formed from HdP mg.	Total turnover in % of HdP added
(*a*) HdP + intact embryo in Ringer-bicarbonate	120	0·32	0·22	54
(*b*) HdP + Ringer-bicarbonate in which the embryos had been shaken 120 min.	30	0	0	0
(*c*) HdP + brei of embryos used for (*b*)	30	0·07	0·10	17

In contrast to the enzyme system of glucolysis proper, the aldolase of the embryo is readily water-soluble, and the transformation of hexosediphosphate into triosephosphate goes on in aqueous extracts.

Exp. 319. 6-day embryos were made into a brei under ice-cold conditions and ground (2 parts to 3 parts) with ice-cold distilled water, centrifuged and the centrifugate used for the experiment. To 1 ml. extract 0·5 ml. solution was added containing 1·24 mg. HdP P.

	Inorganic P mg.	P after alkali hydrolysis mg.	Alkali-labile P formed mg.
0 min.	0·05	0·09	—
15 min. at 37°	0·06	0·27	0·17
15 min. at 37° + cozymase	0·05	0·24	0·15

It was impressive that even after the addition of cozymase this fresh embryo-extract only formed triosephosphate and no inorganic phosphate. According to Vestin [1936] and D. M. Needham [1936] cozymase preparations can substitute perfectly in muscle extract for adenylic acid, as phosphate transporter. Meyerhof & Ohlmeyer [1937] have also shown that it plays an irreplaceable part in the

dismutation of triosephosphate to phosphoglyceric acid; here acting as hydrogen transporter. Therefore the embryo's failure to carry the breakdown further than triosephosphate cannot be due to the lack of cozymase.

We accordingly proceeded to investigate the later stages of the phosphorylation cycles. In general we did not succeed in demonstrating any enzymes in the embryo which form phosphoglyceric acid from triosephosphate. But all the enzymes concerned in the breakdown of phosphoglyceric acid are present, as is shown by the following experiments: the reversible transformation of phosphoglyceric into phosphopyruvic acid, the transport of phosphate from phosphopyruvic acid to adenylic acid with the formation of adenylpyrophosphate and finally its dephosphorylation with the appearance of inorganic phosphate or phosphagen.

Exp. 325. Ninety-six 3-day embryos were ground with their own weight of distilled water, left 1 hr. at room temperature, and centrifuged. The extract was stored for 3 days at 0°. 2 ml. extract were added to 2·9 ml. of a solution containing 0·68 mg. inorganic P; 1·61 mg. phosphoglyceric acid P; 2·0 mg. Mg. Time of experiment 5 hr.; temp. 37°.

Conditions	Inorganic P mg.	Increase inorganic P mg.	% of added PG P	Easily hydrolysable P (HCl: 180 min. − inorganic P) mg.	Phosphopyruvic P appearing mg.	% of added PG P	Total turnover, % of added PG P
0 min.	0·81	—	—	0·92	—	—	—
5 hr.	0·81	0·0	0	1·47	0·55	34	34
5 hr. + 0·3 mg. APP P	1·45	0·64	40	1·84*	0·62	39	79

* Including 0·3 APP P.

From this experiment it is clear that even in the 3-day chick embryo enzymes are present which can convert phosphoglyceric acid into phosphopyruvic acid (transformation of difficultly hydrolysable into easily hydrolysable phosphorus) and can dephosphorylate the phosphopyruvic acid in the presence of adenylpyrophosphate. It is remarkable that, although the experiment lasted 5 hr., and plenty of magnesium was present, there was no increase whatever in the inorganic phosphate when adenylpyrophosphate was absent.

The individual components of this reaction chain were repeatedly tested in brei, washed brei and extracts, and could always be demonstrated. From each step a few sample experiments may be given.

The reversibility of the phosphoglyceric $\rightleftharpoons$ phosphopyruvic acid transformation was shown as follows.

Exp. 317. 0·5 ml. of 6-day embryo brei, previously stored for 48 hr. at 0° (corresponding to 1 embryo), added to 0·25 ml. of a solution containing 0·46 mg. phosphopyruvic P, and 0·05 mg. Mg. After 30 min. at room temperature 0·1 mg. of difficultly hydrolysable P (phosphoglyceric P) had been formed.

Exp. 322. By working at 37° this enzyme could be shown to be active even in a dilution of the brei 1 to 10. 0·3 ml. of 4-day embryo brei, previously stored for 24 hr. at 0° (1 ml. corresponding to 2 embryos), was added to 2·8 ml. of a solution containing 1·25 mg. phosphopyruvic P; 0·68 mg. inorganic P; 1 mg. Mg; 0·2 ml. sodium bicarbonate 2·6%.

	Inorganic P mg.	Inorganic P + P hydrolysable in 60 min. (*N* HCl) mg.	Increase in difficultly hydrolysable P (phosphoglyceric P) mg.	% turnover to phosphoglyceric P
0 min.	1·10	2·35	—	—
60 min. at 37°	1·22	1·86	0·49	39

Thus at a 1 to 10 dilution of 24-hr.-old enzyme solution 39% of the added phosphopyruvic acid was transformed into phosphoglyceric acid. This is of much interest since the equilibrium between these two acids is known in muscle extract to be extremely sensitive to dilution or ageing [Lehmann, 1935, 2]. This fact, as will be seen later, is important in relation to fluoride inhibition of embryo glycolysis.

Since, as the foregoing experiments (319, 325 etc.) have shown, all the enzymes of the phosphorylating cycles that can be demonstrated in embryo at all, can be demonstrated equally well in aqueous extracts (in contrast to glucolysis proper), it was of interest to see whether this last enzyme, known to be one of the weakest of the whole system, could also be found in the residual protein mass from the centrifugate.

Exp. 320. 5-day embryos were ground to a brei and washed twice with 10 times their volume of ice-cold distilled water, for 30 min. The second extract having been removed on the centrifuge, the residue was diluted with distilled water until 1 ml. of the mixture corresponded to 2 embryos.

1 ml. of the mixture was added to 5 ml. of a solution containing 1·25 mg. phosphopyruvic P; 1·25 mg. inorganic P; 0·25 mg. Mg; 0·3 ml. 2·6% bicarbonate.

	Inorganic P mg.	Inorganic P + P hydrolysable in 60 min. *N* HCl mg.	Increase in difficultly hydrolysable P (phospho-glyceric P) mg.	% turnover to phospho-glyceric P
0 min.	1·24	2·48	—	—
20 min. at 37°	1·33	2·12	0·22	18

The transport of phosphorus from phosphopyruvic acid to adenylic acid (adenosinemonophosphate) was examined with special attention since it is known that in some of the invertebrates this substance cannot act as phosphorus-acceptor from phosphopyruvic acid, but is replaced by adenosinediphosphate (Lehmann [1935, 2] for the crayfish; Lohmann [1936] for the octopus). The result might therefore have recapitulatory significance. It should be noted that owing to the different hydrolysis rates of phosphopyruvic acid and the easily hydrolysable phosphorus of adenylpyrophosphate it is possible to calculate the proportion of each in the 7 min. fraction. In this, adenylpyrophosphate is hydrolysed 100%, phosphopyruvic acid only about 50%, the remainder of phosphopyruvic will be the difference between the 60 and the 7 min. values.

Exp. 307. Twenty-eight 6-day embryos were ground to a brei, 5 ml. of ice-cold 0·9% KCl added, and the mixture left half an hour in ice, then centrifuged and inactivated by leaving a further half-hour at room temperature. After 2 days' storage at 0° the extract was used. 1 ml. was added to 2 ml. of a solution containing 1·2 mg. phosphopyruvic P; 0·5 mg. adenylic acid P; 0·7 mg. inorganic P; 2·0 mg. Mg; 0·1 *M*/2 bicarbonate. Experiment done at room temperature.

	Inorganic P mg.	Increase of inorganic P mg.	7 min. P + inorganic P mg.	Increase in adenyl-pyrophos-phate P mg.	60 min. P + inorganic P mg.	Total P (in-cinerated) mg.	Turn-over; decrease of PP P mg.	Turn-over % of original PP P
0 min.	0·87	—	1·50	—	2·08	2·23	—	—
30 min.	1·17	0·30	1·74	0·24	2·00	2·24	0·62	50

It is clear, therefore, that 0·24 mg. of adenylpyrophosphate phosphorus has been formed at the expense of phosphopyruvic acid phosphorus, and therefore that adenylic acid can act as transporter of phosphopyruvic phosphorus in the embryo. Furthermore 0·3 mg. of inorganic phosphorus has been formed. Only

0·08 mg. difficultly hydrolysable, phosphoglyceric, phosphorus has been formed, no doubt because the adenylic acid is rapidly phosphorylated. This enzyme also could be demonstrated in the residual protein mass after aqueous extraction.

Exp. 318. 6-day embryos were ground to a brei and extracted as in Exp. 320. The residual protein mass was used after storage at 0° for 24 hr., and diluted to the same concentration as before. 0·5 ml. of the mixture was added to 1·05 ml. of a solution containing 0·82 mg. phosphopyruvic P; 0·5 mg. Mg; 0·62 mg. inorganic P. Experiment done at 37°.

	Inorganic P mg.	Increase of inorganic P mg.	7 min. P + inorganic P mg.	Increase in adenyl-pyrophosphate P mg.	60 min. P + inorganic P mg.	Increase of difficultly hydrolysable P mg.	Total P (incinerated) mg.
0 min.	0·73	—	1·24	—	1·54	—	1·57
30 min.	0·80	0·07	1·20	0·0	1·42	0·09	1·54
30 min. + 0·25 mg. adenylic acid P	1·03	0·30	1·42	0·27	1·54*	0·06	1·74

* Including 0·05 adenylic acid P hydrolysed in 60 min.

In the absence of adenylic acid, very little phosphoglyceric acid is formed and very little inorganic phosphate. In the presence of adenylic acid, 0·3 mg. of inorganic P is freed from phosphopyruvic acid (37 %) and 0·27 mg. of adenylpyrophosphate P (another 33 %) is formed. The total turnover from phosphopyruvic acid to adenylic acid, therefore, since the inorganic phosphate has passed through adenylpyrophosphate, amounts to 70 %.

In a separate experiment the dephosphorylation of adenylpyrophosphate was shown.

Exp. 316. Four 6-day embryos were made into a brei and inactivated by leaving at room temperature. 0·8 ml. of the brei, corresponding to 1·2 intact embryos, was added to 0·32 ml. of adenylpyrophosphate solution containing 0·20 mg. 7 min. P. The mixture was left 30 min. at 37°.

	Inorganic P mg.	7 min. P + inorganic P mg.	Dephosphorylated 7 min. P mg.
0 min.	0·13	0·34	—
30 min.	0·32	0·37	0·19

Thus 100 % of the adenylpyrophosphate was dephosphorylated. The stationary behaviour of the sum of the 7 min. P and the inorganic P shows that the increase of the latter cannot have come from other sources than the adenylpyrophosphate added.

We also investigated the question whether in the early embryo also the Parnas reaction occurs, that is to say, the transport of phosphorus from phosphopyruvic acid to creatine with adenylic acid as carrier. We shall give further details on this question elsewhere [Lehmann & Needham, 1937], for it has aspects of recapitulatory interest; here we only wish to give one experiment showing that aqueous extracts of embryo can perform the Parnas reaction.

Exp. 324. Forty-eight 5-day embryos were ground in an ice-cold mortar with sand and 12 ml. of ice-cold distilled water added. After 1 hr. standing in ice, the mixture was centrifuged and 15 ml. of extract obtained. The extract was then held at 0° for 4 days to damage the adenylpyrophosphatase by ageing. To 3 ml. of the extract were added 3·5 ml. containing 1·10 mg. phosphopyruvic P; 0·2 mg. 7 min. adenylpyrophosphate P (except in one case as shown below); 0·68 mg. inorganic P; 0·8 mg. Mg.

	Inorganic and phosphagen P mg.	Increase of inorganic and phosphagen P mg.	Increase of phosphagen P mg.	60 min. P + inorganic and phosphagen P mg.	Increase of difficultly hydrolysable P, minus correction for adenylic P added mg.	Total P (incinerated) mg.
0 min.	0·97	—	—	2·17	—	2·44
40 min. at 40°*	1·06	0·09	—	1·67	0·30	—
40 min. at 40°	1·17	0·20	—	1·93	0·24	—
40 min. at 40° + 10 mg. creatine	1·49	0·52	**0·32**	2·19	0·0	—

* Adenylpyrophosphate absent.

In the absence of adenylic acid 0·3 mg. phosphoglyceric acid was formed; in its presence 0·24 mg. was formed; but if creatine was also present, no phosphoglyceric acid at all was formed. Creatine is thus rapidly phosphorylated, the phosphopyruvic acid steadily decreasing, and the conditions for phosphoglyceric acid formation are made less favourable. When adenylic acid is absent the inorganic + phosphagen phosphorus increases by 0·09 mg.; when it is present 0·11 mg. more is formed. This may be partly phosphagen phosphorus, since the embryo extract will contain a little creatine. On further addition of creatine, the inorganic + phosphagen phosphorus goes up to 0·32 mg. more than before, a rise which must be regarded as wholly due to creatinephosphate.

Lastly, we must consider the esterification of glycogen. We were never successful in bringing this about, though we have reason to believe (see p. 1227) that it is not absolutely impossible. Whether adenylpyrophosphate, or cozymase, were present or not, and in the presence or absence of fluoride or iodoacetate, we never observed any decrease of inorganic phosphate.

The blockage at the triosephosphate stage

The evidence given in the preceding section shows that all the enzymes concerned in the phosphorylation cycles are present in the chick embryo during the first week of its development, with the exception of those effecting (*a*) the esterification of glycogen with inorganic phosphate in the presence of coenzyme, (*b*) the dismutation of triosephosphate to phosphoglyceric acid. To this last statement, however, we must now add a qualification, namely, that in about 10 % of our experiments hexosediphosphate was nearly completely broken down on the addition of coenzyme. This observation, of course, is in agreement with the results found in the manometric experiments described earlier in this paper, where coenzymes were added, and also with the occasional results found in the cases of glycogen and glucosemonophosphate without addition of coenzymes by Needham & Nowiński [1937].

In the following three experiments, we show, first, the usual course of events, in which triosephosphate accumulates as the experiment continues; secondly, a case in which the step was successfully passed but, fluoride being present, phosphoglyceric acid accumulated; thirdly, one of the rare cases in which the whole of the process was passed through.

Exp. 343. Fourteen 6-day embryos were rapidly ground under ice-cold conditions and the brei was diluted to 10 ml. with distilled water. 30 min. after the opening of the first egg, to each ml. of the brei 2·3 ml. of solution were added containing 2 mg. hexosediphosphate P; 0·3 ml. 1·3 % bicarbonate and 0·1 ml. 2*N* sodium pyruvate.

Experiment done at 37°

Conditions	Inorganic P mg.	Alkali-labile P + inorganic P mg.	Triosephosphate P mg.
0 min.	0·12	0·17	0·05
60 min.	0·60	1·01	0·41
60 min. + 0·4 mg. adenylic acid P	0·65	1·03	0·38
60 min. + 0·4 mg. AA P + cozymase	0·21	0·61	0·40
60 min. + 0·4 mg. AA P + 10 mg. glyceraldehyde	0·51	0·86	0·35
60 min. + 0·4 mg. AA P + 12 mg. methylglyoxal	0·26	0·70	0·44
60 min. + 0·4 mg. AA P + 2·5 mg. glutathione	0·55	1·02	0·47
60 min. + 0·4 mg. AA P + 5 mg. glucose	0·46	0·94	0·48

This experiment, one of many, is reproduced here largely to show that, under all possible conditions, no considerable dephosphorylation of hexosediphosphate occurs, but an accumulation of triosephosphate. Even with a liberal supply of pyruvic acid (necessary for the dismutation) and in the presence of cozymase, the state of affairs does not alter. Yet it is necessary to remark that under these very conditions maximum glucolysis takes place. Furthermore, attention may be drawn to the slight unspecific dephosphorylation of hexosediphosphate which always occurs, even in the presence of fluoride. This may be a non-enzymic dephosphorylation of the triosephosphate in trichloroacetic acid. What is probably the same phenomenon has been observed by Tsuzuki [1936] working on tumour; there it may be also due to the action of unspecific phosphatases [cf. Takahashi, 1935].

The next experiment is one of the few where we found difficultly hydrolysable phosphorus (phosphoglyceric phosphorus) formed at the expense of hexosediphosphate.

Exp. 332. 6-day embryos made into a brei under ice-cold conditions and used 35 min. after opening of the first egg. 3 ml. (corresponding to 3·5 embryos) were added to 1·5 ml. of a solution, which in all cases contained 1·17 mg. hexosediphosphate P and 1 mg. Mg.

Results in mg. P

	Inorganic P	Increase in inorganic P	Easily hydrolysable P (180 min.) + inorganic P	Easily hydrolysable P (HdP and triose P)	Total P (incinerated)	Change in difficultly hydrolysable P	Newly-formed difficultly hydrolysable P (phosphoglyceric P)
(1) 0 min.	1·18	—	2·34	1·16	3·98	—	—
(2) 60 min.	1·81	0·63	2·68	0·87	—	−0·34	—
(3) 60 min. + *M*/100 NaF	1·59	0·41	2·95	1·36	—	−0·61	—
(4) 60 min. + 1·2 mg. adenylic acid P	2·54	1·36	2·91*	0·37	—	−0·57	—
(5) 60 min. + 1·2 mg. AA P + *M*/100 NaF	1·66	0·48	2·36*	0·70	—	−0·02	**+0·55**

* After subtraction of 0·24 mg. adenylic acid P (20% of that added).

Explanation of results. Sample 2 shows that some of the difficultly hydrolysable P (not identical with phosphoglyceric P) in the embryo, is broken down in an hour, and as sample 3 shows, this process is not inhibited by fluoride. Hexosediphosphate is practically not dephosphorylated, except in sample 4, where adenylic acid is added. In sample 5, where adenylic acid and fluoride are both present, inorganic P appears as usual from the same source as in samples 2 and 3, but the loss of difficultly hydrolysable P previously seen is masked by the breakdown of hexosediphosphate arrested in the phosphoglyceric stage by the fluoride present.

This experiment requires some comment. In the first place, it is shown that here the addition of adenylic acid increases the breakdown of hexosediphosphate, leading to an increase of inorganic phosphate at the expense of the easily hydrolysable phosphate. This process is sharply inhibited by fluoride, just as in muscle, and in the place of inorganic phosphate difficultly hydrolysable phosphorus (phosphoglyceric phosphorus) accumulates. Hence there has been a fermentation of hexosediphosphate through the triosephosphate stage. It will also be noted that, just as in the experiments of Needham *et al.* [1937], the barium-precipitable difficultly hydrolysable phosphorus already in the embryo breaks down, a process which is quite unaffected by fluoride. We do not believe that this fraction has much to do with carbohydrate metabolism.

The last experiment to be mentioned in this connexion is one of the rare cases in which, by the addition of adenylpyrophosphate, hexosediphosphate was completely broken down. It parallels, therefore, the fitful appearance of glycogen and hexosediphosphate breakdown in the manometric experiments already described.

Exp. 331. 5-day embryos dropped directly from the eggs into 3·5 ml. of a 0·9% NaCl solution at 37° containing in each tube 2 mg. hexosediphosphate P. The embryo hearts continued to beat in the solution, during gentle shaking.

Conditions	Inorganic P mg.	Increase in inorganic P mg.
0 min. embryo	0·50	—
30 min. embryo	0·59	0·09
30 min. embryo + allantois	0·63	0·13
30 min. embryo + 0·1 mg. 7 min. P APP	2·66	2·16

Presence of coenzyme of phosphorus transport

The fact that so small an amount of adenylpyrophosphate was added in Exp. 331 and yet proved so effective, permits a calculation regarding the amount of effective coenzyme present in the embryo itself. A fifth-day embryo has a volume of 0·15 ml., so that the amount of adenylic acid phosphorus here added to the embryo interior was 2γ. The living embryo must therefore contain something less of adenylic acid phosphorus than this, whether free or part of the cozymase molecule.

Before taking up the discussion we will add another experiment which shows how small and insufficient, even if present, the amount of dephosphorylating coenzyme must be.

Exp. 346. Five 8-day embryos ground under ice-cold conditions and diluted with ice-cold distilled water to 10 ml. brei. 1 ml. corresponded to half an embryo. From opening the first egg to the addition of the brei to substrates at 37°: 4 min. 1 ml. brei added to 0·7 ml. solution containing 0·1 ml. stock bicarbonate. Time 30 min., except initial. Results in mg. P.

Conditions	Inorganic P	Increase in inorganic P	Increase in inorganic P from phospho-pyruvic P	7 min. P + inorganic P	APP P formed	P hydrolysable in 60 min. + inorganic P
0 min. brei alone	0·14	—	—	—	—	—
30 min. brei alone	0·28	0·14	—	—	—	—
30 min. brei + 0·55 mg. phosphopyruvic P	0·45	0·31	0·17	0·69	—	0·82
30 min. brei + 0·55 mg. phosphopyruvic P	0·45	0·31	0·17	0·68	—	0·81
30 min. brei + 0·55 mg. PP P + 0·4 mg. adenylic acid P	0·68	0·54	**0·40**	0·84	**0·16**	0·97*

* Of this figure, 0·15 mg. is adenylic acid P.

Thus when adenylic acid was added, the amount of dephosphorylated phosphopyruvic acid was 100 % of that added; of this 73 % was found as inorganic phosphorus and 27 % as adenylpyrophosphate phosphorus. On the other hand, in the absence of adenylic acid only 30 % was broken down and found as inorganic phosphate. Of this small change, too, something must be attributed to the action of the unspecific phosphatases [cf. Takahashi, 1935].

Discussion

The manometric experiments contained in the first part of this paper, and the work on the enzymes of the phosphorylation cycles contained in the second part, explain each other. The marked preference of the chick embryo for glucose rather than glycogen or hexosediphosphate as substrate, and the somewhat small and variable results obtained when coenzymes are added to the latter in manometric experiments, can now be clearly referred to a deficiency of the phosphorylation machinery at four distinct places:

(1) The enzyme esterifying glycogen.

(2) The enzyme system dismuting triosephosphate with pyruvate to phosphoglyceric and lactic acids.

(3) Lack of adenylpyrophosphate.

(4) Lack of cozymase.

We do not suggest that these enzyme and coenzyme deficiencies are absolute, for occasionally a certain activity may be shown, especially if coenzymes are added. The conception, however, is reached that in early embryonic life the phosphorylation machinery is not yet fully laid down. We would refer, in this connexion, to facts already mentioned in the first paper of this series, which show that in chicks towards the end of development and even at birth in mammals the normal phosphorylation has not established itself in the muscle.

The state of affairs in the young embryo can hardly be appraised without comparison with what is known of tumour and of adult brain.

A reference to Table IV in the first paper of this series (p. 1172) illustrates the well-known fact that adult mammalian brain tissue breaks down glucose in preference to glycogen or hexosediphosphate. There is in the literature, however, one exception to this generalization, namely, the work of Takasaka [1927], who obtained in his experiments on brain nearly as much lactic acid from glycogen as from glucose. In the series of papers on brain metabolism by Holmes & Ashford [Ashford & Holmes, 1929; 1931; Holmes, 1933; Ashford, 1933; 1934] it was not held that phosphorylation had no place in the carbohydrate metabolism of that tissue, but much evidence was adduced in favour of the existence of a non-phosphorylating path. However, v. Euler *et al.* [1936], using acetone powders, and independently Mazza & Malaguzzi-Valeri [1935], in extracts, were able to demonstrate the presence of the whole of the enzyme system responsible for the phosphorylation cycles. The Swedish authors also claimed that glucose itself passed through the phosphorylation mechanisms. It must be admitted that the figures of lactic acid productions, given by v. Euler and his collaborators, who worked with water extracts concentrated as acetone powders, were very small. Geiger [1935], on the other hand, had only very small glycogen breakdowns by brain brei. In view of the work on the embryo recorded in the present series of papers, and the findings of Tsuzuki [1936] on tumour tissue, it appears that the glucolytic enzyme (or enzymes) is not so soluble in water as the enzymes of the phosphorylation cycles. The most probable conclusion is that the phosphorylation machinery exists in adult brain, and in a more efficient form than we could find in the embryo.

The state of affairs in tumour is not dissimilar. There also glucose is preferred as substrate to glycogen or hexosediphosphate [see Table IV of Needham & Nowiński, 1937, p. 1172]. Yet many investigators [Boyland & Mawson, 1934; Boyland & Boyland, 1935; Mawson, 1936; Scharles *et al.* 1935, 1, 2] have found in tumour the complete phosphorylation system, just as in muscle. Most of these, however, admit that the course of carbohydrate breakdown in tumours has certain peculiarities, and other workers find the peculiarities to be quite far-reaching. In particular, Caló [1935] and Tsuzuki [1936] found great inefficiency in the conversion of pyruvic into lactic acid by dismutation with 3-carbon phosphoric esters; precisely the step which we find to be in most cases impossible for the embryo. This similarity between the imperfectly organized phosphorylation system of embryo and that of tumour is of no little biological interest.

VI. GLUCOLYSIS WITHOUT PHOSPHORYLATION

Introduction

Work already described in the foregoing papers of this series has given many indications that the glucolysis of the chick embryo, contrary to what has been established for some adult tissues, does not involve the phosphorylation of hexoses. We are now to describe experiments designed expressly to settle the question of the existence of a non-phosphorylating glycolysis. The subject is by no means one of mainly embryological interest, for the preference of the embryo for glucose as opposed to glycogen or hexosediphosphate is so strong that it offers itself as a valuable material whereby eventually the mechanism of non-phosphorylating glucolysis may be elucidated.

The starting-point of our observations was that under no one of a variety of conditions could we observe any esterification of phosphate in the presence of glycogen or glucose, although the latter, unlike the former, is vigorously broken down by intact embryo or embryo brei. At no stage of glucose breakdown could any variation of the inorganic phosphate level be observed, even if abundant coenzyme and excess of phosphate were added. Nor did the presence of fluoride or iodoacetate have any effect upon this situation.

Let us take as an example of such experiments one in which a brei of 5-day old embryos was made (Exps. 147 and 308) by grinding in an ice-cold mortar followed by immediate addition of the substrates. Acid formation (manometrically measured) from glycogen did not exceed the autoglycolysis, but glucose and methylglyoxal were powerfully fermented. Yet the inorganic phosphate level was in all cases exactly the same, even when inorganic phosphate and adenylpyrophosphate were added, with or without fluoride $M/50$.

	mg. lactic acid formed in 2 hr.	mg. inorganic P change
Brei alone	0·12	0·03
Brei + glucose	1·42	0·05
Brei + methylglyoxal	0·80	—
Brei + glycogen	0·16	0·07
Brei + glycogen + APP + P + Mg	—	0·02
Brei + glycogen + APP + P + Mg + Fl	—	0·04

N.B. Changes in inorganic P not exceeding 0·05 mg. are not regarded as significant, under the conditions of this experiment. 0·5 ml. brei (corresponding to 2 embryos); 10 mg. glucose; 7 mg. glycogen; 5 mg. methylglyoxal; P = 0·62 mg. inorganic phosphate (Na_2HPO_4); APP = 0·1 mg. P hydrolysable in 7 min.; Mg = 0·2 mg. Mg as $MgCl_2$; Fl = $M/50$ sodium fluoride.

Effects on glucolysis of addition and removal of free phosphate

Although it was thought improbable that the lack of free phosphate could be a factor limiting the glucolytic rate of intact embryos, a few experiments were performed to test the point. Glucosamine, a substance of relevance in view of its occurrence in the egg-proteins [see Needham & Nowiński, 1937, p. 1169], was also tested in the presence of added phosphate. In neither case was there any increase in the glycolytic rate (Table II).

Table II. *Comparison of glycolyses in presence and absence of phosphate; intact embryos*

		$Q_L^{N_2}$			
Exp.	Age days	Substrate alone	Substrate + inorganic phosphate 0·0012 *M* (equivalent to normal Ringer)	Substrate + phosphate 0·0024 *M*	Substrate + phosphate 0·0047 *M*
			Glucose		
37	3½	+17·14	+18·14	+21·34	+20·04
38	2¾	+32·92	+26·22	+25·02	+19·72
39	4¼	+18·80	+17·67	+14·00	+12·30
			Glucosamine		
45	4¼	+ 0·0	+ 0·3	+ 0·0	—
46	2¾	+ 0·0	—	+ 0·0	+ 0·0

In their work on the glycolytic mechanisms of mammalian brain, which is thought to possess both phosphorylating and non-phosphorylating mechanisms, Ashford & Holmes [1929] studied the effect of eliminating all free phosphate as nearly as possible by the use of calcium chloride. They found that the formation of lactic acid from glucose proceeded normally after such treatment, and that subsequent addition of inorganic phosphate had practically no effect on the glycolysis. Although we know that dialysed muscle extract, for instance, which is practically free from inorganic phosphate, can glycolyse glycogen perfectly if adenylpyrophosphate is present, nevertheless a serious decrease of glycolytic rate occurs if inorganic phosphate is absent.

We endeavoured to apply this calcium method to the chick embryo. It proved exceptionally difficult to free the embryo from inorganic phosphate in this way. Preliminary shaking at 38° in N_2/CO_2 gas mixture for half an hour in presence of $M/22$ $CaCl_2$, as well as allowing the glucolysis to proceed in its presence, did not succeed in removing all the free phosphate. Nor did the substitution of beryllium chloride for calcium chloride [Kay & Skill, 1934] lead to any better result, although beryllium phosphate is much less soluble than calcium phosphate in solutions rich in carbon dioxide. It was then thought that in younger embryos the cells might be more permeable to the calcium ion, so that by using them and at the same time increasing the strength of the calcium chloride, the desired effect might be obtained. As Exps. 14 and 15 show (Table III), this actually happened; the reduction of glucolysis rate was very small in the presence of calcium ten times as strong as that previously employed, and yet the free phosphate had been reduced to a trace. A similar effect was seen in another experiment where the embryos were made into a brei.

Table III. *Glucolysis after elimination of free inorganic phosphate by precipitation with Ca*

Abbreviations. Bo: borate-Ringer at *p*H 8·15. Bo-Ca, 12·0: borate-Ringer containing 1 ml. 12% $CaCl_2$/g. wet wt. embryos. Usual R: Ringer-bicarbonate (Krebs-Henseleit) as used in all other experiments. Ca-R, 12·0: Ringer-bicarbonate with similar addition of 12% $CaCl_2$.

Exp.	Age in days	Condition	Nature of preliminary treatment, duration ½ hr., anaerobic, 38°	Nature of medium during glucolysis	No. of parallel exp.	Average $Q_L^{N_2}$	Result of phosphate test on extracted embryos after the experiment
14	3¼	Intact	Bo alone	Usual R	1	+ 7·6	
			Bo-Ca, 12·0	Ca-R, 12·0	2	+ 8·5	Faintest traces only
15	3¾	Intact	Bo alone	Usual R	1	+10·8	
			Bo-Ca, 12·0	Ca-R, 12·0	1	+ 9·9	Trace only
16	4	Brei	Bo alone	Usual R	2	+ 7·5	
15*a*	4	Brei	Bo-Ca, 12·0	Ca-R, 12·0	2	+ 5·5	Traces only

These results serve as further contributory evidence against the intervention of phosphorylation in embryo glucolysis.

Effects of addition and removal of adenylpyrophosphate, adenylic acid and cozymase

As already mentioned [Needham *et al.*, 1937, p. 1200] there exists in the chick embryo a certain amount of barium-precipitable phosphorus hydrolysed by HCl in 7 min., of which it is to be presumed that a good deal, at any rate, is adenylpyrophosphate. Furthermore, it was seen (p. 1212) that, although the Warburg-Christian hexosemonophosphate codehydrogenase (coenzyme II) is always present throughout development, the presence of cozymase itself (coenzyme I) is very doubtful. If present, it must exist only in the smallest amounts.

The study of glucose breakdown in embryos therefore necessitated the addition of adenylpyrophosphate, adenylic acid and cozymase to glucolysing embryos in view of the possibility that their glucolytic rate might thereby be increased. Conversely the removal of adenylpyrophosphate by inactivation or dialysis was studied. It may be said at once that we were never able to observe an appreciable effect of any of these substances on glucolytic rate, whether of intact embryos, brei, or acetone powders prepared according to the method of v. Euler *et al.* [1936]. Sample experiments are the following.[1]

Exp. 173. Effect of adenylpyrophosphate on glucolysis of intact 4-day embryos, $Q_L^{N_2}$ glucose +15·1, glucose + adenylpyrophosphate +12·3.

Exp. 77. Effect of adenylpyrophosphate on glucolysis of intact 4-day embryos, in presence of added inorganic phosphate (twice and four times the usual amount in the Ringer), $Q_L^{N_2}$:

	Phosphate Ringer	Phosphate Ringer × 2	Phosphate Ringer × 4
Glucose	+18·4	+18·5	+16·2
Glucose + adenylpyrophosphate	+21·5	+14·8	+21·0

[1] In the following experiments, except when otherwise stated, the following conventions hold good:

Material. 2·0 ml. of embryo brei; made by grinding in a mortar under ice-cold conditions, but

Exp. 157. Effect of adenylpyrophosphate on glucolysis of intact 5½-day embryos, in presence of added magnesium; $Q_L^{N_2}$, glucose +14·9, glucose + adenylpyrophosphate and Mg +10·1.

Exp. 168. Effect of cozymase on glucolysis of intact 4¾-day embryos; μl. in 2 hr.; glucose 294; glucose + cozymase 288, 299; mannose 291; mannose + cozymase 332.

Exp. 173. Effect of cozymase on glucolysis of intact 4-day embryos; $Q_L^{N_2}$, glucose +15·1; glucose + cozymase + 10·5.

Exp. 160. Effect of adenylpyrophosphate on glucolysis of brei of 5-day embryos, in presence of 1 mg. inorganic P and 0·5 mg. Mg; μl. in 2½ hr.; glucose 37; glucose + adenylpyrophosphate 33.

Exp. 162. Effect of cozymase on glucolysis of 6½-day brei; glucose 212 μl. in 3 hr.; glucose + cozymase 214 μl. in 3 hr.

Exp. 155. Effect of magnesium alone on glucolysis of 6-day embryo brei; μl. in 2 hr.; glucose 721; glucose + Mg 688.

Exp. 163. Effect of cozymase on glucolysis of 6½-day embryo acetone powder; μl. in 2 hr.; glucose 60; glucose + cozymase 36.

Exp. 165. Effect of adenylic acid and adenylpyrophosphate on glucolysis of 6½-day embryo acetone powder; μl. in 1¼ hr.; glucose 139; glucose + adenylic acid 151; glucose + adenylpyrophosphate 141; glucose + cozymase 169.

Glucolysis is equally unaffected by allowing brei to stand at room temperature for a length of time sufficient to destroy any adenylpyrophosphate contained in it.

Exp. 183. Brei of 9-day embryos allowed to wait an hour at 18° for inactivation; μl. in 1 hr.; glucose before inactivation 800; glucose after inactivation 792; autoglycolysis 360.

Finally, during the course of a number of dialysis experiments made in another connexion (see below), it was invariably found that a large glucolysis could occur after a dialysis sufficient to remove all coenzyme, and that furthermore when the glucolytic activity seriously declined, it could not be restored at all either by adenylpyrophosphate or cozymase or magnesium.

Exp. 192. Brei of 6-day embryos dialysed against tap water for 16 hr. at 0°; an aliquot part maintained undialysed at the same temperature; μl. in 1½ hr.; (undialysed) glucose 515; (dialysed) glucose 6; glucose + adenylpyrophosphate 4.

Exp. 194. Brei of 9-day embryos dialysed against tap water for 13 hr. at 0°. μl. in 1½ hr.; (undialysed) glucose 269; (dialysed) glucose 34; glucose + Mg 41, 45; glucose + Mg + inorganic P 58.

Exp. 197. Brei of 8-day embryos dialysed against tap water for 1½ hr. at 0°, then against a further lot of tap water for 1½ hr.; μl. in 1½ hr.; (undialysed) autoglycolysis 149; glucose 637; (dialysed) autoglycolysis 92; glucose 390.

Exp. 215. Brei of 8-day embryos dialysed against 0·45% KCl for 23 hr. at 0°.

	μl.
Brei alone	3
Brei + glucose	61
Brei + glucose + cozymase	43
Brei + glucose + cozymase + Mg	53

not with sand; undiluted but buffered with stock bicarbonate in the usual proportions as if normal Ringer; kept, if necessary, in ice. Or 1–3 weighed intact embryos.

Glucose. 0·2 ml. of 2·5% to make 5 mg. per cup or just over 0·01 *M*.

Adenylpyrophosphate. 0·2 ml. containing 0·2 mg. of P hydrolysable in 7 min.

Adenylic acid. 0·2 ml. containing 0·1 mg. organic P.

Cozymase. Prepared by the method of Myrbäck [1933] slightly modified; 0·2 ml. containing 0·2 mg. organic P.

Magnesium. 0·1–0·3 ml. containing 0·7 mg. Mg as magnesium chloride in 0·3 ml.

Hexosediphosphate. 0·2 ml. of 0·08 *M*, giving 5·2 mg. in the cup.

Hexosemonophosphate. 0·2 ml. of 0·11 *M*, giving 6 mg. in the cup.

Glycogen. 0·2 ml. of 2·5%.

Inorganic phosphate. 0·2 ml. of 0·075 *M*.

Exp. 206. Brei of 6-day embryos dialysed against 0·9% KCl for 19 hr. at 0°, one change, after 2 hr.; μl. in 2 hr.; autoglycolysis 46; glucose 411.

Exp. 209. Brei of 7-day embryos dialysed against Ringer solution (no bicarbonate) at 0° for 40 hr.; μl. in 1½ hr.; autolgycolysis 105; glucose 650.

The last two of these experiments, typical of others, are particularly striking, since, after a prolonged dialysis, amply sufficient to remove free adenyl compounds, powerful glucolyses are observed. It could, however, be urged that a certain fraction of cozymase or other adenyl compound may remain bound to the structure of the disintegrated cells, and so unable to pass through the wall of the dialysing sac (cf. the work of Parnas & Lutwak-Mann [1935] on the protein precipitate of muscle). This possibility could only be tested by experiments of another sort, shortly to be described.

Fluoride inhibition

The next line of attack to be pursued was that of the action of fluoride, in the hope that the two paths of carbohydrate breakdown might be selectively sensitive to this inhibitor. The results, which showed in a very clear-cut way that this is actually the case, are condensed as follows:

Exp. 123. 4½-day embryos were suspended in Ca-free Ringer-bicarbonate and glucolysis was measured manometrically.

	No. of exps.	$Q_L^{N_2}$
Glucose	2	+8·40
Glucose + *M*/200 NaF	2	+3·25
Glucose + *M*/150 NaF	1	+2·30
Glucose + *M*/100 NaF	2	+0·71

Exp. 223. 8-day embryos were made into a brei under ice-cold conditions. 1·4 ml. brei were mixed with 1·2 ml. solution in each cup containing varying amounts of fluoride, and in all cases 4 mg. glucose, the whole buffered to the usual extent with bicarbonate. Glucolysis was measured manometrically. Meanwhile the same brei with the same varying additions of fluoride was incubated with 1·9 mg. of phosphoglyceric acid P and (in some cases) with 0·5 mg. adenylic acid P.

	μl. in 75 min. (after tipping)
Autoglycolysis	60
Autoglycolysis + *M*/50 NaF	110
Glucolysis	397
Glucolysis + *M*/200 NaF	264
Glucolysis + *M*/100 NaF	76
Glucolysis + *M*/50 NaF	54

Additions to brei	Inorganic P mg.	Easily hydrolysable P – inorganic P (after subtraction of adenylic acid P) mg.
0 min. + adenylic acid + phosphoglyceric	0·20	0·34
120 min.; no addition	0·41	0·08
120 min. + *M*/50 NaF	0·56	0·11
120 min. + adenylic acid + phosphoglyceric	1·40	0·40
120 min. + adenylic acid + phosphoglyceric + *M*/200 NaF	0·39	0·08
120 min. + adenylic acid + phosphoglyceric + *M*/100 NaF	0·32	0·05
120 min. + adenylic acid + phosphoglyceric + *M*/50 NaF	0·41	0·15

These relationships are shown graphically in Fig. 5, where it will be seen, first, that the progress of the inhibition with increasing fluoride concentration on the glucolysis of the intact embryo is in good agreement with the figures earlier obtained by Dickens [cit. in Needham, 1932], and secondly that the

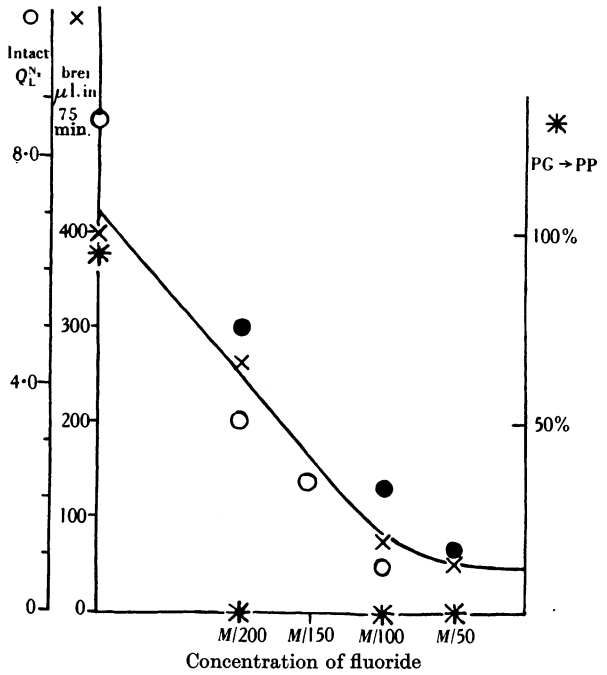

Fig. 5. Comparison of the anaerobic glucolysis rates of intact embryos and embryo brei with the phosphoglyceric acid → phosphopyruvic acid reaction, at various concentrations of fluoride. The inhibition would appear complete if the autoglycolysis were subtracted.

• Dickens's values. o ×, * Our values.

inhibition is exactly the same upon brei as upon the intact embryo, although the amount of enzyme present in the cup is perhaps ten times as much in the case of brei as in the case of intact embryo. Particular attention is drawn to the fact that the glucolysis of the embryo is very resistant to fluoride, for at $M/200$ where the formation of lactic acid from glycogen in muscle is 90 % inhibited [Lohmann, 1931], embryo glucolysis is only inhibited between 40 and 50 %. It is of much interest that the glucolysis of adult brain [Ashford & Holmes, 1929; Dickens & Šimer, 1929] is more sensitive than embryo glucolysis, 80 % being lost at $M/200$ fluoride. In the last-mentioned paper, indeed, a number of tissues were examined for the sensitivity of their glucolyses to fluoride; it was found that, whereas rat sarcoma, brain and retina were nearly as sensitive as muscle, rat testis and kidney gave resistances of the same order as that shown by the chick embryo. Dickens & Šimer were tempted to the generalization that wherever the initial glucolysis rate was high, there the fluoride sensitivity was great, and vice versa. This was supported by some of their own experiments on different samples of human carcinoma, but is somewhat controverted by the results described here for the chick embryo, which as is well known has a high glucolytic rate. They also noticed that the effect of fluoride was small on the aerobic glycolysis of rat testis, but not on that of rat sarcoma.

The bearing of this on the question of whether glucose passes through the phosphorylation route may be considerable, since, as Exp. 223 above demonstrates, the inhibition of the conversion of phosphoglyceric acid into phosphopyruvic acid by the embryo was already complete at $M/200$ fluoride. The conclusion is thus inescapable that two separate ways of breakdown exist in the embryo, one phosphorylating, the other not, and we would especially emphasize, in view of the known variability of inhibitions with fluoride (perhaps due to varying amounts of calcium in the tissue), that our experiments were performed on samples of the same brei at the same time.

It is of interest that Meyerhof [1932] found that lactic acid formation from glucose by haemolysate free from erythrocyte structure was more sensitive to fluoride than lactic acid formation by the cells themselves. He attributed this to permeability differences, but we would suggest that it may be accounted for by the low solubility of the non-phosphorylating system in water.

The combined effect of hexokinase and fluoride or glyceraldehyde

All the facts which have so far been described in this series of papers are consonant with the conclusion that in the embryo there are two possible paths of carbohydrate catabolism (1) the path of phosphorylation, essentially similar to that in muscle and yeast, but seriously deficient at four distinct places, so that glycogen and hexosediphosphate are not vigorously broken down, (2) a non-phosphorylating path of glucose breakdown, closely bound to the cell structure, but yet exceedingly active. The differential fluoride inhibition, described in the last section, goes far to prove that this is so. No proof has yet been given, however, that in the developing egg itself glucose is not phosphorylated and passed through the phosphorylation route. It was thought that particularly convincing evidence on this point could be obtained by the combined use of hexokinase and fluoride or glyceraldehyde.

Hexokinase [Meyerhof, 1927; 1936, 1] is the enzyme or apo-enzyme ("heterophosphatese" [Müller, 1933; v. Euler & Adler, 1934; 1935] isolated from yeast which converts glucose into hexosemonophosphate prior to the yeast fermentation of glucose. In the absence of such a "phosphatese", and if the phosphorylation route is the only one present, as in muscle extract free from cell structure, glucose is not broken down. Glyceraldehyde, as we have already seen [Needham & Nowiński, 1937, p. 1178] is a specific inhibitor for glucose, but not hexosediphosphate, breakdown in animal tissues. If now we block the phosphorylation route by a carefully chosen fluoride concentration, and if at the same time we insert hexokinase into the system, the breakdown of glucose should be reduced or stopped. The hexokinase should shunt it into the phosphorylation route and there it should remain, ultimately in the form of phosphoglyceric acid, without being able to give rise to any lactic acid. Conversely, by the action of glyceraldehyde we may block the non-phosphorylation path, but not the phosphorylation path. Hence the combined action of hexokinase and glyceraldehyde should, if the phosphorylation path is a real alternative for glucose breakdown, lead to a reversal of the usual glyceraldehyde inhibition, lactic acid being formed from phosphorylated hexose. If, on the other hand, no such lifting of the inhibition took place, it would be possible to go further and to say that in all probability, even in the intact egg, the embryo's glucolysis is not performed with the aid of the phosphorylation cycles.

Such shunting experiments, in which the glucose is placed under conditions favourable to phosphorylation if such a process is possible, would also allow of

the exclusion of the possibility, not covered by any of the previous experiments, that glucose breakdown may occur with very small phosphate changes. Addition of intermediates, it may be urged, may lead to unphysiological accumulations of esters, which in the nascent state might have been broken down.

It may be noted that in all cases brei made with distilled water to abolish the cell-integrity was used; hence it cannot be argued that hexokinase had no entrance to the glucolytic system.

Before describing some of these experiments, mention must be made of two minor but necessary controls:

(*a*) test of the activity of the hexokinase preparation,

(*b*) proof that glyceraldehyde does not inhibit hexosediphosphate breakdown.

Exp. 213*a*. Hexokinase prepared from yeast according to the method of Meyerhof [1927]. To 3 ml. of freshly prepared frog muscle extract 0·3 ml. of 2·5% glucose and 0·5 ml. of water or hexokinase solution were added.

	Glucose, mg. (Hagedorn-Jensen)
0 min. + hexokinase	10·05
35 min.	10·03
35 min. + hexokinase	8·30

There was also a decrease in inorganic phosphate. The disappearance of about 2 mg. of glucose shows that the activity is of about the same order as that of Meyerhof's preparations.

Exp. 213*b*. The observation of Holmes [1934] that glyceraldehyde does not inhibit the breakdown of glycogen or of glucose + hexokinase in muscle extract was repeated using hexosediphosphate as substrate. Fresh frog muscle extract was set up with substrate and inhibitor as follows (room temperature; 0·8 mg. hexosediphosphate):

	Inorganic P mg.	Increase in inorganic P mg.
0 min. + HdP	0·35	—
45 min. + HdP	0·48	0·13
45 min. + HdP + 0·6 mg. glyceraldehyde	0·44	0·09
45 min. + HdP + 2·5 mg. glyceraldehyde	0·49	0·14

We also confirmed the observation of Holmes on glucose + hexokinase. Glyceraldehyde had no inhibitory effect; on the contrary, glucose breakdown was somewhat increased; no doubt because the glyceraldehyde formed hexosemonophosphate with the triosephosphate arising [Meyerhof *et al.* 1936].

We can now proceed to describe, first, the combined effect of hexokinase and fluoride, secondly, the combined effect of hexokinase and glyceraldehyde.

From the preceding section it may be concluded that the best fluoride concentration to choose for inhibiting the phosphorylating but not the non-phosphorylating path is $M/200$, and this was used in the following experiment.

Exp. 195. 7-day embryos were ground to a brei under ice-cold conditions, and 2·0 ml. were added to each cup. Fluoride, where added, $M/200$; inorganic phosphate, where added, 0·025 M, i.e. five times as strong as that present in the experiments described in the first section of this paper. Hexokinase 0·5 ml. of the preparation (HK). Lactic acid formation measured manometrically.

	μl. in 2½ hr.
Brei + glucose	761
Brei + glucose + HK	720
Brei + glucose + NaF	490
Brei + glucose + inorganic P	717
Brei + glucose + inorganic P + NaF	362 (inhibition 50%)
Brei + glucose + inorganic P + HK	635
Brei + glucose + inorganic P + HK + NaF	285 (inhibition 55%)

Thus it is quite plain that the inhibition by fluoride is exactly the same whether in the presence or in the absence of hexokinase (55 %; 50 %). Yet if it had been possible to shunt the glucose into the phosphorylating route, the inhibition should have been much greater, perhaps even complete, in the presence of hexokinase. The fact that it is exactly the same in either case, and that the value it reaches at this fluoride concentration is the same as that shown for glucose breakdown without hexokinase in Fig. 5 must mean that hexokinase cannot transfer glucose to the phosphorylation route in competition with the non-phosphorylation route. Perhaps it might, however, if it were aided by the blockage of the latter with glyceraldehyde. This brings us to the next type of experiment.

Exp. 216. 7-day embryos were ground to a brei under ice-cold conditions and used as in the experiment just described, with the exception that *M*/200 and *M*/40 glyceraldehyde were added in some cases. The former concentration was equivalent to that used by Mendel [1929] in his original experiments with glyceraldehyde. As shown before [Needham & Nowiński, 1937, p. 1179] the inhibition caused by concentrations of this order is partly reversed after the lapse of about an hour. In the table here, therefore, inhibitions are calculated for the first hour as well as for the whole 2½ hr. Glucose 2·5 mg. in all cases.

	Whole period μl.	First hour only μl.	Inhibitions: Whole period %	Inhibitions: First hour only %
HK + glucose (no brei)	3	—	—	—
Brei alone	140	—	—	—
Brei + HK	94	—	—	—
Brei + glucose	631	525	73·2	41·0
Brei + glucose + *M*/40 glyceraldehyde	168	—		
Brei + glucose + *M*/200 glyceraldehyde	595	311		
Brei + glucose + HK	562	406	66·5	45·5
Brei + glucose + HK + *M*/40 glyceraldehyde	189	—		
Brei + glucose + HK + *M*/200 glyceraldehyde	500	221		

From this we see that the inhibition by glyceraldehyde, at whichever concentration or time it be taken, is exactly the same whether hexokinase be present or not. This must imply that however favourable the conditions—as here owing to blockage of the non-phosphorylating path—glucolysis cannot proceed through the phosphorylating path.

Lest it should be suggested that in the preceding experiment insufficient inorganic phosphate was present to allow of the thorough esterification of the glucose, we made a special experiment on the combined action of hexokinase and glyceraldehyde in the presence of extra inorganic phosphate.

Exp. 218. 7-day embryo brei in ice made as before. To 1·6 ml. brei were added, in the appropriate cases, 5 mg. glucose; 0·2 ml. glyceraldehyde 1·25 % (to make *M*/40), or water; 0·5 ml. hexokinase solution; and inorganic phosphate to make the same molar concentration as in Exp. 195 (hexokinase-fluoride). This experiment was conducted somewhat differently from the preceding one in that instead of tipping both glucose and glyceraldehyde, here glucose was present from the beginning, and only in those cups in which glyceraldehyde was tipped did the glucolysis fall off sharply.

	μl. in 1¼ hr.	Inhibition %
Brei + inorganic P	116	—
Brei + inorganic P + HK	94	—
Brei + inorganic P + glucose	611	72·8
Brei + inorganic P + glucose + glyceraldehyde	166	
Brei + inorganic P + glucose + HK	683	77·8
Brei + inorganic P + glucose + HK + glyceraldehyde	152	
Brei + glucose	515	68·9
Brei + glucose + glyceraldehyde	160	
Brei + glucose + HK	631	73·2
Brei + glucose + HK + glyceraldehyde	169	

Lack of inorganic phosphate cannot therefore be held responsible for the failure of the glucose to enter the phosphorylation route. In all cases the inhibitions are the same.

In examining the foregoing experiments it may be observed that in several instances hexokinase seems to produce a slight diminution (amounting perhaps to 20 or 30 μl.) when compared with controls without hexokinase. It might be thought that this represents a small fraction of glucose which is phosphorylated and ends blocked as phosphoglyceric acid. We do not believe, however, that the amount of glucose which is esterified in these experiments is ever more than minimum, since in Exp. 218 we followed up the glucolytic periods by phosphate estimations and determinations of triosephosphate by hydrolysis to methylglyoxal and inorganic phosphorus according to the method of Ariyama [1928]. In no circumstances was there any decrease in inorganic phosphate as might have been expected had there been any considerable esterification and triosephosphate formation; nor was any appreciable amount of triose to be found other than the glyceraldehyde itself, which presumably, as in the experiments described in the first paper of this series, remained for the most part unaltered.

The combined effect of hexosediphosphate and glyceraldehyde

If the accumulations of triosephosphate from hexosediphosphate described on pp. 1217–1218 were allowed to occur in the presence of glyceraldehyde, then according to the findings of Meyerhof *et al.* [1936] the triosephosphate should combine with the glyceraldehyde, forming hexosemonophosphate and the inhibition of glucolysis should be removed. The following figures show that this is exactly what happens. Glyceraldehyde inhibition in the presence of hexosediphosphate is much less efficient than in its absence.

		6–7 day embryo brei μl./ml. brei			% inhibition	
Exp.	HdP conc.	Alone	+ glyceraldehyde	+ glyceraldehyde + hexosediphosphate	+ G	+ G + HdP
243	*M*/130	315	38	123	88	61
244	*M*/65	151	24	74	84	51
245	*M*/29	204	54	89	73	56

N.B. Glyceraldehyde concentration always 2·5 mg./ml. brei.

These experiments afford further proof both for triosephosphate accumulation and for a non-phosphorylating path inhibited by *dl*-glyceraldehyde, for if

hexosediphosphate were formed from glucose in the normal course of breakdown, glyceraldehyde would not inhibit it. That the reversal of glyceraldehyde inhibition by hexosediphosphate is not complete would be expected from the fact that the reaction

$$1\ \text{Hexosediphosphate} \rightleftharpoons \text{Triosephosphate} + \text{glyceraldehyde} \rightarrow \text{hexosemonophosphate}$$

is an equilibrium one. As has been already shown [Needham & Nowiński, 1937, p. 1176], hexosemonophosphate is unattacked by the embryo, hence, after a certain point is reached at constant temperature, the disappearance of glyceraldehyde practically ceases.

Discussion

The general conclusion which emerges from this paper and the preceding ones in the series is that the chick embryo in the early stages of its development possesses at least two paths of carbohydrate breakdown, the non-phosphorylating route of glucose and the phosphorylating route of glycogen. The latter, moreover, appears to be seriously deficient at several points, showing that at an early stage of ontogenesis the machinery of the phosphorylation cycles is not yet fully installed. In the absence of fuller information about the machinery of non-phosphorylating glucolysis we cannot give any answer to the question proposed at the opening of the first paper of this series, namely, whether glucolysis proper is not a fundamentally simpler process than the protein and fat combustion which succeed it later in the ontogenetic process. The conception of it as a simpler process is at any rate not adversely affected by any of the results we have obtained, and it clearly exists in maximally active form at a time when phosphorylation breakdown can only proceed with great difficulty, if at all. In this connexion the absence of glycogen as a reserve material from the eggs of birds is interesting; we know [lit. in Needham, 1931] that glycogen is synthesized only slowly during incubation and, until midway through development, mainly in the yolk-sac and not in the embryo. Nevertheless the rise of the phosphorylation system seems to lag much behind the beginning of the glycogenic function of the liver; the latter dates from the eleventh day of incubation, while, as we have seen, even the chick's muscles on the fifteenth day do not show the expected predominance of phosphorylation, and the work of Schönfelder [1935] bears this out in the case of mammalian muscle at and shortly after birth. There may also perhaps be some connexion between the non-phosphorylating character of the glucolysis of the early embryo and the very high glycolytic rate which itexhibits.

Although it may be that non-phosphorylating glucolysis is a simpler process than either phosphorylating carbohydrate breakdown or protein or fat catabolism, we wish to avoid the suggestion that any recapitulatory significance attaches to it. The mere existence of phosphorylation in the classical case of the yeasts is sufficient to require this. However, the work of Reiner *et al.* [1936] is of interest in this connexion, for they found that trypanosomes produce anaerobically from every molecule of glucose one molecule of glycerol and one of pyruvic acid, with no indications of phosphorylation. This gives significance to the failure of Krijgsman [1936] to find any glycogen in trypanosomes.

From what has gone before it will be evident that we are forced by our experiments to join the number of those recent investigators who admit the existence of non-phosphorylated carbohydrate breakdown, e.g. Ashford & Holmes [1929] for mammalian brain; Gaddie & Stewart [1934] for mammalian heart; Nord *et al.* [1936] and Nilsson & Alm [1936] for yeast; above all Geiger [1935], who showed glutathione to be the coenzyme of non-phosphorylating

breakdown, as adenylpyrophosphate is that of phosphorylating breakdown. The failure of Meyerhof & Kiessling [1936] to confirm his results may have been due to the fact that they used muscle extract incapable of breaking down glucose except when shunted into the phosphorylation path by hexokinase. The non-phosphorylating glucolytic mechanism is, as we have seen, very difficult to obtain in tissue extract, and extracts would be expected to differ according to the efficiency of removal of cell debris by centrifuging. In the presence of hexokinase no activating effect of glutathione would be expected.

The non-phosphorylating glucolytic mechanism can really only be studied in those tissues which need no hexokinase for glucolysis. Haarmann [1932] has already given a list of such tissues. We only wish to add the suggestion, prompted by a view of the values of $Q_L^{N_2}$ in the literature, that just those tissues and materials (brain, retina, tumour, embryo etc.) which have a high anaerobic glycolytic rate, have also:

(1) a more or less strong preference for glucose as substrate without addition of hexokinase;

(2) a sensitivity of this glucolysis to glyceraldehyde;

(3) a marked inefficiency of phosphorylating breakdown.

Finally, we would venture the suggestion that the meaning of the double path of carbohydrate catabolism may lie in a relationship with continuity and discontinuity of function. If we examine the distribution of the two paths in different kinds of muscles, we find that in skeletal muscle phosphorylation predominates, in cardiac muscle phosphorylation is known to be weaker and glucolysis stronger, while in unstriated muscle glucolysis predominates. This suggests that phosphorylation is associated with discontinuous function, where sudden large calls upon energy stores (phosphoric esters) have to be satisfied; non-phosphorylating glucolysis with functions more continuous (cardiac activity, brain function etc.) depending sharply on the glucose level in the blood, well enough balanced even in invertebrates. Glucolysis in muscle might thus explain continuous functions hitherto enigmatic: the tonic contraction of molluscan muscle, catatonic rigidity in human schizophrenia, hypnosis etc. In spite of the breaks in growth curves to which attention has been drawn by Brody [1927] and others, embryonic development must in the long run be regarded as a continuous process.

VII. EXPERIMENTS ON THE NATURE OF NON-PHOSPHORYLATING GLUCOLYSIS

Introduction

In view of the evidence given in the former papers of this series, especially the last, we shall consider henceforward that glucolysis without phosphorylation has been demonstrated in the chick embryo, and experiments designed to elucidate the mechanism of this process will be described. We shall consider first the results of dialysis experiments, and then the status of dihydroxyacetone, methylglyoxal and other substances as possible intermediates.

GLUTATHIONE AND GLUCOLYSIS AFTER DIALYSIS

A considerable number of dialysis experiments were carried out, using collodion tubing previously treated for half an hour with $N/10$ HCl to get rid of traces of copper. In the course of these experiments, it was found, as has already been described (p. 1229), that neither adenylpyrophosphate nor cozymase would restore the lost activity. Reduced glutathione, on the other hand, produced a recovery which varied according to the conditions from the limits of detectability to some 80%. But what was remarkable about this recovery effect with glutathione was that it often took place in brei (provided that a sufficient quantity of glutathione was added) which still contained enough glutathione to allow of maximum glyoxalase activity. Conversely, in some cases dialysis had removed all the glutathione present, for glyoxalase activity was minimum and could be restored proportionally by increasing additions of glutathione, yet similar additions did not succeed in recovering the glucolytic power of the brei. Both these situations are difficult to interpret, but they point in one direction, namely, that the as yet unknown factors governing glucolysis are different from those governing glyoxalase activity, the glucolytic system being more vulnerable to damage by strong dialysis than the glyoxalase.

To illustrate this, a few examples may be given, taking the latter situation first. In Exp. 189 9-day embryos were made into a brei under ice-cold conditions as described in other experiments and dialysed against tap water for 15 hr. at 0°. Lactic acid productions, manometrically measured, were then as follows:

		μl. for 1·9 ml. brei 1 hr.	
	No addition	With 5 mg. glucose	With 7·2 mg. methylglyoxal
Brei	69	76	129
Brei + 0·5 mg. GSH	—	77	532
Brei + 1·0 mg. GSH	—	64	—
Brei + 2·5 mg. GSH	—	58	620

In this experiment, therefore, the methylglyoxalase was practically inactivated by the dialysis, and successive amounts of glutathione restored it with some regularity (see Fig. 6), but the same amounts had no effect on the glucolysis. The converse of this is seen in two experiments where the dialysis was short. Here, the glucolysis had been more severely inactivated than the glyoxalase activity and was restored by glutathione, while the glyoxalase was not.

Exps. 196 and 197. Brei of 8-day embryos made as usual, and part of it maintained undialysed at 0° for 4 hr. while the rest was dialysed against tap water for the same period. In Exp. 197 the tap water was changed after 1½ hr. and the total dialysis went on only for 3 hr.

		μl. for 2 ml. brei, 1½ hr.	
	No addition	With 5 mg. glucose	With 7·2 mg. methylglyoxal
Exp. 196:			
Undialysed brei	105	354	150
Dialysed brei	66	70	111
Dialysed brei + 1 mg. GSH	—	126	116
Exp. 197:			
Undialysed brei	149	637	322
Dialysed brei	92	390	242
Dialysed brei + 1 mg. GSH	—	507	251

Thus in both cases glyoxalase activity was not greatly reduced by the short dialysis and was correspondingly not restored by addition of glutathione, but the glucolytic activity was considerably reduced and also considerably restored (even to about 80 % of normal).

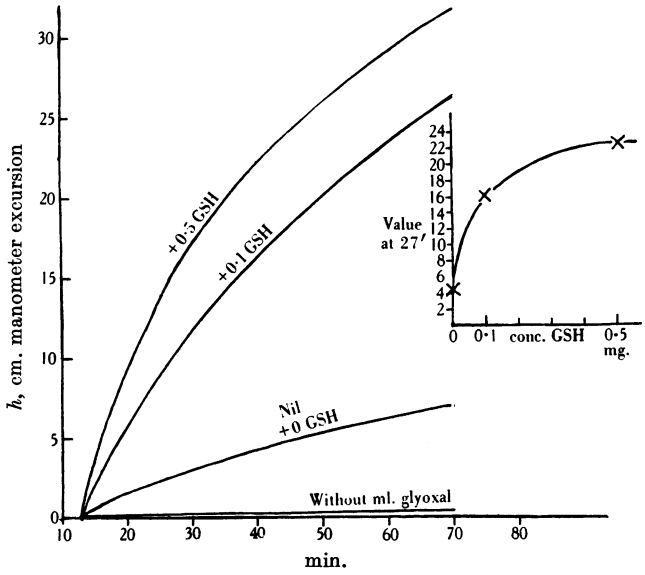

Fig. 6. Reactivation of methylglyoxalase of embryo by glutathione after dialysis of brei.

So far the experiments suggested, since glucolysis and glyoxalase activity showed an independence of each other in their reactions to dialysis and glutathione addition, that glyoxalase and methylglyoxal were not involved in the main route of non-phosphorylating glycolysis. It appeared likely, however, that glutathione is connected with glucolysis itself quite apart from glyoxalase. That glutathione is the coenzyme of glyoxalase is, of course, well known from the work of Lohmann [1932], Giršavičius [1933] and Giršavičius & Heyfetz [1936].

If after short dialysis, then, there is sufficient glutathione left for glyoxalase but not for glucolysis, it might be thought that in Exp. 189 (see above) the glucolytic enzyme systems had been injured by the removal of ions, and that therefore if dialysis was conducted against physiological saline or Ringer solution, and a sufficiently large amount of glutathione added at the end of a prolonged dialysis period, both the systems might be brought back to activity

Exp. 206. Ice-cold brei of 6½-day embryos dialysed against 0·9 % KCl for 19 hr. at 0°, the saline changed at the end of 2 hr. (20 ml. brei against 3 l.).

	μl. from 2 ml. brei in 2 hr.
Brei alone	46
Brei + 9·0 mg. GSH	60
Brei + glucose	411
Brei + glucose + 0·9 mg. GSH	443
Brei + glucose + 3·6 mg. GSH	493
Brei + glucose + 9·0 mg. GSH	651
Brei + glucose + 7·2 mg. GSH + 2·5 mg. glyceraldehyde	143
Brei + 7 mg. methylglyoxal	128
Brei + 7 mg. methylglyoxal + 0·9 mg. GSH	243

again. This could not always be done, but we may give one of the experiments as somewhat instructive; Exp. 206.

It is to be noted that the addition of glyceraldehyde causes a fall of 508 μl. and thus abolishes not only the 365 μl. of glucolysis present before the addition of glutathione, but also the 240 μl. by which the glutathione had increased the original value. We take this to be evidence that the increases caused by glutathione, wherever they are observed, are really due to the formation of lactic acid from glucose and not to any other process.

In view of the variability of the present results, we append a table (Table IV) showing the whole series. From this it is to be concluded that whether the

Table IV. *Dialysis of embryo brei and restoration of glucolysis by glutathione*

Exp. no.	196	197	189	192	194	198	204	206	209	212	214	215
Hours dialysis ...	4	3	15	16	13	14	20	19	40	40	64	23
Undialysed control:												
Brei alone	105	149	—	—	—	—	—	—	—	—	14	—
Brei + G	354	637	—	515	269	—	—	—	—	—	176	—
Brei + MG	150	322	—	305	—	—	—	—	—	—	—	—
Dialysed:												
Brei alone	66	92	69	—	—	33	54	46	105	44	0	3
Brei + G	70	390	76	36	34	88	67	411	650	387	—	61
Brei + G + 0·5 GSH	—	—	77	—	—	—	—	—	—	—	—	—
Brei + G + 0·9 GSH	—	—	—	—	—	98	83	443	745	—	—	76
Brei + G + 1·0 GSH	126	507	64	30	44	—	—	—	—	—	—	—
Brei + G + 2·5 GSH	—	—	58	—	—	—	—	—	—	—	—	—
Brei + G + 3·6 GSH	—	—	—	—	—	—	98	493	743	505	0	—
Brei + G + 5·4 GSH	—	—	—	—	—	141	—	—	—	—	—	—
Brei + G + 9·0 GSH	—	—	—	—	—	—	102	651	786	582	—	63
Brei + G + 7·2 GSH + 2·5 GY	—	—	—	—	—	—	—	143	151	—	—	—
Brei + MG	111	242	129	97	—	125	56	128	144	177	34	13
Brei + MG + 1–3 GSH	116	251	454	—	—	113	411	243	216	196	235	40
Percentage increase from brei alone to brei with largest GSH addition	+80	+30	—	—	+23	+38	+52	+59	+21	+34	—	—

Note. Dialysis media used—tap water for all experiments save 206 where 0·9% KCl was used; 215 0·45% KCl; and 209, 212, 214 Ringer solution.

Abbreviations. All figures in the above table are in μl. and are comparable vertically, not horizontally, since the experimental periods were different. G, 5 mg. glucose; MG, 7 mg. methylglyoxal; GY, glyceraldehyde; GSH, reduced glutathione; values for these last given in mg. in each case. Volume of cup contents always 2·6 ml.

glyoxalase is slightly, partly or fully reactivatable, there may be increases of from nil to 80%, with an average of 45%, in the glucolysis when the largest amounts of glutathione are added.

That glutathione may play an important part in glucolysis has for some time past been suspected. Its action as the coenzyme of glyoxalase led Geiger [1935] to try if it would restore glucolytic activity which had been destroyed by dialysis. On tissue extracts prepared with 0·5% KCl and dialysed for 8–12 hr. at 0° against the same concentration of KCl, his results were closely similar to those now reported for the chick embryo, save that usually they were of a higher order of magnitude. Glutathione increased lactic acid formation of mammalian muscle extract from glucose up to twenty times, and large increases of this kind were also found with extracts of brain, liver, heart muscle and uterus. On the other hand, adenylpyrophosphate produced no increases at all when glucose was the substrate (cf. our results described earlier in this paper) but did so—as would be expected—when glycogen was the substrate. We

attribute the relatively low figures here reported to our dialysis of brei instead of extract, an unsatisfactory procedure, but one to which we were forced to resort owing to the complete failure up to the present time of all attempts to obtain active glucolysis in embryo extract. We have already mentioned that a similar attachment of the glucolytic enzyme system to the cell structure has been observed by some workers on tumour also [e.g. Tsuzuki, 1936]. Moreover, owing to the sensitive nature of our glucolytic system, we could not use severe dialysis conditions. But that our dialyses were not unsuccessful is shown by all those cases where the glyoxalase was inactivated and reactivated.

It is of interest, too, that Geiger found the concentration of reduced glutathione necessary for full activation of the glucolysis of brain brei to be 25 mg./100 ml.; this was about equivalent to the lowest concentration used by us. Our experiments do not as yet permit of any conclusion as to whether the concentration of glutathione required for full activation of glucolysis in the chick embryo is the same as that required for full activation of glyoxalase in the embryo. In connexion with the amounts of glutathione ($10^{-3}M - 10^{-2}M$) needed for reactivation, it may be added that the glutathione concentration of embryo tissues is known to be very high, declining with age [Bierich & Rosenbohm, 1935; and many other workers]. The experiments described in this section indicate, in agreement with those of Geiger, that glutathione is in some way concerned with glucolysis, though not with glycogen glycolysis. They certainly do not prove that methylglyoxal is not an intermediate in glucolysis, though they point in that direction.

It would perhaps be plausible to suppose that the action of the glutathione is due rather to an indirect effect on the oxidation-reduction potential level of the brei than to any more definite coenzyme function. But experiments with other substances (Exp. 222, cysteine; Exp. 235, crystalline vitamin B_1; Exp. 235*a*, ascorbic acid) did not support this possibility, for no positive action could be obtained with them. Nor was any restoration effected by the addition of pyruvate or glycerate.

Methylglyoxal as an intermediate in glucolysis

If methylglyoxal were really an intermediate product in the non-phosphorylating glucolytic process, it would be reasonable to suppose that the breakdowns of the two substances might interfere with each other. Tests of aldehydes other than glyceraldehyde for possible inhibitory action on glucolysis [Needham & Nowiński, 1937, p. 1180] prompted the enquiry whether methylglyoxal too would inhibit glucolysis, either by competing for an aldehydophil linkage on a part of the enzyme system, or, if it were really an intermediate, by blocking the later stages of the glucolytic path. If the former possibility were true, glucolysis would be self-inhibitory, supposing that methylglyoxal were ever produced in considerable amount. Were either possibility the actual fact, simultaneous addition of glucose and methylglyoxal to active tissue would be expected to lead to a relative delay in the breakdown of the glucose, and this would manifest itself as an initial incompleteness of the summation of the lactic acid formations.

The results of the following experiments show that if glucose and methylglyoxal are suddenly given together to an active embryo brei, the subsequent summation which occurs is almost perfect. Methylglyoxal (synthetic) is therefore neither an inhibitor of, nor an intermediate in, the glucolysis of the embryo.

Exp. 220. 6½-day embryos made into brei under ice-cold conditions; each ml. of the brei corresponded to 2 embryos. To 2 ml. of brei were added 2 mg. of glucose and 2 mg. methylglyoxal.

In separate cups the glucose and methylglyoxal were given alone, and autoglycolysis was followed. After successive intervals of time, one manometer after another was removed from the bath, trichloroacetic acid added, and the mixture held at 0° for estimations of glucose (Hagedorn-Jensen) and methylglyoxal (Ariyama). Substrates tipped after 40 min. autoglycolysis.

Manometric data

	min.	μl.	Substrate utilization % of possible
Brei alone	140	89	—
Brei + glucose	140	467	98
Brei + methylglyoxal	140	373	78
Brei + glucose + methylglyoxal	10	135	14
	20	249	26
	30	384	40
	50	516	54
	90	627	65
	140	686	72

Summations

Percentages of possible sum in different manometers

							Av.
First 10 min.	120	90	111	102	89	125	106
First 20 min.	—	—	90	94	92	96	93
First 30 min.	—	—	90	96	97	94	94
Whole period	—	—	—	—	—	90	90

Estimations of glucose and methylglyoxal

Min.	Methylglyoxal mg. per cup	Glucose mg. per cup
0	2·0	3·05
10	0·68	2·56
20	0·31	2·26
30	0·29	1·92
50	0·20	1·50
90	0·25	1·06
140	0·20	1·07

Velocities

Time in min.	Methylglyoxal	Glucose	Calc. sum	Obs. sum
7	685	231	916	1020
19	663	395	1058	1112
29	126	390	516	474
39	—	366	—	324
49	—	336	—	333
69	102	399	501	345
119	42	49	91	67

From the above figures the conclusion follows that glucose breakdown proceeds practically unhindered in the presence of equal amounts of methylglyoxal. This is made still more convincing by the curves of disappearance of the two substrates, shown in Fig. 7, and by the velocity curves, shown in Fig. 8. It will be seen that, although the glucose reaches its final value (not zero, on account of other reducing substances in the embryo) some 60 min. later than does the methylglyoxal, nevertheless there is no period of waiting observable, during which the methylglyoxal would be holding up the glucolysis. The velocity curves (Fig. 8), calculated in μl. per hour at each stage, show another phenomenon also, namely, the induction period of glucolysis, already discussed (p. 1194).

The above argument depends on one important assumption, namely, that the glyoxalase was completely saturated with its substrate. If this were not so, extra amounts of methylglyoxal derived from the breakdown of glucose could

be handled without leading to any hold-up of the general process. A test, however, demonstrated that this assumption was entirely justified; Exp. 221.

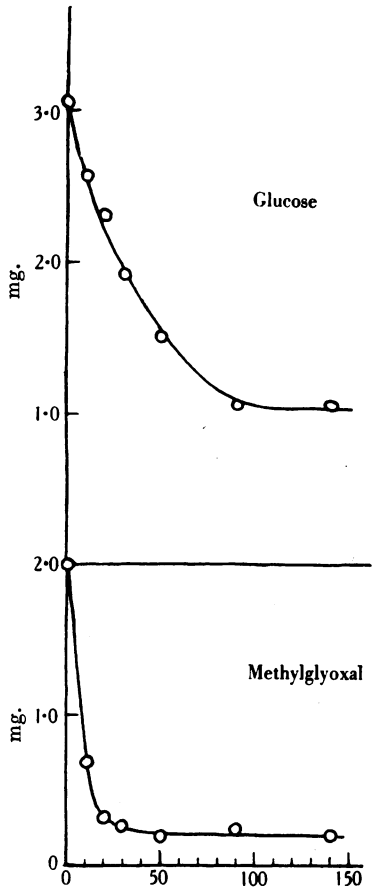

Fig. 7.

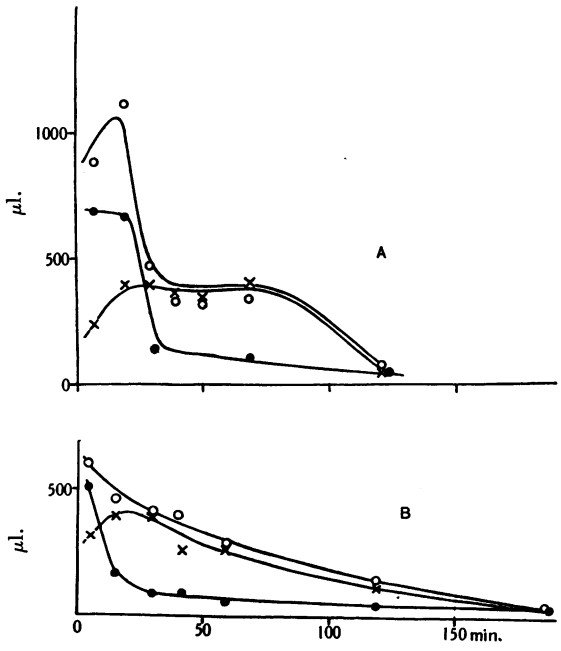

Fig. 8.

Fig. 7. Curves of simultaneous disappearance of the two substrates, glucose and methylglyoxal, during a competition experiment; explanation in text.

Fig. 8. Velocity curves of simultaneous disappearance of the two substrates, glucose and methylglyoxal, during a competition experiment; explanation in text. The induction period of glucolysis is seen in both experiments. × glucose, • methylglyoxal, o total. A. Exp. 220. Large amount of methylglyoxal. B. Exp. 219. Small amount of methylglyoxal.

Exp. 221. 8½-day embryo brei prepared exactly as in the experiment last described. To 2 ml. were added, by tipping, in some cases 2 mg. (as in the previous experiment), in other cases 4 mg. methylglyoxal.

	Methylglyoxal	
	2 mg.	4 mg.
First 10 min.	150	150
Next 25 min.	99	318
Next 30 min.	38	31
Next 30 min.	29	23

The initial velocities were therefore identical and the acid formations linear for the first 25 min., falling off afterwards. The enzyme was therefore fully saturated.

The main experiment was repeated, varying the proportions of glucose and methylglyoxal.

Exp. 219. 6½-day embryo brei prepared and treated exactly as in Exp. 220 except that 0·07 mg. of methylglyoxal wa added to the appropriate cups instead of 2 mg. Substrates tipped after 30 min. autoglycolysis.

Manometric data

	min.	µl.	Substrate utilization % of possible
Brei alone	187	103	—
Brei + glucose	187	461	97
Brei + methylglyoxal	187	204	100
Brei + glucose + methylglyoxal	15	122	19
	30	252	39
	60	396	61
	120	501	77
	187	594	91

Summations

min.	Percentages of possible sum
15	82
30	100
60	101
120	96
187	107
	Av. 99

Velocities

Time in min.	Methylglyoxal	Glucose	Calc. sum	Obs. sum
5	504	312	816	750
15	162	396	558	455
30	84	396	480	417
42	90	255	345	400
59	53	261	314	283
119	42	118	160	160
187	35	24	59	40

The velocities here shown are graphically represented in Fig. 8.

Gluconic acid and 3-carbon compounds other than methylglyoxal as intermediates in glucolysis

Loebel [1925] and later Dickens & Šimer [1929] found that tissues were able to bring about the conversion of dihydroxyacetone into lactic acid. The latter authors also made the interesting observation that the transformation proceeded with undiminished speed even when the whole of the sodium chloride in the Ringer solution was replaced by sodium fluoride. In view of the fact that dihydroxyacetonephosphate is now regarded as an important intermediate compound in phosphorylating glycolysis [Meyerhof & Lohmann, 1934], we thought it desirable to make some experiments with dihydroxyacetone on the embryo. We did not indeed believe that dihydroxyacetone would prove to be an intermediate because it is difficult to see how a ketotriose could arise from glucose. Moreover, the fermentation of dihydroxyacetone and its phosphorylation by a yeast (*S. Ludwigii*) was found by Lehmann [1935, 1] to involve primary polymerization followed by hexosediphosphate formation.

We first found (Exp. 152) that the embryo was incapable of producing any appreciable amount of manometrically measurable lactic acid from dihydroxyacetone, whether in the presence or absence of inorganic phosphate, added as in the trials with glucose and glucosamine mentioned on p. 1228. About 10 μl. more than the autoglycolysis, in lieu of about 205 for glucose, could be attributed to traces of methylglyoxal present in the dihydroxyacetone. This quite negative result leads us to question the freedom from methylglyoxal of the dihydroxyacetone samples employed by the earlier workers.

To get an orientation on the metabolic properties of dihydroxyacetone, we made an experiment in which possible competition with, or inhibition of, glucose breakdown was tested, together with any effect of the combination of dihydroxyacetone with glyceraldehyde.

Exp. 205. 7-day embryo brei made under ice-cold conditions, buffered with bicarbonate as usual. To 1·8 ml. of the brei were added in various combinations 7·5 mg. glucose; 2·5 mg. glyceraldehyde; 7 mg. dihydroxyacetone.

	μl. in 3 hr.
Brei alone	289
Brei + glyceraldehyde	278
Brei + glucose	1158
Brei + glucose + glyceraldehyde	265
Brei + methylglyoxal	740
Brei + methylglyoxal + glyceraldehyde	660
Brei + dihydroxyacetone	321
Brei + dihydroxyacetone + glyceraldehyde	307
Brei + glucose + dihydroxyacetone	1209
Brei + methylglyoxal + dihydroxyacetone	589

This shows, then, (1) that glucose breakdown is not inhibited by dihydroxyacetone, (2) that dihydroxyacetone is not itself converted into lactic acid (allowance being made for small traces of methylglyoxal in the sample), (3) that dihydroxyacetone and glyceraldehyde together do not form significant amounts of lactic acid, (4) that glyoxalase is inhibited only very slightly by either glyceraldehyde or dihydroxyacetone.

It is not likely, therefore, that glucolysis proceeds according to a scheme similar to that generally accepted for phosphorylating glycolysis [see Meyerhof, 1936, 2; Parnas, 1936; Needham, D. M. 1937], only without the phosphate groups. Nor can the embryo convert dihydroxyacetone into methylglyoxal, for, if it did, a marked lactic acid production would result.

Following an obvious suggestion from the phosphorylation schemes, we tested the combined effect of dihydroxyacetone and pyruvic acid.

Exp. 208. Ice-cold 6¼-day embryo brei buffered as usual and 2 ml. added to either 12·2 mg sodium pyruvate or 12·3 mg. dihydroxyacetone, or both, by tipping after 20 min. autoglycolysis.

	μl. in 2 hr.
Brei alone	96
Brei + pyruvate	168
Brei + dihydroxyacetone	196
Brei + pyruvate + dihydroxyacetone	224

Had this been the real method of lactic acid formation in glucolysis, we could have expected well over 1000 μl. from the quantities added; instead only 100 were produced. The rise due to pyruvate alone is curious, but we had seen

this phenomenon before, in Exps. 144–146, where the following figures had been obtained manometrically:

	Q_L (intact 5-day embryo)		
	Autoglycolysis	+NaPy	Diff.
Exp. 144	+0·48	+0·70	0·22
Exp. 145	+0·60	+0·95	0·35
Exp. 146	+0·67	+0·80	0·13

This effect is easily reproducible on brei (cf. Exps. 236 and 246). It resembles the pyruvate acceleration of glycolysis seen in several adult tissues and tumours by Mendel *et al.* [1931] and by Rosenthal [1932]. It must, of course, always be remembered that pyruvate solutions, if not absolutely fresh, may contain methylglyoxal.

In order to follow further the metabolism of these possible intermediate substances, an experiment was carried out by the method of Ariyama [1928] which estimates methylglyoxal colorimetrically and, after hydrolysis to methylglyoxal, dihydroxyacetone and glyceraldehyde [Meyerhof & Lohmann, 1934]. A brei previously subjected to prolonged dialysis was chosen, in order to give opportunity for the accumulation of any intermediate products possible.

Exps. 209 and 210. 7-day embryo brei dialysed against Ringer without bicarbonate for 40 hr. at 0°. At the end of that time the glucolytic power and glyoxalase activity were manometrically tested; considerable glucolysis remained which could, however, be increased by the addition of glutathione, and both fractions abolished by glyceraldehyde. Methylglyoxalase still functional but increased by glutathione (refer to Table IV for the figures). Samples of this brei (2 ml.) were incubated with various substances at 37° for 1 hr., Ariyama estimations being made before and after this period.

	min.	Ariyama estimations direct (methylglyoxal)	Ariyama estimations after ½ hr. hydrolysis with *N* HCl in a sealed tube in boiling water-bath (methylglyoxal + dihydroxyacetone + glyceraldehyde)
Brei + glucose, 50 mg.	0	0	0
	60	0	0
Brei + glucose, 50 mg. + glyceraldehyde, 2·5	0	0	2·18
	60	0	2·32
Brei + dihydroxyacetone, 24·5 mg.	0	2·54	21·60
	60	0·98	21·48
Brei + dihydroxyacetone 12 mg. + glyceraldehyde 12 mg.	0	2·50	24·32
	60	1·11	22·96
Brei + dihydroxyacetone 24·5 mg. + glutathione 1·8 mg.	0	2·52	23·24
	60	0	24·76

From this we conclude (1) that nothing estimable by the Ariyama method accumulates during a glucolysis in which about 3 mg. of glucose disappear to acid; (2) that during the almost complete inhibition of this by glyceraldehyde, the latter remains almost unchanged [cf. Needham & Nowiński, 1937, p. 1178]; (3) that dihydroxyacetone and glyceraldehyde remain together unchanged when incubated with the brei; (4) that glutathione has no effect upon the added dihydroxyacetone; finally (5) that the sample of dihydroxyacetone used by us contained some 10% of methylglyoxal, the disappearance of which was accelerated by the simultaneous presence of glutathione. In the second line of the table, the glyceraldehyde referred to was proved to be glyceraldehyde and not dihydroxyacetone by fractional hydrolysis in sealed tubes in *N* HCl, since 65% was broken down in 10 min. and nearly 100% in 30 min. in both cases

[Meyerhof & Lohmann, 1934]. Glyceraldehyde is therefore not converted into dihydroxyacetone by the embryo.

In the preceding paragraph it was said that the *dl*-glyceraldehyde remained almost unchanged during inhibition of glucolysis. Inspection of the figures of the last experiment, however, shows that there was a loss of glyceraldehyde of approximately 10%. We believe that this small change is real and cannot be attributed wholly to an uncertainty of the experimental method. In another experiment, where particular care was taken to test the point, a diminution of 12·5% of the glyceraldehyde originally present was observed during a period of 2 hr. glucolysis during which the inhibition amounted to 88% (Exp. 243). These small diminutions were, it was found, considerably increased if substrate was absent. Thus in Exp. 241, during an hour's autoglycolysis, the glyceraldehyde fell from 2·6 to 1·4 mg. (a decrease of 46%) and this disappearance was completely abolished if 5 mg. pyruvate were present. Such a decrease of glyceraldehyde in the absence of glucose or other substrate would well account for the effect noted by Needham & Nowiński [1937, p. 1168] in which the autoglycolysis is increased about 14% by glyceraldehyde, and this shows that an acid, probably lactic acid, is formed. We shall return to this point in the discussion.

A number of experiments were made with other possible intermediates. In Exp. 221 no lactic acid was formed (manometric) when glycerol and glycerol + sodium glycerate were added to fresh embryo brei. In Exp. 245*a*, sodium gluconate formed no new acid (manometric) and no triose (Ariyama).

It could be supposed that the non-phosphorylating glucolysis followed a course parallel with that of the Embden-Meyerhof-Parnas cycles. Thus from glucose two molecules of *d*-glyceraldehyde would be formed and each of these would combine with one molecule of pyruvate and one of water to give one molecule of lactate and one of glycerate. The glycerate would yield pyruvate again and so the process would go on. This scheme, however, can hardly represent the facts, for we have found it impossible to obtain lactic acid from a mixture of glyceraldehyde and sodium pyruvate or glycerate in the presence of active embryo brei. The following is a sample experiment (Exp. 234):

	μl./hr./ml. brei of 7-day embryos
Brei alone	175
Brei + glucose	577
Brei + glyceraldehyde + pyruvate	179
Brei + glyceraldehyde + glycerate	166

Further proof that this scheme cannot account for the facts was afforded by experiments in which lactic acid was estimated chemically. Here it appeared that neither pyruvate nor glycerate formed lactic acid anaerobically when incubated with fresh embryo brei.

	mg. lactic acid formed
Exp. 250 (2 hr.):	
Brei alone	1·8
Brei + glucose	9·5
Brei + pyruvate	1·8
Brei + glycerate	1·5
Exp. 250*a* (1 hr.):	
Brei alone	1·2
Brei + glucose	2·9
Brei + glyceraldehyde	1·1
Brei + glyceraldehyde + pyruvate	1·4

Here the acid formation from glyceraldehyde was also negligible.

Discussion

It will be seen that in spite of our substantial agreement with Geiger, we are not inclined to accept the view that methylglyoxal is an intermediate in non-phosphorylating glucolysis. The accumulations of methylglyoxal found by Neuberg [lit. in Neuberg & Kobel, 1925], and recently in dog muscle by Aubel & Simon [1934], may be due to the non-enzymic conversion of triosephosphate into methylglyoxal, as suggested by Meyerhof & Kiessling [1935]. We regard the specific finding of Neuberg *et al.* [1930] of methylglyoxal formation from hexosediphosphate by acetone powders of chick embryo as certainly explicable in this way, especially as we have demonstrated the activity of aldolase (converting hexosediphosphate into triosephosphate) in the embryo.

The following possible intermediates have also been excluded by our experiments: gluconic acid, glycerol, glyceric acid, pyruvic acid and dihydroxyacetone. *dl*-Glyceraldehyde appears to form small amounts of acid, but in general its capacity to do so is very slight. Various combinations of these substances have no greater effect than the substances alone, e.g. glyceraldehyde + pyruvate does not give lactic acid.

Nothing, however, in this or the preceding papers of the series, is incompatible with the suggestion that in non-phosphorylating glucolysis glucose give first two molecules of *d*-glyceraldehyde by a movement of one hydrogen atom from carbon atom 3 to 4, and that these two molecules then produce two molecules of lactic acid by rearrangements of the hydrogen and hydroxyl groups at carbon atoms 1, 3, 4 and 6. The considerable intramolecular oxidation-reductions involved in this scheme might explain the high demand for glutathione. The suggestion would follow that the inhibiting action of *dl*-glyceraldehyde is due to the *l*-glyceraldehyde and not to both isomerides. The fact that a small disappearance of glyceraldehyde seems to occur, especially in the absence of glucose, would agree with this hypothesis, which has the further advantage of admitting of relatively easy experimental test.

At first sight the active mannolysis of the embryo would seem to militate against this view, for from mannose one molecule of *d*- and one of *l*-glyceraldehyde would be formed. However, it is thought that mannose can be converted into glucose readily enough by tissues. Neuberg & Mayer [1902] obtained much *l*-glucose in the urine of rabbits after ingestion of *l*-mannose, and it has long been known that mannose will form glycogen [Cremer, 1892; Rosenfeld, 1900]. *In vitro*, if the solution is slightly alkaline, mannose spontaneously passes into glucose.

Summary

1. In spite of the use of the most delicate methods available, coenzyme I (the cozymase of Harden & v. Euler) could not be demonstrated in the chick embryo. On the other hand, coenzyme II (the hexosemonophosphate codehydrogenase of Warburg) was found to be present in all probability throughout development and even in traces in the yolk. Phosphorus-transporting coenzyme could be demonstrated in the embryo, but in very small amounts.

2. When phosphorus transporters such as adenylic acid, adenylpyrophosphate, and cozymase are added to intact embryo or embryo brei given glycogen or hexosediphosphate, a certain breakdown of these substrates may occur, but it is always small and not always demonstrable. The addition of extra magnesium or inorganic phosphate makes no difference. If an effect is seen, it is never of long duration; the glycolysis quickly falling again.

3. Aldolase (zymohexase), which converts hexosediphosphate into triosephosphate, is present in the chick embryo.

4. The triosephosphate so formed accumulates, even when intact embryos are used. The conclusion is drawn that hexosediphosphate must penetrate the cell-walls and be transformed within into triosephosphate.

5. The failure of the embryo to carry the breakdown of hexosediphosphate further than triosephosphate cannot be due simply to lack of sufficient cozymase. It must therefore be due to lack or insufficiency of the enzyme system converting triosephosphate into phosphoglyceric acid.

6. Glycerophosphate with either pyruvate, phosphopyruvate or phosphoglycerate gives very little more lactic acid than that due to autoglycolysis.

7. All the enzymes, however, concerned in the breakdown of phosphoglyceric acid are present in the embryo; the reversible transformation of phosphoglyceric into phosphopyruvic acid, the transport of phosphorus from phosphopyruvic acid to adenylic acid with the formation of adenylpyrophosphate, and finally its dephosphorylation with the appearance of phosphagen or inorganic phosphate, were demonstrated. The Parnas reaction is certainly present.

8. The esterification of glycogen could not be demonstrated.

9. All the enzymes of the phosphorylation route which could be demonstrated in embryo at all, could be demonstrated in aqueous extracts. This is in strong contrast with the glucolytic system.

10. The preceding paragraphs must be qualified by the statement that in about 10% of our experiments hexosediphosphate was broken down to a considerable extent. This observation is in agreement with the occasional utilization of glycogen and phosphorylated hexoses reported in the first paper of this series, and with the weak effects obtained on the addition of phosphorus transporters. Glucose was always vigorously fermented whether the phosphorylation system was feebly present or not.

11. All the facts summarized above and in the previous papers of this series may be interpreted upon the hypothesis that in the chick embryo there are two separate routes of carbohydrate breakdown (i) a non-phosphorylating glucolysis mechanism, very active, and closely bound to the cell structure, and (ii) a phosphorylating mechanism closely similar to that in muscle, dealing with glycogen and hexosediphosphate, but of low activity because deficient in four distinct places (*a*) the enzyme esterifying glycogen, (*b*) the dismutase forming phosphoglycerate and lactate from triosephosphate and pyruvate, (*c*) lack of adenylpyrophosphate, (*d*) lack of cozymase. It would thus appear that, in early embryonic development, the phosphorylation machinery has not yet been fully laid down. Hence it is of interest that a not dissimilar state of affairs has been described for some tumours.

12. Under no conditions could any esterification of carbohydrate be observed during active glucolysis.

13. The glucolytic rate is not affected either by the addition of inorganic phosphate or by its almost complete removal by calcium or beryllium.

14. Nor is the glucolytic rate affected by either the addition of adenylpyrophosphate, adenylic acid or cozymase, or their removal by means of dialysis.

15. The progress of fluoride inhibition according to the concentration of fluoride varies with the system studied. At *M*/200 NaF the conversion of phosphoglyceric acid into phosphopyruvic acid is completely suppressed, while the glucolysis of the same material at the same time is only 45% suppressed. This is regarded as further strong evidence for the existence of two routes of breakdown, one phosphorylating, the other not.

16. Proof of the existence of the two routes of breakdown may be obtained by the combined use of hexokinase (the phosphorylase of yeast which phosphorylates glucose) and fluoride or *dl*-glyceraldehyde. If, by a carefully chosen fluoride concentration, the phosphorylation route is blocked but not the non-phosphorylation route, then the inhibition of glucolysis should be greater in the presence of hexokinase than in its absence, since it should shunt the glucose into the phosphorylation route, where its breakdown beyond phosphoglyceric acid should be blocked. Conversely, glyceraldehyde will block the non-phosphorylation route but not the phosphorylation route, hence in the presence of hexokinase the inhibition of glucolysis should be less than in its absence, since all the glucose should be shunted through the phosphorylation route. Yet in all cases fluoride and glyceraldehyde inhibitions are exactly the same whether hexokinase is present or not. It is therefore concluded not only that two paths of carbohydrate breakdown exist, but that glucose breakdown in the living embryo goes on wholly without phosphorylation.

17. Inhibition of glucolysis by *dl*-glyceraldehyde is partially reversed by the presence of hexosediphosphate. The triosephosphate which accumulates at the expense of the latter combines with the glyceraldehyde to form hexosemonophosphate. Glyceraldehyde may therefore be used generally as a specific inhibitor to distinguish non-phosphorylated glucolysis from that in which hexosediphosphate occurs as intermediate.

18. Attention is drawn to a correlation which seems to exist between (i) relatively high $Q_L^{N_2}$, (ii) more or less pronounced preference for glucose as substrate without addition of hexokinase, (iii) sensitivity to inhibition by *dl*-glyceraldehyde, (iv) inefficiency of the phosphorylation path of glucolysis.

19. By dialysis of embryo brei, glucolysis may be inactivated. It may be restored up to 80% by the addition of glutathione (10^{-3}–$10^{-2}M$), whether the methylglyoxalase present has been irreversibly inactivated or not. This suggests that methylglyoxal is not an intermediate in glucolysis.

20. Glutathione cannot be replaced for this purpose by cysteine, ascorbic acid, vitamin B_1 or pyruvic acid. There is no connexion between the inhibition of glucolysis by glyceraldehyde and the amount of glutathione present.

21. If glucose and methylglyoxal, in amounts sufficient to saturate the enzymes, are suddenly given together to an active embryo brei, the subsequent summation of lactic acid production which occurs is almost perfect. In so far as it can be tested by the use of synthetic methylglyoxal, methylglyoxal is not, therefore, an intermediate in glucolysis.

22. Gluconic acid, glycerol, glyceric acid, pyruvic acid and dihydroxyacetone appear also not to be intermediates in non-phosphorylating glucolysis.

23. Optically active glyceraldehyde cannot yet be excluded as an intermediate.

The thanks of the authors are due to Dr André Lwoff and Dr David Green for their very kind co-operation in the tests for coenzymes. The authors wish to express their thanks to Dr Dorothy Needham for interest and counsel, to Miss Mary Cross for certain glucose estimations, and to Miss Jean Innes, who prepared some of the cozymase used in the work. One of us (H. L.) is much indebted to Sir F. G. Hopkins for the hospitality of his laboratory and to the British Fund for German Jewry for a maintenance grant.

REFERENCES

Ariyama (1928). *J. biol. Chem.* **77**, 359.
Ashford (1933). *Biochem. J.* **27**, 903.
—— (1934). *Biochem. J.* **28**, 2229.
—— & Holmes (1929). *Biochem. J.* **23**, 748.
—— —— (1931). *Biochem. J.* **25**, 2028.
Aubel & Simon (1934). *C.R. Soc. Biol., Paris*, **114**, 905; **115**, 373, 1178.
Baldwin & Needham (1933). *J. exp. Biol.* **10**, 105.
Bierich & Rosenbohm (1935). *Hoppe-Seyl. Z.* **231**, 47.
Boyland & Boyland (1935). *Biochem. J.* **29**, 1910.
—— & Mawson (1934). *Biochem. J.* **28**, 1409.
Brody (1927). *J. gen. Physiol.* **10**, 637.
Caló (1935). *Acta Cancrologia, Budapest*, **1**, 1.
Cremer (1892). *Z. Biol.* **29**, 484.
Dickens & Šimer (1929). *Biochem. J.* **23**, 936.
v. Euler & Adler (1934). *Hoppe-Seyl. Z.* **226**, 195.
—— —— (1935). *Hoppe-Seyl. Z.* **235**, 122.
—— & Johannsson (1928). *Hoppe-Seyl. Z.* **178**, 209.
—— Günther & Vestin (1936). *Hoppe-Seyl. Z.* **240**, 265.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Friedemann, Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
Gaddie & Stewart (1934). *J. Physiol.* **80**, 457.
Geiger (1935). *Biochem. J.* **29**, 811.
Giršavičius (1933). *Biochem. Z.* **260**, 278.
—— & Heyfetz (1936). *Biochemia, Moscow*, **1**, 525.
Green (1936). *Biochem. J.* **30**, 2095.
Haarmann (1932). *Biochem. Z.* **255**, 103, 142, 203; **256**, 326.
Holmes (1933). *Biochem. J.* **27**, 523.
—— (1934). *Ann. Rev. Biochem.* **3**, 395.
Kay & Skill (1934). *Biochem. J.* **28**, 1222.
Kraut & Bumm (1928). *Hoppe-Seyl. Z.* **177**, 125.
Krijgsman (1936). *Z. vergl. Physiol.* **23**, 664.
Lehmann (1935, 1). *Biochem. Z.* **277**, 261.
—— (1935, 2). *Biochem. Z.* **281**, 271.
—— & Needham (1937). *J. exp. Biol.* (in the Press).
Loebel (1925). *Biochem. Z.* **161**, 219.
Lohmann (1928). *Biochem. Z.* **194**, 306.
—— (1931). *Biochem. Z.* **236**, 444.
—— (1932). *Biochem. Z.* **254**, 332.
—— (1936). *Biochem. Z.* **286**, 28.
Lwoff, A. & Lwoff, M. (1936). *C.R. Acad. Sci., Paris*, **203**, 520, 896.
—— —— (1937). *Proc. roy. Soc.* B, **122**, 352, 360.
Mawson (1936). *Biochem. J.* **30**, 1592.
Mazza & Malaguzzi-Valeri (1935). *Boll. Soc. ital. Biol. sper.* **10**, 722.
Mendel (1929). *Klin. Wschr.* **8**, 169.
—— Bauch & Strelitz (1931). *Klin. Wschr.* **10**, 118.
Meyerhof (1927). *Biochem. Z.* **178**, 395, 462; **183**, 186.
—— (1932). *Biochem. Z.* **246**, 249.
—— (1935). *Biochem. Z.* **277**, 77.
—— (1936, 1). *Naturwissenschaften*, **23**, 850.
—— (1936, 2). *Curr. Sci.* **4**, 669.
—— & Kiessling (1936). *Biochem. Z.* **283**, 83.
—— & Lohmann (1934). *Biochem. Z.* **271**, 89.
—— Lohmann & Schuster (1936). *Biochem. Z.* **286**, 319.

Meyerhof & Ohlmeyer (1937). *Naturwissenschaften*, **24**, 741.
Müller (1933). *Biochem. Z.* **262**, 240.
Myrbäck (1933). *Ergebn. Enzymforsch.* **2**, 139.
Needham, D. M. (1936). *C.R. Acad. Sci., Paris*, **203**, 615.
—— (1937). *Ann. Rev. Biochem.* **6**, 395
—— & v. Heyningen (1935). *Biochem. J.* **29**, 2040.
Needham, J. (1931). Chemical Embryology. (Camb. Univ. Press.)
—— (1932). *Proc. roy. Soc.* B, **112**, 114.
—— & Nowiński (1937). *Biochem. J.* **31**, 1165.
—— —— Dixon & Cook (1937). *Biochem. J.* **31**, 1185.
Neuberg & Kobel (1925). *Z. angew. Chem.* **38**, 761.
—— —— & Laser (1930). *Z. Krebsforsch.* **32**, 92.
—— & Mayer (1902). *Hoppe-Seyl. Z.* **37**, 530.
Nilsson & Alm (1936). *Biochem. Z.* **286**, 254, 273.
Nord, Dammann & Hofstetter (1936). *Biochem. Z.* **285**, 241.
Ostern, Baranowski & Reis (1935). *Biochem. Z.* **279**, 85.
Parnas (1936). *Bull. Soc. chim. biol., Paris*, **18**, 53.
—— & Lutwak-Mann (1935). *Biochem. Z.* **278**, 11.
Reiner, Smythe & Pedlow (1936). *J. biol. Chem.* **113**, 75.
Rosenfeld (1900). *Zbl. inn. Med.* **7**, 1.
Rosenthal (1932). *Z. Krebsforsch.* **38**, 216.
Scharles, Baker & Salter (1935, 1). *Biochem. J.* **29**, 1927.
—— —— —— (1935, 2). *Amer. J. Cancer*, **25**, 122.
Schönfelder (1935). *Arch. exp. Path. Pharmak.* **180**, 24.
Sym, Nilsson & v. Euler (1930). *Hoppe-Seyl. Z.* **190**, 228.
Takasaka (1927). *Biochem. Z.* **184**, 390.
Takahashi (1935). *J. Biochem., Tokyo*, **22**, 303.
Tsuzuki (1936). *J. Biochem., Tokyo*, **23**, 421.
Vestin (1936). *Hoppe-Seyl. Z.* **240**, 99.
Warburg & Christian (1931). *Biochem. Z.* **242**, 206.
Waterman (1925). *Brit. J. exp. Path.* **6**, 300.

Note added 2 May 1937. While this paper was in the press two communications appeared recording the activation of glucose breakdown in the absence of hexokinase by adenylic acid and cozymase in tumour [Boyland, Boyland & Greville, *Biochem. J.* 1937, **31**, 461], and in brain [Adler, Calvet, v. Euler & Gunther, *Naturwissenschaften*, 1937, **25**, 282]. Although we ourselves only bring forward evidence that the glucose breakdown in embryo cannot go through the phosphorylation route, we suggest that the glucose breakdown in brain and tumour cannot to any great extent go that way either. The fact that in tumour and brain the oxidoreduction (dismutation) between pyruvic acid and triose-phosphate is found by these investigators to be unimpaired makes the elucidation of glucose breakdown naturally more difficult than in embryo where the general defect of phosphorylation facilitates the demonstration of glucolysis proper. The fact that glyceraldehyde inhibits glucolysis in brain and tumour clearly shows that during glucose breakdown (cf. p. 1236) no hexosediphosphate can occur, since otherwise the glyceraldehyde would be removed by the dihydroxyacetone-phosphate formed from the former. In connexion with this we would refer to control experiments where in muscle extract with hexokinase the phosphorylated glucose breakdown is not inhibited with glyceraldehyde but rather slightly increased.

In Table III of the paper of Boyland, Boyland & Greville 1 ml. extract of tumour produced per g. tissue per hr. from 10 mg. glucose, without adenylic

acid 5·0 mg. lactic acid, with adenylic acid, 5·8 mg.; from 10 mg. glycogen, without adenylic acid, nil, with adenylic acid, 6·0 mg. This seems to us to confirm our findings on the embryo that adenylic acid is without influence upon glucolysis proper. The transient manometric effects of cozymase described in the same paper could surely be due to glycogen originally present in the extracts.

The experiments of the Stockholm investigators suffer from the extreme inactivity of their acetone powders of brain extracts. This is even lower than their glucose breakdown without hexokinase in muscle extract, although brain is a strongly glucolysing tissue. The experimental figures do not yet therefore allow of the conclusion that even in brain the glucose breakdown goes through phosphorylation. In 2 hr. 3 ml. of their enzyme preparation + 1 ml. substrate used 0·47 mg. of 15 mg. glucose added, yet the blank without glucose showed 0·57 mg. lactic acid formation. Nor can we regard their statement that glyceraldehyde has no effect on glucolysis in muscle extract as substantiated. In their experiment with muscle extract the effects of cozymase and adenylic acid on lactic acid formation in the presence of glucose (2·45 mg. lactic acid; blank 1·22 mg.; blank + glucose alone 1·38 mg.) might equally well have been obtained in the absence of glucose, since the high blank suggests a high content of glycogen or phosphoric esters in the enzyme preparation. An influence of glyceraldehyde would not therefore be expected. As we have already said (p. 1236), the effect of glyceraldehyde on glucolysis can only be shown if phosphorylated formation of lactic acid is not going on at the same time, as in this case the high blank suggests that it is.