CCXXXIV. INTERMEDIARY CARBOHYDRATE METABOLISM IN EMBRYONIC LIFE

VIII. GLYCERALDEHYDE AND GLUCOLYSIS

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(*Received 31 July 1937*)

MENDEL [1929] made the important discovery that the glucolysis of tumour tissue was very strongly inhibited by *dl*-glyceraldehyde. At a concentration of 10^{-3} *M* it was already maximum. Later it was found [Mendel *et al.* 1931] that this inhibition could be reversed by small amounts of pyruvate, and other investigators reported it for various other tissues such as brain. Since then glyceraldehydephosphate, first synthesized by Fischer & Baer [1932], was found by Embden *et al.* [1933] to give lactic acid readily in the presence of muscle enzymes, and we know now that it occupies an important position in the cycles of phosphorylating fermentation. Hence it is rather remarkable that hitherto there has been no further study of the inhibition of glucolysis by glyceraldehyde itself.

That *dl*-glyceraldehyde strongly inhibits the glucolysis of the chick embryo has already been shown in the first paper of this series [Needham & Nowiński, 1937]. It was noticed at the same time, however, that the inhibition was never quite complete (av. 93%), and that in the presence of glyceraldehyde the autoglycolysis was raised (from 10 to 23%). The meaning of this was not immediately obvious since other agents, such as fluoride, can considerably raise the autoglycolysis, but in a later work [Needham & Lehmann, 1937] it was found that intact embryo or embryo brei can cause glyceraldehyde (estimated directly by the method of Ariyama [1928]) to disappear. If glucolysis was allowed to proceed simultaneously some 12% of the added quantity disappeared but in the absence of glucolysis a disappearance of as much as 46% was recorded. In this paper it was also shown that the glyceraldehyde inhibition can be reversed by hexosediphosphate, since the triosephosphate formed by the aldolase removes the glyceraldehyde according to the reaction of Meyerhof *et al.* [1936] forming hexosemonophosphate. It was pointed out that glyceraldehyde must thus be regarded as a specific inhibitor for non-phosphorylating glucolysis, since if phosphorylating fermentation were taking place at the same time its effect could not be apparent.

The object of the present work, therefore, was to elucidate the mechanism of the *dl*-glyceraldehyde inhibition and to determine whether and in what way glyceraldehyde takes part in non-phosphorylating glucolysis.

GLYCERALDEHYDE USED

The *dl*-glyceraldehyde used was the Schering-Kahlbaum product.

A sample of optically active glyceraldehyde was very kindly placed at our disposal by Prof. H. O. L. Fischer and Dr F. E. Baer. It was prepared from dihydroxyacetone-mannitol according to the method of Fischer & Baer [1936].

The substance was sent to us in concentrated solution from Basel by aeroplane, and was in our hands 48 hr. after its preparation. The solution contained 101 mg. glyceraldehyde per ml. and had an optical rotation of $[\alpha]_D^{20°} + 9{\cdot}9°$. In the course of the following 10 days this value fell slowly to $[\alpha]_D^{20°} + 8{\cdot}0°$, after which it remained nearly constant. The purest preparations of Fischer & Baer's *d*-glyceraldehyde had $[\alpha]_D^{20°} + 14{\cdot}0°$. The question therefore arose whether the loss of rotation was due to polymerization or racemization. The fact that the number of reducing groups remained constant and that the amounts of dihydroxyacetone and methylglyoxal present were nil and negligible respectively showed that the cause of the falling optical activity was a slow racemization.

Solution tested	mg. methylglyoxal by the Ariyama method
Blank	0
30 mg. optically active glyceraldehyde 16 days old	0·125 (0·4%)
30 mg. optically inactive glyceraldehyde 3 days old	0

Solution tested	mg. calculated from reducing power (Hagedorn-Jensen method)
0·05 mg. optically active glyceraldehyde	0·05
0·05 mg. *dl*-glyceraldehyde	0·045
0·10 mg. glucose	0·10
0·10 mg. optically active glyceraldehyde	0·088
0·10 mg. *dl*-glyceraldehyde	0·088
0·20 mg. glucose	0·21
0·15 mg. optically active glyceraldehyde	0·13
0·15 mg. *dl*-glyceraldehyde	0·135
0·30 mg. glucose	0·29

The optically inactive glyceraldehyde solution and the glucose solution were 1 day old. The optically active glyceraldehyde solution was 14 days old. The rotation of the optically active solution was $[\alpha]_D^{20°} + 8{\cdot}0°$, i.e. 57% of the maximum value.

To exclude any possible hydrolysis of polymerized glyceraldehyde which could have occurred during the boiling in the Hagedorn-Jensen method, we checked the reducing power further by the method of Macleod & Robison [1929] which is carried out at room temperature, estimates only aldose groups and thus allows of the exclusion of dihydroxyacetone as impurity.

Solution tested	mg. calculated from reducing power (Macleod-Robison method)
0·5 mg. optically active glyceraldehyde	0·50
0·5 mg. *dl*-glyceraldehyde	0·50
1·5 mg. glucose	1·50

These control experiments show that dihydroxyacetone was not present as impurity, that methylglyoxal was present only to a negligible extent, and that polymerization was not taking place. The fall of optical activity was therefore due to racemization and the daily determination of $[\alpha]_D^{20°}$ informed us of the exact amount of *d*- and *l*-isomerides in our optically active solution at any given time. The percentage of *d*-glyceraldehyde in our solution, if $[\alpha]_D^{20°}$ be x, was therefore

$$50\left(1+\frac{x}{14}\right),$$

since the maximum rotation is $+14{\cdot}0°$. Thus when the rotation was $+8{\cdot}0°$ the amount of *d*-glyceraldehyde was 78·5% and that of *l*-glyceraldehyde was 21·5%.

Relative activities of the stereoisomerides; *l*-glyceraldehyde as the inhibitor

On adding optically active glyceraldehyde to intact embryos or embryo brei in the presence of glucose it was immediately seen that inhibition took place. But in order to examine it closely it was necessary to construct curves showing the effect at various concentrations. These experiments, which were all made

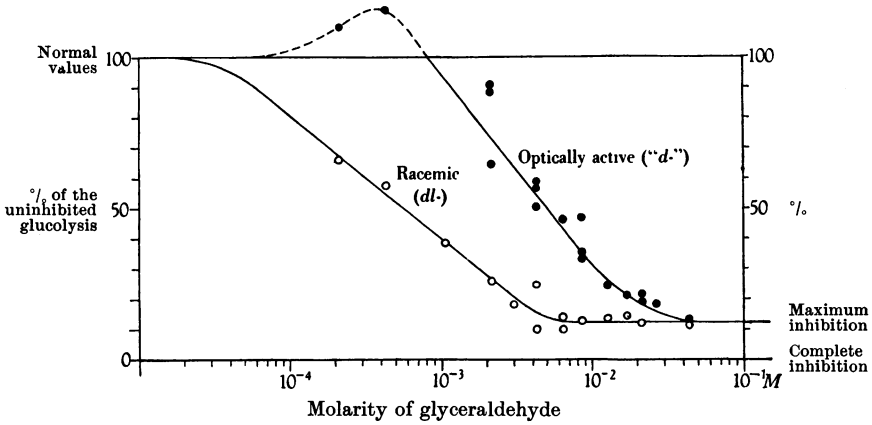

Fig. 1. Glyceraldehyde inhibition of embryonic glucolysis.

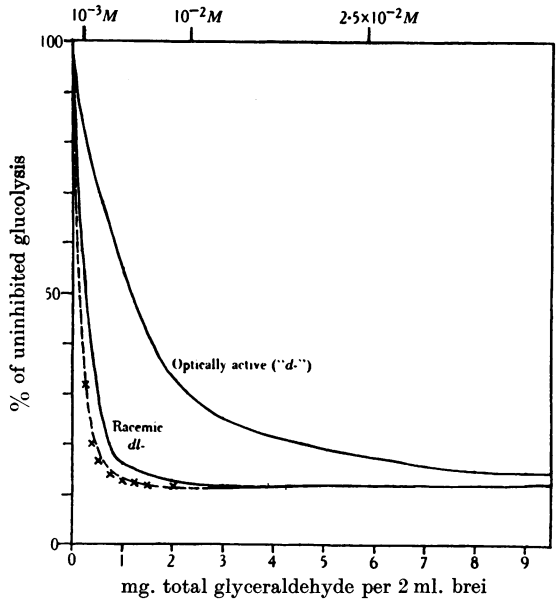

Fig. 2. Demonstration that *l*-glyceraldehyde is the inhibitor of glucolysis. The broken line is drawn for *l*-glyceraldehyde by calculation from the known composition of the optically active mixture. × = ½ the *dl*-values.

with 4½-day intact embryos, and lasted for a standard time (3 hr.), are graphically summarized in Fig. 1. The percentages of the $Q_L^{N_2}$ without inhibitor, at the various

concentrations, are plotted on an arithlog scale ranging from 10^{-5} to 10^{-1} *M*. It can at once be seen that the racemic optically inactive glyceraldehyde was a much more efficient inhibitor of embryonic glucolysis than the optically active glyceraldehyde. For example, at a concentration of $2 \cdot 10^{-3} M$ the former inhibited the glucolysis 75 % while the latter only inhibited it 25 %. Similarly, the inhibition by the former became maximum at $4 \cdot 10^{-3} M$, that by the latter at $4 \cdot 10^{-2} M$. Thus the inhibition was most powerful where most *l*-glyceraldehyde was present. The only simple explanation of the facts was that *l*-glyceraldehyde inhibits glucolysis and *d*-glyceraldehyde not at all.

This could be demonstrated in the following way. If the glyceraldehyde inhibition was always proportional not to the total amount of glyceraldehyde added but to the amount of *l*-glyceraldehyde added, then knowing the amount of *l*-glyceraldehyde in the optically active solution used each day it should be possible to calculate the theoretical inhibition curve for a solution of pure *l*-glyceraldehyde, and this should be identical with a curve showing at every point the inhibition attained with double the amount of *dl*-glyceraldehyde. As Fig. 2 shows, this is just what happens. For example, a solution of *dl*-glyceraldehyde (containing 50 % *l*-glyceraldehyde) should be up to 2·3 times more powerful than a solution of *d*-glyceraldehyde of rotation $[\alpha]_D^{20^\circ} + 8 \cdot 0^\circ$ (containing only 21·5 % *l*-glyceraldehyde).

It appears, therefore, that *l*-glyceraldehyde must be regarded as solely responsible for the phenomenon of glyceraldehyde inhibition.

The formation of lactic acid from glyceraldehyde

Inhibition of glucolysis with glyceraldehyde has always been reported as incomplete. At the highest concentrations used by Mendel [1929] in the case of tumour, it was not above 90 %. Fig. 1 of the present paper demonstrates the same phenomenon; maximum inhibition of embryonic glucolysis is just under 90 %. We now found that if the concentration of glyceraldehyde which produces maximum inhibition of glucolysis is present with embryo enzymes alone, the same amount of lactic acid is formed as when glucose is present too. The apparently incomplete inhibition of lactic acid formation from glucose in the presence of glyceraldehyde is therefore due to lactic acid formation from glyceraldehyde itself. Thus the 100 % inhibition of glucolysis is masked.

Exp. 259a. Fresh brei from 7-day embryos; 2·0 ml. brei per manometric cup + 0·6 ml. substrate; time 1 hr. 45 min. at 37°. Bicarbonate etc. as in previous papers.

	μl. CO_2 formation	μl. CO_2 from substrate
Brei alone	61	—
Brei + 10 mg. glucose	730	669
Brei + 10 mg. glucose + 30 mg. glyceraldehyde	248	187
Brei + 30 mg. glyceraldehyde	285	224
Brei + 2·3 mg. pyruvic acid as pyruvate	90	29
Brei + 30 mg. glyceraldehyde + 2·3 mg. pyruvic acid as pyruvate	295	234

N.B. Glyceraldehyde here used contained 79 % *d*- and 21 % *l*-forms.

This experiment shows that glyceraldehyde itself produces acid, whether glucose is present (and powerfully inhibited) or not. That this formation of carbon dioxide was due to real acid production was shown by control experiments in which glyceraldehyde was added to embryo brei in the manometer to which no bicarbonate had been added.

The velocity of the acid formation is shown in Fig. 3. It can be seen that glyceraldehyde and glyceraldehyde + glucose have identical kinetics of acid development. That added pyruvic acid cannot increase the slow acid formation

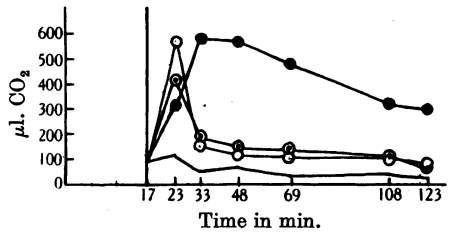

Fig. 3. (Exp. 259 *a*.) Velocity of acid formation µl./hr./2 ml. brei.
○ Glyceraldehyde alone. ● Glucose alone.
⊙ Glyceraldehyde + glucose. — Autoglycolysis.

from glyceraldehyde indicates that a dismutation such as that observed in the phosphorylation scheme between glyceraldehydephosphate and pyruvate is unlikely to be responsible. It may be recalled that this reaction was one of those parts of the phosphorylation machinery which was found not to be fully laid down in the embryo [Needham & Lehmann, 1937].

The production of lactic acid from glyceraldehyde was further checked by lactic acid estimations according to Friedemann *et al.* [1927]. In the following experiment a satisfactory correspondence was attained between the amounts of lactic acid calculated from the manometer readings and chemically measured.

Exp. 258. In each cup 2·0 ml. brei, equivalent to half one 7-day embryo. Bicarbonate and gas mixture as usual; time 4 hr. Glyceraldehyde contained 79 % *d*-, 21 % *l*-forms.

mg. glyceraldehyde added to brei	µl. CO_2 formed	µl. CO_2 formed from substrate*	Lactic acid, mg. Calc.	Found	Found, as from substrate
0	134	—	—	0·4	—
10	362	203	0·7	0·8	0·4
20	412	227	0·8	0·9	0·5
30	529	318	1·3	1·7	1·3

* Including tipping correction.

In this last experiment it was striking that no saturation of the enzyme was reached, 30 mg. glyceraldehyde giving more gas output than 20, 20 than 10. But control experiments with Ringer and boiled embryo brei alone gave no acid formation from glyceraldehyde. The observed lactic acid formation therefore involves a true enzymic process.

Since glutathione is the coenzyme of lactic acid formation from methylglyoxal, and from glucose if broken down without phosphorylation, the question arose whether lactic acid formation from glyceraldehyde needed glutathione as

Exp. 259 b. Brei from 7-day embryos dialysed 14 hr. at 0° against tap water, the water being changed several times. Glyceraldehyde 79 % *d*-, 21 % *l*-forms.

	µl. gas output in 1 hr. 40 min.
Brei alone	5
Brei + 30 mg. glyceraldehyde	134
Brei + 30 mg. glyceraldehyde + $6{\cdot}5 \times 10^{-3}$ glutathione	407

coenzyme. Experiments with dialysed brei in which the cell integrity had previously been destroyed by grinding in distilled water, showed that this was in fact the case (Exp. 259 *b*).

Another experiment, in which the quantity of glutathione used was varied, follows:

Exp. 260. Brei from 7-day embryos dialysed 18 hr. at 0° against distilled water. Glyceraldehyde as before.

	μl. gas output per hr.
Brei alone	0
Brei + 30 mg. glyceraldehyde	34·5
Brei + 30 mg. glyceraldehyde + $6{\cdot}5 \times 10^{-4}$ glutathione	72·5
Brei + 30 mg. glyceraldehyde + $1{\cdot}3 \times 10^{-3}$ glutathione	94
Brei + 30 mg. glyceraldehyde + $6{\cdot}5 \times 10^{-3}$ glutathione	180

Is glyceraldehyde an intermediate in glucolysis?

Since glyceraldehyde gave lactic acid in a true enzymic process, quantitatively dependent upon glutathione as coenzyme, the question arose whether glyceraldehyde might be regarded as an intermediate in glucolysis, or whether alternatively either glucose itself or methylglyoxal might be regarded as intermediates in lactic acid formation from glyceraldehyde. As lactic acid formation from glyceraldehyde is much slower than from glucose or methylglyoxal, it might be thought that one of the latter alternatives was the more probable. Nevertheless we investigated both possibilities. The relative slowness and incompleteness of lactic acid formation from glyceraldehyde might be due to the lack of some linkage or reaction partner.

An experiment may first be given in which competition between glucose and glyceraldehyde was tried. It was impossible to make such an experiment entirely satisfactory since although the enzyme could easily be saturated with glucose, this was not the case, as has already been shown, for glyceraldehyde. More lactic acid was formed the more glyceraldehyde was present, up to 30 mg. per cup (2·6 ml.). Moreover, since our glyceraldehyde contained 21 % of the *l*-form, which inhibits glucolysis, an amount of glyceraldehyde had to be chosen which did not contain enough of the *l*-form to inhibit entirely the glucolysis.

Exp. 269. 2 ml. 7-day embryo brei, plus 0·6 ml. substrate per cup.

	μl. CO_2 formed from substrate		
	0–60 min.	60–150 min.	Whole period
I. Brei + 0·8 *d*-, 0·2 *l*- mg. glyceraldehyde	35	22	58
II. Brei + 5 mg. glucose + 0·8 *d*-, 0·2 *l*- mg. glyceraldehyde	120	94	215
III. Brei + 5 mg. glucose + 0·2 *d*-, 0·2 *l*- mg. glyceraldehyde	83	54	136
IV. Brei + 5 mg. glucose	160	126	286
III + I	118	76	194
II	120	94	215

Thus III gives the amount of gas ouput from glucolysis in the presence of 0·2 mg. *l*- and 0·2 mg. *d*-glyceraldehyde. As against IV an inhibition of 25 % is observed. If now the concentration of *l*-glyceraldehyde is maintained as before ($8{\cdot}5 \times 10^{-4} M$) while a further 0·6 mg. *d*-glyceraldehyde is added, making the total amount of *d*- 0·8 mg., and the result compared with the gas output shown by brei in the presence of 0·2 *l*- and 0·8 *d*-glyceraldehyde alone, then if the paths

of lactic acid production from glucose and glyceraldehyde are different, III + I should be equal to II. This is seen to be actually the case.

These results give suggestive evidence against the participation of the glyceraldehyde → lactic acid reaction in glucolysis, but rather more conclusive experiments are available.

We used many of the possible "Abfangsmittel" or trapping substances known in the literature as combining with aldehydes, in order to fix glyceraldehyde during the process of glucolysis, but never with success. Nor did we ever find that glyceraldehyde accumulated (Ariyama method) during periods of glucolysis strongly inhibited by iodoacetate or fluoride. We wish to record here only our experiments with dihydroxyacetonephosphate as the fixing agent since this is the most physiological. The enzyme aldolase [Meyerhof *et al.* 1936] which has been shown to be present in embryo [Needham & Lehmann, 1937] combines dihydroxyacetonephosphate with aldehydes. It thus forms dihydroxyacetonephosphate plus glyceraldehydephosphate (the two triosephosphates) from fructosediphosphate, or combines dihydroxyacetonephosphate with glyceraldehyde to hexosemonophosphate. Both these processes have been shown to exist in the embryo. If therefore glyceraldehyde were formed during vigorous glucolysis, and hexosediphosphate (forming triosephosphate) were added to the embryo at the same time, this glyceraldehyde should be partially removed and a concurrent loss of triosephosphate and gain of hexosemonophosphate should be observed.

The following experiment was designed to test the matter.

Exp. 268. 2 ml. brei from 6-day-old embryos + 2 ml. *M*/20 acetate buffer at *p*H 6·8, maintained 3 hr. in evacuated Thunberg tubes at 37°.

I. Brei + 2·8 mg. hexosediphosphate P with 0·2 mg. inorganic P.
II. Brei + 20 mg. glucose.
III. Brei + 20 mg. glucose + 2·8 mg. hexosediphosphate P with 0·2 mg. inorganic P.

	Inorganic P mg.	P after alkali hydrol. mg.	Triose P mg.	P after 180 min. hydrol. mg.	HdP P mg.
I	1·07	1·66	0·59	3·81	2·15
II	0·8	0·8	0	—	—
III	0·07	1·66	0·59	3·81	2·15

There is therefore not the slightest difference in the distribution of triosephosphate, whether glucose is present or not.

A second experiment may be adduced in which fluoride was added on account of the possibility that, just as in phosphorylated breakdown, fluoride blockage occurs after the formation of two triosephosphate molecules from one of hexosediphosphate, so here it might occur after the formation of two triose molecules from one of glucose. Possibly glyceraldehyde might have been formed from glucose but might have suffered so quick a removal to lactic acid that no opportunity would arise for a reaction with triosephosphate. We did not consider this very probable since in 3 hr. even a slow process ought, according to the law of mass action, to have manifested itself. The details are shown in Exp. 277 on p. 1920.

Thus the addition of 10 mg. glyceraldehyde (VIII) diminishes the triosephosphate formed from the hexosediphosphate by more than 50%; proof that the reaction proceeds readily in our conditions. Yet on the addition of 10 mg. glucose (VI) the triosephosphate level is exactly the same as in the absence of

Exp. 277. Three 6-day, three 7-day and one 8-day embryos were ground to a brei with distilled water (15 ml.). Samples contained 1 ml. brei, 2 ml. substrate, *M*/100 phosphate buffer at *p*H 7·2, and *M*/100 fluoride. Left for 1 hr. in evacuated Thunberg tubes at 37°.

	Time min.	Additions	Inorganic P mg.	Alkali-labile + inorganic P mg.	Increase in alkali-labile (triose-phosphate) P mg.
I	0	Nil	1·25	1·25	—
II	0	HdP	1·42	1·41	—
III	60	Nil	1·24	1·23	—
IV	60	HdP	1·49	1·95	+0·56
V	60	Glucose	1·27	1·27	0
VI	60	Glucose + HdP	1·52	2·07	+0·55
VII	60	Glyceraldehyde	1·17	1·26	+0·09
VIII	60	Glyceraldehyde + HdP	1·49	1·71	+0·22

Amounts added: hexosediphosphate P 2·8 mg. with 0·2 mg. inorganic P, glucose 10 mg., glyceraldehyde 8 mg. *d*-, 2 mg. *l*-forms.

any glycolysable substrate (IV). We regard this very sensitive enzymic test for glyceraldehyde as particularly convincing evidence that during glucolysis no stable glyceraldehyde, indeed no aldehyde of any kind, is formed.

We may here remark that the phosphoric ester deriving from this reaction between triosephosphate and glyceraldehyde was apparently not the easily hydrolysable Neuberg ester, but that 66% of the P lost from the alkali-labile fraction were found in the fraction (total P by incineration − P hydrolysed in 180 min. in *N* HCl), just as would be expected if the Robison ester had been formed.

Is glucose an intermediate in glyceraldehyde fermentation?

The next question which arose was whether the slow enzymic breakdown of glyceraldehyde was due to a corresponding slow condensation of two molecules to one of glucose, similar to the well-known condensations of trioses to hexoses *in vitro* described by Emil Fischer [Fischer & Tafel, 1887; 1890]. For the fermentation of the ketotriose dihydroxyacetone by *Saccharomyces Ludwigii*, it was shown [Lehmann, 1935] that both hexosemonophosphate and hexosediphosphate appeared before the dihydroxyacetonephosphate was formed, thus indicating that a primary polymerization of dihydroxyacetone is necessary. In this case the lactic acid formation from glyceraldehyde ought to be inhibited by the same poisons and to the same degree as glucolysis itself. In the earlier communications of this series [Needham & Lehmann, 1937], it has been pointed out that glucolysis is inhibited by fluoride, but at a concentration different from that which stops the formation of phosphopyruvic from phosphoglyceric acid, an essential step in the phosphorylation scheme. At $5 \times 10^{-3} M$ the latter is entirely inhibited; the former only about 45%. Hitherto the inhibition of embryonic glucolysis proper by iodoacetate has not been studied (although some work has been done on its effect on embryonic respiration [Needham, 1932]). Since the reaction in the phosphorylation scheme affected by iodoacetate (the dismutation between triosephosphate and pyruvate) is precisely one of those most feeble or quite lacking in the embryo, we cannot compare the effects of iodoacetate upon phosphorylation and upon glucolysis proper in the embryo itself. But we may mention that the glucolysis of the embryo is a good deal more sensitive to iodoacetate than the phosphorylating glucolysis of muscle extract. In the case of the former, complete inhibition may be attained with *M*/20,000 iodoacetate; for the latter *M*/2000 is necessary.

Exp. 263. Brei of 8-day embryos. In each manometric cup 2 ml. of brei and 0·6 ml. substrate and additions. Iodoacetate (IAA).

	Gas output per hr. μl.
Brei alone	62
Brei + 16 mg. glucose	520
Brei + 16 mg. glucose + 2×10^{-3} *M* IAA	13
Brei + 16 mg. glucose + 1×10^{-3} *M* IAA	15
Brei + 16 mg. glucose + 5×10^{-4} *M* IAA	21
Brei + 16 mg. glucose + 2×10^{-4} *M* IAA	18
Brei + 16 mg. glucose + 1×10^{-4} *M* IAA	51

It may be remarked that this effect of iodoacetate on glucolysis can hardly be due to combination with the —SH groups of the molecules of glutathione (though this has been shown to be a necessary coenzyme of glucolysis), partly because it shows itself at such low concentrations, and partly because it was not possible to reverse it by adding glutathione, in whatever amount.

On the other hand the formation of lactic acid from glyceraldehyde was extremely insensitive to iodoacetate. Details of an experiment follow.

Exp. 265. Brei of 7-day embryos. In each manometric cup 2 ml. of brei and 0·6 ml. of substrate and additions. Glyceraldehyde (24 mg. *d*-, 6 mg. *l*-forms) in all cups.

	Gas output per hr. μl.	
	Alone	In the presence of $6{\cdot}5 \times 10^{-3}$ *M* glutathione
Brei alone	61	108
Brei alone + 4×10^{-3} *M* IAA	44	102
Brei alone + 2×10^{-3} *M* IAA	59	119
Brei alone + 1×10^{-3} *M* IAA	74	112
Brei alone + 1×10^{-4} *M* IAA	57	118

It was noteworthy that the very slight inhibition obtained with *M*/250 iodoacetate was completely reversed by adding glutathione. Moreover, the formation of lactic acid from glyceraldehyde was increased by the addition of glutathione even in this undialysed brei.

Another experiment was carried out to test the sensitivity to fluoride of lactic acid formation from glyceraldehyde. The action of fluoride as an inhibitor of glucolysis has already been discussed. There was not the slightest effect of *M*/50 fluoride on glyceraldehyde fermentation.

Exp. 279a. Brei of 7-day embryos. Glyceraldehyde 20 mg. (80% *d*-, 20% *l*-) added to each cup.

	Gas output per hr. μl.
Brei alone	37
Brei + glyceraldehyde	118
Brei + glyceraldehyde + *M*/50 fluoride	138

The increased gas output in the presence of fluoride may be referred to the property which this substance has of increasing the autoglycolysis [Needham & Nowiński, 1937]. In sum, it would appear that condensation of glyceraldehyde to glucose followed by glucolysis cannot be the process involved in the fermentation of glyceraldehyde.

IS METHYLGLYOXAL AN INTERMEDIATE IN GLYCERALDEHYDE FERMENTATION?

The feeble inhibition of glyceraldehyde fermentation by iodoacetate and its reversibility by glutathione called to mind the action of iodoacetate on methylglyoxalase. We therefore investigated the possibility that glyceraldehyde might be converted into lactic acid by way of methylglyoxal.

We first found that if glyceraldehyde was added to an enzyme system saturated with methylglyoxal, no additional lactic acid production was observed.

Exp. 279b. Brei of 8-day embryos; 2 ml. brei and 0·6 ml. additions in each manometric cup. 20 mg. glyceraldehyde contained 16 mg. *d*- and 4 mg. *l*-forms.

	Gas output in 25 min. μl.
Brei alone	24
Brei + 2 mg. methylglyoxal (*M*/100)	182
Brei + 16 mg. glyceraldehyde	87
Brei + 2 mg. methylglyoxal + 16 mg. glyceraldehyde	192

The velocity curves of this experiment are reproduced in Fig. 4. It may be seen that although at the beginning the velocity of the glyceraldehyde → lactic acid

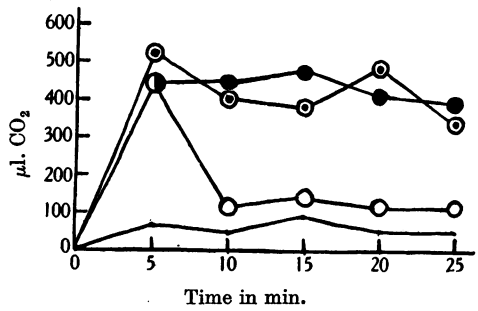

Fig. 4. (Exp. 279*b*.) Velocity of acid-formation μl./hr./2 ml. brei.

○ Glyceraldehyde (16 mg. *d*-, 4 mg. *l*-forms) alone. ● Methylglyoxal alone (2 mg.).
⊙ Glyceraldehyde + methylglyoxal. — Autoglycolysis.

reaction is just as high as the methylglyoxal → lactic acid (saturated) reaction, no summation of both reactions occurred. This strongly indicates that methylglyoxal is an intermediate in glyceraldehyde fermentation by the embryo.

The next step was to obtain direct evidence as to whether, and under what conditions, methylglyoxal is formed from glyceraldehyde. We were able to show that when glyceraldehyde is shaken with embryo brei at 37° a substance is formed which gives a strong colour reaction in Ariyama's method for methylglyoxal estimation.

Exp. 279c. 20 mg. glyceraldehyde (16 mg. *d*-, 4 mg. *l*-forms) in 2·6 ml. water or brei of 7-day embryos, shaken for 1 hr. at 37°.

	Methylglyoxal (Ariyama) mg.
Glyceraldehyde, initial	0·08
Glyceraldehyde in Ringer-bicarbonate	0·85
Glyceraldehyde + 2 ml. brei	0·44

But the substance was formed in the same or, as here, even greater amount, in the absence of the enzyme systems, simply by shaking at 37°, because the

methylglyoxal was not removed by glyoxalase to lactic acid. Since the trichloroacetic acid extract gave typical iodoform crystals after treatment with alkaline iodine solution, it contained the $CH_3.CO$ group. Since no acid was formed (manometer readings) and there was no diminution in the number of aldehyde groups (Macleod-Robison method [1929]), the substance formed could only be methylglyoxal. Control experiments showed that under our conditions no non-enzymic conversion of methylglyoxal into lactic acid took place. We may therefore divide the glyceraldehyde fermentation into two parts:

$$\underset{\text{Non-enzymic}}{CH_2OH.CHOH.CHO \rightarrow CH_3.CO.CHO} + \underset{\text{Enzymic}}{H_2O \rightarrow CH_3.CHOH.COOH.}$$

We are now in a position to return to Figs. 1 and 3 in order to explain certain peculiarities which were not before remarked upon. In Fig. 1 it will be seen that low concentrations of optically active glyceraldehyde not only do not inhibit glucolysis, but actually appear to increase it. This is due to the fact that at such concentrations the amount of *l*-glyceraldehyde present is so small as to have no effect upon the glucolysis, while at the same time the *d*-glyceraldehyde forms methylglyoxal, the fermentation of which summates with the lactic acid production from glucose. If only *d*-glyceraldehyde were added the total lactic acid production would remain at some level above that due to glucose alone, dependent on the velocities etc. of the processes of glyceraldehyde fermentation already mentioned. With *dl*-glyceraldehyde the effect is not seen because, at the concentration of *l*-isomeride at which no inhibition of glucolysis is produced, insufficient glyceraldehyde is present to show it.

Secondly, the initial velocity of glyceraldehyde fermentation, seen in Fig. 3, is high because, while the substance was still in the side bulb of the manometer cup during equilibration in the thermostat and before tipping, a considerable amount of methylglyoxal was formed. This quickly broke down under the influence of glyoxalase.

Discussion

The fact that *l*-glyceraldehyde inhibits glucolysis, and that *d*-glyceraldehyde does not, seems to us to be very significant with regard to the mechanism of glucose breakdown, since *l*-glyceraldehyde has the same configuration as the naturally occurring *l*-lactic acid, sarcolactic acid, formed in the body from *d*-glucose.

That glyceraldehyde is not an intermediate in glucolysis is in agreement with the old findings of Sansum & Woodyatt [1916] who reached a similar result in the course of their investigations of carbohydrate metabolism in diabetes. "Conceptions of the breakdown of glucose", they said, "in which the process is depicted as though it proceeded from one fixed molecule to another, are fundamentally inadequate."

It will be remembered that the present series of investigations on embryonic carbohydrate breakdown took their origin from the question whether glycolysis in early developmental stages was not a different and simpler process than that described in the well-known scheme of Embden-Meyerhof-Parnas. In previous work [Needham & Lehmann, 1937] the following were excluded as stable intermediates of glucolysis proper; not only hexosediphosphate, triosephosphate, phosphoglyceric and phosphopyruvic acids, but also gluconic acid, glyceric acid, glycerol, pyruvic acid and pyruvic aldehyde (methylglyoxal). Glyceraldehyde now joins this group of substances.

The exclusion of so many three-carbon compounds as possible intermediates reminds us that possibly a process of glucose breakdown other than the formation

of stable intermediates of this kind may be at work. We might recur to the work of Nef [1907; 1910] who obtained glucose from fructose *in vitro* on treatment with strong alkali:

$$CH_2OH.C{:}O.CHOH.CHOH.CHOH.CH_2OH \rightarrow CH_2OH.CHOH.COH{:}COH.CHOH.CH_2OH.$$

He therefore suggested that a shift of the double bond along the carbon chain might be an essential step in enzymic glucose breakdown. A temporary optical inactivation of an asymmetric carbon atom by the formation of a double bond, e.g.

$$CH_2OH.CHOH.COH{:}COH.COH{:}CH_2 + H_2O,$$

and its reactivation as the unsaturated linkage is removed, would make the derivation of *l*-lactic acid (sarcolactic acid) from *d*-glucose somewhat more comprehensible. The final steps would then involve dismutation, hydration, and scission at 3:4. It is interesting that certain sugars with double bonds in the chain have been shown to be readily metabolized by the mammal [Freudenberg, 1933; Freudenberg & Felton, 1933]. It has recently been shown that a Cannizzaro reaction between two aldehydes may proceed in a medium containing D_2O without the appearance of any deuterium in the reaction products [Bonhöffer & Fredenhagen, 1937]. Exchange of atoms in the reaction had thus been entirely intramolecular. It would be interesting to know whether the lactic acid formed from glucose by the glucolytic process studied in this series of papers, if in deuterium-containing solution, would show the presence of heavy hydrogen or not. The enolization and dismutation involved in a mechanism such as that just envisaged might account for the sensitivity of glucolysis proper to fluoride and iodoacetate, which also inhibit (though in different concentrations) the enolase and the dismutase in the phosphorylation machinery.

Summary

1. The inhibition of embryonic glucolysis by *dl*-glyceraldehyde is due to the *l*-compound only, which corresponds in its configuration to the *l*-lactic acid deriving from *d*-glucose in the body. The inhibition is complete at $2{\cdot}5 \times 10^{-3} M$.

2. Embryo brei forms lactic acid from glyceraldehyde itself in a reaction for which glutathione is a necessary coenzyme. This obscures the 100% inhibition of glucolysis by glyceraldehyde.

3. Glyceraldehyde cannot be an intermediate of glucose breakdown because during glucolysis no formation of hexosemonophosphate from added triose-phosphate is observed, though this reaction readily takes place if glyceraldehyde is added.

4. Condensation to glucose cannot be an intermediate step in glyceraldehyde "fermentation", since it is not inhibited by iodoacetate and fluoride in amounts sufficient to inhibit glucolysis.

5. Glyceraldehyde forms methylglyoxal non-enzymically if shaken at *p*H 7·2 at 37°, and this is then converted into lactic acid by glyoxalase.

6. The possibility of a glucolysis without stable intermediates is discussed.

The authors take the opportunity of warmly thanking Prof. Fischer and Dr Baer for their gift of optically active glyceraldehyde. One of us (H. L.) is indebted to the British Fund for German Jewry for a maintenance grant. A grant from the Government Grant Committee of the Royal Society partially defrayed the costs of this research.

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Note added 11 October 1937. We would like to suggest that the action of glutathione on lactic acid formation from hexosediphosphate in organ extracts at 55° [Mawson, *Biochem. J.* 1937, **31**, 1657] is due to a non-enzymic transformation of triosephosphate into methylglyoxal [cf. Needham & Lehmann, *Biochem. J.* 1937, **31**, 1249]. Aldolase, which forms triosephosphate from hexosediphosphate, is stable at that temperature, and under such conditions the equilibrium is much in favour of the former. At the same time the former is very unstable in alkaline solutions at high temperature, decomposing non-enzymically to triose and phosphate. The triose so formed decomposes non-enzymically to methylglyoxal, and methylglyoxalase, which is resistant to rise of temperature, then converts it into lactic acid. Hence the stimulating effect of added glutathione. We believe that Mawson's effect is due to non-enzymic transformations, the second one of which would be identical with that described in the present paper.