On the Mechanism of the Inhibition of Glycolysis by Glyceraldehyde

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(Received 29 August 1950)

Since the discovery by Mendel (1929) of the inhibition by 0.001M-glyceraldehyde (GA) of lactic acid formation from glucose in tumour slices, the mechanism of this inhibition has been the subject of much experimentation and discussion. Very varied results are recorded by different workers using slices or brei from various tissues, or tissue extracts with or without added yeast hexokinase; on the whole, the conclusion may be drawn that with systems using glucose, the glycolysis is very sensitive to GA while with glycogen or starch as source, the inhibition arises only with much higher concentrations of GA. The rather numerous exceptions to this rule are probably to be explained on the following grounds: (a) only monomeric GA inhibits glucose breakdown. This was not realized before the work of Lehmann & Needham (1938), and earlier workers may have used one form or the other or a mixture of both. Lehmann & Needham also found that a high concentration of the dimeric form ($3.0\text{--}6.7 \times 10^{-2}\text{M}$) inhibits phosphorylase. (b) Stickland (1941), using muscle extract reinforced with yeast hexokinase, found that the inhibition of glucose breakdown by about 0.003M-GA can be prevented by a small excess of hexokinase. In earlier work the need for this strict control of the amount of added hexokinase was not understood.

Stickland (1941), taking account of both these precautions, made a careful study of the glycolysis of both starch and glucose, in the presence of GA, in muscle extract. The systems differed only in that with glucose the minimal amount of hexokinase necessary to obtain a steady glycolysis was added. Using only 0.003M-GA, 85–95% inhibition was found with glucose or fructose, while 0.03M-GA was needed with starch to obtain even 5–30% inhibition. Since the enzymes acting in the two cases are the same with the exception of the phosphorylase and the hexokinase concerned in the initial phosphorylations, Stickland concluded that GA must affect the phosphorylation of glucose by hexokinase. However, later experiments (Stickland, 1942), designed to test directly the effect of GA upon yeast hexokinase, showed no inhibition even with GA concentrations as high as 0.05M.

After the discovery by Warburg & Christian (1939) and by Dixon & Caputto (1945) that GA can act as a substrate for the triosephosphate dehydrogenase

(TPD) of yeast and muscle, but is acted upon much more slowly than glyceraldehydephosphate (GAP) (at about $\frac{1}{300}$ th of the rate in the case of muscle), the suggestion arose that GA might inhibit the oxidation of GAP by competing with it for the enzyme. In tissues or in a system such as that used by Stickland, where only catalytic amounts of adenosinetriphosphate (ATP) are present, glycolysis depends upon continual rephosphorylation of the adenosinediphosphate (ADP) formed; inhibition of TPD will interfere with this process. The need for this replenishment of ATP is twice as great when glucose rather than glycogen is the substrate; in the presence of adenosinetriphosphatase, as in muscle extract, the effect of even partial inhibition of TPD might well be to diminish or stop the phosphorylation of glucose through lack of ATP.

The possibility that GA can inhibit TPD in this way was tested by two lines of approach (a) indirectly by experiments using the same system as Stickland; (b) directly by experiments on pure TPD. As will be described, good evidence was obtained for this inhibition, particularly by the D-form of the aldehyde.

When this work was nearing completion a paper appeared by Rudney (1949) describing the inhibition by L-glyceraldehyde of the hexokinase of various tissues and extracts. In view of the discrepancy between these observations and those of Stickland, the question of hexokinase inhibition by GA was further examined; the degree of inhibition with muscle hexokinase was found to depend on the concentration of sugar present.

EXPERIMENTAL

Tissue extracts and enzyme preparations. (a) Rabbit-muscle extract (Meyerhof, 1926) was used for all the glycolysis experiments. The cooled muscle was minced and extracted with 1.5 times its weight of ice-cold water. After standing at 0° for 30 min., the mass was strained through cheese cloth. The filtrate was dialysed in cellophan tubing for 20 hr. against 20 vol. distilled water at 4°. The resulting cloudy fluid (a bulky precipitate appeared during dialysis and its removal led to great loss of activity) was used without centrifugation, being well mixed before samples were taken; it was stored at 4° and kept its activity during several days.

(b) The hexokinase was prepared from baker's yeast by the method of Meyerhof (1927). It was precipitated by 50% (v/v) ethanol, dried, and stored in a desiccator. For the

solution, which was made up fresh each day, 10 mg. were rubbed up with 1 ml. water and the insoluble residue was removed by centrifugation.

(c) The crystalline triosephosphate dehydrogenase was made by the method of Cori, Slein & Cori (1947). It was stored at 4° in 66% saturated $(\text{NH}_4)_2\text{SO}_4$ solution. Before use, it was centrifuged and the residue taken up in the desired medium.

(d) In the preparation of rat-muscle extract, used in measuring hexokinase activity, the cooled leg muscles were finely chopped with scissors, then ground in a mortar with 1.5 times the weight of ice-cold water. This procedure was carried out in a cold room. The brei was strained through fine handkerchief cotton and the filtrate used as quickly as possible.

Chemical preparations. The ATP was made by the method of Lohmann as described by Needham (1942) and the ADP by the method of Bailey (1949). The coenzyme I was made in some cases by the method of Williamson & Green (1940), in others by a modification of this method due to Ochoa (1948). The D-glyceraldehyde was kindly supplied by Mr B. C. Loughman, who prepared it by the method of Baer (Baer, Grosheintz & Fischer, 1939; Baer, 1945). We are indebted to Mr Loughman for the following characterization: the dimedone derivative (crystallizing in long prisms) had m.p. 196° and $[\alpha]_D^{25} + 195^\circ$ (in pure dry ethanol). The D-GA was stored as a semicrystalline syrup, slightly acid with acetic acid, at 4°; before use the pH of the solution was adjusted with 0.1 N-NaOH, using the glass electrode.

The glyceraldehydephosphate (GAP) was prepared by the method of Baer & Fischer (1943); much of it was kindly made for us by Mlle Bianca Tchoubar.

The phosphopyruvic acid was kindly supplied by Dr C. S. Hanes.

The sodium pyruvate was purified by the method of Robertson (1942).

Estimation methods. Glycolysis was followed by the method of Warburg. Lactic acid estimations were by the method of Friedemann, Cotonio & Schaffer (1927); glucose estimations by the method of Nelson (1944); phosphate estimations by the method of Fiske & Subbarow (1925); and fructose by the colorimetric method of Cole, Hanes, Jackson & Loughman (1951). Glyceraldehyde was estimated in the solutions by the method of Willstätter & Schudel, as described by Browne & Zerban (1941); when it had to be estimated in the presence of glucose, Rudney's (1949) modification of Nelson's method was used.

RESULTS

Effects of DL-glyceraldehyde in experiments with muscle extract

The system used was very similar to that described by Stickland (1941). The muscle extract (1.2 ml.) was made up to a final volume of 2.4 ml. This contained sodium phosphate, pH 7.6, 0.025 M; sodium bicarbonate, 0.26 g./100 ml.; glucose or glycogen, 0.32 g./100 ml.; ATP, 5×10^{-4} M; coenzyme I, 4×10^{-5} M; magnesium chloride, 4×10^{-3} M. Preliminary experiments were done with each new muscle extract to find the minimal amount of hexokinase which would give a steady glycolysis rate

over 30–60 min.; 0.05–0.15 ml. of the solution described was usually needed. 95% nitrogen–5% carbon dioxide (v/v) was passed through the Warburg flasks and the experiments were carried out at 37°. Previous workers had shown that inhibition comes on slowly, and when GA was used, unless otherwise stated, it was added to the mixture immediately before the muscle extract; all vessels were then gassed for 5 min., and equilibrated in the bath for 10 min. before readings were begun.

Effect of glyceraldehyde on glucose utilization. Fig. 1 (curves A and B) shows an experiment in which, with 0.0036 M-GA, inhibition of carbon dioxide production was practically complete from the beginning.

Estimations of glucose gave the following results:

	Glucose present (mg./sample)	
	At 0 min.	At 60 min.
Control	8.1	4.35
With 0.0036 M-GA	8.1	8.1

This shows that the inhibition under these conditions affects the first stage of glycolysis, the phosphorylation of glucose by hexokinase. However, the next experiment indicates, in confirmation of Stickland's results, that the inhibition is not directly upon hexokinase activity.

Fig. 1. Lactic acid formation from glucose in muscle extract + yeast hexokinase system. Curves A and C without GA; curve B with 0.0036 M-GA; curve D with 0.002 M-GA.

Curves C and D, in Fig. 1, shows the onset of inhibition in another extract when 0.002 M-GA was used. It will be seen that there is about 75% inhibition after 10 min. and that after 20 min. the inhibition is complete. Two extra manometers were set up, one with and one without GA, similar in all respects to those giving curves C and D, except that 0.06 M-fluoride was present and 0.004 M-ATP instead

of 0.0005M was used; the required amount of ATP was placed in the side bulb. For initial examination of reducing power 0.5 ml. was removed from each vessel, and the manometers were gassed and equilibrated as usual. 10 min. after this the ATP was tipped in, and after another 10 min. period, samples again taken out. Half the usual amount of glucose was used in this experiment.

In this experiment no separate estimation was made of the GA present at the beginning and end of the experiment; but other experiments showed that with muscle extract the change in GA concentration is negligible; the change in reducing power may therefore be put down to utilization of glucose. It will be seen that under these conditions, where the ATP concentration is not limiting hexokinase activity, there is not more than 10% inhibition.

	Glucose utilization (mg./sample) in the presence of GA, with high ATP concentration		
	Initial	Final	Used
Control	4.34	3.79	0.55
With 0.002M-GA	5.25	4.75	0.50

Experiments were also done with the high ATP concentrations over a longer period (40 min.) and in absence of fluoride, lactic acid formation being measured chemically. The increased ATP lowered the inhibition by GA, but the effect was small, no doubt because the ATP is rapidly removed by ATPase:

	Lactic acid formation (mg./sample)	
	0.0005M-ATP	0.002M-ATP
Control	2.72	4.25
With 0.004M-GA	0.55	1.64

The inhibitions were thus 80 and 61% with 0.0005 and 0.002M-ATP respectively.

Effect of iodoacetate. When the glycolytic system described was treated with a low concentration of iodoacetate (5×10^{-4} M) a state resembling that obtaining with GA could be produced, i.e. in which glucose breakdown was markedly inhibited, while glycogen breakdown was little affected. A typical experiment is shown in Fig. 2. The rate of glycolysis with glycogen was usually found to be less than the rate with glucose + hexokinase; it could be increased to some extent by addition of creatine (5 mg./sample). It might be expected that inhibitions in the slowly acting system might be less readily obtained, simply because the enzyme concerned did not so readily become the limiting factor; but, as the curves show, with the same extract the rate of glucose breakdown can be diminished by the iodoacetate far below that of glycogen breakdown, although the latter may start at a slower pace. Since

TPD is the enzyme in the glycolytic series known to be most sensitive to iodoacetate, the effects described here, so similar to those obtained with GA, may be attributed to its partial inhibition.

Fig. 2. Effect of 5×10^{-4} M-iodoacetate on lactic acid production from glucose and from glycogen in muscle extract + yeast hexokinase system. Curve A, glucose control; curve B, glycogen control; curve C, glycogen + iodoacetate; curve D, glucose + iodoacetate.

Effect of increased coenzyme I concentration. It was first observed by Mendel, Bauch & Strelitz (1931) that the inhibition of glycolysis by 0.001M-GA in tumour slices can be markedly reduced by addition of traces of pyruvate and completely prevented by 0.001M-pyruvate. Stickland (1941), using the muscle extract + hexokinase system with 0.003M-GA, found the inhibition of 92% reduced to 4% by 0.0015M-pyruvate, and he showed that the oxido-reduction

glyceraldehyde + pyruvate $\rightarrow$ glyceric acid + lactate goes on so slowly at these low concentrations as to be quite insufficient as a means of removing the GA and thus reducing its toxic effect.

If the inhibition of glycolysis in this system is due to partial inhibition of TPD, the effect of the added pyruvate could be to increase the turnover at the TPD by providing for more rapid reoxidation of the reduced Co I. If this were the case, addition of Co I itself should have the same effect in alleviating the inhibition. As Fig. 3 shows, it was indeed found that increasing the Co I concentration about ten times (to 3.3×10^{-4} M) overcame the inhibition due to 0.004M-GA. The increased Co I concentration has practically no effect on the rate of glycolysis in the control. This does not necessarily mean, however, that the TPD in the control is saturated with respect to Co I—we suggest that triosephosphate dehydrogenation is not the limiting reaction in the control, but has become so after the GA treatment. Anything

then enabling the partly inhibited enzyme to work with better efficiency is important in staving off the inhibition of glycolysis.

Effect of glyceraldehyde on enolase and pyruvic phosphokinase. Meyerhof & Wilson (1948) state that in one case at any rate (rat sarcoma) where traces of pyruvate greatly increase glycolysis in tissue extracts, this effect is due to a deficiency in enolase. The possibility was therefore considered that in the present experiments the striking effect of pyruvate

The source of the pyruvic phosphokinase was muscle powder made by precipitation of muscle extract with 3 vol. of acetone, filtering and drying rapidly. An extract was made with 25 mg./ml. water and centrifuged. Samples (0.5 ml.) of this extract were treated in the following way: to the control were added 0.1 ml. 0.2M-phosphate buffer, pH 7.5 and 0.15 ml. water, while to the other were added buffer and 0.15 ml. 0.05M-GA. The samples were then kept at 30° for 10 min.

Fig. 3. Effect of increased Co I concentration upon lactic acid formation from glucose in muscle extract + yeast hexokinase in the presence and absence of 0.004M-GA. ○—○—○, no GA, 3.3×10^{-4} M-CoI; ●—○—○, no GA, 4×10^{-5} M-CoI; ○—○, 0.004M-GA, 3.3×10^{-4} M-CoI; ●—●, 0.004M-GA, 4×10^{-5} M-CoI.

addition might depend on partial inhibition by the GA of enzymes responsible for converting phosphoglycerate into pyruvate. Two of these were tested, enolase and pyruvic phosphokinase, but no indication of inhibition was found.

The enolase used was a freeze-dried protein fraction kindly supplied by Miss A. B. Askonas who obtained it after precipitation of muscle extract between 0 and -5° with ethanol between 51 and 54% (v/v). A solution was made of 10 mg. in 2 ml. and centrifuged. The enzyme solution (0.1 ml.) was used in a final volume of 3 ml. with 0.07M-phosphate, pH 6.7, 0.003M-magnesium sulphate, 0.01 g./100 ml. glycine and 0.006M-2-phosphoglycerate. Under these conditions the rate of reaction was found to be proportional to the enzyme concentration. The enzyme was mixed with the reagents except for the phosphoglycerate, and both control and sample + GA (0.0033M) were allowed to stand 10 min. at 37°. The temperature was then adjusted to 20°, and the solutions were transferred to cells of the Beckman spectrophotometer; the reaction was started by adding the phosphoglycerate, and the rate of phosphopyruvate formation was followed by measuring the increase in optical density at 240 mμ. (Warburg & Christian, 1941). One of the experiments is shown in Fig. 4.

Fig. 4. Effect of GA (0.0033M) on enolase: formation of phosphopyruvate from 2-phosphoglycerate. ○—○, no GA; ●—●, + GA.

Fig. 5. Effect of GA (0.0066M) on pyruvic phosphokinase: formation of pyruvate from phosphopyruvate. ○—○, no GA; ×—×, + GA.

The activities of these pretreated enzyme solutions were then tested in the following system: 0.3 ml. of each was used in a final volume of 1.6 ml. with 0.025M-phosphopyruvate, 0.012M-ADP, 0.02M-phosphate, pH 7.5, 0.006M-magnesium chloride, and 0.025M-fluoride. The final concentration of GA with the GA-treated enzyme was 0.006M. The experiment was carried out at 30°, and 0.3 ml. samples were removed at

intervals into 1 ml. 5% (w/v) trichloroacetic acid. The pyruvate formed was estimated by means of lactic dehydrogenase and reduced coenzyme I in the Beckman spectrophotometer, by measuring the decrease in density at 340 m μ . A final volume of 3 ml. was used, containing 0.1 ml. of the unknown solution, 1 mg. reduced coenzyme I, 0.05 mg. lactic dehydrogenase (a freeze-dried protein fraction made by Miss A. B. Askonas from muscle extract by precipitation with ethanol 23.5–31% (v/v) between 0 and –5°) and 0.03 M-phosphate buffer, pH 7.5. The results of a typical experiment are shown in Fig. 5.

Effect of added triosephosphate dehydrogenase. If the inhibition of lactic acid formation from glucose in the muscle extract + hexokinase system is due to partial inhibition of TPD, it might be expected that addition of extra TPD would delay the onset of the inhibition. This was indeed found to be the case.

TPD suspension of crystalline protein (0.5 ml.) in 66% saturated $\text{NH}_4(\text{SO}_4)$ was centrifuged at 20,000 rev./min. at 0°. The residue was dissolved in 0.6 ml. 0.03 M-pyrophosphate buffer, containing 0.0033 M-cysteine, pH 7.2; this solution was dialysed in cellophan tubing against 100 ml. of the same buffer at 0° for 1.5 hr. in an atmosphere of N_2 . This TPD solution (0.3 ml.) was added to the usual system; 0.3 ml. of the pyrophosphate-cysteine buffer was added to the control.

One such experiment is illustrated in Fig. 6. GA was present from the beginning in two of the four Warburg vessels. A marked retardation by added

the quantity of enzyme in the system by more than about 30–50%.

Experiments with crystalline triosephosphate dehydrogenase

In this part of the work the effect of the presence of DL-GA and of D-GA upon the rate of reduction of Co I by GAP in the presence of TPD was studied; the increase in reduced coenzyme concentration being measured by following the increase in density at 340 m μ . in the Beckman spectrophotometer. Conditions were adjusted to ensure that the rate of reaction was proportional to the amount of enzyme present. Arsenate was added to prevent accumulation of diphosphoglyceric acid and pyrophosphate buffer was used to minimize the effects upon the enzyme of any traces of heavy metals. The pH was 8.5, the optimal for TPD activity, unless otherwise stated. TPD is well known to be a sulphhydryl enzyme; it is active without added cysteine, but in the presence of cysteine, the activity may be increased some fivefold. This increase in activity comes on gradually and is probably due to reduction of S—S linkages in the enzyme. On account of these progressive changes, and of the non-reproducible changes taking place in the enzyme solution on standing in absence of cysteine (see below), the observations were always made in groups of four Beckman cells. All four measurements were made within a few seconds of each other. Readings were taken at 0.5 min. intervals for 3 min., the rates of reaction at zero time calculated (cf. Rapkine, Shugar & Siminovitch, 1950) and the percentage inhibition calculated therefrom.

Fig. 6. Effect of added TPD upon lactic acid formation from glucose in muscle extract + hexokinase system in the presence and absence of 0.003 M-GA. ○—○, Control + 0.3 ml. pyrophosphate cysteine buffer; ○---○, control + 0.3 ml. TPD in pyrophosphate-cysteine buffer; ●—●, + GA + 0.3 ml. pyrophosphate-cysteine buffer; ●---●, + GA + 0.3 ml. TPD in pyrophosphate-cysteine buffer.

TPD of the inhibition was observed; the addition of the extra enzyme made no difference to the rate of the control. About 0.5 mg. enzyme protein was added, an amount which probably did not increase

Fig. 7. Reduction of Co I by GA. Concentration of DL-GA, 0.0033 M. Concentration of Co I, 5×10^{-5} M. Temperature 25°, pH 8.5.

Non-enzymic reduction of coenzyme I by glyceraldehyde. It was observed in the course of this work that when Co I is allowed to stand with GA at pH 8.5 and room temperature, there is a gradual increase in absorption at 340 m μ . Fig. 7 shows the rate of the reaction with the usual concentration of Co I and 0.033 M-GA. At pH 7.0–7.5 the reaction is quite insignificant. Since most of the experiments described here were at pH 8.5, it is clear that a

correction is needed to allow for this non-enzymic reaction; the correction is small (about 0.01 in $\log I_0/I$) for the 3 min. experiments, but may become important in experiments of longer duration and with larger concentrations of either GA or Co I. A reaction of this sort between GAP and Co I was hardly detectable; on the other hand, reaction with benzaldehyde seems comparable in rate to that with GA.

Effect of DL-glyceraldehyde upon oxidation of glyceraldehydephosphate by triosephosphate dehydrogenase in absence of cysteine. The system used consisted of 10^{-4} M-GAP, 10^{-4} M-Co I, 6×10^{-3} M-arsenate, 0.033M-sodium pyrophosphate, pH 8.5, and 40–100 μ g. TPD contained in 3 ml. The reaction was always started by addition of the GAP.

Fig. 8 and Table 1 illustrate the effect of the presence of 0.033–0.0033M-DL-GA in this system. It will be seen that the inhibition comes on at once, but even with the highest GA concentrations does not exceed 35–40%. Attempts were made to increase this inhibition by allowing the samples to stand, with and without GA, for some minutes before addition of GAP. It was found, however, that quite apart from GA effects, the enzyme under these conditions at room temperature is not stable and its activity decreases or even occasionally increases. There were indications that the presence of GA sometimes afforded protection from the inactivation, and inhibitory effects of the GA might thus be masked. These attempts were therefore given up.

Some experiments of longer duration were done in which the GA was not added until the reaction in the presence of GAP was already in progress. That the inhibition comes on as well under these conditions is shown in curves A and B in Fig. 9. Curve C shows the

increase in optical density in presence of only GA. If the correction due to the GA reaction, enzymic and non-enzymic, is taken into account, although it cannot be accurately assessed since the enzymic GA reaction is probably inhibited by the presence of GAP, the inhibition observed in curve B is even more marked and reaches approximately 25% at the end of 12 min.

Fig. 8. Effect of various concentrations of DL-GA on oxidation of GAP by TPD. No cysteine present. Temp. 25°, pH 8.5.

Effects of DL-glyceraldehyde upon oxidation of glyceraldehydephosphate by triosephosphate dehydrogenase in presence of cysteine. In these experiments, 6×10^{-4} M-cysteine was present, and the system already described, with the exception of the co-enzyme and GAP, was allowed to stand until full activity was reached (10–20 min.). Only about 10 μ g. of TPD were needed as the activity of the

Table 1. Inhibition, by various concentrations of DL-glyceraldehyde, of the oxidation of glyceraldehyde-phosphate by triosephosphate dehydrogenase

Exp. no.	Glyceraldehyde (M)	pH	Activities at zero time expressed in units of optical density		Inhibition (%)
			GAP alone	GAP plus GA	
1	0.033	8.5	0.170	0.116	34.0
2	0.033	8.5	0.382	0.246	35.6
	0.033	—	—	0.263	31.2
3	0.05	8.5	0.305	0.182	40.6
	0.033	—	—	0.213	30.0
	0.016	—	—	0.230	24.5
4	0.01	8.5	0.144	0.102	29.2
	0.0066	—	—	0.110	23.5
	0.0033	—	—	0.120	16.6
5	0.033	8.5	0.168	0.097	42.3
	0.016	—	—	0.115	31.5
	0.003	—	—	0.130	22.5
6	0.033	7.5	0.067	0.056	16.5
7	0.033	7.5	0.068	0.054	20.5

enzyme was so much greater. Coenzyme was then added to all the cells and the reaction started, with or without GA, by the addition of GAP. Under these conditions, very little, if any, inhibition was observed during the 3 min. of the test.

Fig. 9. Effect of addition of DL-GA (0.0033M) after GAP oxidation has begun, pH 8.5, temp. 25. No cysteine present. A, GAP alone; B, GAP + GA; C, GA alone.

However, when the DL-GA was added at the same time as the cysteine, and the TPD plus GA plus cysteine were allowed to stand as before, large inhibitions were found. Fig. 10 shows one set of experiments where up to 75% inhibition was obtained with 0.02M-GA. Similar results were obtained when the enzyme was first activated with cysteine, and the GA then added and allowed to remain in contact with the enzyme for various lengths of time before the test. It was then seen that the inhibition was a function of time (Table 2).

It was further found that these large inhibitions, even when up to 80%, could be reversed by subsequent addition of larger amounts of cysteine (final concentration 0.033M).

Seven cells contained the usual reaction mixture, and TPD plus cysteine (6.6×10^{-4} M in the cell) was added to all of them. After 30 min., the reaction in cell 1 was measured by addition of Co I and GAP as usual, and GA (0.0033M) was added to cells 5-7. At 60 min., the reaction in cells 2 and 5 was measured

and a concentrated solution of cysteine (0.033M in the cell) was added to cells 4 and 7. After 15 min., the reaction in cells 3, 4, 6 and 7 was measured. The results of cells 1-3 (Table 3) show that in the absence of GA no appreciable change in activity in presence of cysteine occurred through the duration of the experiment. The activity in cell 4 indicates that these results represent maximum activities of TPD since an increased concentration of cysteine did not appreciably alter the rates obtained. The rate of

Fig. 10. Effect of DL-GA on oxidation of GAP by TPD, pH 8.5, temp. 25°, in presence of 6.6×10^{-5} M-cysteine. Cysteine and GA added 15 min. before measurement of activity.

Table 2. Effect of time on the inhibition of triose-phosphate dehydrogenase by 0.007M-DL-glyceraldehyde in the presence of 6×10^{-4} M-cysteine

(Cysteine added at time 0; GA added after 27 min.)

Time (min.)	Activities at zero time expressed in units of optical density	
	Without GA	With GA
34	0.362	0.287
43	—	0.214
53	—	0.042

reaction in cell 5 represents the decrease in activity in the presence of GA after 30 min. contact and in cell 6 after 45 min. contact. The increase in activity found in cell 7 clearly demonstrates that much of this loss can be recovered by addition of cysteine; in other words, that the inactivation is reversible. These results will be discussed more fully later in this section.

Table 3. *Reversibility of the glyceraldehyde inhibition of cysteine-activated triosephosphate dehydrogenase*(System as usual + 6.6×10^{-4} M-cysteine; reaction started by the addition of CoI and glyceraldehydophosphate.)

Additions	Activities at zero time, expressed in units of optical density, when reaction was started at		
	30 min.	60 min.	75 min.
None	0.842	0.801	0.721
At 60 min. cysteine to final concentration of 0.033 M	—	—	0.716
At 30 min. glyceraldehyde to final concentration of 0.0033 M	—	0.305	0.028
At 30 min. glyceraldehyde to final concentration of 0.0033 M; at 60 min. cysteine to final concentration of 0.033 M	—	—	0.492

Since it is known that many aldehydes react reversibly with cysteine, combining probably through either the —SH or —NH₂ groups or both (see, for example, Schubert, 1935, 1936) the possibility was considered that, in presence of cysteine, the inhibitory effect of GA might be due to removal of free cysteine. This possibility is rendered unlikely by the fact that D-GA was found to have an inhibitory effect as great as twice the amount of DL-GA; it seems that the inhibitory effect is probably due entirely to the D isomer and it is unlikely that there would be any marked difference in rate of reaction of the two isomers with free cysteine.

Fig. 11. Effect of D-GA and DL-GA on the oxidation of GAP by TPD; pH 8.5, temp. 25°. No cysteine present.

Experiments with D-glyceraldehyde. When this optical isomer became available studies were made to compare its effect quantitatively with that of DL-GA on GAP oxidation by TPD, both in the absence and presence of cysteine. Some of these results are shown in Tables 4 and 5 and in Figs. 11 and 12. Fig. 12 includes two sets of curves, one at pH 8.5, the other at pH 7.5. It is clear that the inhibition occurs also at the lower pH, and is indeed more marked.

This was the first time that an inhibitory effect of the D-form had been found. In the experiments of Needham & Lehmann (1937) on 4.5-day chick embryos, and of Mendel, Strelitz & Mundell (1938) on

tumour slices, no inhibition was found with the D-form at concentrations where the DL-form was very effective; the inhibition in these cases was obviously due to the L-component.

An experiment was also done with D-GA in the absence of cysteine, to test the effect of GAP concentration upon the GA inhibition. Fig. 13 shows that when the GAP concentration was doubled, the inhibition was halved.

Fig. 12. Effect of D-GA and DL-GA on the oxidation of GAP by TPD, pH 8.5 and 7.5, temp. 25°, in presence of 6.6×10^{-4} M-cysteine. GA and cysteine added 15 min. before measurement of activity. —, pH 8.5; - - - -, pH 7.5.

It is evident that a systematic kinetic study of TPD, with and without cysteine in the presence of the two substrates GAP and GA, would yield interesting results. The data so far collected in the present work are consonant with the following picture. In the absence of cysteine, reaction takes place at the active centres, D-GA acting as a competitive inhibitor with the GAP. When cysteine is present, a number of S—S groups are reduced and the activity of the enzyme is greatly increased. It is suggested that now the most marked effect of the GA is to put these —SH groups out of action by

Table 4. *Effect of various concentrations of DL-glyceraldehyde and D-glyceraldehyde on the activity of triosephosphate dehydrogenase*

Exp. no.	Form of glyceraldehyde concentration (M)	Concentration (M) as DL-glyceraldehyde	Activities at zero time expressed in terms of optical density		Inhibition (%)
			GAP alone	GAP plus GA	
1	D 0.014	0.014	0.125	0.080	36.6
	DL 0.016	0.008	—	0.114	9.1
	DL 0.033	0.016	—	0.103	17.5
2	D 0.0066	0.0066	0.142	0.108	24.0
	DL 0.0066	0.0033	—	0.125	12.0
	DL 0.0135	0.0067	—	0.117	18.0
3	DL 0.0033	0.0016	0.233	0.213	8.0
	D 0.0025	0.0025	—	0.203	13.0
	DL 0.01	0.005	—	0.160	32.0
4	DL 0.003	0.0015	0.278	0.285	0.0
	D 0.003	0.003	—	0.215	23.0
	DL 0.009	0.0045	—	0.208	27.0
5	D 0.0018	0.0018	0.267	0.242	10.0
	DL 0.006	0.003	—	0.194	28.0
	D 0.058	0.0058	—	0.141	47.0

Table 5. *Effect of various concentrations of DL-glyceraldehyde and D-glyceraldehyde in presence of 6×10^{-4} M-cysteine on the activity of triosephosphate dehydrogenase*

Exp. no.	Form of glyceraldehyde and concentration (M)	Concentration (M) as D-glyceraldehyde	Time of contact of GA and cysteine-activated TPD (min.)	Activities at zero time expressed in terms of optical density		Inhibition (%)
				GAP alone	GAP plus GA	
1	D 0.002	0.002	8	0.359	0.361	0
	DL 0.005	0.0025	—	—	0.343	5
	D 0.006	0.006	—	—	0.110	69
2	D 0.002	0.002	15	0.383	0.293	24
	DL 0.005	0.0025	—	—	0.183	52
	D 0.006	0.006	—	—	0.121	69
3	DL 0.0032	0.0016	15	0.254	0.202	18
	D 0.0032	0.0032	—	—	0.179	27
	DL 0.0092	0.0046	—	—	0.166	32
4	DL 0.0032	0.0016	15	0.365	0.300	18
	D 0.0032	0.0032	—	—	0.208	43
	DL 0.0092	0.0046	—	—	0.156	57

combining with them. This does not preclude concurrent competitive inhibition at the active centres. With the very high GA concentrations, the —SH groups produced by the cysteine are probably practically all obliterated and the activity falls to about that of GA inhibited cysteine-free enzyme.

There is obviously a great difference in behaviour of GA with and without cysteine; in the former case, inhibition appears at once but never goes very far, while in the latter case, inhibition comes on gradually but can reach 80–85%. This difference in behaviour might be tentatively correlated with competition of the GA with GAP in the cysteine-free enzyme, and predominant reaction of the GA with —SH groups in the cysteine-activated enzyme.

Fig. 13. Effect of GAP concentration on the GA inhibition of the oxidation of GAP by TPD; pH 8.5, temp. 25°. No cysteine present.

*Experiments on the inhibition of hexokinase
by glyceraldehyde*

In the paper by Rudney (1949) experiments were carried out on extracts from brain, tumour and muscle, in the presence of a high concentration of ATP and of fluoride, to test the effect of GA on hexokinase. 0.0045–0.002M-DL-glyceraldehyde gave 50–85% inhibition, varying somewhat with the different tissues. Yeast hexokinase was rather less sensitive, showing not more than 60% inhibition, even with 0.006M-DL-glyceraldehyde. D-Glyceraldehyde tested on brain extract caused no inhibition, so that the effect must be due to the L-component.

An important difference between the tests used by Rudney and our own for the effect of GA on hexokinase activity lay in the glucose concentration. Rudney used 0.0025M-glucose while we used 0.01M. A series of experiments was therefore carried out with rat-muscle extract under conditions similar to those of Rudney, except that the glucose concentration was varied. The results are given in Table 6. GA was also estimated in all cases; the changes were very small and within experimental error, so that the

change in reducing power was due to change in glucose content. The protective effect of increased glucose concentration on hexokinase activity in the presence of GA is clearly seen.

Some experiments were also carried out with fructose (Table 7); with this substrate, higher concentrations are needed to afford protection, which was only partial at 0.006M with 0.002M-GA. With the yeast hexokinase preparation of Meyerhof (1927), Stickland (1942) found no inhibition in presence of 0.02M-fructose, with 0.02M-GA. Lardy, Wiebelhaus & Mann (1950) find that L-sorbose-1-phosphate, at a concentration of only 0.0001M, inhibits hexokinase activity in brain extracts. It is to be expected that this substance would be formed under the action of aldolase from dihydroxyacetone-phosphate and L-GA, and in inhibition following treatment with L-GA, the L-sorbose-1-phosphate may usually be the true toxic agent.

*Effects of D-glyceraldehyde in experiments
with muscle extract and hexokinase*

In view of the proof that L-GA inhibits hexokinase while D-GA inhibits TPD it became necessary to test the effect of D-GA on the system used by

Table 6. *Effect of DL-glyceraldehyde upon the activity of muscle hexokinase in the presence of various concentrations of glucose*

(0.8 ml. Rat-muscle extract was used in a final volume of 2.4 ml. The other components were NaHCO₃, 0.26 g./100 ml.; MgCl₂, 0.003M; ATP, 0.005M; NaF, 0.08M. The Warburg vessels were filled with 95% N₂, 5% CO₂ and were incubated at 30° for 12 min. 1 ml. samples were removed at the beginning and end of the experiment.)

Exp. no.	Glucose (M)	Glyceraldehyde (M)	Glucose disappearing		Inhibition with GA (%)
			In control	In sample and GA	
1	0.006	0.003	0.537	0.528	None
	0.0025	0.003	0.508	0.129	75
2	0.0033	0.003	0.744	0.211	70
3	0.007	0.003	0.566	0.393	30
	0.005	0.003	0.566	0.278	50
	0.004	0.003	0.489	0.182	63
4	0.004	0.002	0.393	0.388	None
	0.0024	0.002	0.201	0.144	30
	0.0014	0.002	0.110	0	100

Table 7. *Effect of DL-glyceraldehyde upon the activity of muscle hexokinase in the presence of various concentrations of fructose*

(Components of the system as in Table 5, and experiments carried out in the same way, except that ATP concentration was 0.0066M. GA, 0.002M.)

Exp. no.	Fructose (M)	Fructose disappearing (mg.)		Inhibition with GA (%)
		In control	In sample and GA	
1	0.004	0.36	0	100
	0.0025	0.24	0	100
	0.0017	0.21	0	100
2	0.004	0.18	0	—
	0.005	0.18	0	—
	0.006	0.36	0.16	56

Stickland. It was found that D-GA does inhibit glycolysis in this system; its effect is rather greater than that of an equivalent amount of aldehyde in the racemic form, but not so great as the effect of twice the amount of racemic aldehyde. This indicates that the L-form is also having an effect, but to a less degree. It is doubtful whether, in the presence of the glucose concentrations used, this effect can be on hexokinase. Perhaps still another enzyme is affected by the L-isomer. Fig. 14 illustrates these experiments.

Fig. 14. Effect of D-GA and DL-GA on lactic acid formation in the muscle extract + hexokinase system.

An attempt was made to show the inhibition of TPD in muscle extract by spectrophotometric measurements. A 1 in 200 dilution of the extract (0.2 ml.) was used in the Beckman cells in place of the pure TPD with and without GA. Reduction of coenzyme proceeded with only a small inhibition for the first few minutes. Thereafter, although the density continued to increase slowly for a while with the GA-treated, a reversal took place in the control, the density becoming less. Clearly re-oxidation of reduced coenzyme was proceeding in the control but less so in the sample with GA-treated extract. This re-oxidation was unaffected by the presence of 0.016M-fluoride, as was shown in another experiment, and is probably to be put down to glycerophosphate dehydrogenase. It would thus appear that in the GA-treated extract, glycerophosphate dehydrogenase is inhibited. This circumstance makes it difficult to determine, by this method, whether TPD is also inhibited.

DISCUSSION

The results described may help to explain the fact that glycolysis in tissues has usually been found more sensitive to DL-glyceraldehyde than glycolysis in extracts. At the glucose concentrations generally

used, the hexokinase of extracts, to judge by results with yeast and muscle extracts, would be protected; but with tissues, the glucose concentration at the enzyme surfaces within the cells may be much lower than that in the external medium. Further work is needed, however, to explain the mechanism of the glucose protection. Lardy *et al.* (1950) report that, with brain extract, increase in glucose concentration from 0.001 to 0.035M has no effect on the percentage inhibition of hexokinase by 8×10^{-5} M-sorbose-1-phosphate. Possibly when glucose prevents inhibition of hexokinase it does so, not by keeping a competitive inhibitor off the hexokinase, but by acting itself as a competitive inhibitor of aldolase, and thus slowing formation of the hexokinase inhibitor. Evidence for slight inhibition of aldolase by glucose and fructose has been given by Herbert, Gordon, Subrahmanyam & Green (1940) in the presence of 0.02M-fructosediphosphate; with minimal fructose-diphosphate concentration, the inhibition might be much greater.

Inhibition of glycolysis by D-glyceraldehyde, via the partial inhibition of triosephosphate dehydrogenase, seems only to become important when there is high adenosinetriphosphatase activity in the system. Thus in the yeast hexokinase + muscle extract system, greater inhibition was found with D-glyceraldehyde than with an equivalent amount of DL-glyceraldehyde. In tissues, on the other hand, where adenosinetriphosphatase activity is localized and controlled, D-glyceraldehyde has comparatively little effect. It should be noted that both Needham & Lehmann (1937) and Mendel *et al.* (1938) observed some inhibition with D-glyceraldehyde; thus Needham & Lehmann (1937) found 25–30% inhibition with D-glyceraldehyde when DL-glyceraldehyde, at twice the concentration of total aldehyde, was giving about 85% inhibition. They explained the inhibition with D-glyceraldehyde as due to the presence of a small percentage of the L-isomer, since the optical rotation had fallen below that of the freshly prepared specimen. Baer & Fischer (1939) have since shown, however, that racemization cannot be the cause of the fall in optical activity, and there is, therefore, no reason to assume that the small inhibitions found are not due to D-glyceraldehyde itself.

The effect of pyruvate upon glycolysis in tissues inhibited by glyceraldehyde shows remarkable variation from one tissue to another and in the same tissue after different treatment. Thus striking alleviation of the inhibition is shown in the muscle extract-hexokinase system used by Stickland (1941) and in tumour slices (Mendel *et al.* 1931). But there is no effect on the inhibition in brain slices or extract (Baker, 1938). Rudney (1949) found no alleviation in brain, tumour and muscle extracts of the inhibition of hexokinase.

A possible mechanism for the pyruvate effect when D-glyceraldehyde is the inhibitor has been suggested earlier in this paper. Lardy *et al.* (1950) have suggested that when L-sorbose-1-phosphate is the inhibitor, pyruvate alleviation could be explained by supposing that increase in the ratio coenzyme I/reduced coenzyme I would favour oxidation of glyceraldehydephosphate rather than condensation with L-glyceraldehyde. Since both these suggested mechanisms depend on altering the balance in the activities of two enzyme systems, the variability of the response to pyruvate probably reflects only a variability in relative concentration of these enzymes in different tissues, or in their extractability when extracts are concerned.

SUMMARY

1. It is suggested that inhibition of triosephosphate dehydrogenase may play an important part in the well known inhibition of glycolysis by glyceraldehyde.

2. In a system consisting of muscle extract reinforced with yeast hexokinase, the inhibition can be delayed by addition of pure triosephosphate dehydrogenase. Other indirect evidence indicating the part played by inhibition of glyceraldehydephosphate oxidation in this system is also given.

3. The reduction of coenzyme I by glyceraldehydephosphate in presence of pure triosephosphate dehydrogenase is inhibited by DL-glyceraldehyde. This happens with the enzyme in absence of cysteine

and also after cysteine activation. In the absence of cysteine, the degree of inhibition was found to depend upon the concentration of glyceraldehydephosphate. The inhibition of the cysteine-activated enzyme could be largely overcome by addition of more cysteine.

4. When D-glyceraldehyde was compared with DL-glyceraldehyde, it appeared that the inhibition of the pure enzyme was entirely due to the D-form. With the system consisting of muscle extract with yeast hexokinase, D-glyceraldehyde caused inhibition of glycolysis, but the effect was not as great as that of twice the amount of DL-glyceraldehyde.

5. In the latter system there was some evidence that glycerophosphate dehydrogenase was also inhibited by D-glyceraldehyde.

6. The inhibition of the hexokinase of rat-muscle extract by DL-glyceraldehyde was confirmed. It was found that the degree of inhibition depends on the concentration of glucose present; in the presence of 0.002M-glyceraldehyde the enzyme was entirely protected by 0.004M-glucose. About three times this concentration of fructose is necessary to protect the hexokinase.

One of us (D. M. N.) gratefully acknowledges the hospitality of the departments of the late M. Louis Rapkine and of M. André Lwoff at the Pasteur Institute, Paris; and also the receipt of a personal grant from the Medical Research Council.

We are deeply indebted to M. Rapkine for the benefit of much discussion.

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