LIII. INTERMEDIARY CARBOHYDRATE METABOLISM.

THE EFFECT OF SODIUM IODOACETATE ON GLYOXALASE.

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Introduction.

The keystone of a theory of intermediary carbohydrate metabolism, put forward by Dakin and Dudley [1913, 3; 1914] is the ketonic aldehyde, methylglyoxal. The postulation of this substance as the catabolic precursor and anabolic successor of lactic acid ($CH_3.CO.CHO \rightleftharpoons CH_3.CHOH.COOH$) correlates in an intelligible scheme many of the known metabolic changes of carbohydrates.

The discovery of the enzyme glyoxalase [Dakin and Dudley, 1913, 1], which, detectable in all mammalian tissues with the exception of the pancreas, rapidly converts methylglyoxal into lactic acid, provided substantial support for the theory, and a large body of evidence, subsequently collected, has upheld the opinion that methylglyoxal is, in fact, an intermediary compound in normal glycolysis [see *e.g.* Toenniessen and Fischer, 1926; Ariyama, 1928, 2].

Now Lundsgaard recently [1930, 1] independently rediscovered the fact, first noted by Pohl [1887], that the muscles of animals, poisoned with iodoacetic (or bromoacetic) acid, form no lactic acid under conditions in which normal muscle produces considerable amounts of this substance. Here then is a simple chemical substance which prevents the normal breakdown of carbohydrate, presumably by interfering with one or more of the catabolic reactions involved, and possibly, therefore, with that brought about by glyoxalase. The experimental examination of this possibility, which forms the basis of this paper, proves that sodium iodoacetate is in fact a powerful inhibitor of the action of glyoxalase.

Experimental.

In all the experiments here mentioned the breast-muscle of chickens was used as the source of the enzyme, since this tissue yields highly active extracts.

The technique of determining glyoxalase activity was that of Dakin and Dudley [1913, 4] in which the muscle extract is incubated with phenylglyoxal

in the presence of an excess of freshly precipitated calcium carbonate. *l*-Mandelic acid is produced, and a determination of its optical rotation gives a measure of the enzymic activity.

Glyoxalase is very sensitive to acids [Dakin and Dudley, 1913, 2] and it is for this reason that calcium carbonate is used to neutralise the acid formed during the reaction. It was obviously not permissible, therefore, in experiments on the effect of iodoacetic acid on the enzyme, to add the acid itself, and solutions of the neutral sodium salt were employed.

Numerous experiments concerning the effect of this salt on the action of glyoxalase *in vitro* were performed; in the interests of economy of space only one of these is described, which displays all the points of interest revealed in earlier experiments from which the best conditions for demonstrating the inhibitory effect of sodium iodoacetate were evolved.

Effect of sodium iodoacetate on the action of glyoxalase in vitro.

The minced breast-muscle of two chickens, which had been killed with chloroform, was digested with five times its weight of distilled water with frequent stirring at room-temperature for 2 hours. The extract was then filtered through muslin.

In the meantime a solution containing 3 mg. iodoacetic acid, neutralised with its equivalent of sodium hydroxide, per cc., a solution containing 20 mg. phenylglyoxal per cc., and an aqueous suspension of freshly precipitated calcium carbonate had been prepared.

All these liquids were first warmed to 37° and then in each of 8 flasks were placed 10 cc. of calcium carbonate suspension, 50 cc. of muscle extract and 2 drops of toluene. Each of the flasks 1–5 then received 10 cc. of sodium iodoacetate solution, and each of the flasks 6–8 (controls) 10 cc. of water. The flasks were kept at 37° and 10 cc. of phenylglyoxal solution were added to the series as follows: immediately to flasks 1 and 6; after 1 hr. to flask 2; after 2 hrs. to flasks 3 and 7; after 3 hrs. to flask 4; after 4 hrs. to flasks 5 and 8.

Each flask was withdrawn from the hot-room 20 hrs. after it received its phenylglyoxal, and the contents were worked up as follows.

25 g. ammonium sulphate were added and the flask was heated in a boiling water-bath for 5 mins. To the cooled contents were added cautiously 8 cc. of syrupy phosphoric acid. After standing for 30 mins. the liquid was filtered through a Büchner funnel; the precipitate was not washed. The filtrate was extracted 4 times with 10 cc. quantities of ether, and the combined extract was washed with 5 cc. water. The ether was then removed *in vacuo* and the residue dissolved in 7 cc. water. After filtration through paper in which a very small amount of charcoal had been placed the rotation of the Hg_{5461} line was read in a 1 dm. tube.

The experimental result is given in Fig. 1.

The points on curve *A* represent the rotations of the *l*-mandelic acid formed

in flasks 6, 7 and 8 and indicate that no appreciable loss of enzymic activity occurs when a glyoxalase solution is incubated in the presence of calcium carbonate at 37° for 4 hrs. before adding the substrate. The points on curve *B* represent the rotations of the *l*-mandelic acid formed in flasks 1–5 and the curve shows the rate of inactivation of glyoxalase by sodium iodoacetate under the prescribed experimental conditions.

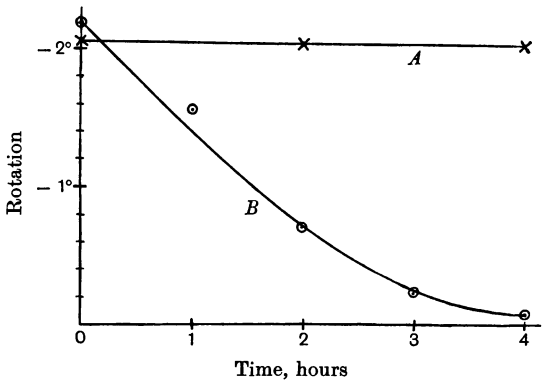

Fig. 1. *A* is the control glyoxalase curve. *B* shows the inactivation of glyoxalase by sodium iodoacetate.

To ensure comparable results in experiments of this kind the technique must be rigidly constant.

It is advisable to use flasks of equal capacity and shape. They should be large enough to allow the reaction mixture to lie in a shallow layer so that the calcium carbonate, which sinks to the bottom, may act reasonably efficiently as neutraliser of the acid formed. In these experiments 350 cc. Erlenmeyer flasks were employed.

The amount of toluene added as an antiseptic should be small and accurately measured since this substance itself has a definite inhibitory action on glyoxalase [Dakin and Dudley, 1913, 4].

The ether used for extracting the mandelic acid in experiments where iodoacetate has been added should be reasonably pure. Ether containing much peroxide liberates free iodine during the extraction. If this occurs a few drops of thiosulphate solution should be added to the water used for washing the combined ether extract.

Effect of sodium iodoacetate on glyoxalase in vivo.

4 cc. of a solution of sodium iodoacetate, equivalent to 26 mg. iodoacetic acid, were injected under the skin of the back into a chicken weighing 385 g. The first symptoms were noticed after 20 mins., the bird becoming lethargic. Its weakness steadily increased, until after $2\frac{3}{4}$ hrs. it died apparently from circulatory failure. A normal control bird of about the same weight was killed with chloroform and the breast-muscle of each was minced and digested for $2\frac{1}{2}$ hrs. at room temperature with five times its weight of distilled water. 50 cc. of each extract, filtered through muslin, were incubated at 37° for 16 hrs. with 10 cc. of calcium carbonate suspension and 10 cc. of a solution containing 0·2 g. phenylglyoxal, 2 drops of toluene being added to each flask as antiseptic. The mandelic acid formed was extracted in the manner already

described and the rotations were observed under the same conditions as in the previous experiment.

A second experiment in which a bird weighing 610 g. received 40 mg. iodoacetic acid as sodium salt in 4 cc. water was performed. The poisoned bird died 3¼ hrs. after the injection, and a control bird was killed for comparison at the same time. The rotations of the *l*-mandelic acid formed in the two experiments were as follows:

Normal chickens	Poisoned chickens
−2·06°	−1·24°
−2·02°	−1·19°

Although in many instances it is not justifiable to take tissues of one normal animal as controls against which to compare corresponding tissues of an experimental animal, it is the writer's experience that, with a rigid adherence to technique, the glyoxalase activity of a given tissue in a series of normal animals of the same kind is remarkably constant. The results quoted above are therefore considered to be significant, and the effect of poisoning with iodoacetic acid appears to have reduced the glyoxalase activity of the breast-muscle of these chickens nearly to one-half that of the same muscle of normal birds at a time when death supervenes.

Discussion.

The experiments described in this communication demonstrate that sodium iodoacetate is a powerful inhibitor of the tissue enzyme which converts methylglyoxal into lactic acid.

The significance of this observation is considerably enhanced by Lundsgaard's investigations which have shown that sodium iodoacetate is not a general enzymic poison; it disturbs neither glycogenolysis nor the normal breakdown of phosphagen in a muscle in which it has completely suppressed the formation of lactic acid [Lundsgaard, 1930, 2]. In particular is it noteworthy that it does not interfere with the actions of the carbohydrate-splitting enzymes invertase and ptyalin, and, whilst it prevents the alcoholic fermentation of yeast, the oxidative metabolism is unaffected by it [Lundsgaard, 1930, 2, 3]. In the light of these interesting experiments on the scope of its action Lundsgaard forms the opinion that sodium iodoacetate inhibits specifically the process of glycolysis, interfering, therefore, at some stage of the degradation of glucose to lactic acid. The work here presented indicates a specific inhibition of the reaction which has been postulated as that immediately concerned with the production of lactic acid in tissues, thus supporting at the same time Lundsgaard's conclusion and the view that methylglyoxal is indeed the immediate precursor of lactic acid in the normal glycolytic chain of reactions.

Another inhibitor of the action of glyoxalase has been known for some time. In the course of their early work on glyoxalase Dakin and Dudley [1913, 4] observed that pancreatic extracts inhibited the action of the enzyme,

and they named the factor "antiglyoxalase." It is very interesting to note that both antiglyoxalase and sodium iodoacetate exert their effect on the enzyme in the same manner; neither inhibitor acts instantaneously, the degree of inhibition increasing with the length of time of contact between inhibitor and enzyme. The curve shown in Fig. 1 is of precisely the same type as is obtained in similar experiments with antiglyoxalase, and could be closely imitated by using an appropriate pancreatic extract.

Some time after Dakin and Dudley's description of antiglyoxalase Winfield and Hopkins [1915] announced that pancreatic extract was capable of inhibiting the formation of lactic acid in muscle.

The assumption of identity between antiglyoxalase and Winfield and Hopkins's factor was rendered difficult at that time mainly by their statement that the latter was heat-stable (and could therefore be neither trypsin, amylopsin nor lipase) whilst Dakin and Dudley had found that antiglyoxalase was readily destroyed by heat. Further differences appeared when Foster [1925] claimed to have separated them by means of 70 % alcohol in which the lactic acid-inhibiting factor was said to be soluble whilst antiglyoxalase was not. On this point, however, her evidence does not carry full conviction, for in several of her experiments the alcoholic extracts displayed antiglyoxalase action. It is true that this was slight in comparison with the inhibition of lactic acid formation in muscle caused by these preparations, but since she allowed her extracts to act on glyoxalase solutions for only 1 hr. before adding the substrate, it is clear, remembering that antiglyoxalase develops its inhibitory effect in a manner closely similar to that shown for iodoacetate (Fig. 1), that only slight inhibition would be expected, and that this would bear no simple quantitative relationship to the results which she obtained on the inhibition of lactic acid production in muscle.

Some confirmation of the inhibitory factor's heat-stability, as reported by Winfield and Hopkins, was perhaps provided by the partial survival of activity in her preparations after autoclaving for 20 mins. at 120°, although it should be noted that they were tested only with respect to antiglyoxalase.

When phenylglyoxal is incubated in muscle extracts which have been inactivated with respect to glyoxalase, or in pancreatic extracts which contain none of this enzyme, a yellow colour, or yellow precipitate, appears. Foster considered that this phenomenon most probably indicated that pancreatic extract acted on the substrate rather than on the enzyme, and doubted the existence of "antiglyoxalase" in the sense postulated by Dakin and Dudley. This conclusion has been negatived by Ariyama [1928, 1], and recently Giršavičius [1930] has stated that the production of the yellow colour is entirely independent of the antiglyoxalase activity of the pancreas. Being apparently unaware of Dakin and Dudley's observation [1913, 1] that phenylglyoxal readily condenses with arginine, ornithine, histidine and lysine, yielding sparingly soluble yellow compounds, he rediscovered the clue to the proper explanation of the phenomenon, which is undoubtedly caused by

condensation of phenylglyoxal with basic amino-acids either in, or arising from, the protein of the tissue extracts.

Further investigations into the nature of the substance in pancreatic extracts which inhibits the formation of lactic acid by muscle have led McCullagh [1928] and Case and McCullagh [1928] to the conclusion that it is merely amylase, which acts by virtue of its power of preventing the formation of hexosephosphates, an explanation which has been challenged by Harrison and Mellanby [1930].

This conclusion is incompatible with Winfield and Hopkins's original observation on the heat-stability of the factor and with Foster's statement concerning its solubility in 70 % alcohol, for Harrison and Mellanby found that such solutions were practically devoid of amylolytic action.

As the problem stands at present, therefore, assuming the accuracy of the experimental observations briefly reviewed, it appears possible that there may be more than one mechanism whereby pancreatic extracts prevent the formation of lactic acid in muscle.

The significance and importance of antiglyoxalase in this connection is apparent from the work of Toenniessen and Fischer [1926; see also Ariyama, 1928, 2]. They have presented strong evidence that in a mixture of minced muscle and pancreas (or in mixed extracts of these organs) to which sodium fructosediphosphate had been added, methylglyoxal accumulated in detectable amounts. Similar experiments, using sodium iodoacetate as inhibitor, would be of considerable interest. According to Case and McCullagh's results, pancreatic amylase (or some associated substance) may interfere at an earlier stage of the degradation of carbohydrate; and the fact that a simple salt, sodium iodoacetate, inhibits lactic acid formation in muscle and the glyoxalase activity of muscle extracts, suggests the idea that there may occur in the pancreas a relatively simple compound, possibly heat-stable and soluble in 70 % alcohol, which also exerts an inhibitory power on glyoxalase, or on some other enzyme concerned in the process of normal glycolysis.

If this proved to be so, the conflict of evidence on the subject which has arisen in connection with this problem would be resolved.

In any event, however, there is good ground for the belief that the antiglyoxalase of pancreatic extracts does inhibit the formation of lactic acid in muscle by virtue of its inhibitory action on glyoxalase; and the fact that sodium iodoacetate, which suppresses the formation of lactic acid in muscle, also inhibits the action of glyoxalase, provides significant support to that belief.

Summary.

1. Sodium iodoacetate, which Lundsgaard has shown to suppress lactic acid formation in muscle, is a powerful inhibitor of the enzyme glyoxalase which converts methylglyoxal into lactic acid. The available evidence supports the view that sodium iodoacetate owes its power of interfering with normal glycolysis to this fact.

2. The probability that methylglyoxal is the immediate precursor of lactic acid in the glycolytic chain of reactions is strengthened by this observation.

3. The inhibitory actions of sodium iodoacetate and pancreatic anti-glyoxalase on glyoxalase are similar in character: the significance of this finding is discussed in connection with the conflicting reports on the nature of the factor in pancreatic extracts which inhibits the formation of lactic acid in muscle.

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