LIII. STUDIES ON ANTIGLYOXALASE.

I. THE ACTION OF PANCREATIC EXTRACT ON PHENYLGLYOXAL.

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While investigating the distribution of glyoxalase in various animal tissues, Dakin and Dudley [1913] found that the pancreas is distinguished from all other animal organs in that it not only contains no glyoxalase itself, but also inhibits the glyoxalase action of other tissues with which it is incubated. They assumed the existence in pancreas of an inhibiting substance which they called antiglyoxalase. Some further work on antiglyoxalase has been published by the same authors [1914], by Foster [1925], by Kuhn and Heckscher [1926] and by Ariyama [1928]. Nothing definite is known, however, about the nature and the mode of action of antiglyoxalase, and the interest which attaches to the transformations of methylglyoxal as probable important steps in the intermediary metabolism of carbohydrates seemed to warrant a further investigation of the problem. Both Dakin and Dudley, and Ariyama are agreed that the inhibiting action of antiglyoxalase develops gradually when the pancreatic extract is incubated together with a glyoxalase solution. The fullest inhibition is only obtained when the phenyl- or methylglyoxal is added after several hours' incubation of glyoxalase with antiglyoxalase. This seems to indicate that the inhibition is due to a reaction of the inhibiting factor with the enzyme, but it is not possible to decide on the present evidence whether glyoxalase is destroyed in the process or merely inactivated by combination with antiglyoxalase. Foster came to a different conclusion regarding the mode of action of antiglyoxalase. This author observed that whenever phenylglyoxal is incubated with pancreas, with or without addition of other tissue extracts, an orange colour develops in the solution. Owing to an apparent parallelism between the intensity of the colour developed and the intensity of the inhibition found, she suggested that the inhibiting action of pancreas might be due to the combination of some substance present in it with the glyoxal, forming a coloured compound resistant to the action of glyoxalase. The action of antiglyoxalase would thus be not on the enzyme but on the substrate. While the present work fully confirms Foster's observation, it will be seen that the phenomenon described by her is entirely independent of the antiglyoxalase action of pancreas.

Technique.

Dakin and Dudley, and Foster followed the action of glyoxalase by estimating polarimetrically the mandelic acid formed. Kuhn and Heckscher estimated methylglyoxal in the solution by an iodimetric method and thus followed its disappearance, whilst Ariyama used for the same purpose a colorimetric method based on the reduction of Benedict's uric acid reagent by glyoxals in presence of KCN. It was thought that a manometric method, based on the principle of Warburg's method for following glycolysis, might prove more rapid and less laborious. Warburg's technique had already been used by Meyerhof [1925] for following the transformation of methylglyoxal into lactic acid by tissue slices. As no Warburg manometers were available and the work had to be done with manometers of the Barcroft type, it was first necessary to adapt the technique so as to allow it to be used with the Barcroft manometer. Warburg's method consists in measuring manometrically the CO_2 expelled from a solution containing $NaHCO_3$ by the lactic acid produced. To adjust the p_H it is necessary to equilibrate the solution against a CO_2 atmosphere, the CO_2 tension required depending on the $NaHCO_3$ concentration and on the p_H desired. The Barcroft manometer cannot be filled with a gas by passing it through the cups as in Warburg's method. Instead it is necessary to evacuate it and fill it with the gas required, a process which has to be repeated at least twice, to assure a fairly complete replacement of the air. Filling the manometers in this way with air-CO_2 mixtures did not lead to constant results, and the following technique was adopted after some trials.

The manometers are connected with the pump and with an aspirator bottle filled with CO_2 from a cylinder. They are also connected with a mercury manometer and with a gas burette, used for checking from time to time the purity of the CO_2. The Barcroft manometers are twice evacuated and filled with CO_2; thereupon they are again connected with the pump and the pressure in the whole system is lowered to the tension of CO_2 desired. The process is followed by means of the mercury manometer. A correction has of course to be applied for the difference in water-vapour tension in the Barcroft cups at 38° and in the connections with the mercury manometer at room temperature. Now the manometer cups are lowered into the thermostat in the usual way, and the shaking mechanism is started. After about 5 minutes the taps of the manometers are closed. Though pressures of about one-fifth of an atmosphere within the cups have been used most of the time, no trouble has been experienced with leakages. The $NaHCO_3$ and CO_2 concentrations required were calculated on the basis of the Henderson-Hasselbalch formula, modified by Hastings and Sendroy [1925] so as to take account of the activities. Four Barcroft manometers were used for each experiment, run as two duplicate pairs, a necessary precaution since duplicates, whilst usually agreeing within about 3–5 %, were occasionally found to diverge more widely. Both cups of the manometer contained the same amounts of water, $NaHCO_3$

solution, enzyme, etc. A small glass cup with a platinum hook was hung on the edge of the KOH tube of the manometer cup. That in the right-hand cup contained the glyoxal solution (usually 0·3–0·6 cc.) whilst that in the left cup contained an equal volume of water. After the taps have been closed, as described above, the initial reading is taken and the small cups are upset by slightly lifting the manometer and tapping with the finger.

Results and discussion.

Fig. 1 shows the effect of adding pancreatic extract to a glyoxalase solution in a Barcroft manometer. Minced liver, extracted with about five times its volume of water, was used (as also in the following experiments) as glyoxalase solution. The pancreatic extract was prepared in a similar manner. The curve without pancreas was obtained with 0·6 cc. liver extract in the cups, for the curve with pancreas 0·6 cc. liver extract and 1·0 cc. pancreatic extract were used. It will be seen that, instead of inhibiting, the pancreas seems at first actually to accelerate the reaction, and only after about 2 hours does the

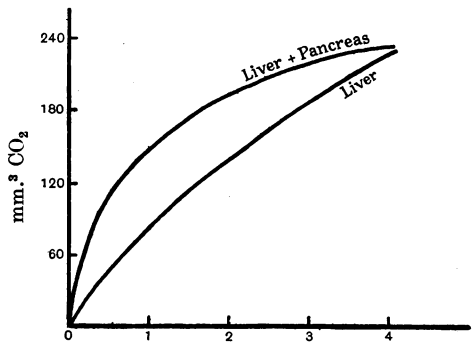

Fig. 1.

reaction velocity with pancreas begin to fall off compared to that with liver alone. Controls were thereupon made with pancreas alone, and it was found that this causes on addition of phenylglyoxal an evolution of CO_2, that is, an acid production, of the same order approximately as an equal volume of liver extract. Hence the effect described seems to be due to the action of the pancreas superposed on that of the liver-glyoxalase, rather than to an accelerating action of the former on the latter. Ariyama, measuring the methylglyoxal disappearance, also found an apparent acceleration at first after adding pancreas, and several hours were required before inhibition began to develop. He suggests that some glyoxalase may be present in pancreas, without bringing any evidence to support this suggestion, beyond the fact that some methylglyoxal disappears on incubation with pancreatin alone. To test whether the acid production on addition of phenylglyoxal to pancreatic extract is due to a transformation of the former into mandelic acid, pancreatic extract was incubated with phenylglyoxal in comparatively large scale

experiments and an attempt was made to isolate mandelic acid by the method described by Dakin and Dudley [1914]. Several such experiments were made, but all with completely negative results. Instead, it was found that, as described by Foster, an orange or brownish colour develops in the solution and on longer incubation with high initial phenylglyoxal concentrations a fairly copious yellow precipitate is deposited. The precipitate, after centrifuging off, was found to consist of a substance readily soluble in alcohol or ether but only very sparingly in water. Attempts to recrystallise it from ether proved fruitless, as a yellow oil remained which on standing gradually solidified to a resinous substance. That the action of the pancreatic extract on phenylglyoxal is not due to an enzyme follows from the fact that prolonged boiling or even autoclaving does not affect its activity. Even 2 hours' boiling

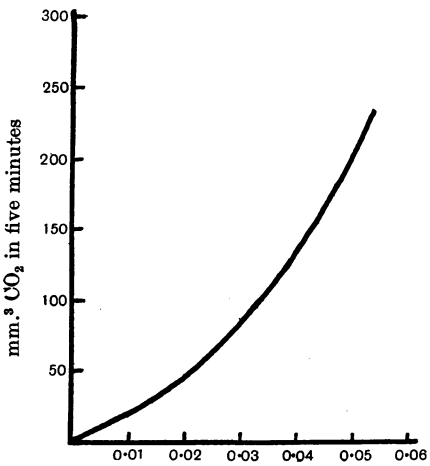

Fig. 2.

with about 20 % HCl or KOH does not cause more than a 20 % decrease in activity. In Fig. 2 the effect of variations in the concentration of phenylglyoxal on the velocity of its reaction with pancreas is shown. The molar concentration of phenylglyoxal in the solution is plotted against the CO_2 (in mm.³) evolved in 5 minutes. The shape of the curve suggests that two molecules of phenylglyoxal may enter into the reaction. On the other hand, the reaction velocity is strictly proportional to the amount of pancreatic extract taken. The effect of changing the p_H between 7·0 and 7·9 was investigated and a rapid rise in reaction velocity with increasing alkalinity was found. Below p_H 7·0 the reaction is negligible. The substance in pancreas, responsible for the reaction with phenylglyoxal, is destroyed by treating with $NaNO_2$ and acetic acid, which suggests that we are dealing with an amino-compound.

It is clear that any reaction involving the condensation of $-NH_2$ groups with a glyoxal must lead to an increase in the acidity of the solution and

hence to an evolution of CO_2. The effect would somewhat resemble that made use of in Sørensen's method of amino-acid titration.

It is well known that phenylglyoxal reacts with ammonia and amines by condensation. Pinner [1902, 1905] identified the products of the action of ammonia on phenylglyoxal as consisting partly of 1-benzoyl-4-phenyliminazole and partly of 1 : 4-diphenyl-3-hydroxypyrazine. The products of the action of amino-acids on glyoxals have not yet been investigated. Neuberg and Kobel [1927] describe the action of amino-acids and related compounds on methylglyoxal. On heating the solution they observed extensive decomposition, with production of CO_2 and acetaldehyde. In the cold they only observed a change in rotation and formation of "melanin."

Attempts were made to isolate from the pancreas the substance or substances responsible for the reaction. It proved possible to precipitate with tannic acid much of the protein and peptone present in the extract, without markedly diminishing its power of reacting with phenylglyoxal. The tannic acid was removed by baryta. On treating the tannic acid filtrate with phosphotungstic acid the substances looked for were completely precipitated. This is evidence in favour of the supposition that we are dealing with amino-compounds. Repeated attempts to isolate these substances from the phosphotungstic acid precipitate by the usual procedure proved fruitless. The occurrence of the substances in question in other sources than pancreas was then investigated. It was found that liver, muscle and kidney are completely inactivated by 5 or 10 minutes' boiling. On the other hand, boiled yeast extract and boiled extract of testis were found to possess an activity similar to that of pancreas. Pancreas, yeast and testis are known to be particularly rich in histones, containing a high proportion of diamino-acids. On allowing phenylglyoxal to act on various amino-acids in Barcroft manometers, as described above, it was found that whilst monoamino-acids are inactive, the diamino-acids, histidine, arginine, and lysine give a reaction very similar to that obtained with pancreas. Both acid production and the development of an orange or yellow colour were observed.

Reacting as an aldehyde, phenylglyoxal should of course condense with monoamino-acids in a way analogous to the condensation obtained with formaldehyde. Using, in the Barcroft method described, formaldehyde or acetaldehyde, reaction rates too rapid to be measured were obtained both with mono- and with di-amino-acids. No colour develops in the solution when formaldehyde or acetaldehyde reacts with an amino-acid, and this, together with the fact that only diamino-acids react with phenylglyoxal seems to show that the two reactions are quite distinct. The curve shown in Fig. 2, indicating that two molecules of phenylglyoxal may enter the reaction, confirms this view. It is interesting to note that the substances obtained in this reaction somewhat resemble, in their physical properties, the products of the action of ammonia on phenylglyoxal, as described by Pinner. According to Mr Pirie (unpublished communication) chloral hydrate produces no shift of

p_H in a solution of alanine. It seems at any rate possible that the fact that phenylglyoxal exists as a hydrate is an important factor in determining the reactions described in this paper.

Whilst it has been shown that the diamino-acids give with phenylglyoxal a reaction similar to that obtained with pancreatic extract, the question remains whether the diamino-acids present in the extract are sufficient to account for the whole of the reaction obtained. A number of experiments were made, in which the amino-nitrogen and the reaction velocity with phenylglyoxal of a boiled pancreatic extract and of a histidine solution were compared. The histidine solution was found to possess only two-thirds of the activity of the pancreatic extract, whilst it contained 1·4 times as much amino-nitrogen. As the whole of the free amino-nitrogen of the pancreatic extract cannot be derived from diamino-acids the disparity of the reaction rate and the diamino-acid concentration must be still greater than is indicated by the above figures. No special search was made for other substances in pancreas giving the same reaction. But it was found that glycylglycine shows the reaction, and so does liver juice after partial acid hydrolysis. Hence it appears that polypeptides are also capable of reacting with phenylglyoxal. No quantitative experiments were attempted to show whether the polypeptides plus diamino-acids could account for the whole of the reaction obtained with pancreatic extract, or whether some further substances have to be taken into account.

That the antiglyoxalase action of pancreatic extracts cannot be due to removal of the glyoxal by the reaction described in this paper, as suggested by Foster, seems to follow from a number of results described in the literature. Neither yeast nor testis extracts show any antiglyoxalase activity (Dakin and Dudley). Both Ariyama and Kuhn and Heckscher observed antiglyoxalase action while following methylglyoxal disappearance and not, like Dakin and Dudley or Foster, mandelic acid production. Finally the complete thermostability and easy dialysability of the substances responsible for the reaction here described disagree with the properties ascribed to antiglyoxalase by the above workers. The problem is being further investigated.

Summary.

1. A method is described for following acid production from phenylglyoxal by means of the Barcroft manometer.

2. A production of acid, similar at first sight to that obtained with phenylglyoxal and liver extract, is obtained when phenylglyoxal is added to a pancreatic extract.

3. It is shown that this acid production is not due to a transformation into mandelic acid, but to a reaction with amino-compounds, partly diamino-acids, with formation of a sparingly soluble orange substance. The reaction seems to be identical with that described by Foster.

4. The diamino-acids present in pancreatic extract cannot account for the whole of the reaction obtained, but it is found that polypeptides, including those of monoamino-acids, also react, while the monoamino-acids themselves do not.

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REFERENCES.

Ariyama (1928). *J. Biol. Chem.* **77**, 359.
Dakin and Dudley (1913). *J. Biol. Chem.* **15**, 463.
—— —— (1914). *J. Biol. Chem.* **16**, 505.
Foster (1925). *Biochem. J.* **19**, 757.
Hastings and Sendroy (1925). *J. Biol. Chem.* **65**, 444.
Kuhn and Heckscher (1926). *Z. physiol. Chem.* **160**, 154.
Meyerhof (1925). *Biochem. Z.* **159**, 433.
Neuberg and Kobel (1927). *Biochem. Z.* **185**, 477.
Pinner (1902). *Ber. deutsch. chem. Ges.* **35**, 4131.
—— (1905). *Ber. deutsch. chem. Ges.* **38**, 1531.