CXCV. STUDIES ON ANTIGLYOXALASE.

II. METHODS OF FOLLOWING GLYOXALASE ACTION.

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Criticism of the manometric method.

IN a previous paper [Giršavičius, 1930] an attempt at following glyoxalase action by a manometric method was described. It was thought that the evolution of CO_2 in a bicarbonate- CO_2 buffer system would provide a convenient means of following the production of lactic or mandelic acid under the action of glyoxalase. It was soon found, however, that a gas evolution, indistinguishable from that caused by the investigated reaction, takes place when phenylglyoxal and pancreatic extract are brought together. Further investigation showed that this reaction is not abolished by first boiling the pancreatic extract, and that boiled extracts of testis and of yeast show the same phenomenon, whilst boiled liver and kidney extracts do not. As the reaction is abolished after treating the tissue extract with nitrous acid, or after precipitation with phosphotungstic acid, it was considered to be due to amino-compounds. It was then shown that, although monoamino-acids do not cause more than a slight gas evolution with phenylglyoxal, diamino-acids cause a rapid gas evolution; the conclusion was drawn that diamino-acids or related compounds, or polypeptides containing a high proportion of diamino-acids, are responsible for the reaction in question. Later work has shown, however, that proteins and peptones also cause a gas evolution in the Barcroft manometer, and that this reaction is apparently characteristic for all protein derivatives, although with monoamino-acids it is comparatively slow.

The difference between liver and kidney extracts on the one hand and extracts of pancreas, yeast and testis on the other hand seems to be due to differences in the amount of non-heat-coagulable N they contain, rather than to the proportion of diamino-acids entering into their composition.

Exp. 1. In the following experiment the effects of liver and of pancreatin on phenyl- and on methyl-glyoxal were compared. 0.084 *M* solutions of phenyl- and methyl-glyoxal were prepared, making use of the modified Friedemann method described in this paper.

0.5 cc. of these was tipped into the following solutions:

- (1) with liver extract,
 - (a) 1 cc. liver extract, 1 cc. water, 0.7 cc. sodium bicarbonate solution, and, in the small cup, 0.5 cc. phenylglyoxal.
 - (b) like (a), but methylglyoxal instead of phenylglyoxal;
- (2) with pancreatin solution (2 % pancreatin B.D.H.),
 - (a) 1 cc. pancreatin solution instead of liver extract, otherwise as (1, a),
 - (b) like (a), but with methylglyoxal.

All solutions had a p_H of 7.6. The results are given in mm.³ CO₂.

Minutes	(1 a)	(1 b)	(2 a)	(2 b)
4	110	60	37	14
8	154	78	47	20
15	198	98	55	29
21	225	114	—	34

Both with liver and with pancreatin the reaction proceeds at almost exactly twice the rate with phenylglyoxal as with methylglyoxal. This seems to show that not only the reaction with pancreatin, but also the reaction with liver observed under these conditions, is largely the protein reaction, rather than the reaction due to glyoxalase.

It is clear from the foregoing results that the manometric method cannot be used for following glyoxalase action. In no case is it possible to be certain how much of the reaction observed is due to glyoxalase and how much to the proteins and protein derivatives present in the solution.

In a recent paper, Lohmann [1931] has used the manometric method for a study of the co-enzyme of glyoxalase. From his results he concludes that glyoxalase preparations inactivated by dialysis, and, to a somewhat lesser extent, fresh glyoxalase preparations, are activated by the addition of a "co-enzyme" preparation. This he obtained by treating a trichloroacetic acid extract of yeast with lead acetate and mercuric nitrate. Unfortunately he gives no control figures for the effect of this preparation itself on methylglyoxal; it is therefore impossible to judge from the results he reports whether he was dealing with a true co-enzyme, or whether the activation observed was really a summation of two reactions (as when pancreatin is added to liver extract).

Since the manometric method had thus proved itself unsuitable as a means of investigating glyoxalase action, a different method had to be sought. Two ways of attacking this problem presented themselves. One could either, as Dakin and Dudley (and in some of their work Kuhn and Heckscher [1926, 1, 2] had done, estimate polarimetrically the mandelic acid produced from phenylglyoxal; or, like Kuhn and Heckscher in the main part of their work, Ariyama [1928] and Sakuma [1930], follow, by some suitable method, the disappearance of the glyoxal. As the estimation of the acid is rather laborious, the second way was chosen. It was clear, of course, that the reaction with proteins would have to be taken into account, and might interfere considerably; but whether this

effect would be large enough to make this method as unsuitable as the manometric method, could not be decided *a priori*—especially as such a method had already been used by the above authors. The first problem was to find a satisfactory way of estimating glyoxals.

Modification of Friedemann's method for use as a micro-method.

Two methods were available for estimating glyoxals; the iodimetric method of Kuhn and Heckscher, and the colorimetric method of Ariyama¹. Of these the former seemed less satisfactory, as it is not quite specific, but mainly because of the two-hour periods required before each titration. Ariyama's method has the disadvantage that it can only be used within narrow limits of concentration with one standard. A rapid titrimetric method seemed preferable, if it could be designed. Friedemann [1927] had shown that glyoxals are quantitatively oxidised by hydrogen peroxide in alkaline solution giving two equivalents of acid for each mol. of glyoxal. Phenylglyoxal, for instance, is oxidised to an equimolar mixture of benzoic and formic acids. Friedemann based on these facts a method for estimating various glyoxals by titrating the acids produced. This method has not been applied in actual practice, except by Ariyama, who used it to standardise his methylglyoxal solutions. It seemed probable that if it could be modified into a micro-method of sufficient accuracy and sensitivity, Friedemann's method would have the advantage of being very rapid and simple to carry out.

For accurate acidimetric titration of dilute solutions it is necessary to determine the end-point with reference to a standard. It is also necessary to control the indicator concentration, which can be done in two ways: either the standard is diluted parallel with the titrated fluid, or else more indicator is added during the titration, so as to keep its concentration constant. The first method is preferable, as coloured solutions can be titrated fairly accurately by its use. It is convenient to carry out the titrations in front of a box with a plate glass front illuminated from inside.

The following solutions are placed in five boiling-tubes: *A*, *B*, *s*, *1*, *2*.

A, CO₂-free water, containing 1 cc. phenolphthalein per 10 cc., and adjusted to a rose-pink colour with a few drops of *N*/50 NaOH.

B, 20 vol. H₂O₂ diluted 1 : 5, containing 1 cc. phenolphthalein per 10 cc., and adjusted to the same colour as *A*. The colour of this solution gradually fades on standing, owing to destruction of the indicator; from time to time a few drops of phenolphthalein have to be added, to readjust the colour.

¹ Recently Barrenscheen and Dreguss [1931] have published another method for the estimation of glyoxals. They claim it to be very specific, and, judging by their figures, it seems to be more sensitive and perhaps more accurate than the method described in this paper. On the other hand, it is far more cumbersome. Where specificity, or great accuracy at high dilution, are required, the method of Barrenscheen and Dreguss (or Ariyama's method) will be preferable. Where these considerations are less important, the method here described will probably be sufficiently accurate and at the same time simpler and more rapid.

s, *1*, *2*, receive 10 cc. each of the solution to be titrated, and 1 cc. phenolphthalein. *s*, which serves as standard for the titration, and the two other tubes, are then adjusted to the same colour as *A* and *B*.

1 cc. *A* is now added to *s*, and 1 cc. *B* to *1* and *2*. If the solution contains any methyl- or phenyl-glyoxal the colour in *1* and *2* will fade or disappear immediately on adding the hydrogen peroxide. *1* is then titrated with *N*/50 NaOH to a permanent red colour. The same amount of NaOH is added to *2* (it is convenient to have two burettes attached to the bottle containing the NaOH); to *s* is added the same volume of CO₂-free water. After standing about a minute, tubes *1* and *2* are adjusted to the colour of *s* with *N*/50 HCl, again with addition of CO₂-free water to *s*. It is clear that the sharpness of the end point will depend upon the absence of substances exerting a buffer action within the *p_H* range of the indicator used. With phenolphthalein as indicator phosphate has little buffering power. The buffer action of borate can be shifted into a lower *p_H* range by adding a glycerol solution to tubes *s*, *1*, *2*. On the other hand, carbonates present in the original solution, and CO₂ absorbed from the atmosphere, definitely diminish the sharpness of the readings. The figures of Table I may serve to illustrate the accuracy obtainable.

Table I.

Phenylglyoxal cc.	NaOH (found) cc.	NaOH (average) cc.	NaOH (calculated) cc.
3	6.08	6.06	—
3	6.02		
3	6.09		
1	2.03	2.01	2.02
1	1.99		
0.5	1.00	1.02	1.01
0.5	1.04		
0.5	1.05		
0.5	1.01		
5	2.10	2.13	—
5	2.16		
5	2.16		
5	2.13		
5	2.10		
2	0.84	0.86	0.85
2	0.87		
2	0.88		
2	0.84		
1	0.44	(0.43)	0.42
1	0.43		
1	0.42		
1	(0.61)		
1	0.42		

As 1 mol. of the various glyoxals gives rise to 2 equivalents of acid, 1 equivalent of NaOH corresponds to 36 g. methylglyoxal or 67 g. phenylglyoxal. 1 cc. *N*/50 NaOH corresponds to 0.72 mg. methylglyoxal, or 1.34 mg. phenylglyoxal.

For the estimation of phenylglyoxal in solutions containing tissue extracts, 25 cc. are pipetted into 50 cc. volumetric flasks, containing 2 cc. 10 % trichloroacetic acid. After standing for some minutes the liquids are made up

to the mark and filtered through dry pleated filters (Dreverhoff's "Rapid Filtrierpapier" is very convenient) into small dry flasks. Samples of 10 cc. are then pipetted out for analysis, and treated as described above. Separate experiments have shown that the concentration of phenylglyoxal is not altered by precipitating protein in the solution and filtering.

The main defect of the present method lies in its lack of specificity. Acetaldehyde does not interfere. Although it is gradually attacked under the conditions of this method, the reaction is so slow that even high concentrations of acetaldehyde introduce no appreciable error. On the other hand pyruvic acid and dihydroxyacetone behave exactly like methyl- and phenyl-glyoxal, and it is possible that this method may be of some use in their estimation, where confusion is out of the question. The possible confusion with dihydroxyacetone, and particularly with pyruvic acid, of course diminishes considerably the usefulness of the method for the estimation of glyoxals.

Investigation of glyoxalase action by following glyoxal disappearance.

In the work now to be described the modified Friedemann method has been made use of for following the disappearance of phenylglyoxal under the action of glyoxalase and under various other conditions. The following typical experiment on the action of pancreas will serve to illustrate the method as used in practice.

Exp. 2.

Solution (1): 20 cc. liver extract, 20 cc. phosphate buffer, $p_H = 7.6$, 20 cc. water.
Solution (2): like solution (1), but instead of water 20 cc. pancreatic extract.

These solutions were incubated overnight at 38° . 25 cc. phenylglyoxal solution were then added to each and samples were taken for analysis immediately and after 40 and 80 minutes. The following are the results, the figures being the differences between the initial and final titrations (cc. $N/50$ NaOH):

Initial phenylglyoxal = 6.49 cc. NaOH.

Minutes	(1)		(2)	
40	0.20	0.29	0.54	0.68
80	—	0.67	0.84	0.86

It will be seen that in the present experiment not only does the addition of pancreas not cause any inhibition, but in its presence actually more phenylglyoxal disappears than without it. The following experiment explains this phenomenon.

Exp. 3.

Solution (1): 50 cc. liver extract, 15 cc. buffer, 60 cc. water.

Solution (2): 50 cc. water, 15 cc. buffer, 60 cc. 0.4 % pancreatin B.D.H.

Solution (3): 50 cc. liver extract, 15 cc. buffer, 60 cc. pancreatin.

The solutions were incubated for $5\frac{1}{2}$ hours. 50 cc. of each were then pipetted into flasks containing 20 cc. buffer and 10 cc. phenylglyoxal solution. Samples were taken immediately and after 1 hour.

¹ In this as in all following experiments toluene was added as an antiseptic.

Initial phenylglyoxal = 4.27 cc. *N*/50 NaOH.

Phenylglyoxal destroyed after 1 hour equals (in cc. *N*/50 NaOH):

(1)		(2)		(3)	
0.95	0.93	0.19	0.18	1.17	1.10

It will be seen that the destruction of phenylglyoxal by liver and by pancreatin alone have been exactly summed, when both were incubated together; there was neither inhibition nor acceleration. It is, however, possible to demonstrate antiglyoxalase action, as the following experiments with a different sample of pancreatin show.

Exp. 4.

Solution (1): 50 cc. liver, 20 cc. water, 20 cc. buffer.

Solution (2): 50 cc. water, 20 cc. pancreatin solution, 20 cc. buffer.

Solution (3): 50 cc. liver, 20 cc. pancreatin solution, 20 cc. buffer.

After incubation overnight 30 cc. of these solutions were pipetted into flasks containing 20 cc. buffer and 10 cc. phenylglyoxal solution. Samples were taken immediately, after 35 minutes and after 1 hour and 30 minutes.

Initial phenylglyoxal = 2.58 cc. *N*/50 NaOH.

Minutes	(1)	(2)	(3)	(3)-(2)
35	0.52	0.06	0.39	0.33
150	1.50	0.33	0.76	0.43

In the fourth column the figures obtained by subtracting the phenylglyoxal destruction by pancreatin alone from the phenylglyoxal destruction by liver *plus* pancreatin, are tabulated. Whether this correction is applied or not, the figures show a considerable inhibition.

Exp. 5. In this experiment a larger amount of pancreatin was used. The solutions were treated as in the preceding experiment; the results alone will therefore be given.

Initial phenylglyoxal = 6.92 cc. *N*/50 NaOH.

The amounts of phenylglyoxal destroyed after 1 hour equal:

(1) Liver alone	(2) Pancreatin alone	(3) Liver <i>plus</i> pancreatin	
2.26	2.04	2.36	2.33

If the destruction of phenylglyoxal by pancreatin is quantitatively summed in (3) with the residual activity of the liver extract, almost complete inhibition is shown by these figures. This experiment, however, also shows clearly the reason why this method was finally abandoned as unsuitable for the study of glyoxalase action. The destruction of phenylglyoxal by pancreas alone is far too large to be ignored, in fact the correction that has to be applied for it is in many experiments considerably larger than the effect investigated—the inhibition by antiglyoxalase. It is impossible, under these conditions, to obtain reliable results.

Moreover, other experiments have shown that, as with the gas evolution measured manometrically, the destruction of phenylglyoxal by pancreatin and

pancreatic extracts is only a special case of a reaction with proteins and their derivatives. It is clear therefore that not only has the destruction of phenylglyoxal by liver treated with pancreas to be corrected for the destruction by pancreas alone, but, which is even more important, a considerable and not readily assessable proportion of the phenylglyoxal destruction by liver extracts (and those of other tissues) is due, not to glyoxalase, but to a reaction with the tissue proteins. This makes it impossible to interpret with any certainty the results obtained. It can be concluded from these experiments that it is impossible to follow glyoxalase action in more than the roughest qualitative manner by following the disappearance of the substrate. Glyoxalase can only be studied reliably by estimating the lactic or mandelic acid produced.

Much of the work so far done on glyoxalase (Kuhn and Heckscher, Ariyama, Sakuma) has depended on the estimation of glyoxals by various methods. The results obtained in this way have to be accepted with some care, as they cannot be relied upon to represent actual glyoxalase action. Only the work carried out with Dakin and Dudley's technique is free from this particular objection.

The rather irregular kinetics found by Kuhn and Heckscher [1926, 1] in their study of methylglyoxalase, and the variable results these authors obtained, for instance on dialysing their extracts, are probably partly to be explained by the phenomena described above. The p_H -activity curves obtained by Kuhn and Heckscher (fall in activity beyond p_H 8), as well as the diminishing activity of their extracts on standing, show that their results are at any rate partly, perhaps mainly, due to glyoxalase action. Non-enzymic methylglyoxal destruction must, however, have gone on simultaneously, so that their results cannot be interpreted with certainty. Kuhn and Heckscher [1926, 2] also noted the destruction of methylglyoxal by boiled tissue extracts, causing an apparent acceleration of glyoxalase, but did not follow up this observation. Sakuma's work calls for similar comments. This author observed the destruction of methylglyoxal by proteins and various amino-compounds. According to his observations the reaction is a rather complex one; simultaneously with the disappearance of methylglyoxal there is an equivalent production of NH_3 and CO_2 . This probably explains the gas evolution observed when using the manometric method, which in the previous paper of this series was tentatively, and apparently erroneously, ascribed to the action of some acid compound of phenylglyoxal and an amino-acid. Whilst Sakuma has studied this reaction in some detail, he has failed to see that it greatly diminishes the value of his work on the distribution of glyoxalase, and on the "co-enzyme" of glyoxalase. That such a co-enzyme is active in boiled tissue extracts, like those of Sakuma, is possible. In view of the work of the Neuberg school it is even quite probable. His results do not, however, prove the existence of such a co-enzyme, especially as he gives no control figures for the destruction of methylglyoxal by his "co-enzyme" solutions alone. In his Table V, for instance, boiled extracts of liver, muscle, and kidney are added to a liver and to a muscle extract. The percentage activations obtained differ widely, being far greater with the weakly active muscle extract. If, however, the differences, in other words the excess

activities when boiled extracts are added, are compared instead, it is found that each of the boiled extracts produces practically the same increase in activity, whether it is added to the liver or to the muscle extract. Again, if the results of his Table VI, where increasing amounts of boiled liver juice are added to liver extracts, dialysed for various periods of time, are plotted, it will be seen that the points fall practically on parallel straight lines, differing only in their point of origin, according to the activity of the liver extracts themselves. These results are more consistent with a summation of activities, as in Exp. 3 and others of the present paper, than with a co-enzyme activation. At any rate the question is still open. The inhibition by phosphate and the exceedingly interesting acceleration by sugars, which Sakuma reports, are more likely to be properties of glyoxalase itself. But all these require re-investigation.

It will be seen from the present paper, that only results obtained by estimating the acid produced can give a true picture of glyoxalase action. So far the polarimetric estimation of mandelic acid is the only method available for this purpose. This method, as used by Dakin and Dudley, is rather cumbersome. Some improvements have therefore been introduced, and will be described in a subsequent paper, together with the results obtained.

Summary.

1. Manometric methods, based on Warburg's glycolytic technique, are unsuitable for the study of glyoxalase as glyoxals cause a gas evolution with proteins and their derivatives.

2. A volumetric method for the estimation of glyoxals is described. This method makes use of the fact, discovered by Friedemann, that glyoxals are oxidised to two equivalents of acid by H_2O_2 in alkaline solution.

3. By means of this method it is shown that, owing to its relatively high rate, the reaction between glyoxals and proteins cannot be ignored, or even adequately corrected for. This makes results obtained by following glyoxal disappearance very unreliable as expressions of glyoxalase action. In this connection some of the work of other authors, who used similar methods, is discussed critically.

4. It follows that glyoxalase action can only be reliably investigated by estimating the acid produced.

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