XX. SOME PROPERTIES OF THE ENZYME GLYOXALASE.

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TECHNIQUE.

IN a previous paper [Giršavičius, 1931] it has been shown that, owing to a reaction of proteins with methyl- and phenyl-glyoxal, methods based on the estimation of these two substances are unsuitable for the study of glyoxalase. Reliable information about the action of this enzyme can only be obtained by studying the rate of formation of the reaction product—lactic or mandelic acid. Of the various techniques so far used for the investigation of glyoxalase only Dakin and Dudley's method of estimating mandelic acid polarimetrically [Dakin and Dudley, 1913] can be regarded as satisfactory. This method has however two disadvantages: it is rather laborious, involving four successive extractions with ether, and it is insufficiently sensitive. The second point is very important. In most enzyme kinetic studies it is essential to measure reaction velocities under conditions as nearly invariant as possible. With glyoxalase, even more than with most other enzymes, this means measuring the reaction velocity in the course of a small percentage transformation of the substrate. For the kinetics of glyoxalase are complicated by two factors: the non-enzymic reaction in which the substrate is rapidly destroyed, and the production of an acid from a neutral substance by the action of the enzyme.

To shorten the necessary reaction time, and yet obtain sufficiently large polarimetric readings, the volume to which the mandelic acid has to be made up for polarimetry has been diminished in the present work by using 2 dm. polarimeter tubes, holding 3–3.5 cc. Further, the rotation of the mandelic acid has been increased by the addition of uranyl nitrate. As discovered by Walden [1897] uranyl nitrate increases the rotation of mandelic acid 2.5-fold under suitable conditions. In the present experiments a 10 % solution of uranyl nitrate (12.75 g. $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ per 100 cc.) in 1 % NaOH was used. The mandelic acid solution is evaporated down, the residue is taken up with 4.5 cc. uranyl nitrate solution and filtered. The rotations obtained under these conditions are not directly proportional to the quantities of *l*-mandelic acid used, but increase less rapidly. In Table I some figures are given, from which a calibration chart can be constructed, and corrections, to be applied to the readings, obtained.

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Table I.

<i>l</i> -Mandelic acid solution cc.	Rotation
5	- 2·37°
5	- 2·32°
5	- 2·34°
10	- 4·46°
10	- 4·38°
25	- 10·08°
25	- 10·16°

Taken up in 4·5 cc. water, after evaporation, 5 cc. of the solution gave a rotation of $-0\cdot98^\circ$. The rotation is thus found to be increased 2·40 times at a concentration corresponding to a rotation of -1° in water. The increase at other concentrations can be calculated by means of the above table, since the rotation in water increases linearly at the dilutions here considered. In these experiments, as well as in the following ones of this paper, the polarimetric readings refer to the yellow line of the Hg spectrum ($\lambda = 577$). Using the green line ($\lambda = 546$) a rotation increase of 2·50 (at -1°) is obtained; the rotation in water is itself larger at the lower wave-length. Nevertheless the yellow line was used in these experiments, the light of the green line being absorbed more strongly by the solutions used. Only in the following, earlier, experiment were the readings made at $\lambda = 546$.

Fig. 1.

In Fig. 1 the results of an experiment on the rotation of mandelic acid in a uranyl acetate solution with varying quantities of alkali are recorded. This experiment was done before the present technique had been worked out, and the experimental conditions are different. It shows however that a 2·5-fold increase in rotation is about maximal. 5 cc. of a *l*-mandelic acid solution (the *l*-mandelic acid used in these experiments was prepared from amygdalin) with varying additions of 10 % KOH were made up to 50 cc. Excess of solid uranyl acetate, which according to Critical International Tables is soluble to 7·8 %, was added. After standing overnight the solutions were filtered and the

rotations read in a 4 dm. tube. The same quantity of mandelic acid gave in 50 cc. water a rotation of -1.21° .

The extraction method has also been modified, so as to diminish the number of extractions required, and at the same time obtain more nearly colourless extracts. The last point is specially important with the semimicro-polarimetry used in this work. At the time when lactic acid extraction used to be an integral part of lactic acid estimation this process had been markedly improved by the substitution of amyl alcohol for ether [Ohlsson, 1916]. To see whether a similar improvement was possible in the estimation of mandelic acid some experiments were made on its distribution between amyl alcohol and concentrated ammonium sulphate (acidified with H_2SO_4). A partition coefficient of about 28 was found¹, so that a single extraction with three times the volume of amyl alcohol should remove 98–99 % of mandelic acid from an ammonium sulphate solution. From the amyl alcohol the mandelic acid can be recovered by extracting twice with 20 cc. of 0.2 *N* NaOH. This leaves about 1–1.5 % mandelic acid in the amyl alcohol, as shown by the following experiment (among others). Varying quantities of mandelic acid solution had been extracted with 300 cc. lots of amyl alcohol. After running out the aqueous layers and washing the walls and stems of the separating funnels with 20 cc. of water, the amyl alcohol layers were shaken twice with 20 cc. lots of NaOH (about 1.5 mins. each time), then washed with 10 cc. water. The two NaOH extracts and the last washing were collected together as *a*. The process was repeated with one shaking with NaOH and one washing: *b*. (1 *a*) -3.72° ; (1 *b*) -0.06° ; (2 *a*) -6.89° ; (2 *b*) -0.09° .

Irregular results were at first obtained, which turned out to be due to the effect of small amounts of ammonium sulphate solution clinging to the walls of the separating funnel and passing into the NaOH extract. Ammonium sulphate diminishes the rotation of mandelic acid in uranyl nitrate solution. These remnants of ammonium sulphate are removed by washing with 10 cc. of water before extracting with NaOH. The partition coefficient of mandelic acid between amyl alcohol and water has been found to be about 3.8, so that 10 cc. of water would remove less than 1 % of mandelic acid from 300 cc. amyl alcohol.

The extraction is now carried out as follows. 100 cc. of the ammonium sulphate solution, containing the mandelic acid, are pipetted into 300 cc. amyl alcohol (coloured with Sudan III to emphasise the boundary) in a 500 cc. separating funnel. This is shaken for 2–3 mins. After separation the aqueous layer is run out. By a circular shake drops of the aqueous solution clinging to the walls are forced to the bottom; this is also run out. Now 10 cc. water are pipetted into the funnel, which is briefly shaken. After the water has been run out the stem of the funnel is rinsed with a jet of water. 20 cc. 0.2 *N* NaOH are pipetted into the funnel, which is shaken for about $1\frac{1}{2}$ mins. This process is repeated. The walls of the funnel are now washed with 20 cc. of water. The two

¹ No great accuracy is claimed for this figure, as the mandelic acid in the aqueous phase could not be determined directly, but had to be estimated as the difference between initial and final concentration in the alcohol, that is, as the small difference between two relatively large figures.

NaOH extracts and the final washing are run into a 3 in. crystallising dish. Between the extractions the stem of the funnel is washed out with a jet of water into the same dish. To the joint extracts about 1 cc. of 0.1 % aqueous solution of *p*-nitrophenol is added and 5 cc. *N* H₂SO₄. The neutralisation is finished with 0.2 *N* H₂SO₄. (Because of the high CO₂ content of the solutions it seems more convenient to over-acidify them at first, and finish the neutralisation with CO₂-free NaOH and 0.2 *N* H₂SO₄ after partial evaporation.) The solutions are evaporated on a boiling water-bath under a current of air. The dry residues are treated with 4.5 cc. uranyl nitrate solution, filtered, and the rotations are read as described above. The mandelic acid is not racemised during the evaporation, nor is its rotation affected by the Na₂SO₄ formed in the neutralisation. The following experiment illustrates the method. Samples of 10 cc. *l*-mandelic acid solution + 90 cc. concentrated ammonium sulphate (acidified) were treated as described. Readings: - 3.27°; - 3.38°; - 3.25°. 10 cc. of the solution neutralised and evaporated directly gave - 3.51°. 94 %, with a maximum deviation of ± 2.4 %, were recovered in these experiments.

For the estimation of mandelic acid in liver extracts Dakin and Dudley's method of deproteinising was used, in a slightly modified form. To 100 cc. of the liver extract 5 cc. of 60 % (by volume) H₂SO₄ were added and 70 g. of solid ammonium sulphate. After heating on a water-bath the solutions were made up to 150 cc. with concentrated ammonium sulphate solution and filtered. 100 cc. of the filtrate were treated as described. Very surprisingly it has been found that this method of deproteinising causes a considerable loss of mandelic acid. Details will not be reported here as the matter is still under investigation; in a fairly extensive series of experiments a recovery of 76.8 % of *l*-mandelic acid added has been found, with a probable error of ± 4.2 %.

ENZYME EXPERIMENTS.

In the following experiments the above technique has been used for the estimation of mandelic acid. The liver extracts used were prepared by shaking minced ox liver (fresh from the butcher) for an hour on the shaking machine with five times the weight of water, then filtering through muslin. The extracts were stored in the refrigerator and generally used 12 hours after they had been prepared. The first experiment deals with the effect of toluene on glyoxalase. Dakin and Dudley [1913] have stated that glyoxalase action is inhibited by toluene. Kuhn and Heckscher [1926], using methylglyoxal, and estimating the rate of disappearance of the substrate, have come to the opposite conclusion. They think that the inhibition in Dakin and Dudley's experiments may have been due to extraction of phenylglyoxal from the aqueous solution by the toluene.

Solution (1): 50 cc. liver extract, 20 cc. 10 % CaCO₃ suspension, 20 cc. water, 10 cc. phenylglyoxal solution (about 15 mg. per cc.).

Solution (2): like (1) but shaken with 2 cc. toluene before adding the phenylglyoxal.

Both solutions were incubated for $5\frac{1}{2}$ hours at 37° . Readings: (1) -2.70° ; -2.64° . (2) -1.96° ; -2.04° . There is an inhibition of 20.5 %. This can scarcely be accounted for by extraction of the substrate, but the question needs further investigation. All subsequent experiments were done without addition of an antiseptic. Dakin and Dudley state that phenylglyoxal itself acts as a mild antiseptic. In the present experiments no obvious signs of putrefactive changes were present after incubation.

The next point that needed investigation was the effect of suitable buffer substances on the activity of glyoxalase. According to Kuhn and Heckscher glyoxalase has a p_H optimum at about 8.2. At this p_H borate and phosphate buffer can be used (though with little buffering power), also the diethyl barbiturate buffer recently introduced by Michaelis [1931]. In the following experiment the activities of the enzyme in presence of various concentrations of these buffer-substances are compared.

Table II.

	Liver extract cc.	Na barbitone cc.	Water cc.	Phenyl- glyoxal cc.	Initial p_H	Final p_H
1	140	175	—	35	8.10	8.03
2	140	75	100	35	8.10	7.83
3	140	35	140	35	8.02	7.56
Phosphate						
4	140	175	—	35	—	7.87
5	140	75	100	35	8.05	7.79
6	140	35	140	35	7.98	7.66
Borate						
7	140	175	—	35	—	7.85
8	140	75	100	35	—	7.75
9	140	35	140	35	7.88	7.37

Fig. 2.

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The solutions were incubated for $16\frac{1}{2}$ hours at 26.5° . The diethyl barbiturate (barbitone) buffer was $0.070\text{ }M$ in barbitone, the phosphate buffer (Sørensen) was $0.2\text{ }M$ in PO_4 , the borate buffer (Palitzsch) $0.2\text{ }M$ in BO_2 . The results obtained are plotted in Fig. 2. On the abscissa the M buffer concentrations are plotted, in the terms just described. Unfortunately they are not strictly comparable in this way, which assumes that each buffer solution contains only one molecular species. In fact the diethyl barbiturate buffer contains both the undissociated acid and its ion, the phosphate buffer contains both HPO_4'' and $\text{H}_2\text{PO}_4'$ ions, whilst in the borate buffer several, partly polymeric, molecular and ionic species are present. How much of the inhibition found is due to one or other ion or molecule in the solution cannot be decided without further investigation, so that the inhibitions produced by the various buffers cannot be compared exactly. It appears however that borate has little inhibiting action, phosphate inhibits the enzyme markedly, whilst diethyl barbiturate has a strong inhibiting action. That glyoxalase is inhibited by phosphate has been shown by Sakuma [1930]. It is remarkable that an enzyme, which presumably is a part of the glycolytic system, should be sensitive to phosphate. When

Fig. 3.

extracting the solutions containing borate it was found that boric acid follows mandelic acid through the extraction process. As no way could be found of separating the two substances, and as boric acid diminishes the rotation of mandelic acid, the following artifice was used. The final uranyl nitrate solutions containing mandelic acid and some boric acid were saturated with solid H_3BO_3 . The diminution in rotation so produced was determined separately, and corrected for. The results, however, are not as consistent as with the other solutions. Although diethyl barbiturate inhibits more strongly than the other buffers, its greater buffering power in what was believed to be the optimum pH range of glyoxalase led to its being used in the following experiment on the

p_H -activity curve of the enzyme. The solutions consisted of 100 cc. liver extract, 30 cc. diethyl barbiturate solution (0.1 M), 25 cc. phenylglyoxal solution, enough 0.1 N H_2SO_4 to give the desired p_H , and enough water to bring the volume up to 250 cc. (The quantities of H_2SO_4 to be added were calculated on the basis of the titration curve of the buffer (Michaelis). In fact the actual initial p_H values of the mixtures came out considerably lower than intended, owing, apparently, to the buffering power of the neutral liver extract.) The solutions were incubated for 17½ hours at 26.5°. The p_H was again determined at the end of the incubation. As Fig. 3 shows, a very considerable fall in p_H has taken place during the reaction. The optimum seems to lie between p_H 6 and 7; its exact position and the shape of the p_H -activity curve of glyoxalase cannot, of course, be deduced from these figures. The difference of the optimum p_H range found in this experiment from that reported by Kuhn and Heckscher must be at any rate partly due to the fact that these authors only determined the initial p_H in their experiment. The fact that they used phosphate buffer, with little buffering power at the higher p_H values, must also have contributed to the discrepancy. On the other hand part of the shift into the acid range observed in this experiment, as compared with Kuhn and Heckscher's observations, may of course be due to a specific action of the buffer used. This will have to be further investigated.

SUMMARY.

1. A method is described for estimating active mandelic acid in tissue extracts, by extracting with amyl alcohol and enhancing the rotation with uranyl nitrate.

2. By means of this method the effect of toluene and of various buffer substances on glyoxalase has been investigated, as well as the p_H -activity curve of the enzyme.

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