XXII. THE QUANTITATIVE DETERMINATION OF GLYOXALASE IN THE BLOOD AND SOME OF ITS APPLICATIONS.

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In previous communications [Dakin and Dudley, 1913, 1914] the properties of the enzyme glyoxalase, which converts α -ketonic aldehydes into α -hydroxy-acids according to the general scheme:

have been discussed. It has been demonstrated that the enzyme, which is widely distributed, rapidly converts methylglyoxal into lactic acid, and is therefore most probably of importance in the intermediary metabolism of carbohydrates. In order to investigate more closely some problems connected with the enzyme a quantitative method of estimation was desirable, and the object of the present paper is to describe such a method and some of the results obtained by its aid.

On account of the difficulty of the quantitative extraction of lactic acid from liquids containing protein and the laboriousness of its determination, methylglyoxal could not be used with any hope of success as substrate in a quantitative method for estimating glyoxalase activity. The previous qualitative work on the enzyme was greatly facilitated by the employment of phenylglyoxal as substrate. The ease with which the optically active mandelic acid produced by the action of the enzyme can be extracted from digestion mixtures containing protein and the high value of the specific rotation of the acid, render quick determinations of the activity of glyoxalase preparations possible. Accordingly a quantitative method was devised, using phenylglyoxal as substrate, which was in principle essentially the same as the method employed in previous qualitative work on the enzyme.

The primary object of the quantitative estimation of glyoxalase was the comparison of its activity in the living animal under varying conditions in the hope of gaining more detailed knowledge as to its function in carbohydrate metabolism. Blood was therefore chosen as the tissue in which to determine the activity of the enzyme, since its withdrawal from time to time in small quantities is without effect on the general condition of the animal, and comparisons between equal volumes of different specimens can be accurately made.

The method consists in allowing 5 cc. of defibrinated blood to act on phenylglyoxal in the presence of calcium carbonate at 37° for 20–24 hours. The protein is then precipitated by adding solid ammonium sulphate and heating. The mixture, after acidification, is filtered, and the protein precipitate is washed free from mandelic acid, which is then extracted from the filtrate with ether and determined polarimetrically.

It was found at the outset that the concentration of phenylglyoxal is by no means a matter of indifference. It exhibits a toxic effect on the enzyme in concentrations even as low as 0.80 %. It is therefore necessary to have a standard concentration in all quantitative determinations. It was further shown that the method is not applicable without modification to amounts of blood greater than 7 cc., mainly because of the difficulty of washing the bulky protein precipitate free from mandelic acid. Amounts of blood from 1 to 7 cc. give amounts of active mandelic acid, measured polarimetrically, in simple proportion to the quantity of blood taken.

By means of this method the fact has been established that the “glyoxalase value” for the blood of different animals of the same species is remarkably constant, and is also definite for the particular species. Comparisons between species show definite differences in the glyoxalase values of the blood. In descending order of glyoxalase activity the species so far examined arrange themselves thus:

Dog, Ox, Rabbit, Sheep, Pig.

The order in which these animals appear is interesting. There appears to be a rough parallelism between the glyoxalase activity and the sugar tolerance of the animals. It has been shown by Hunter and Hill [1914] that “the sheep’s capacity to deal with subcutaneously administered glucose is greatly inferior to that of the dog,” while Carlson and Drennan [1912] have observed an extraordinarily low sugar tolerance in the case of the pig. It will be noticed that the sheep and the pig show the lowest values for blood

glyoxalase in the various species so far examined, whilst the value for the dog is the highest. Experiments of Underhill and Closson [1906] have demonstrated that the dog can utilise large quantities of glucose introduced parenterally without developing a glycosuria. They have shown, too, that the rabbit is able to assimilate much glucose injected subcutaneously; indeed under the experimental conditions observed by them its sugar tolerance appears to be similar to that of the dog. But apparently no accurate determinations of the limits of sugar tolerance in these animals have been made. If the glyoxalase of the blood is any guide to the sugar tolerance of an animal one would expect the rabbit to occupy an intermediate position between the dog and the sheep. It certainly can assimilate more parenterally administered glucose than the sheep, but its efficiency as compared with the dog has not been determined.

Without doubt the sugar tolerance of an animal must be governed by the interplay of many complicated factors, and of these glyoxalase activity may be one, so that the apparent parallelism between the sugar tolerance of an animal and the glyoxalase value of its blood may not be entirely fortuitous.

Experiments on the glyoxalase activity of the blood in various forms of glycosuria are in progress and will be reported shortly.

EXPERIMENTAL.

Quantitative determination of glyoxalase in 5 cc. of blood.

Before the blood is shed a 250 cc. conical flask containing 10 cc. of a suspension of 2.75 g. chalk in 100 cc. water is placed in an incubator at 37°. A 1.2 % solution of phenylglyoxal hydrate is also heated to 37°. The experimental animal is then bled and the blood is whipped until free from fibrin, when it is strained through muslin. 10 cc. of the phenylglyoxal solution (0.12 g. phenylglyoxal hydrate) are then well mixed with the chalk suspension and 5 cc. of the blood are finally added. It is important to make the additions in this order since most phenylglyoxal preparations give distinctly acid solutions. This acidity is removed by the chalk before the blood is added and so can do no damage to the glyoxalase, which has been shown to be very sensitive to acid. The mixture is then incubated at 37° for 20–24 hours. Experiments have been performed which show that the action is complete in this period. Phenylglyoxal displays distinctly anti-septic properties, so that the addition of toluene is unnecessary. In no case in the performance of many experiments has any bacterial activity been

observed. After completion of the reaction 25 g. ammonium sulphate are added to the mixture, which is then heated in a boiling water-bath for 3–4 minutes. The protein is thereby precipitated. After cooling, 12 cc. of 50 % phosphoric acid are added and the liquid is allowed to stand, with frequent shaking, for half an hour. The protein precipitate is then filtered on a Buchner funnel and washed five times with 10 cc. 40 % ammonium sulphate solution. These washings must be performed slowly, each washing being allowed to soak well into the precipitate before being sucked through. A water-clear filtrate is obtained which is extracted four times with small quantities of ether (10–12 cc.). Each ether layer is washed with distilled water (about 3 cc.). The mandelic acid is completely removed by this process. The ether is then removed by distillation and a crystalline residue of mandelic acid is obtained. This is dried in a desiccator over sulphuric acid and then dissolved in 12 cc. distilled water, solution being accelerated by warming on a water-bath. A pinch of animal charcoal is added to the hot solution, which is first allowed to cool and is then filtered. The small amount of fat which usually appears in the ether residue is completely removed by these means. The solution is then examined polarimetrically in a 2 dm. tube, the reading giving a measure of the amount of *l*-mandelic acid formed, and so of the glyoxalase content of the blood.

Experiments to show the effects of increasing concentration of phenylglyoxal and of dilution, and the completeness of removal of mandelic acid from the protein precipitate.

Sheep's blood was used in this series. The technique was that described in the standard method outlined above.

Expt.	Chalk cc.	Water cc.	Phenyl- glyoxal cc.	Blood cc.	Total vol. cc.	α	<i>l</i> -mandelic acid, g.
1.	10	30	5 (0.1 g.)	5	50	–0.66°	0.0251
2.	10	25	10 (0.2 g.)	5	50	–0.62°	0.0235
3.	10	25	10 (0.2 g.)	5	50	–0.63°	0.0239
4.	10	15	20 (0.4 g.)	5	50	–0.45°	0.0171
5.	10	75	10 (0.2 g.)	5	100	–0.60°	0.0228

After the usual filtration and washing of the precipitate in Experiment 2, the protein was subjected to five further washings by 10 cc. 40 % ammonium sulphate solution and these washings were extracted four times with ether. No mandelic acid was obtained by this treatment, showing that the acid had been completely removed by the previous manipulations.

Experiments 2 and 3 serve to demonstrate how closely duplicate determinations agree.

Experiment 4 shows definitely the toxic effect of higher concentrations of phenylglyoxal on the enzyme.

Experiment 5 shows that dilution of the reaction mixture to twice the volume taken in standard experiments is without appreciable effect on the result.

Experiments showing the applicability of the method for amounts of blood from 1 to 7 cc. and its failure for larger quantities.

The first series was carried out with ox blood, the second with sheep's blood. I am indebted to Dr H. E. Woodman for the second series.

OX BLOOD.

Expt.	Chalk cc.	Water cc.	Phenyl- glyoxal cc.	Blood cc.	α	<i>l</i> -mandelic acid, g.
1.	10	29	10 (0.2 g.)	1	-0.19°	0.0072
2.	10	26	10 (0.2 g.)	4	-0.88°	0.0334
3.	10	25	10 (0.2 g.)	5	-1.04°	0.0395
4.	10	25	10 (0.2 g.)	5	-1.03°	0.0391
5.	10	23	10 (0.2 g.)	7	-1.31°	0.0497
6.	10	20	10 (0.2 g.)	10	-1.06°	0.0403
7.	10	15	10 (0.2 g.)	15	-0.84°	0.0319
8.	10	10	10 (0.2 g.)	20	-0.56°	0.0213

9. Protein precipitate of 8, further washed with 50 cc. 40 % ammonium sulphate solution, filtrate extracted with ether: a quantity of mandelic acid was extracted which, examined under standard conditions, gave $\alpha = -0.40^\circ$.

SHEEP'S BLOOD.

Expt.	Chalk cc.	Water cc.	Phenyl- glyoxal cc.	Blood cc.	α	<i>l</i> -mandelic acid, g.
1.	10	29	10 (0.1 g.)	1	-0.12°	0.0045
2.	10	27	10 (0.1 g.)	3	-0.35°	0.0133
3.	10	25	10 (0.1 g.)	5	-0.63°	0.0239
4.	10	23	10 (0.1 g.)	7	-0.79°	0.0300
5.	10	21	10 (0.1 g.)	9	-0.57°	0.0216

Experiments 1 to 5 of the first series and 1 to 4 of the second show that the amount of *l*-mandelic acid produced is proportional to the amount of enzyme taken. The effect of adsorption of mandelic acid by the protein precipitate is beginning to be felt when 7 cc. of blood are taken, and with higher quantities the results are of no value.

Experiments showing the rate of the reaction under the conditions of the standard determination.

A series of experiments, using 5 cc. of dog's blood in each, carried out according to the standard method, were interrupted at various intervals and afforded information with regard to the progress of the reaction.

Action stopped after	1	2	4	6	10	20	23 hours
$\alpha =$	-0.28	-0.39	-0.57	-0.67	-0.83	-0.99	-0.99°

It will be seen that the velocity of the reaction is large at first, diminishing considerably in the later stages, and that under the conditions of experiment the reaction is complete in 20 hours.

Normal glyoxalase values for various animals.

5 cc. of freshly-shed, defibrinated blood were used in all these determinations, which were carried out in accordance with the standard method.

Dog. The following rotations for *l*-mandelic acid obtained by the action of 5 cc. dog's blood in the cases of seven different animals, most of the experiments being performed in duplicate, serve to show how constant is the value of the glyoxalase content of the blood in a particular species. The dogs, some male and some female, were of different breeds.

Dog	1	2	3	4	5	6	7
$\alpha =$	$\begin{cases} -1.05^\circ \\ -1.04^\circ \end{cases}$	-1.04°	$\begin{cases} -1.02^\circ \\ -1.03^\circ \end{cases}$	$\begin{cases} -1.08^\circ \\ -1.00^\circ \end{cases}$	$\begin{cases} -1.07^\circ \\ -1.06^\circ \end{cases}$	$\begin{cases} -1.03^\circ \\ -1.03^\circ \end{cases}$	-1.05°

Mean value for $\alpha = -1.04^\circ = 0.0395$ g. *l*-mandelic acid.

Ox. The blood of two animals was investigated.

Mean value for $\alpha = -1.03^\circ = 0.0382$ g. *l*-mandelic acid.

Rabbit. Four animals were used.

Mean value for $\alpha = -0.74^\circ = 0.0281$ g. *l*-mandelic acid.

Sheep. Duplicate determinations were made on the blood of two animals.

Mean value for $\alpha = -0.63^\circ = 0.0239$ g. *l*-mandelic acid.

Pig. Two animals were investigated.

Mean value for $\alpha = -0.61^\circ = 0.0232$ g. *l*-mandelic acid.

SUMMARY.

A method for the quantitative determination of the glyoxalase activity of blood is described.

It is shown that the glyoxalase of the blood is remarkably constant in different animals of the same species under normal conditions. It is further demonstrated that different species display definite differences in the glyoxalase activity of the blood, and attention is drawn to the observation that animals displaying high glyoxalase values are those having high sugar tolerance, whilst those with lower glyoxalase activities have less well developed powers of assimilating sugar.

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