CLIV. THE PRODUCT OF THE OXIDATION OF GLUCOSE BY GLUCOSE DEHYDROGENASE.

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RECENTLY, a description was given of the preparation from the livers of various animals of a new enzyme, glucose dehydrogenase, which brought about the oxidation of glucose, apparently with the formation of an acid [Harrison, 1931]. The present paper deals with the isolation and identification of this oxidation product.

Glucose in presence of the dehydrogenase is not oxidised by molecular oxygen, but is oxidised in presence of methylene blue. It was shown that the aerobic oxidation of glucose could be brought about by the dehydrogenase if the oxygen-activating system cytochrome and indophenol oxidase were present. Since it was difficult to prepare sufficient cytochrome for a large scale oxidation of glucose, the oxidation was carried out in presence of air, using methylene blue as an intermediary carrier. The methylene blue is reduced to leuco-methylene blue which is then rapidly oxidised back to methylene blue by the oxygen of the air. The methylene blue thus acts as a carrier between the activated hydrogen of the glucose and the oxygen of the air.

In order to obtain a clue as to the nature of the oxidation product, a number of preliminary experiments were carried out in the Barcroft differential manometer to determine the relationship between the amount of acid formed and the amount of oxygen taken up. The dehydrogenase preparation and methylene blue were put into both vessels of the apparatus, and glucose solution was added to one vessel and an equal volume of water to the other vessel. The enzyme was the dialysed preparation from acetone-liver [Harrison, 1931], equivalent to about 5 g. of acetone-liver in each vessel. The final concentration of methylene blue was 1 in 2500 and of glucose 0.25 *M*. After shaking for about 3 hours at 37°, the oxygen uptake was measured, and the solution in the vessel containing glucose was titrated with *N*/50 NaOH to the same p_H as that of the solution containing no glucose. In spite of the presence of methylene blue, this could be carried out sufficiently accurately by withdrawing a very small amount of solution on the end of a glass rod and using an external indicator such as phenol red. The titration was checked electrometrically using hydrogen electrodes.

As the result of a number of determinations, an average ratio of slightly less than one atom of oxygen to one equivalent of acid formed was obtained. This rendered improbable the formation of a number of acids such as glycuronic,

keto-gluconic or saccharic acid, all of which require more than one atom of oxygen per equivalent of acid. Determination of lactic acid in the solutions with and without glucose had already shown that no lactic acid is formed from glucose during the oxidation [Harrison, 1931]. Experiments on a rather larger scale showed that after removal of protein from the solution, negative nitroprusside and guaiacol tests were obtained for pyruvic acid, and a negative naphthoresorcinol test was obtained for glycuronic acid, though strongly positive tests were given on adding traces of these acids to the solution.

By comparing the amount of precipitate in the solutions with and without added glucose, it was found that the oxidation product of glucose was precipitated by basic lead acetate but not by normal lead acetate. This again excluded as main products of the reaction a number of acids such as malic, tartaric, citric, succinic, malonic and glyceric acids.

On decomposing the basic lead acid precipitate and treating the liberated acid with CaCO_3 , a calcium salt was obtained which was soluble in water but which was precipitated by alcohol. The acid did not reduce Fehling's solution but reduced hot ammoniacal silver nitrate. In view of the elimination of the acids mentioned above, these properties, coupled with the observation that one equivalent of acid is formed for each atom of oxygen taken up, suggested that gluconic acid might very possibly be the oxidation product. By carrying out the oxidation on a large scale, it was found possible to prepare and isolate derivatives of the acid which were found to be identical with the corresponding derivatives of *d*-gluconic acid.

The methods used for isolation differed somewhat in different experiments, but the technique used in the experiment to be described proved to be the most satisfactory, and details of the other experiments will therefore be omitted.

The glucose dehydrogenase was prepared by water extraction of 450 g. of acetone-liver, followed by dialysis and precipitation by saturation with ammonium sulphate exactly as previously described [Harrison, 1931]. The dry preparation was ground up, put into two large wet collodion sacs and dialysed into solution against distilled water in the ice-chest. After adjusting to p_{H} 7.4 and centrifuging, the volume of the enzyme solution was 130 cc.¹ 125 cc. of this solution were mixed with 25 cc. of 2*M* glucose (B.D.H. dextrose) and 37.5 cc. of methylene blue (1 in 500), the mixture being put into corked 250 cc. conical flasks and shaken fairly rapidly in a water-bath at 37°. The flasks were opened at frequent intervals and filled with fresh air.

At the same time, two Barcroft differential manometers were used to measure the oxygen uptake. The right-hand vessels each contained 2 cc. enzyme and 0.6 cc. methylene blue. The experimental apparatus contained in addition 0.4 cc. 2*M* glucose, while the control apparatus which measured the residual oxygen uptake of the enzyme preparation received 0.4 cc. water. The side tubes

¹ Recent experiments by Mann [1932] have shown that an activator is concerned in the oxidation of glucose by glucose dehydrogenase. The enzyme preparation described above contains both the dehydrogenase and the activator.

of the vessels contained NaOH to absorb CO_2 , and the vessels were shaken in the same water-bath and for the same length of time as the conical flasks.

After four hours, the oxygen uptake in the Barcroft apparatus was measured, and the flasks were opened. Incubation of samples on agar plates showed that the solutions were almost sterile and that the oxidation of glucose was therefore not due to bacteria.

The solutions in the flasks, together with the contents of the Barcroft vessel containing glucose, were brought to approximately p_{H} 7.6 and the protein was precipitated by the addition of absolute alcohol to a concentration of 80 %. After filtering under suction, the residue was ground up with 100 cc. of water, filtered and washed. The latter filtrate was precipitated with 80 % alcohol and filtered, using a trace of kieselguhr on the filter-paper. The precipitate was ground with a little water, acidified to p_{H} 4 with acetic acid, filtered, precipitated with 80 % alcohol and again filtered. All the alcoholic filtrates were combined and evaporated down under reduced pressure to about 150 cc.

The solution was brought to p_{H} 7.6, allowed to stand overnight to permit of the hydrolysis of any lactone, and precipitated by adding a saturated solution of basic lead acetate together with a little NaOH until a drop of either produced no further precipitate (p_{H} approximately 7.8). The precipitate was suspended in water, stirred well with acetic acid and then brought almost to neutrality by adding NaOH. The precipitate, consisting largely of PbSO_4 (from $(\text{NH}_4)_2\text{SO}_4$ contained in the enzyme preparation) was centrifuged off, washed, and the clear solution and washings were made alkaline and reprecipitated with basic lead acetate. The precipitate was washed twice with water containing a little basic lead acetate. All the washings and the solution left from the first precipitate were combined, freed from lead with H_2SO_4 and $\text{Ba}(\text{OH})_2$, concentrated under reduced pressure and reprecipitated with basic lead acetate. (The solutions were never allowed to stand in presence of basic lead acetate in order to avoid the risk of decomposition of any glucose in the solution.) The combined basic lead acetate precipitates were suspended in water and treated with slight excess of H_2SO_4 to remove lead, the excess of H_2SO_4 being exactly removed with baryta. The clear filtrate was evaporated to dryness over solid NaOH in a vacuum desiccator, and most of the acetic acid was removed by reducing the pressure to about 0.5 mm. Hg. (The methylene blue had been almost completely removed at this stage since it was not adsorbed on the lead precipitate.) The residue was dissolved in a little water, stirred with 0.2 g. CaCO_3 and left overnight over NaOH. The mixture was then heated on the water-bath for several hours, stirring at intervals, and when nearly neutral a few drops of lime water were added until the solution was permanently just neutral to phenol red. The solution was filtered and the calcium salt was precipitated with 80 % alcohol in a total volume of about 50 cc. After standing overnight in the ice-chest, the precipitate was centrifuged off and dissolved in about 2 cc. of warm water, and warm alcohol was added to a concentration of 30 %. On standing overnight the calcium salt crystallised out and was filtered and washed with a little

cold 30 % alcohol and dried over H_2SO_4 . The mother-liquor and washings were evaporated down and again allowed to crystallise. By repeating this process four times, assisting crystallisation each time by seeding the solution with a crystal from the previous crop, a total of 255 mg. of crystalline salt was obtained, the crystals consisting of clusters of microscopic needles resembling the highly characteristic crystals of the calcium salt of gluconic acid.

By heating a portion of the free acid on the water-bath with phenylhydrazine and acetic acid [Fischer and Passmore, 1889], crystals were obtained on cooling which, after dissolving in hot water, decolorising with a little charcoal and recrystallising once from very dilute acetic acid and three times from hot water, commenced to decompose at 196° on rapidly heating and melted completely at 200° . A sample of the phenylhydrazone of gluconic acid prepared from an authentic specimen of the acid melted at the same temperature, and the melting-point was not changed by mixing the two specimens together. The two samples of crystals were apparently identical under the microscope, being microscopic, square-ended prisms with straight extinction. Further confirmation that the acid is gluconic acid was obtained by preparing the cinchonine salt which after recrystallising from 95 % alcohol had m.p. $185\text{--}186^\circ$. Micro-analysis (Schoeller) of the phenylhydrazone: C, 50.38, 50.50; H, 6.22, 6.28 %. (Calculated for $\text{C}_{12}\text{H}_{18}\text{O}_6\text{N}_2$: C, 50.35; H, 6.29 %.)

There appears to be no doubt, therefore, that gluconic acid is formed by the oxidation of glucose in presence of glucose dehydrogenase.

In order to determine whether the acid were *laevo*- or *dextro*-gluconic acid, 150 mg. of the dry, recrystallised Ca salt were dissolved in water, made up to 5.0 cc., and the rotation measured in a 1 dm. tube at 20° . Taking the average of a number of closely agreeing readings, the specific rotation $[\alpha]_D^{20}$ was calculated to be $+8^\circ$. The values given in the literature for Ca *d*-gluconate range from $+6^\circ$ to $+10^\circ$. As would be expected, therefore, the *dextro*-form of gluconic acid is formed by the oxidation of *d*-glucose by the enzyme.

The oxygen uptake during the oxidation was equivalent to the formation of 424 mg. of calcium gluconate (after subtracting the residual oxygen uptake of the enzyme alone). The amount actually isolated in the crystalline state was 255 mg. which is equivalent to just over 60 % of the oxygen uptake observed. In dealing with such small amounts, the isolation is by no means quantitative, and it seems quite likely, therefore, that *d*-gluconic acid is the sole product of this oxidation of glucose.

DISCUSSION.

The oxidation of glucose at the potential aldehyde group with the formation of gluconic acid is readily brought about by a number of oxidising agents and would seem at first sight a probable method of breakdown of glucose in the body. The results reported in the literature of feeding and perfusion experiments with gluconic acid are rather contradictory, but there appears to be a certain amount of evidence that some utilisation of gluconic acid takes place. Experi-

ments in which unchanged gluconic acid was recovered in the urine after feeding the acid may possibly be explained by the rapid elimination of the acid by the kidneys after feeding in relatively large doses, this preventing a quantitative utilisation of the acid.

While, therefore, gluconic acid may be a normal intermediary in the oxidation of glucose, an alternative possibility might be that the gluconic acid which has been isolated in these experiments may arise from some labile oxidation product of glucose which normally, in the intact cell, becomes further oxidised instead of appearing as gluconic acid. A similar hypothesis has been brought forward by Meyerhof [1930] to explain the conversion by muscle enzyme of glycogen to lactic acid *via* hexosemonophosphate. In this case, although hexosemonophosphate can be isolated during the reaction, yet glycogen is broken down by the enzyme more rapidly than the supposed intermediary, hexosemonophosphate. Meyerhof explains this by assuming that the true breakdown path is *via* an unknown labile form of hexosemonophosphate which, if not immediately converted into lactic acid, may appear as stable hexosemonophosphate.

I put these possibilities forward only as mere suggestions, but in view of the experiments I have described and of the high concentration of glucose dehydrogenase in the liver, it seems difficult to escape from the conclusion that in the body, part, at least, of the glucose metabolised must go through gluconic acid or some precursor of this acid during its oxidation.

It is interesting that glucose dehydrogenase and glucose oxidase, the enzyme prepared from *Aspergillus niger* by Müller [1928], both convert glucose into gluconic acid; for, as previously pointed out [Harrison, 1931], the two enzymes differ fundamentally in properties, the oxidase being able to utilise oxygen but not methylene blue as the hydrogen acceptor, while the dehydrogenase can use methylene blue but not oxygen directly.

SUMMARY.

The product of the oxidation of glucose by glucose dehydrogenase has been isolated and shown to be *d*-gluconic acid.

The amount of acid isolated as the crystalline calcium salt was equivalent to over 60 % of the amount formed as calculated from the oxygen uptake, and *d*-gluconic acid is probably the sole product of the oxidation.

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