CXXXIV. THE PRODUCTION OF GLUCOSONE FROM CARBOHYDRATES BY ENZYMIC ACTION

BY CECIL ROBERTSON BOND, EDWIN COULTHARD KNIGHT
AND THOMAS KENNEDY WALKER

From the College of Technology, University of Manchester

(Received 7 May 1937)

RECENT studies of the biological oxidation of glucose have considerably extended our information concerning the first steps in its utilization by various organisms. Conversion into gluconic acid has been effected by the agency of an aerobic oxidase extracted from the mycelium of *Aspergillus niger* and of *Penicillium glaucum* [Müller, 1931] and by the action of a dehydrogenase present in liver extracts, which functions in conjunction with a co-enzyme [Harrison, 1931]. The latter author concluded that the oxidation of glucose via gluconic acid can occur in the body as an independent alternative method of glucose breakdown, for the liver is rich in glucose dehydrogenase. Under the action of *Acetobacter suboxydans* glucose yields 5-ketogluconic acid [Kluyver & de Leeuw, 1924], and the latter acid together with 6-aldehydogluconic acid (*l*-guluronic acid) has been obtained by the use of *Bacterium gluconicum* [Bernhauer & Irrgang, 1935]. In later experiments with the latter organism Bernhauer & Görlich [1935] detected the formation of a third 6-carbon acid which proved to be 2-ketogluconic acid. The production of this substance is of interest from the fact that its keto-group must have been derived from the oxidation of a secondary carbinol group in the *trans*-position to another secondary carbinol group. From observations of the action of the sorbose bacterium on polyhydric alcohols Bertrand [1904] concluded that in order that oxidation of the alcohol molecule could be effected it was necessary to have present two secondary alcoholic groups in the *cis*-position with respect to each other and standing next to the terminal $\text{—CH}_2\text{OH}$ group. This generalization evidently cannot be extended to cases of related compounds in which the terminal group is not $\text{—CH}_2\text{OH}$, since this detection of 2-ketogluconic acid as a product of the oxidation of glucose constitutes the second case to be reported of the biological oxidation of a secondary alcoholic group lying between the end carbon atom of the chain and another secondary alcoholic group to which it is in the *trans*-position. The first case of this kind to be noted was that of the production of glucosone by a mould belonging to the *flavus* section of the *flavus-oryzae* series of the Aspergilli when placed in contact with a glucose solution in the presence of toluene [Walker, 1932]. The formation of this 6-carbon derivative of glucose by enzymic agency had not been recorded previously and the case is of interest in view of the suggestions of Hynd [1927] that glucosone may be the first product of the utilization of glucose in the animal body and that insulin may play the part of an oxidase catalysing the conversion, suggestions with which Dixon & Harrison [1932] are not in agreement, since they were unable to detect glucosone in the blood of rabbits in hypoglycaemic convulsions following a large overdose of insulin. These authors were unable also to demonstrate the presence of glucosone in fresh liver, but they stated that the possibility of glucosone acting as an intermediary cannot be excluded entirely in view of the fact that it can be

formed by enzymic action as described by Walker [1932]. Later, Berkeley [1933] brought forward evidence that glucosone is produced from glucose by the action of an oxidase system present in the crystalline style of the mollusc *Saxidomus giganteus*, and also by the operation of a dehydrogenase system, the activity of which depends on the joint action of a substance contained in the style and another contained in the diatomaceous food material of the mollusc, as was shown by bringing them together in the presence of methylene blue. It is suggested by Berkeley that the first-mentioned oxidase system serves as a hydrogen acceptor to promote the action of the dehydrogenase system.

In view of these observations it was considered of interest to investigate further the production of glucosone by mycological agency and experiments have now been made with different moulds using as substrates starch and various sugars and related substances. Formation of glucosone has now been observed in the cases of two moulds when allowed to act upon soluble starch, maltose, sucrose and glucose, whilst negative or doubtful results were obtained with lactose, mannose, fructose, xylose, mannitol and glycerol. Further, it has been found that the appearance of glucosone is observed only when the mould mycelium is subjected to the action of plasmolytic agents and, hitherto, it has not been possible to obtain positive results with enzyme preparations extracted from the mycelium. Hence, it is not yet possible to decide whether the oxidation is the result of one specific enzyme or whether it is due to the activity of the residue of an enzyme complex, a component of which is inhibited or destroyed by the plasmolytic agent.

EXPERIMENTAL

The mould employed for purposes of the greater part of the experimental work belongs to the *flavus* section of the *flavus-oryzae* series of the Aspergilli, and it has been described, with morphological details, in a previous communication [Challenger *et al.* 1931]. It was cultivated on aqueous media containing glucose and the following mixture of inorganic salts, recommended by Kinoshita [1927]: NH_4NO_3 , 0.04%; KH_2PO_4 , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%. In preliminary experiments, conical flasks of 1500 ml. capacity were charged each with 300 ml. of this medium to which glucose (5%) had been added. After sterilization in a current of steam for 20 min. on each of three successive days the contents of the flasks were sown with spores of the mould and incubated at 28° for 4 days. At the end of this time sporulation was in progress and the medium was then poured away from beneath the mycelia and was replaced by sterile tap water at 28°; subsequently, the flasks were returned to the incubator for a period of about 6 hr., after which the water was poured away and replaced by fresh sterile water, also at 28°. The cultures were maintained overnight at 28° and the following morning the mycelia were given a third washing with sterile water with which they were allowed to remain in contact for about three hours. This process served to remove from the cells of the mould absorbed sugar and diffusible metabolites (for example kojic acid, which this strain synthesizes from sugar), and it is probable that the period of starvation was sufficient to cause appreciable utilization of reserve materials in the cells. The final washing-water extracted only the merest trace of kojic acid as indicated by the very feeble coloration given with ferric chloride. A sterile 5% aqueous solution of glucose at 28° was then emulsified with toluene (1.5%) by shaking, and by means of a large pipette this emulsion was introduced quickly in lots of 300 ml. beneath the mycelial mat in each flask, and incubation was continued at 28°. In later experiments a temperature of 30° was employed, both previous and subsequent to the washing

of the mycelium. In these operations of washing the mould care was exercised to avoid wetting the top surface of the mycelium, and it was found to be important that the latter was not unduly bruised or torn during the necessary manipulation of the cultures. After the glucose-toluene medium had been in the flasks for a short time the toluene separated and was absorbed at the underneath surface of the mould, wetting ultimately the whole of the mycelium and giving it a darkened appearance. This wetting of the mould by the toluene is a necessary condition and may cause the mycelial cover to sink slightly in the liquid, but this does not appear to be disadvantageous at this stage provided that the mould does not sink to the bottom of the flask. Also, it may be necessary to add a little more toluene after 2 or 3 days, to replace that lost by evaporation.

After incubation in the presence of toluene for 4 days, samples of the liquid were withdrawn by pipettes from the flasks and tested by shaking with equal volumes of a cold 5% aqueous solution of phenylhydrazine acetate. Small quantities of yellowish brown flocculent precipitate were observed after periods of shaking varying from thirty seconds to about 3 min.

Similar tests which were made with cultures to which no toluene had been added yielded no precipitates, and a 5% aqueous solution of glucose either with or without toluene gave no precipitate with phenylhydrazine acetate solution in the cold, even after standing for 5 hr. Larger volumes of the solutions from the culture flasks were then combined, treated with an appropriate quantity of phenylhydrazine acetate in the cold and shaken for 5 min., after which the precipitate which had separated was removed, washed at the pump first with dilute acetic acid and then with water, and dried in a desiccator. After several crystallizations from ethyl alcohol diluted with a little water the material had constant M.P. 204–205° alone or in admixture with a pure and authentic specimen of glucosazone. (Found: N, 15.9%. $C_{18}H_{22}O_4N_4$ requires N, 15.64%.)

Identification of glucosone as anhydrogluco-o-diaminobenzene, a condensation product with o-phenylenediamine

(a) Griess & Harrow [1887] found that when equimolecular proportions of glucose and the acetate of *o*-phenylenediamine were brought together in very concentrated solution and left to stand for 8 days at 30°, a crystalline azine, which they termed anhydrogluco-*o*-diaminobenzene,¹ crystallized from the solution.

Later, Fischer [1889] stated that the same substance could be obtained by warming for a few minutes an aqueous solution of glucosone and *o*-phenylenediamine in the absence of acid. We have used this azine as a further means of identification of glucosone in culture media, and for purposes of comparison it was prepared by the method of Griess & Harrow. The crude crystalline product was decolorized by charcoal and purified by several recrystallizations from much hot water. After drying at 100° it melted at 194°. Neither Griess & Harrow nor Fischer recorded the M.P.

(b) Glucosone was prepared by the method of Fischer [1889; 1890] from 50 g. of pure glucosazone and was obtained (yield 8 g.) as a light-brown syrup which, after standing at 0.2 mm. over phosphorus pentoxide in a desiccator, set to an amorphous solid. That this material was in a high state of purity was shown from the weight of glucosazone which an aliquot portion yielded immediately on mixing with the appropriate quantity of phenylhydrazine acetate solution. A quantity (3.5 g.) of the glucosone was taken up in 1000 ml. of cold water and to this solution were added successively 5 g. of *o*-phenylenediamine dissolved in the minimum quantity of cold water and 10 ml. of 10% sodium

¹ A modern designation of this substance would be 2-(tetrahydroxy-*n*-butyl)quinoxaline.

hydroxide solution. After vigorous shaking for a few minutes, small amber-coloured prisms commenced to separate. After standing for several hours these were collected, washed with water, dried in air and recrystallized first from ethyl alcohol and then from methyl alcohol; yield, 1.4 g. The m.p. was 194° and was not depressed by admixture with anhydrogluco-*o*-diaminobenzene prepared by the method of Griess & Harrow.

(c) A quantity (930 ml.) of a 5% solution of glucose which had been submitted for 5 days to the action of the mould in the presence of toluene was mixed with an aqueous solution of 5 g. of *o*-phenylenediamine and the whole was made slightly alkaline by the addition of about 10 ml. of a 10% solution of sodium hydroxide. The colour of the mixture darkened somewhat and, after standing for about 20 min., the separation of small prisms commenced and was completed after several hours. These were removed and on recrystallization (yield 1.6 g.) melted at 194° alone or in admixture with either of the products obtained as described under (a) and (b). (Found: N, 11.5; 11.4%. $C_{12}H_{14}O_4N_2$ requires N, 11.2%.)

(d) A 5% aqueous solution of glucose was treated with an appropriate quantity of an aqueous solution of *o*-phenylenediamine and was then rendered slightly alkaline with a few ml. of dilute sodium hydroxide and allowed to stand in the cold for 24 hr. No condensation product was deposited under these conditions.

For the greater part of the experimental work, however, condensation with phenylhydrazine acetate was employed as a means of obtaining rough comparisons of the quantities of glucosone formed in the various culture media and, in order to obtain some idea of the degree of accuracy of this method, an experiment was first carried out with a series of solutions consisting of known weights of glucosone dissolved in 5% aqueous solutions of glucose. The concentrations of glucosone which were thus employed covered a range from 0.05 to 0.35%, and over this range it was found that addition of an appropriate volume of a 5% solution of phenylhydrazine acetate yielded within 15 min. a weight of glucosazone representing about 20% of the quantity of glucosone present initially in solution. After standing for 3 hr. the yield had increased in all cases to about 35% of that possible from the weight of glucosone employed. Since the concentration of glucosone as produced enzymically in the various experiments was usually within the limits 0.05–0.3%, it is probable that the rough estimations of glucosone which were made subsequently by addition of phenylhydrazine acetate to aliquot volumes of the culture media are representative of only about one-third of the glucosone which was actually produced in each case, and this point should be borne in mind, therefore, in considering the values shown in the Tables. Later, use was made of 2:4-dinitrophenylhydrazine which, according to Dixon & Harrison [1932], will give a precipitate with a 0.03% solution of glucosone. It was found that whereas phenylhydrazine would cause the precipitation of only about one-third of the glucosone from a solution of the latter at a concentration of 0.1%, under similar conditions 2:4-dinitrophenylhydrazine precipitated 58% of the glucosone as the 2:4-dinitrophenylosazone of glucose (m.p. 252–253°). In using 2:4-dinitrophenylhydrazine the precipitate was filtered off after about 90 min., since longer standing in the presence of glucose brought down traces of a precipitate due to the latter substance. In a blank experiment in which 100 ml. of an aqueous 0.5% solution of glucose were mixed with 50 ml. of the reagent (0.5 g. 2:4-dinitrophenylhydrazine in 30 ml. 2*N* HCl [Case & Cook, 1931]) and the whole allowed to stand in the cold, no precipitate had appeared during the first 2 hr. but, subsequently, material separated and after 3 days 0.0055 g. of precipitate was obtained.

*Determination of optimum conditions for the conversion
of glucose into glucosone*

(1) *Time required for accumulation of glucosone in highest yield.* This was found usually to be from 4 to 6 days (at 30°) from the time when the mycelium was placed in contact with the glucose-toluene mixture.

(2) *Concentration of substrate.* The initial work was done with 5% aqueous solutions of glucose plus toluene, but it was found that higher percentage yields were obtained from solutions of 0.5 to 1% concentration, the time being 6 days and the temperature 30°. The subsequent experiments in presence of toluene were all conducted with 0.5% solutions of the substrate (the mycelia used for these trials being first developed, as usual, on 5% glucose-salts medium).

(3) *Hydrogen ion concentration.* The effects due to this factor are shown in Table I. The duration of the experiment was 6 days at 30°.

Table I

pH	g. of glucosazone precipitated from 100 ml. of medium	% conversion of glucose into glucosone, calculated from g. glucosazone precipitated
2.5	—	—
3.0	0.030	3.0
4.0	0.064	6.4
5.0	0.072	7.2
6.0	0.086	8.6
7.0	0.078	7.8
8.0	0.076	7.6

(4) *Temperature.* The effects of changes over the range of biologically normal temperatures were slight but generally it was found advantageous not to incubate at temperatures higher than 30° in order to restrict loss of toluene by evaporation. The figures shown in Table II are from estimations made after standing in the presence of toluene for 5 days.

Table II

Temperature (° C.)	g. of glucosazone precipitated from 100 ml. of medium	% conversion of glucose into glucosone, calculated from g. glucosazone precipitated
18	0.0762	7.70
25	0.0784	7.92
30	0.0802	8.10

(5) *Effects of the age of the mycelium prior to replacement of the medium by the glucose-salts mixture.* Mycelial mats which were respectively 4, 5, 6 and 8 days old when washed free from the glucose-salts medium were placed in contact with the glucose-toluene mixture and maintained at 30° for 5 days, at which time the estimations shown in Table III were performed.

Table III

Age of mycelium in days	g. of glucosazone precipitated from 100 ml. of medium	% conversion of glucose into glucosone, calculated from g. glucosazone precipitated
4	0.0790	7.98
5	0.0804	8.08
6	0.0830	8.39
8	0.0810	8.19

(6) *Effects of different plasmolytic agents.* Bromobenzene (1.5%) was nearly as effective as toluene, and with chloroform at the same concentration the yield of glucosone was somewhat lower and the time of its formation somewhat longer than in corresponding trials with toluene.

No glucosone was formed in the presence of sodium chloride at a concentration of 7%.

Trials with different species of Aspergillus and of Penicillium.

Experiments were made in order to obtain some idea as to how general is the capability of glucosone formation amongst species of mould fungi. Since these trials were only on a small scale with about ten species of each genus the results obtained were in no way conclusive, but they indicated that capability of glucosone formation is not a common biochemical characteristic among the *Aspergilli* and *Penicillia*. Apart from the mould used originally in this investigation (a specimen belonging to the *flavus* section of the *flavus-oryzae* series of the *Aspergilli*) only one other species, namely, *Aspergillus parasiticus* Speare, was found to possess the power to form glucosone, and the yields obtained were very close to those given by the first-named organism. Now both these moulds produce kojic acid in good yield from glucose and, accordingly, it appears possible that the capability of forming glucosone may be a property restricted to those species of mould fungi which normally transform glucose into kojic acid.

In order to test this suggestion the following specimens were obtained from the National Collection of Type Cultures, Lister Institute: (1) *A. tamarii* Kita, (2) *A. effusus* Tiraboschi, (3) *P. Daleae* Zaleski and (4) *A. oryzae* Ahlberg, all of which had been shown previously to yield kojic acid when cultivated on Czapek-Dox medium containing glucose [Birkinshaw *et al.* 1931]. Of these moulds only (1) and (2) gave kojic acid within 14 days when sown on glucose-Kinoshita salts medium, and none of them yielded glucosone when the washed mycelia were placed on glucose solutions in the presence of toluene. Cultures of these moulds raised on other media also failed to convert glucose into glucosone when treated under our experimental conditions. The results of these experiments must therefore be regarded as indefinite, particularly in view of the well-known fact that some of the biochemical characteristics of micro-organisms are liable to undergo modification during protracted periods of laboratory cultivation on artificial media.

Formation of glucosone from soluble starch, maltose and sucrose

In place of glucose as the substrate the following sugars and related substances were separately employed: soluble starch, maltose, sucrose, lactose, mannose, fructose, mannitol, xylose and glycerol. In all cases the mould was first grown on Kinoshita salts solution containing 5% of glucose, and the washed mycelium was subsequently floated on an aqueous solution (0.5 to 1%) of the substance under investigation, plus 1.5% of toluene.

Appreciable yields of glucosone were obtained only from soluble starch, maltose and sucrose. Several of the other substances gave traces of material yielding precipitates with phenylhydrazine acetate, but in no case was there sufficient for purification and identification. The glucosone from the starch, maltose and sucrose was identified by precipitation as glucosazone and also by condensation with *o*-phenylenediamine. The yields and analytical figures for the specimens of anhydrogluco-*o*-diaminobenzene thus obtained are given in Table IV.

Table IV

Substrate at 0.5 % concen- tration	% converted into glucosone, calcu- lated from weight of glucosazone precipitated	M.P. of o-phe- nylenediamine derivative (° C.)	Mixed M.P. of o-phenylene- diamine deri- vative with authentic specimen (° C.)	Found on analysis:		
				C	H	N
Soluble starch	15.12	193	193.5	57.72	5.45	11.41
Maltose	17.12	193.5	193.5	57.40	5.65	11.12
Sucrose	13.60	193	193.5	57.81	5.78	11.01

$C_{12}H_{14}O_4N_2$ requires: C, 57.6; H, 5.6; N, 11.2%.

The yields from these three carbohydrates are thus seen to be much larger than those obtained from glucose.

*Action of a mould of the flavus section of the
flavus-oryzae group on glucosone*

The fact that production of glucosone by the action of plasmolysed mould mycelia on glucose had been observed only in the cases of two species which were known to give rise to good yields of kojic acid when grown on glucose raises the question as to whether, in the conversion of the latter sugar into kojic acid, glucosone may not be a normal intermediate product. In order to obtain further information on this point the mould which had been used largely in the experiments on glucosone formation was sown on a medium of Kinoshita salts solution (15 ml. in each of six boiling-tubes) to which 5% of pure glucosone had been added subsequent to sterilization. Growth and sporulation were quite normal and after 8 days samples from the tubes gave strongly positive reactions (wine-red colour) for kojic acid when treated with a drop of ferric chloride solution. After 14 days the combined solutions were worked up but yielded only a few mg. of crystalline kojic acid. The smallness of the yield is a fact which does not support the possibility that glucosone may be a normal intermediate product in the production of kojic acid from glucose by this mould. The fact that glucosone can be converted into kojic acid is not without interest, however, since it must be assumed that fission of the glucosone molecule must first occur followed by condensation of fission products to kojic acid, or to a 6-carbon compound capable of conversion into kojic acid. Assuming such a mode of fission to yield one molecule of dihydroxyacetone and one molecule of hydroxymethylglyoxal it is conceivable that glucosone could then arise by oxidative condensation between two molecules of the former [cf. Challenger *et al.* 1931] or by condensation of a molecule of dihydroxyacetone with one of hydroxymethylglyoxal, a possibility which would not be out of harmony with the known facts of organic chemistry.

DISCUSSION

The question as to whether the enzyme or enzyme complex responsible for the conversion of glucose into glucosone is strictly specific with respect to this one hexose, or whether it is capable of attacking similarly those sugars to which the group

is common, has not been settled by the present investigation. If the specificity is such that the only essential requirement for enzyme action is the possession within the molecule of a group possessing this configuration, then osone formation should also result from the action of the plasmolysed mould on *d*-galactose, *l*-arabinose and *d*-xylose. The last only of these three sugars has so far been submitted to the action of the mould, but the formation of xylosone was not detected. Failure to form glucosone from lactose would appear to be due to inability of the mould to hydrolyse this disaccharide; similarly *A. niger*, which contains no lactase, cannot convert lactose into citric acid.

SUMMARY

1. Cultures of *A. parasiticus* Speare and of an unnamed mould belonging to the *flavus* section of the *flavus-oryzae* series of the Aspergilli, after plasmolysis by toluene, bromobenzene or chloroform, converted glucose in dilute aqueous solution into glucosone. The optimum conditions for this oxidation were found to be: plasmolysing agent, toluene, about 1.5%; glucose concentration, about 0.5–1.0%; temperature, 30°; pH of solution, 6.0; time, from 4 to 6 days.

2. Using the same procedure it was found that soluble starch, maltose and sucrose also gave rise to glucosone, in every case in much better yields than were obtained from glucose.

3. The glucosone was characterized as phenylglucosazone, as 2:4-dinitrophenylglucosazone and as anhydrogluco-*o*-diaminobenzene.

REFERENCES

- Berkeley (1933). *Biochem. J.* **27**, 1357.
 Bernhauer & Irrgang (1935). *Biochem. Z.* **280**, 360.
 — & Görlich (1935). *Biochem. Z.* **280**, 367.
 Bertrand (1904). *Ann. Chim. (Phys.)*, 8 Série III, 202.
 Birkinshaw, Charles, Lilly & Raistrick (1931). *Philos. Trans. (B)*, **220**, 130.
 Case & Cook (1931). *Biochem. J.* **25**, 1319.
 Challenger, Klein & Walker (1931). *J. chem. Soc.* 16.
 Dixon & Harrison (1932). *Biochem. J.* **26**, 1954.
 Fischer (1889). *Ber. dtsh. chem. Ges.* **22**, 87.
 — (1890). *Ber. dtsh. chem. Ges.* **23**, 2121.
 Griess & Harrow (1887). *Ber. dtsh. chem. Ges.* **20**, 2205.
 Harrison (1931). *Biochem. J.* **25**, 1016.
 Hynd (1927). *Proc. roy. Soc. B*, **101**, 244.
 Kinoshita (1927). *Acta phytochim., Tokyo*, **3**, 31.
 Kluyver & de Leeuw (1924). *Tijdschr. u. Vergelijk. Geneesk.* **10**, Afl. 2–3.
 Müller (1931). *Biochem. Z.* **232**, 423.
 Walker (1932). *Nature, Lond.*, **130**, 582.