CLXXXIII. THE OXIDASE AND DEHYDROGENASE SYSTEMS OF THE CRYSTALLINE STYLE OF MOLLUSCA.

By CYRIL BERKELEY.

From the Pacific Biological Station, Nanaimo, B.C.

(Received June 15th, 1933.)

THE occurrence in the crystalline style of *Saxidomus giganteus*, *Paphia staminea* and *Mya arenaria* of substances having the general properties of enzymes and capable of oxidising some of the reagents commonly employed for the detection of oxidases was recorded in a previous paper [Berkeley, 1923]. Similar substances have since been found in the style of *Pecten maximus*, *Mytilus edulis*, *Ostrea edulis* [Yonge, 1925; 1926, 1]. *Ensis siliqua* [Yonge, 1925; Graham, 1931], *Schizothaerus nuttalli*, *Ostrea lurida*, *Ostrea virginica*, *Ostrea gigas*, *Bankia setacea* and *Cardium corbis* [Berkeley, 1933].

The observations that the oxidation of guaiacum could be brought about by an extract of the style of *Saxidomus giganteus* in the absence of free oxygen and that the style disappeared from animals kept in boiled water in sealed vessels and was regenerated on access of air led me to suggest that the style might play a part in the anaerobic respiration of lamellibranchs first recorded by Collip [1920]. Doubt has been thrown on the suggestion that there is any essential connection between the disappearance of the style and anaerobic respiration by Nelson [1925] and by Yonge [1925; 1926, 2]. Nelson contends that anaerobic conditions slow down the movement of the cilia of the style-sac and the activity of the style-secreting cells; the material of the style dissolved in the stomach is not replaced and the style therefore disappears quite apart from any possible part it might play in supplying oxygen. He also points out that no relation can be found between the sizes of the styles of molluscs living normally under very well and very poorly aerated conditions respectively. Yonge brings forward the same arguments and also shows that the disappearance of the style depends upon the relation between its p_H and that of the gastric fluid. The style has invariably the lower p_H , so that the portion of it which is exposed to the action of the stomach contents is normally dissolved, and its maintenance depends entirely on its continuous renewal by the secreting cells.

Whilst there can be no doubt of the validity of these contentions it still remained possible that an oxidation process brought about by the oxidase set free by the dissolution of the style might lead to the production during anaerobiosis of some or all of the CO_2 on the production of which the evidence for anaerobic respiration rests. This possibility could not be disposed of until the actual physiological reaction brought about by the activity of the oxidase was determined. The present paper deals with an attempt to determine this reaction.

The autoxidisable substance of the oxidase system.

The style of *Saxidomus giganteus* has been used throughout the experiments to be described. It was stated in my previous paper [Berkeley, 1923] that active oxidase preparations were obtained when styles of this clam were dried rapidly

at air temperature *in vacuo*. Further experience has led to a modification of this finding. It is true that the dried material, when freshly prepared, yields active aqueous extracts, but it has been found that it loses the power of so doing after keeping for a few weeks, even in a tightly closed container. The loss of activity is due to the destruction of the autoxidisable component of the oxidase system, and not to that of the peroxidase, since it is restored on addition of hydrogen peroxide to the extracts. It has further been found that, in order to obtain active oxidase preparations from undried styles taken from freshly-dug clams, it is essential that the anterior ends of the styles, which impinge on the gastric shield, be left intact. If these are removed preparations made from the remaining portion of the styles have little¹ or no oxidase activity. They are rendered active by addition of either hydrogen peroxide or of an extract of the anterior end of the style.

This observation led to the conclusion that the autoxidisable component of the oxidase system was in some way connected with the anterior end of the style. In clams which have recently been feeding this end of the style always carries a mass of finely ground food material consisting mainly of bottom-living diatoms. It was suspected that these might be the source of the autoxidisable substance, and experiments were therefore made with aqueous extracts of similar diatoms. A mass of these organisms was scraped off the glass sides of an aquarium tank. The material weighed about 2 g. after draining and drying as completely as possible with filter-paper, and consisted almost entirely of *Navicula* spp. It was ground with a little distilled water until microscopic examination showed that most of the cells were ruptured; more water was added and grinding continued for a further period. The material was then saturated with toluene, the volume made up to 15 cc. and allowed to stand for an hour or so. It was then centrifuged until microscopic examination showed practically no solid particles in suspension. Half a gram of crystalline styles dried *in vacuo* some weeks previously was finely ground and allowed to stand in 15 cc. of distilled water saturated with toluene for 17 hours at air temperature. At the end of this time most of the material had dissolved. The solution was centrifuged and tested for oxidase activity, with and without addition of an equal volume of the diatom extract, by the indophenol reaction and by means of *p*-phenylenediamine, α -naphthol and pyrogallol. The diatom extract, without addition of the style extract, was tested simultaneously. The mixed extracts gave the most rapid and strongest reactions with each reagent. The style extract alone gave a slight indophenol reaction, but did not oxidise the other reagents. The diatom extract alone showed some slight activity towards pyrogallol, but was practically without action on the other reagents. The rather dark yellow-green colour of the diatom extract prevented the satisfactory use of guaiacum as a reagent for these tests owing to the difficulty of detecting the development of blue. A parallel series of tests was carried out, however, using an extract of plankton diatoms (obtained by towing in the open sea and consisting mainly of *Chaetocerus* spp.) made in the same manner. The reactions obtained were similar and, in this case, the guaiacum reaction could be applied since the diatom extract was only slightly coloured. It confirmed the other tests; the style extract alone being quite inactive, the diatom extract very slightly so, and the mixture giving a strong and rapid reaction.

¹ Removal of the anterior end of the styles eliminates the greater part, but not all, of the diatom food-material. Some inevitably remains both on and in the style. This probably accounts for the fact that extracts of styles which have had their anterior ends removed sometimes show slight oxidase activity.

It was evident that the autoxidisable component of the oxidase system of the style was contained in the diatoms adherent to it. A confirmation of this was obtained from clams which had been kept in the sea in a floating cage during a period of the year when the surface water contained little or no plankton. As a consequence the clams had been unable to feed and the anterior ends of their styles were quite free from diatoms. An extract of these styles was entirely without oxidase activity, but it was established by addition of either an extract of diatoms or of hydrogen peroxide.

The nature of the diatom constituent involved has yet to be determined. It does not seem to be as unstable as was to be anticipated from the rather rapid loss of oxidase activity by the dried styles, so long as the diatoms remain intact. Some of the bottom diatom material used in the previous experiments was dried *in vacuo* and kept for some weeks. It was then ground and extracted and a series of tests carried out with the extract to determine its power of inducing oxidase activity in an inactive style extract relatively to that of an extract of undried diatoms. The two diatom extracts were made as nearly as possible of the same concentration. The dry material led to only slightly less oxidising activity than the wet, indicating that little destruction of the autoxidisable substance had resulted from drying and storage. The destruction of the autoxidisable substance is presumably due to oxidation by atmospheric oxygen, and it is probable that this occurs more rapidly in the material occurring naturally on the style than in the intact diatoms owing to the fine state of division to which it has been reduced. Filtration of diatom extracts removes the autoxidisable substance almost entirely.

Since it was not always possible to obtain live clams as they were required, extracts of dried styles, which were inactive without addition of the autoxidisable substance, and of fresh diatoms, made as described above, have been used for a large part of this work. Moreover, this course had the advantages that solutions of greater oxidising activity could be made than were obtained by using fresh styles and that the parts played in their joint action by style extract and diatom extract respectively could be separately studied.

Selection of substrate.

Since all molluscs which have a crystalline style are herbivores [Yonge, 1930], and the style is mainly concerned in grinding food the organic portion of which consists largely of polysaccharides and submitting it to preliminary digestion, it was anticipated that the substance acted upon by the oxidase was a carbohydrate. It has long been known that the style contains an amylolytic enzyme, but there is no evidence that the style alone can carry the hydrolysis of starch to the formation of glucose, indeed evidence to the contrary has been brought forward by Yonge [1926, 1, 2], who showed that maltase is not present in the style of *Ostrea edulis*, though it occurs in the digestive diverticula. The production of glucose certainly occurs after the ground food mixed with style material leaves the stomach, and it is unlikely that any oxidative process would take place until this stage of hydrolysis had been reached. Accordingly it was decided to experiment in the first place with glucose as substrate.

Acid formation.

All attempts to detect the formation of an acid substance by the action of style extract on glucose failed. Freshly dug clams were used for these experiments and care was taken to retain the anterior portion, carrying the diatom

mass, in dissecting out the styles. These were dissolved in distilled water, the p_H of which had been adjusted at 7.0, saturated with toluene, using 1 style (weighing on average 0.2 g.) per 1 cc. of water. Solution was almost complete after standing for a period of 12 hours at air temperature, at the end of which the small amount of undissolved material was removed by centrifuging. Solutions thus prepared reacted strongly with oxidase reagents. Their p_H ranged from 6.2 to 6.0. The p_H of the style of *Saxidomus giganteus* is thus higher than that recorded for other lamellibranchs [Yonge, 1931]. Quantities varying from 1.25 to 5 % of pure glucose were added to tubes each containing 5 cc. of such style solutions and bromocresol purple as indicator. A set of tubes was allowed to stand at air temperature and another in a bath kept at 30° to 35°. No change of p_H could be observed in any of the tubes during periods up to 3 days. It seemed probable that the large amount of protein present in the style extracts exerted so strong a buffering action that an actual development of acid produced no corresponding alteration in p_H . It was found, however, that addition of one drop of a 0.01 % solution of formic acid to any one of the tubes produced a recognisable alteration in the colour of the indicator. It was concluded, therefore, that if the glucose were undergoing oxidation, the process did not result in the production of an acid.

Production of a substance giving the tests for glucosone.

Since glucosone occurs in addition to acids amongst the products of the mild oxidation of glucose an attempt was made to identify this substance as a reaction product of the style extract on glucose. For this purpose a test was necessary which would detect glucosone in small concentration in the presence of relatively large amounts of glucose. A solution containing glucosone was prepared from pure glucose by oxidation with copper acetate, following the method of Evans *et al.* [1928]. Glucosone was not isolated. The solution, after removal of copper, was used direct for the purpose of standardising the tests, and calculations of concentration were made on the assumption that oxidation to glucosone had occurred at the rate indicated by Evans *et al.* under the experimental conditions laid down by them.

A solution of 0.5 g. of *m*-nitrophenylhydrazine in 30 cc. 2*N* HCl was found a satisfactory reagent. Equal volumes of the reagent and of the solution under test are mixed and allowed to stand at 37°. A 0.03 % solution of glucosone produces a precipitate in half an hour. A 5 % solution of glucose is without action in that time. Dixon and Harrison [1932] used a 2 : 4-dinitrophenylhydrazine reagent made up in the same way for the detection of glucosone. The reagents seem to be of about equal delicacy.

It will be shown later that positive reactions were obtained with this test in application to solutions of crystalline style containing glucose, but, since it was not found possible to obtain sufficient of the reaction product to identify it by melting-point or other quantitative means, it was considered advisable to supplement the test by the following two others.

I. *Reduction to fructose and detection by the Seliwanoff test.* Glucosone on reduction by zinc dust and acetic acid yields fructose which can be detected in very dilute solution by means of the Seliwanoff test. Since glucose in relatively concentrated solution reacts to some extent it is necessary to remove most of it from the solutions to be tested. This can be accomplished by oxidation with bromine water, which leaves fructose practically unaltered. The procedure employed is as follows. 1 g. Zn dust and 0.5 cc. of glacial acetic acid are added to 5 cc. of the solution under test and the mixture is heated in a loosely-corked

test-tube standing in boiling water for 2 hours. After cooling the undissolved Zn is filtered off and 20 cc. of bromine water (saturated at air temperature) are added. The mixture is allowed to stand at air temperature in a closed vessel for at least 4 days, after which it is evaporated on a water-bath to 5 cc. An equal volume of 25 % HCl is added to this and a trace of resorcinol and the solution is heated in boiling water for a minute and quickly cooled. The presence of 0.02 % of glucosone produces a quite definite red colour. A 5 % solution of glucose gives none.

II. *Schiff's reaction*. Dixon and Harrison [1932] mention that glucosone gives this reaction. In the experiments to be described equal volumes of the solution under test and of the reagent were taken. The presence of 0.04 % of glucosone is easily detected. Glucose is entirely without action.

The presence of compounds other than glucosone might be indicated by either of these tests, but there would seem to be no other substance resulting from the oxidation of glucose which gives all three. The occurrence of positive reactions in all three cases affords, therefore, strong presumptive evidence of its presence and its formation has been assumed when this has occurred, though it is realised that the finding needs final confirmation by quantitative means.

Before applying the tests it was essential to remove all protein from the solution. This was accomplished by dialysing through cellophane. It was necessary to concentrate the dialysate before testing. Dixon and Harrison [1932] show that solutions of glucosone may be concentrated *in vacuo* in acid solution without appreciable loss. This has been confirmed.

A preliminary experiment carried out with mixed extracts of wet bottom diatoms and dried styles indicated that glucosone was produced. To 10 cc. of an extract of the diatoms made as described in a previous paragraph 0.25 g. of finely ground dried style and 0.5 g. glucose were added, the liquid was saturated with toluene and the mixture shaken at intervals until the style material was dissolved. It was then put into a wide-bottomed flask, in order to expose as large a surface to the air as possible and allowed to stand at air temperature for 48 hours. At the end of this time it was transferred to a small cellophane bag and dialysed against about 50 cc. of distilled water saturated with toluene. The water was changed at 12-hour intervals until, on testing with Fehling's solution, only a slight reducing action was obtained. The united dialysates were concentrated to 10 cc. *in vacuo* at 50–60°, after addition of 0.5 cc. of glacial acetic acid.

The three above-mentioned tests applied to 2 cc. samples of this concentrate gave in each case a positive reaction for glucosone. This has been repeated several times using extracts of both bottom and plankton diatoms, and similar results have been obtained using solutions of undried styles extracted from freshly-dug clams with care to retain the anterior ends carrying the diatoms, but, in the latter case, the glucosone reactions were never so strong as in those from solutions of the same concentration of dried style in diatom extract.

In order to determine to what extent the production of glucosone was to be attributed to the activity of each component of the mixtures, series of experiments were carried out in which each constituent was omitted in turn. The concentrations of the extracts and of glucose were the same as those in the experiments described above. The results obtained are summarised in Table I.

It is evident that the oxidation of glucose to glucosone by the combined extracts of style and diatoms depends upon the presence of the latter. The extract of diatoms alone can bring it about to only a small extent. The reaction proceeds in this respect in a manner precisely analogous to the oxidation of

Table I.

Mixture	<i>m</i> -Nitrophenylhydrazine	Fructose test	Schiff test
Style, bottom diatoms and glucose	Precipitate in 15 mins.	Strong colour	Coloration in 2 mins.
Style, plankton diatoms and glucose	Precipitate in 30 mins.	Strong colour	Coloration in 5 mins.
Style and bottom diatoms	Trace of precipitate in 30 mins.	Very slight reaction	Faint coloration in 6 mins.
Bottom diatoms and glucose	Slight precipitate in 30 mins.	Distinct coloration	Faint colour in 2 mins.
Plankton diatoms and glucose	Slight precipitate in 30 mins.	Fair coloration	Faint colour in 5 mins.
Style and glucose	No reaction	Trace of colour	No reaction

guaiacum or pyrogallol. In the absence of added glucose the glucosone reactions are very slight. Doubtless the trace which is found may be attributed to the glucose produced by the hydrolysis of carbohydrate in the diatom extract.

Presence of a dehydrogenase.

The nature of the oxidation of glucose brought about by the style with its added diatoms, consisting as it does of the removal of hydrogen from the glucose molecule, suggested that a dehydrogenase might be concerned.

An experiment was carried out with a mixture of extracts of bottom diatoms and dried style, made as described above, and arranged to detect the presence of such an enzyme. The liquid was centrifuged as soon as solution of the style material was complete, 5 % of glucose and 1 cc. of a 1/10,000 solution of methylene blue were added, and, after removal of a small sample for colour comparison, the mixture was immediately put into a Van Slyke [1917] CO₂ apparatus and pumped free from dissolved air. After standing *in vacuo* in this apparatus for 24 hours some decoloration of the methylene blue was evident by comparison with the sample removed before exhaustion, but it was difficult to estimate its degree because the blue was obscured by the yellow-green of the diatom pigment. After standing another 24 hours, during which the green colour of the solution became appreciably yellower, it was removed from the apparatus and heated to 98° for 2 minutes to inactivate the enzymes and so avoid risk of oxidation proceeding under the action of the oxidase during dialysis. After cooling the liquid was dialysed and the dialysate concentrated and tested as previously described. *m*-Nitrophenylhydrazine gave a definitely positive reaction for glucosone in 15 minutes and its presence was confirmed by the Schiff test.

Although it was not probable that the glucosone formed in this experiment was due to oxidation under the influence of the oxidase of glucose derived from the diatoms during the dissolution of the style material, since, as has been shown above, only traces are produced in this way, it seemed advisable to eliminate the possibility. The experiment was therefore repeated using plankton diatoms, which give a lightly coloured extract, in which decoloration of methylene blue can be easily followed, and an extract of dry style in water. The glucose was dissolved in the style extract and the diatom extract mixed with it immediately before addition of the methylene blue and exhaustion of air. After standing 6 hours *in vacuo* considerable decoloration had taken place; after 24 hours it was almost complete. 0.5 cc. more methylene blue solution was then added and, after re-exhausting, the solution was allowed to stand a further 24 hours during which further decoloration occurred. Strong reactions for glucosone were given by the concentrated dialysate obtained from this experiment.

Finally a similar experiment was carried out with a solution of undried styles, with their normal complement of attached diatoms, in water (about 3 g. styles to 15 cc. water). Only slight decoloration of the methylene blue took place, and all the glucosone tests were weak though definitely positive.

It was evident therefore that the combined extracts of style and diatoms contained a substance, or substances, which could reduce methylene blue and lead to the production of glucosone from glucose *in vacuo*. It remained to be determined whether these two reactions were connected and how far each constituent of the mixture was essential to them. To this end a series of experiments was carried out in which each constituent of the complete mixture was eliminated in turn. The experiments were maintained *in vacuo* for periods of 48 hours. Both bottom and plankton diatoms were used, but, since the results confirmed one another almost completely, they are not reported separately (Table II).

Table II.

Mixture	Decoloration of methylene blue	<i>m</i> -Nitrophenylhydrazine	Fructose test	Schiff test
Style, diatoms, glucose and methylene blue	Almost complete	Precipitate in 15 mins.	Strong colour	Colour in 6 mins.
Diatoms, glucose and methylene blue	Slight	Trace of precipitate in 30 mins.	Slight colour	Slight colour in 15 mins.
Style, glucose and methylene blue	Slight	Slight turbidity	Slight colour	No reaction
Style, diatoms and methylene blue	None	—	—	—
Glucose and methylene blue	Very slight	No reaction	Very slight colour	No reaction
Style, diatoms and glucose	—	Slight turbidity	Trace of colour	Very slight colour in 15 mins.

It is apparent from these results that the decoloration of methylene blue under the combined action of style and diatoms depends upon the presence of glucose; without it no decoloration occurs. Also that in the absence of methylene blue practically no formation of glucosone occurs, so that methylene blue is evidently essential as hydrogen acceptor for the dehydrogenation of glucose to proceed. It seems reasonable therefore to look upon the two reactions as connected by a dehydrogenase system transferring hydrogen from glucose to methylene blue. The full activity of this dehydrogenase system depends upon the presence of a diatom constituent. The decoloration of methylene blue and the glucosone reactions are all more pronounced in the case of the mixed extracts than in that of either alone. This diatom constituent, unlike that which functions as the autoxidisable component of the oxidase system, becomes inactive in dried diatoms which have been kept for some weeks. An extract of such dried diatoms in which dried styles and glucose had been dissolved did not decolorise methylene blue appreciably faster or more completely than a solution of the same concentration of styles and glucose in water, and the mixture led to only very slight glucosone reactions. Mann [1932] has shown that a hydrogen activator plays a part in the oxidation of glucose by the glucose dehydrogenase isolated by Harrison [1931] from liver. It is probable that such an activator is present in diatoms, but the evidence available does not exclude the possibility that the dehydrogenase is contained in the diatoms and an activator in the style. This point has yet to be investigated.

DISCUSSION.

The observations that glucose is oxidised to glucosone by the activity of the crystalline style in contact with air under the influence of an oxidase system and also out of contact with air under that of a dehydrogenase system seem to demand a common explanation. The most probable one appears to be that the production of glucosone in solutions in contact with air is due to the activity of the dehydrogenase system and that the oxidase system serves merely as hydrogen acceptor to promote this activity, but proof that this is the case awaits the separation of the various substances concerned. That an oxidase system and a dehydrogenase system can thus interact has been shown by Harrison [1931] who was able to promote the oxidation of glucose in contact with air under the influence of his glucose dehydrogenase by means of an oxidase isolated from heart muscle. In this case cytochrome was necessary to serve as carrier between the two activating systems and, if the analogy be complete, some substance performing this function is to be looked for in the crystalline style or its accompanying diatoms.

It is clear that Harrison's glucose dehydrogenase differs essentially from the dehydrogenase of the crystalline style inasmuch as the one leads to the production of gluconic acid, the other to that of glucosone.

The only other enzymes bringing about the oxidation of glucose which have been described appear to be that of Müller [1929] isolated from *Aspergillus niger* and that which occurs in certain bacteria. The former behaves as an oxidase and produces gluconic acid. The latter is inactivated by toluene [Quastel and Wooldridge, 1927]. Both, therefore, differ from the dehydrogenase of the crystalline style.

This appears to be the first time that the production of glucosone as the result of animal, or partly animal, physiological activity has been described; it is indeed the only record of its occurrence as a biological product except that of Walker [1932] who detected it in a glucose medium in which a mould of the *flavus-oryzae* group of *Aspergilli* had been grown.

If the formation of glucosone from glucose is the only reaction brought about by the oxidising activity of the crystalline style, as seems to be the case, the suggestion that the CO_2 produced by clams during anaerobiosis might be attributed to that activity must be abandoned, since CO_2 is not a product of the reaction.

It is realised that the results which have been recorded in this paper are of a preliminary nature. Future work will be directed toward the separation of the several substances concerned in the reactions which have been described, the determination of the conditions governing their interactions, the quantitative identification of the product or products, and the effect of substituting other substrates for glucose.

SUMMARY.

1. The oxidase system of the crystalline style of *Saxidomus giganteus* depends for its activity on at least two components, a peroxidase contained in the style itself and an autoxidisable substance contained in the diatoms constituting the food of the animals.
2. No acid is formed by the action of the oxidase system on glucose.
3. Glucosone appears to be formed by the action, but this needs quantitative confirmation.

4. A dehydrogenase system also occurs whose activity also depends on the joint action of a substance contained in the style and another contained in the diatom food material.

5. Glucosone also appears to be formed by the action of the dehydrogenase system on glucose in the presence of methylene blue *in vacuo*.

6. It is suggested that the oxidase system serves as hydrogen acceptor to promote the action of the dehydrogenase system.

REFERENCES.

- Berkeley (1923). *J. Exp. Zool.* **37**, 477.
 — (1933). *Nature*, **131**, 94.
 Collip (1920). *J. Biol. Chem.* **45**, 23.
 Dixon and Harrison (1932). *Biochem. J.* **26**, 1954.
 Evans, Nicoll, Strouse and Waring (1928). *J. Amer. Chem. Soc.* **50**, 2267.
 Graham (1931). *Trans. Roy. Soc. Edinburgh*, **56**, 725.
 Harrison (1931). *Biochem. J.* **25**, 1016.
 Mann (1932). *Chem. Ind.* **51**, 164.
 Müller (1929). *Biochem. Z.* **213**, 211.
 Nelson (1925). *Biol. Bull.* **49**, 86.
 Quastel and Wooldridge (1927). *Biochem. J.* **21**, 159.
 Van Slyke (1917). *J. Biol. Chem.* **30**, 347.
 Walker (1932). *Nature*, **130**, 582.
 Yonge (1925). *J. Mar. Biol. Assoc.* **13**, 945.
 — (1926, 1). *J. Mar. Biol. Assoc.* **14**, 295.
 — (1926, 2). *Nature*, **117**, 691.
 — (1930). *Nature*, **125**, 444.
 — (1931). *J. Con. Intern. Explor. Mer.* **6**, 187.