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CELLULOLYTIC ACTIVITY IN THE LAMELLIBRANCH CRYSTALLINE STYLE

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Lavine (1946) gives the first report of a lamellibranch style cellulase. Whilst investigating the style amylases of *Mactra* and *Mya*, he dialysed aqueous solutions of crystalline style material through Visking casing (regenerated cellulose) and observed that the casing eroded away. Strips of Visking casing immersed in style solution were also dissolved, but filter paper was little affected. Up till then the existence of cellulolytic activity in the lamellibranch style had been denied by Yonge (1926) and Fox *et al.* (1936), for though Fox did obtain some very slight evidence of cellulose utilization, he dismissed it as negligible.

However, the lamellibranch diet seems to consist mainly of dinoflagellates (in fact the sea mussel *Mytilus californianus* is a selective feeder, rejecting diatoms and ingesting dinoflagellates, see Fox *et al.*, 1936), and since many of these latter organisms are reputed to possess a cellulose cell wall or cuticle, their assimilation would seem to require a cellulase. Cellulases have, of course, been reported in many other Mollusca, e.g. *Pterocera* (Yonge, 1932), *Xylophaga* (Purchon, 1941), *Teredo* (Potts, 1923), and the gastric cellulase of *Helix* has been known for many years.

It seemed, in view of Lavine's findings, that the absence of cellulolytic activity previously reported in the lamellibranchs might have been due to the use of an unsuitable substrate. Boswell (1941) quotes evidence that regenerated cellulose is much more easily utilized in the cellulolytic processes of micro-organisms. In view of the report by Yonge (1926) of the presence of active spirochaetes in the crystalline style of the oyster, *Ostrea edulis*, it seemed worth while to make use of this lamellibranch, and also of the mussel, *Mytilus edulis*, in an investigation of the possible presence of a style cellulase with an action upon regenerated cellulose.

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Methods

The cellulose substrate used was in the form of a finely divided suspension of filter-paper, prepared by the method of Scales as given by Stephenson (1939), The stability of the suspension was found to be improved by passage through

an 'Ormerod' emulsifier. In view of the method used (precipitation from 62·5%, v/v, sulphuric acid) the following tests for possible breakdown products of cellulose were carried out: (*a*) Reducing sugars. Qualitatively by Benedict's reagent and the picramate test. Quantitatively by Somogyi's method (1930). (*b*) Amyloids. Iodine coloration test.

No reducing activity was obtained either in the suspension itself or in filtrates from it, subjected to acid hydrolysis. There were no amyloids present, and no cellulose could be detected in filtrates of the suspension (chlor-zinc-iodine test). The suspension gave a green ring in the Molisch test, changing to the more usual violet coloration after about 15 min. Filtrates gave no Molisch reaction at all.

Utilization of cellulose was followed turbidimetrically in a Harvey absorptiometer (Harvey, 1948), and the units of 'turbidity' given in Tables I and II are those of the absorptiometer scale—which are logarithmic, being based on Beer's Law. The data show that the turbidity of the cellulose suspension alone remains constant over the periods of time used in the experiments, and the turbidity of the suspension was found to be proportional to the amount of cellulose present (cf. Stadi & Riggs, 1943).

The style solutions were made by dissolving crystalline style material from *Mytilus* and *Ostrea* in citrate-phosphate buffer at various pH values in the region of 5·5. Preliminary experiments showed that the optimum pH of the cellulolytic activity lay in this region, which approximates to the pH of the stomach fluid in *Ostrea* (Yonge, 1926). The cellulose in later experiments was also suspended in citrate-phosphate buffer. All experiments were conducted at laboratory temperature, which varied between 15 and 19° C.

Results

Boiling tubes were set up in series, each containing an equal volume of reaction mixture. A typical experiment was as follows, using style material from *O. edulis*.

Tubes A and B, 5 ml. buffer + 3 ml. cellulose suspension.

Tubes C, E and F, 5 ml. style solution + 3 ml. cellulose suspension.

Tubes B and C were boiled for 3 min. to act as controls. The turbidities of the five tubes were measured at intervals and are given in Table I as absorptiometer scale readings.

TABLE I

	Hours								
Tube	0	1	2	3	4	6	7	8	20
A	275	270	269	270	262	270	270	265	268
B	275	270	268	270	262	268	272	269	270
C	408	406	410	406	404	400	402	408	409
E	235	225	207	200	187	176	177	171	146
F	238	223	213	194	184	173	168	164	142

After this experiment, 5 ml. samples from tubes A, B, E and F were taken (shaking well to disperse the suspension as uniformly as possible). Each sample was centrifuged, decanted, and washed, three times, and the residual cellulose then dried to constant weight. The weights are shown below:

Tube	Residual cellulose (mg.)	Tube	Residual cellulose (mg.)
A (control)	14·9	E (active)	10·2
B (control)	15·0	F (active)	8·6

The activity is clearly thermo-labile.

In the case of experiments using material from *Mytilus edulis*, a similar decline in turbidity in tubes containing unboiled style solution and cellulose occurred, but activity was less than with style material from *Ostrea*. The styles from the two animals differ in appearance, and Berkeley (1935) has shown that qualitative differences in the glyco-proteins, at least, of various lamellibranch styles do occur. Since it might have been that the cellulose utilization was merely due to some of the numerous bacteria inhabiting the style, various preservative agents were used in later experiments. 'Merthiolate' (1 in 200,000) and chloroform (0·5–8 ml. of active preparation) did not appear to inhibit cellulose utilization. Xylene and toluene (also 0·5–8 ml. of active preparation) made turbidity measurements impracticable, but after one experiment, using *Mytilus* material, and toluene, the residual cellulose in each of three tubes after 20 hr. activity was collected, washed, dried and weighed. The weights are given below:

Tube	Residual cellulose (mg.)
Control	14·3
Preparation with toluene	8·9
Preparation without toluene	8·6

Thus toluene, at least, does not abolish cellulose utilization.

It was found that reducing substances appeared in active preparations as cellulose disappeared, and in some experiments the increase in such reducing activity, with time, was followed.

In one such experiment, five tubes were set up containing cellulose suspension in buffer and *Mytilus* style material, also in buffer. Relevant controls were set up as before, but this time included a tube with style solution only, to compensate for reducing activity in the style itself. This proved to be a necessary precaution.

The turbidities of tubes containing the whole system showed a regular decline, whilst those of the controls remained constant. After various periods of time, single tubes were withdrawn and activity stopped by the addition of sodium hydroxide solution to a pH above 8·5 (phenol red). The reaction mixtures were filtered and the filtrates retained. Estimation of reducing activity was effected by the Somogyi method (Somogyi, 1930), on 5 ml.

samples from two tubes before, and all five tubes after, acid hydrolysis. This hydrolysis was carried out by heating the samples for 10 hr. at 90° C., after adding five drops of concentrated sulphuric acid. The reducing activity is given below, corrected for reagent and style solution reducing substances, and expressed as milligrams of glucose per millilitre of reaction mixture.

Incubation time (hr.)	Reducing substances	
	Before acid hydrolysis	After acid hydrolysis
0	Nil	Nil
6½	0·051	0·066
19	—	0·116
25	—	0·144
43	0·072	0·166

It will be seen that substances able to reduce the Somogyi reagent are found in the *Mytilus* style preparation, but that the large increase in the reducing power of filtrates after acid hydrolysis suggests that the principal immediate products of cellulose breakdown are soluble compounds with little reducing power.

When active tubes were evacuated or tightly stoppered, the activity was slowed down or stopped. Using style material from *Mytilus*, five experimental tubes were set up (Table II). Control tube B showed no change with time. Boiling the control tube 1 increased the turbidity which then showed no further change with time. After 5 hr. the active tube 3 was tightly stoppered which prevented further reduction of turbidity. In the other two active tubes reduction continued until the end of the experiment.

TABLE II. TURBIDITY, AS LOGARITHMIC SCALE READINGS OF THE ABSORPTIOMETER

Tube	Hours								
	0	1	2	3	5	7	19	22	25
B (control)	363	364	366	365	370	367	367	370	370
1 (control)	662	662	662	663	662	661	662	662	662
3 (active)	432	402	390	384	375	377	370	376	374
4 (active)	417	392	374	371	358	352	327	322	312
5 (active)	412	382	367	362	352	350	314	314	314

From this investigation and from Berkeley's (1933 *a*, *b*) work on style oxidase, presence of glucosone (2-keto-glucose) was expected but neither glucosone nor glucose could be detected, no keto groups could be demonstrated by the Rothera or nitro-prusside tests.

On warming protein-free filtrates of reaction mixtures with phenyl hydrazine hydrochloride, in 2N-hydrochloric acid, a phenyl hydrazone of colourless pinnate crystals could be obtained. After acid hydrolysis, filtrates gave an ozazone resembling glucosazone, and having a decomposition point at 205° C. Further identification of the products of this cellulose utilizing system was not possible in the limited time available.

This investigation extends Lavine's findings of cellulase in *Mactra* and *Mya* to *Ostrea* and *Mytilus*. It has not established an actual relation between the cellulolytic action of the style and the presence of spirochaetes, and in *Mytilus* the bacterial population of the style is not yet known. In view, however, of the known ability of some groups of spirochaetes (see Walker & Warren, 1938) to hydrolyse cellulose to simpler carbohydrates, a connexion between style spirochaetes and cellulolytic action remains an interesting possibility. Morton (1952) discusses this point more fully.

Summary

A method of demonstrating cellulose utilization in biological systems by measurement of the turbidity of a cellulose suspension in a photo-electric absorptiometer is described. The crystalline styles of *Ostrea edulis* and *Mytilus edulis* appear to contain a cellulolytic factor as yet uncharacterized.

REFERENCES

Berkeley, C., 1933*a*. The oxidase and dehydrogenase systems of the crystalline style of Mollusca. *Biochem. Journ.* Vol. 27, pp. 1357–65.

—— 1933*b*. Oxidase of the crystalline style. *Nature, Lond.*, Vol. 131, p. 94.

—— 1935. The chemical composition of the crystalline style and of the gastric shield: with some new observations on the occurrence of the style oxidase. *Biol. Bull. Woods Hole*, Vol. 68, pp. 107–14.

Boswell, J. G., 1941. Biological decomposition of cellulose. *New Phytol.*, Vol. 40, pp. 20–33.

Fox, D. L. *et al.* 1936. The habitat and food of the California sea mussel. *Bull. Scripps Inst. Oceanography, Univ. California Tech. Serv.*, Vol. 4 (1), pp. 1–64.

Harvey, H. W., 1948. The estimation of phosphate and of total phosphorus in sea waters. *Journ. Mar. Biol. Assoc.*, Vol. 27, pp. 337–60.

Lavine, T. F., 1946. A study of the enzymatic and other properties of the crystalline style of clams: evidence for the presence of a cellulase. *Journ. Cell. Comp. Physiol.*, Vol. 28, pp. 183–195.

Morton, J. E., 1952. The role of the crystalline style. *Proc. Malacol. Soc. London*, Vol. 29, pp. 85–92.

Potts, F. A., 1923. The structure and function of the liver of *Teredo*, the ship-worm. *Proc. Camb. Phil. Soc.*, Vol. 1, pp. 1–17.

Purchon, R. D., 1941. On the biology and relationships of the lamellibranch *Xylophaga dorsalis* (Turton). *Journ. Mar. Biol. Assoc.*, Vol. 25, pp. 1–39.

Somogyi, M., 1930. A method for the preparation of blood filtrates for the determination of sugar. *Journ. Biol. Chem.*, Vol. 86, pp. 655–63.

Stadie, W. C., & Riggs, B. C., 1943. A photo-electric method for the determination of peptic activity in gastric juice. *Journ. Biol. Chem.*, Vol. 150, pp. 463–70.

Stephenson, M., 1939. *Bacterial Metabolism.* London.

Walker, E. & Warren, F. L., 1938. Decomposition of cellulose by *Cytophaga*. I. *Biochem. Journ.*, Vol. 32, pp. 31–43.

Yonge, C. M., 1926. The structure and physiology of the organs of feeding and digestion in *Ostrea edulis*. *Journ. Mar. Biol. Assoc.*, Vol. 14, pp. 295–386.

—— 1932. Notes on feeding and digestion in *Pterocera* and *Vermetus*, with a discussion on the occurrence of the crystalline style in the Gastropoda. *Sci. Rep. Gt. Barrier Reef Exped.*, Vol. 1, No. 10, pp. 259–81.