CCVIII. A CELLULASE FROM THE SYMBIOTIC INTESTINAL FLAGELLATES OF TERMITES AND OF THE ROACH, *CRYPTOCERCUS PUNCTULATUS*.

By WILLIAM TRAGER.

From the Department of Tropical Medicine, Harvard University Medical School, Boston, Massachusetts.

(*Received August 16th, 1932.*)

The number of organisms able to utilise cellulose as the sole source of carbohydrate is small, and the number from which it has been possible to isolate a cellulase is smaller still. Karrer *et al.* [1923; 1924; 1925] have done extensive work with a cellulase from the intestinal juice of the vineyard snail, as have Pringsheim and Bauer [1928] and Pringsheim and Leibowitz [1928] with a very similar enzyme obtained from malt. Boynton, Lyman and Miller [1927] were able to demonstrate cellulase somewhere in the anterior portion of the shipworm, *Bankia setacea*. Jewell and Lewis [1918] found lichenase in the alimentary system of each of twenty species of invertebrates. Since lichenin is a form of cellulose [Karrer, Joos and Staub, 1923] the active extracts from these animals would probably also have hydrolysed cellulose. Pringsheim [1912; 1919] showed long ago the presence of a cellulase in cellulose-fermenting bacteria, but this enzyme loses its activity very soon after the death of the bacteria, and has never been obtained in a cell-free condition.

Of all the cellulose-feeding organisms there are few whose diet contains as high a percentage of cellulose as does that of termites. Cleveland [1923; 1924] has shown that these insects can live for long periods of time on a diet of almost pure cellulose, if, and only if, their normal complex intestinal fauna has not been removed. This fauna consists mainly of representatives of two orders of flagellates: the polymastigotes and the hypermastigotes. The great majority of these intestinal protozoa ingest cellulose particles and apparently digest them. They might be presumed to contain a cellulase.

Most termites, on account of their small size, do not furnish very good material for the preparation of enzyme extracts. Recently, however, it has been discovered that the large wood-feeding roach, *Cryptocercus punctulatus*, possesses a fauna of intestinal flagellate symbionts very similar in all respects to those of termites [Cleveland, 1930]. These roaches, obtained through the kindness of Dr L. R. Cleveland, proved to be an excellent source of the enzyme, cellulase, and they were used for most of the work reported in this paper. The

presence of the enzyme has also been demonstrated in the termites, *Reticulitermes flavipes* and *Termopsis angusticollis*, as well as in pure cultures of one of the protozoa from the latter.

Action of the cellulase. Proof that it is secreted by the intestinal protozoa.

It was a simple matter to show that a cellulase was present in the alimentary tract of *Cryptocercus*. Only a single typical experiment need be detailed. The guts of twenty roaches were ground in a mortar with sand to a brown paste. This was diluted with distilled water to 25 cc. giving a light brown suspension, which, after filtration through fine filter-paper, became almost white. The extract, covered with toluene, was dialysed in a collodion bag against running water for 18 hours to rid it of all reducing substances. Two tubes were then prepared, one containing 1 cc. of the extract, the other 1 cc. of the extract with a small amount of reprecipitated, powdered cellulose. The liquid in both was covered with toluene and the tubes were incubated at 35·5°. Tests were made for glucose with Benedict's reagent.

	Benedict's test after	
Tube contents	4 hours	24 hours
1 cc. extract	–	–
1 cc. extract + cellulose	+	+ + +

– means test negative.

An active extract could also be obtained by grinding up the gut of a single roach, diluting to 5 cc. and centrifuging.

In several cases, the digest, after the action of the enzyme on the cellulose had ceased, was evaporated to dryness over a water-bath, and the residue taken up in phenylhydrazine-sodium acetate solution. On boiling, typical glucosazone crystals were obtained. In one experiment these were filtered off, washed and dried, and the melting-point found to be 202–204° (uncorr.).

It next appeared important to determine whether the cellulase was secreted by the protozoa, or by the host, or by both. Insect enzymes are typically produced in the midgut and in the salivary glands [Abbott, 1926; Wigglesworth, 1927]. The flagellates of *Cryptocercus* are present only in the hind-gut [Cleveland, Hall and Sanders, unpublished]. The following experiment was accordingly performed. The salivary glands, fore- and mid-guts, and hind-guts of 5 roaches were carefully removed, and a separate extract made from each. The salivary glands, after having been ground up, were diluted with water to 3 cc., the fore- and mid-guts to 3 cc., and the hind-guts to 15 cc. The extracts were partly cleared by centrifuging.

	Benedict's test after	
Tube contents	1 day	3 days
1 cc. salivary gland extract, 0·5 cc. 95 % alcohol	–	–
Same with cellulose	–	–
1 cc. fore- and mid-gut extract, 0·5 cc. 95 % alcohol	–	trace
Same with cellulose	–	trace
1 cc. hind-gut extract, 0·5 cc. 95 % alcohol	–	–
Same with cellulose	+ +	+ +

The enzyme is thus present only in the hind-gut, a fact which indicates its protozoan origin.

Still more conclusive proof of the protozoan origin of the enzyme was obtained from the following experiment, done in conjunction with Dr L. R. Cleveland. A group of roaches was fed for 1½ months on nearly pure cellulose (Whatman filter-paper No. 40). The roaches were then divided into three groups. One group (*A*) received no further treatment. A second (*B*) was defaunated by keeping the insects at a temperature of 34° for 6 hours, a procedure which in no way injures the roach. The third group (*C*) was defaunated by oxygenation at 4 atmospheres for 2 hours [Cleveland, 1925]. An extract from a roach of group *A* was oxygenated at the same time with the insects of group *C*. The latter, after treatment, were fed on the alimentary canal material of roaches killed and defaunated by keeping them at a temperature of − 10° for 1 hour. This was done so that the group *C* roaches could be reinfected by any bacteria that might have been killed by the oxygen treatment (bacteria are generally very resistant to low temperatures) and thereby have a normal intestinal flora. Extracts were made from each group, using the gut of one roach diluted to 5 cc. and centrifuged, with results as summarised below.

No.	Tube contents	Benedict's test after 1 day	2 days	3 days
1	1 cc. from *A*, 0·5 cc. 95 % alcohol	−	0	0
2	Same as 1 with 0·25 cc. cellulose suspension	+ +	0	0
3	Same as 1 oxygenated 2 hrs., 4 atm.	−	0	0
4	Same as 2 oxygenated 2 hrs., 4 atm.	+ +	0	0
5	1 cc. from *B*, 1 day after treatment, 0·5 cc. 95 % alcohol	−	−	−
6	Same as 5 with 0·25 cc. cellulose suspension	−	−	−
7	1 cc. from *C*, 2 days after treatment, 0·5 cc. 95 % alcohol	−	−	−
8	Same as 7 with 0·25 cc. cellulose suspension	−	−	−

0 means not tested.

It is evident that regardless of the method of defaunation used, the enzyme disappears coincidently with the disappearance of the protozoa, although the treatments which destroy the protozoa injure neither the enzyme extract nor the roach. The roach itself secretes no cellulase, a fact which is very important with respect to the symbiotic nature of the flagellates.

Still further proof that the cellulase is secreted by the flagellates was furnished by the action of an extract prepared from cultures of *Trichomonas termopsidis*, a xylophagous, intestinal flagellate of the termite, *Termopsis angusticollis*. This organism, now in its 22nd subculture, has been cultivated for almost 2 years in an artificial medium containing finely powdered cellulose[1]. The cultures are contaminated by only one species of bacterium, a small bacillus which actively ferments dextrose but is incapable of attacking cellulose. The contents of 20 culture-tubes were centrifuged, and the small mass of protozoa thus obtained was ground with sand, diluted to 2 cc. and cleared by centrifuging. The results were:

Tube contents	Benedict's test after 2·5 hrs.
1 cc. extract, 0·5 cc. phosphate buffer (p_H 5·3), 0·5 cc. water	−
1 cc. extract, 0·5 cc. phosphate buffer (p_H 5·3), 0·5 cc. cellulose suspension	+ +

[1] Full details of the cultivation will be given in a later paper.

Presence of the cellulase in termites.

The guts of 100 *Reticulitermes flavipes* were ground with sand and diluted with water to 3 cc. The enzyme was adsorbed on aluminium hydroxide and eluted with 3 % KH_2PO_4.

Tube contents	Benedict's test after 5 hours
0·5 cc. extract, 0·5 cc. buffer (p_H 5·3), 0·25 cc. cellulose suspension	+ +
Same but without cellulose	–

The gut of a single *Termopsis angusticollis* was ground with sand and diluted with water to 2 cc. This extract also showed typical enzyme action.

Properties and partial purification of the cellulase.

The properties of this protozoan cellulase have been studied in some detail, mainly to provide a basis for comparison of this enzyme with cellulases from other organisms, notably snail-cellulase and malt-cellulase.

Very early in the course of the work it was noted that the physical state of the cellulose had much to do with the ease with which it was attacked by the enzyme. Thus an extract of 25 *Cryptocercus* guts dialysed 24 hours and made up with alcohol and distilled water to 25 cc. containing 30 % alcohol gave these results (incubated at room temperature):

	Benedict's test after		
Tube contents	1 day	2 days	3 days
5 cc. extract, ground filter-paper	–	–	+ +
5 cc. extract, cellulose suspension	+ +	+ + +	0
5 cc. extract	–	–	–

The extract of a single roach gut diluted to 6 cc. with distilled water gave:

Tube contents	Benedict's test after 1 day at room temperature
1 cc. extract, cellulose suspension	+ + +
1 cc. extract, filter-paper	+
1 cc. extract	–

The material referred to as cellulose suspension was prepared by v. Weimann's method in accordance with the directions given by Pringsheim and Bauer [1928]. 1 g. of pure filter-paper was dispersed in a solution of 50 g. of lithium chloride in 50 cc. of water at a temperature of 160°. The solution so obtained was poured into about 600 cc. of cold water, whereupon the cellulose precipitated out in a very fine flocculent form. It was washed and centrifuged about 9 times with water. The final residue was taken up in distilled water to give a suspension having about 6 to 8 mg. of cellulose per cc. The surface of the cellulose exposed to enzyme action by such a preparation is much greater than that exposed by even finely powdered filter-paper and the micellar structure is probably also different. Karrer, Schubert and Wehrli [1925] have shown that both these factors are important with respect to the degree of hydrolysis of

various cellulose preparations by snail-cellulase. Any form of treated cellulose, although chemically identical with native cellulose, was more easily hydrolysed than the latter.

The enzyme concentration also affected the extent of hydrolysis. A series of tubes was prepared in which a watery extract of the cellulase was diluted 1–4, 1–8, 1–16, *etc.* to 1–128 with phosphate buffer at a p_H of 5·3. The results were:

Dilution of extract	Roach units per cc.	Benedict's test after 2 hours
1–4	5/6	+ + + + +
1–8	5/12	+ + + +
1–16	5/24	+ + +
1–32	5/48	+ +
1–64	5/96	+
1–128	5/192	–
1–4 (no cellulose)	5/6	–

One roach unit is the extract from one roach. This term will be used to give an approximate idea of the concentration of material in the various extracts. It has meaning only when preparations from one and the same extract are being compared. Some quantitative results indicating the effect of concentration of enzyme on the time course of the reaction, cellulose → glucose, are given in a later portion of this paper.

The cellulase was inactivated by 10 minutes' immersion in a boiling water-bath, as well as by 1 hour's at 60°. The extracts were treated first and then were mixed with cellulose suspension and allowed to act on it at a temperature of 35° for periods of time up to 24 hours.

It has been possible partially to purify the enzyme extracts. The original watery preparations are always smooth brown suspensions which pass through coarse filter-paper unchanged. Filtration of such an aqueous extract through a Seitz asbestos filter gives a clear colourless filtrate devoid of enzyme. If, however, enough alcohol is first added to the extract to give a 30 % concentration, and the mixture is kept about a day, subsequent filtration through a Seitz filter gives a clear, slightly yellowish filtrate, which does contain the enzyme, although in not nearly as high a concentration as in the unfiltered material. In watery extracts the cellulase appears not to pass through a collodion membrane, as shown below.

Tube contents	Benedict's test after 2 hours	Benedict's test after 1 day
1 cc. extract, 1 cc. phosphate buffer (p_H 5·3), 0·5 cc. cellulose suspension	+ + +	+ + +
1 cc. extract in a collodion bag immersed in 1 cc. phosphate buffer (p_H 5·3) with 0·5 cc. cellulose suspension	–	–

In this respect this cellulase seems to differ from the lichenase of Karrer, Staub and Joos [1924, 1] which did act on lichenin through a collodion membrane. In the present instance the collodion bag was made from Merck's U.S.P. collodion, allowed to dry 10 minutes, fixed in 95 % alcohol and washed in water. The membrane used by Karrer, Staub and Joos may have been prepared in a different manner.

The enzyme was not adsorbed on Merck's powdered animal charcoal, but all the brown material present in the extract could be removed in this way. The cellulase could be adsorbed completely by aluminium hydroxide and eluted with 3 % KH_2PO_4, thus again behaving like snail-cellulase [Karrer, Staub and Joos, 1924, 1]. It could also be precipitated with acetone and dried with ether. The experimental details follow.

One hundred roach guts were ground with sand and diluted with distilled water to 30 cc. The mixture was centrifuged and the supernatant liquid dialysed against running water for 19 hours. Three 5 cc. portions of the extract were now subjected to three different treatments. Part 1 was diluted with distilled water to make 10 cc. To part 2, 5 cc. of acetone were added, giving a brown precipitate which was filtered off, washed twice with ether and allowed to air-dry. Part 3 was treated with aluminium hydroxide paste and filtered. The clear colourless filtrate may be designated as part 3*a*. The residue was washed thrice with 10 cc. of 3 % KH_2PO_4, the final preparation (3) being a colourless, slightly turbid liquid. Test- and control-tubes were now set up in this manner: each control-tube contained 3 cc. of extract, 2 cc. phosphate buffer (p_H 5·3) and 1 cc. of distilled water; each test-tube held the same, except that 1 cc. of cellulose suspension replaced the 1 cc. of water. In the case of part 2 the dry powder was suspended in 6 cc. of water to give a concentration of 1·3 mg. per cc. The material in the tubes was covered with toluene. The tubes were incubated for about 2 hours at 35·5°, and the glucose concentration in each was then determined by the Willstätter-Schudel [1918] iodimetric method [modified by Kline and Acree, 1930]. The results were:

Part No.	Mg. glucose per cc.
1	4·3
2	0·2
3	0·8
3*a*	0

The method of precipitation and drying involves a considerable loss. Adsorption on aluminium hydroxide and elution with KH_2PO_4 also occasion a loss of enzyme due to the incomplete nature of the elution. The activity of the purified enzyme is, however, sufficiently great for its use in quantitative studies.

Effect of enzyme concentration and of temperature.

Because of the restricted amount of material available, quantitative work with this protozoan cellulase has necessarily been limited. Enough results have been obtained to indicate the type of reaction and the way in which it is affected by changes in enzyme concentration and in temperature. The reaction was followed in all cases by the titration of samples, removed at intervals, by the modified Willstätter-Schudel iodimetric method, using 2 cc. of 0·1 *N* iodine solution, 3 cc. of 0·1 *N* NaOH and a 5 cc. Folin burette for the thiosulphate.

The protozoa from 10 *Cryptocercus* were washed and centrifuged in a balanced salt solution in which they could live for long periods of time. The mass

so obtained was ground with sand, diluted to 10 cc. with distilled water and centrifuged. 5 cc. of the extract were mixed with 25 cc. of buffer (p_H 5·3) and 5 cc. of cellulose suspension. The mixture was kept at 35° and 1 cc. samples were removed. The data are plotted in Fig. 1.

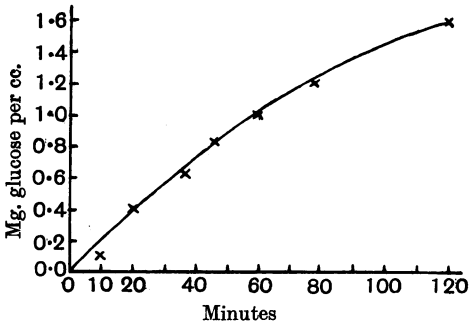

Fig. 1. Hydrolysis of cellulose by an unpurified extract at 35°.

In another experiment the washed protozoa from 30 roaches were similarly extracted. The extract was treated with aluminium hydroxide paste and the enzyme eluted with 30 cc. of 3 % KH_2PO_4. Thus 1 cc. of this preparation contained 1 roach unit. Tubes were set up containing cellulose suspension and enzyme appropriately diluted with phosphate buffer of p_H 5·3. The liquid in each case was covered with toluene and 1 cc. samples were taken. The results are shown graphically in Fig. 2.

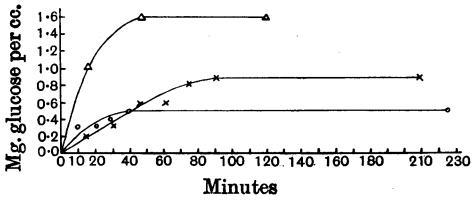

Fig. 2. Effect of enzyme concentration on the hydrolysis of cellulose by a purified extract at 33·5°.

—o— 0·2 roach unit per cc.
—x— 0·5 roach unit per cc.
—△— 0·75 roach unit per cc.

With yet another similarly purified extract, these results were obtained, keeping the concentration of enzyme the same:

Temperature	Mg. glucose per cc. after 2 hours
16°	0·3
21°	0·4
26°	0·7
34°	0·2

A temperature of 26° was thus more favourable, over a period of 2 hours, than any of the lower or higher temperatures tried. For a real analysis of the

temperature effect, it will be necessary to compile a family of curves showing the rates of hydrolysis at various temperatures. The velocity constant of the hydrolysis at each temperature could then be calculated (assuming that the reaction follows the unimolecular law) and the relation of these constants to temperature found.

Cellobiase.

Some cellobiose was prepared from the octa-acetate according to the method of Peterson and Spencer [1927]. It was dissolved in phosphate buffer of p_H 5·3 at a concentration of 0·01 g. per cc., and used in the following experiment.

Washed protozoa from 30 roaches were ground and the mixture centrifuged. The supernatant liquid was made up to 10 cc. with water, giving extract 1, with 3 roach units per cc. 7 cc. of 1 were treated with aluminium hydroxide and the enzyme eluted with 10 cc. of 3 % KH_2PO_4 giving the purified extract 2, with 2 roach units per cc. Two tubes were prepared. One (*A*) contained 2 cc. of 1, 1 cc. of 3 % KH_2PO_4 and 3 cc. of the cellobiose solution to give a mixture having 1 roach unit and 0·005 g. of cellobiose per cc. The other (*B*) held 3 cc. of 2 and 3 cc. of the cellobiose solution giving a mixture with 1 roach unit (purified) and 0·005 g. of cellobiose per cc. The contents of both tubes were covered with toluene. The tubes were incubated at 30·1°. At intervals 1 cc. samples were removed and titrated iodimetrically. The data are summarised in Table I. The figures in column 6 were obtained in the following way. 5 mg.

Table I.

(1) Tube No.	(2) Time (minutes)	(3) Milliequiv. I	(4) Milliequiv. $Na_2S_2O_3$	(5) Total milli-equiv. I used	(6) (see text)	(7) % hydrolysis
A	0	0·1960	0·1466	0·0494	0·0292	0
	15	0·2940	0·2399	0·0541	0·0339	16·2
	30	0·2940	0·2380	0·0560	0·0358	22·8
	45	0·2940	0·2323	0·0617	0·0415	42·7
	135	0·2940	0·2152	0·0788	0·0586	100
B	0	0·2940	0·2599	0·0341	0·0292	0
	15	0·2940	0·2599	0·0341	0·0292	0
	30	0·2940	0·2570	0·0370	0·0321	10
	60	0·2940	0·2513	0·0427	0·0378	30
	120	0·2940	0·2456	0·0484	0·0435	50

of cellobiose react with 0·0292 milliequiv. of iodine. This number was accordingly subtracted from the total milliequiv. used at zero time to give the milliequiv. of iodine used by the mixture itself apart from the cellobiose. This latter figure was then in each case subtracted from the total milliequiv. used to give the numbers in column 6. The reducing power of the mixture itself was presumably due to the presence of glucose and was very much less (almost negligible) in the purified extract than in the unpurified. The percentage hydrolysis was obtained from Table II, constructed after a similar table used by Pringsheim and Bauer [1928]. Fig. 3 illustrates the course of the hydrolysis in tubes *A* and *B*.

About 18 hours after the start of the reaction, the remaining liquid in tube *A* was evaporated to dryness and the residue taken up in phenylhydrazine-

Table II.

% hydrolysis	Mg. cellobiose	Milliequiv. I	Mg. glucose	Milliequiv. I	Total milliequiv.
0	5	0·0292	0	0	0·0292
10	4·5	0·0263	0·525	0·0058	0·0321
20	4	0·0233	1·05	0·0117	0·0350
30	3·5	0·0204	1·575	0·0175	0·0379
40	3	0·0174	2·1	0·0233	0·0407
50	2·5	0·0146	2·625	0·0291	0·0437
60	2	0·0117	3·15	0·0350	0·0467
70	1·5	0·0087	3·675	0·0408	0·0495
80	1	0·0058	4·2	0·0466	0·0524
90	0·5	0·0029	4·725	0·0525	0·0554
100	0	0	5·25	0·0583	0·0583

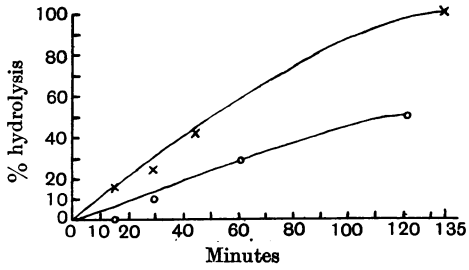

Fig. 3. Hydrolysis of cellobiose at 30·1°.
—×— Unpurified extract (tube *A*).
—o— Same extract purified (tube *B*).

sodium acetate solution. Glucosazone was obtained on boiling and was filtered off while the mixture was hot. The filtrate, on cooling, gave no crystals of cellobiosazone. The remaining liquid in tube *B* was treated in the same manner. In this case both glucosazone and an osazone soluble in hot water (cellobiosazone) were formed.

This protozoan cellulase thus contains, like malt-cellulase and snail-cellulase, a cellobiase.

Summary.

A cellulase has been demonstrated in the symbiotic intestinal flagellates of *Cryptocercus punctulatus*, *Reticulitermes flavipes* and *Termopsis angusticollis*.

The enzyme has also been extracted from one of these flagellates, *Trichomonas termopsidis*, grown for 2 years in pure culture.

The active extract hydrolyses cellulose to glucose. It also hydrolyses cellobiose to glucose. Possibly two enzymes are concerned, a cellulase proper and a cellobiase.

The cellulase complex can be adsorbed on aluminium hydroxide and eluted with 3 % KH_2PO_4. In this and in several other respects it resembles snail-cellulase and malt-cellulase.

Data are presented indicating the way in which the hydrolysis of cellulose by this protozoan cellulase is affected by enzyme concentration and by temperature.

Acknowledgment is due to Dr L. R. Cleveland under whom this work was done, and also to Dr A. E. Navez of the Physiology Department, Harvard University.

REFERENCES.

Abbott (1926). *J. Exp. Zool.* **44**, 219.
Boynton, Lyman and Miller (1927). *J. Biol. Chem.* **75**, 613.
Cleveland (1923). *Proc. Nat. Acad. Sci.* **9**, 424.
—— (1924). *Biol. Bull.* **48**, 289.
—— (1925). *Biol. Bull.* **48**, 309.
—— (1930). *Anat. Rec.* **47**, 293.
—— Hall and Sanders (in manuscript).
Jewell and Lewis (1918). *J. Biol. Chem.* **33**, 161.
Karrer, Joos and Staub (1923). *Helv. Chim. Acta*, **6**, 800.
—— Schubert and Wehrli (1925). *Helv. Chim. Acta*, **8**, 797.
—— Staub and Joos (1924, 1). *Helv. Chim. Acta*, **7**, 144.
—— —— —— (1924, 2). *Helv. Chim. Acta*, **7**, 154.
Kline and Acree (1930). *Bureau of Standards J. Res.* **5**, 1063.
Peterson and Spencer (1927). *J. Amer. Chem. Soc.* **49**, 2822.
Pringsheim (1912). *Z. physiol. Chem.* **78**, 266.
—— (1919). Die Polysaccharide. (Berlin.)
—— and Bauer (1928). *Z. physiol. Chem.* **173**, 188.
—— and Leibowitz (1928). Die Methodik der Fermente III, 902. (Thieme, Leipzig.)
Wigglesworth (1927). *Biochem. J.* **21**, 51.
Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.