CELLULOSE DIGESTION BY THE SILVERFISH *CTENOLEPISMA LINEATA**

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It is the prevailing opinion to-day that the majority of species of animals which feed upon plants or plant products are unable to hydrolyse cellulose in spite of its abundance (Baldwin, 1952), depending instead upon the sugars, starches, fats and proteins present in the plant tissues. This implies the lack of a suitable alimentary enzyme, cellulase, or symbiotic gut micro-organisms which could perform that function. Study of cellulose digestion in animals is hampered by the difficulty of defining the role of the micro-organisms of the gut. If cellulolytic action is found in extract of tissues or in secretions of glands, the question remains whether or not this activity is attributable to micro-organisms; conversely, finding a cellulose-digesting microbe in the intestine of an animal does not prove conclusively its benefit to the animal.

Although observations on the feeding habits of the silverfish have been numerous and date back to Hooke's *Micrographia* (1665), its nutrition has been little investigated. However, Lindsay (1940) found the silverfish *Ctenolepisma longicaudata* would eat any kind of cellulose but preferably the most degraded. He also found that a diet of cellulose alone kept the animal alive for a longer time than if it were starved. He reported obtaining an enrichment culture of cellulose-digesting bacteria from the crop of a single animal, and from this concluded that digestion of cellulose in silverfish is accomplished by symbiotic micro-organisms of the gut.

The object of the present investigation was to define more clearly whether silverfish digest and metabolize cellulose and whether this is accomplished by symbiotic micro-organisms or by enzymes secreted from the cells of the gut. The silverfish *Ctenolepisma lineata* was chosen for this purpose because specimens are easily obtained in numbers on the bark of local *Eucalyptus* trees. They thrive in the laboratory at 25° C. and at a relative humidity of 80%. They were fed on a diet of rolled oats in darkened gallon jars containing a nidus of absorbent cotton. Breeding takes place usually in April and May, at which time the eggs are laid. Young could be grown to maturity under laboratory conditions without difficulty; therefore they served admirably for some of the experiments spanning a long time and involving tests of growth and moulting.

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DEMONSTRATION OF CELLULOSE DIGESTION IN THE SILVERFISH

By determining the respiratory quotient (R.Q.) of silverfish, fed for a month on a cellulose diet (Whatman no. 43 filter-paper), it should be possible to gain indicative evidence of cellulose digestion. For this study three or four silverfish were placed into each of a number of small Warburg flasks attached to small-bore manometers and the gaseous exchange was followed for a day. Readings were taken after the animals had become acclimatized to the prevailing temperature of the water-bath (27·2° C.) and the confined area of the flasks at which time they became relatively quiet and remained so for the remainder of the experiment.

The R.Q. on cellulose was 0·91, 1·09 and 0·92 in a series of three experiments, whereas silverfish, fed for a month on gelatin (Gelfoam) and tested in a similar manner to those on cellulose, gave an R.Q. of 0·75. The results suggest that cellulose-fed animals are metabolizing almost pure carbohydrate.

More definite evidence for digestion of cellulose was obtained by feeding individual silverfish pure cellulose, recovering the faecal pellets as they were dropped, and determining the digestibility coefficient (Roeder, 1953) for the silverfish by the following ratio:

$$\% \text{ digestibility} = \frac{\text{Dry weight of food consumed} - \text{dry weight of excrement}}{\text{Dry weight of food consumed}} \times 100.$$

When digestion is complete no excrement is obtained and the coefficient is 100%. Some typical values for insects include 24% for the silkworm larva, 48·5% for the armyworm, *Prodenia eridania*, and 46·3% for the mealworm, *Tenebrio molitor* (Roeder, 1953). From the data in Table 1 for silverfish fed on cellulose for a month or more, it is evident that the silverfish digests more of the food taken in than the other insects listed above, suggesting that it digests cellulose while the phytophagous insects do not. In this respect it resembles the dairy cow with a coefficient of 72% for dried grass (Maynard, 1937). Not only did the silverfish digest a considerable amount of the cellulose eaten but in many cases they gained weight even when fed on cellulose alone.

The most decisive evidence of cellulose digestion was obtained by feeding silverfish with uniformly labelled ^{14}C cellulose. In these experiments a well-fed adult silverfish (*c.* 15–25 mg.) was put into a standard, single-arm Warburg vessel without a centre well, fitted with a standard taper hollow glass tube which was torch-sealed at the untapered end. A sample of radioactive cellulose weighing several milligrams was included as food for the animal, and the side arm was filled with 0·4 ml. freshly prepared 5% sodium hydroxide. After a month or longer the alkali, which had absorbed the carbon dioxide produced during respiration, was removed and transferred to a conical 12 ml. centrifuge tube to which 6% barium chloride was added dropwise until no further precipitation of barium carbonate was observed. The tube was then centrifuged, the residual alkali decanted, and the precipitate washed several times with 95% ethyl alcohol. The control without an animal served as a blank for carbon dioxide absorbed from the air. The barium carbonate

was spread on aluminum planchets (Calvin *et. al.* 1949) and the radioactivity was measured by a helium-flow, windowless Geiger counter (Tracerlab Autoscaler). The results of these tests are given in Table 2. The high radioactivity of the carbon dioxide respired by the silverfish indicates extensive metabolism of nutrient derived from radioactive cellulose which in turn could happen only if the silverfish had digested cellulose since it was the only food available.

Table 1. *Digestibility coefficients for silverfish fed cellulose for several months*

No.	Initial wet wt. silverfish (mg.)	Dry wt. cellulose consumed (mg.)	Dry wt. of faeces (mg.)	Digestibility coefficient (%)
1	22·21	9·39	1·37	85·4
2	25·21	7·46	1·94	74·2
3	25·38	3·35	0·99	73·2
4	20·01	1·58	0·38	75·9
5	23·51	4·49	0·58	87·0
6	27·25	2·33	0·63	71·7
7	15·67	5·99	0·84	85·8

Table 2. *Radioactivity of* $Ba^{14}CO_3$ *derived from the respiratory* CO_2 *of silverfish fed* ^{14}C *cellulose*

Planchet no.	mg. $BaCO_3$	Counts* per min.	*d*/min./mg. $BaCO_3$	*d*/min./0·1 mM $BaCO_3$
		Experiment 1		
1	4·0	3710	3840	$7·58 \times 10^4$
2	6·5	5150	3380	$6·69 \times 10^4$
3	1·3	1100	3230	$6·37 \times 10^4$
4	2·0	1730	3420	$6·75 \times 10^4$
5	2·1	1750	3300	$6·51 \times 10^4$
6 Back-ground	—	59	—	—
		Experiment 2		
1	2·9	1510	2560	$5·05 \times 10^4$
2	2·0	1200	2830	$5·58 \times 10^4$
3	1·2	855	3220	$6·36 \times 10^4$
4 Back-ground	—	45	—	—

* Counts per minute are presented after correction for self-absorption, coincidence and the efficiency of the counter which was determined in each run by counting a radioactive standard sample; *d* = disintegrations.

Additional evidence for incorporation of radioactive cellulose came from determination of radioactivity of the tissues of the silverfish. For this purpose silverfish were fixed in formol-acetic-alcohol, dehydrated in alcohol, embedded in paraffin and sectioned in the sagittal plane. The paraffin was first removed from the 100 μ thick sections, and then from the resulting dried tissue remnants of the gut were removed under a dissecting microscope, leaving only muscle and glandular tissue. The remaining section, placed under a Geiger counter, gave 1400 counts per

0·46 mg. dry tissue per minute, a count equivalent to 3120 disintegrations per mg. of dry silverfish tissue without the intestine or its contents. This value is many times the background. Incorporation of radioactive carbon could have occurred only if the cellulose had been digested.

LACK OF CELLULOLYTIC ACTION OF SILVERFISH GUT MICRO-ORGANISMS

Since the silverfish used in the experiments described in the first section carry a population of micro-organisms in various parts of the gut, especially in the crop, it was necessary to determine whether the microflora might account for cellulose digestion. When a microfauna exists it consists only of large colourless parasitic sporozoans averaging about fifteen per animal and could be excluded from consideration on this account.

An attempt was therefore made to determine whether cellulolytic microbes occurred in the gut. For this purpose into each of twenty sterile 125 ml. Erlenmeyer flasks containing 75 ml. of Hungate's mineral medium (1950) and containing folded pieces of Whatman's no. 43 filter-paper as a source of cellulose was placed the entire gut of a silverfish, with aseptic precautions. The flasks were incubated at room temperature for over a month. Only seven showed cellulose decomposition, and in these moulds, not bacteria, were responsible. Since moulds were never seen growing in the gut of the silverfish, the best explanation of the results is that during their extensive grazing the silverfish had eaten spores of moulds growing on wood.

Since the silverfish gut contains nutrients other than cellulose, it was possible that micro-organisms might actively hydrolyse cellulose only in the presence of other nutrients. Therefore, forty additional tests were made in which the cellulose was supplemented with protein, carbohydrates, nucleic acid hydrolysate and vitamins in addition to minerals. Various combinations and concentrations, as suggested by demands of various micro-organisms but particularly those digesting cellulose, were tested. Although many bacteria developed in such media they did not decompose cellulose.

Since it was necessary also to exclude the possibility that anaerobic bacteria might decompose the cellulose in the gut of the silverfish, twenty-six tests were made following methods devised by Hungate (1950) for detection of anaerobic cellulolytic micro-organisms. These tests were negative, and it became evident that anaerobic conditions were unfavourable for the silverfish microflora, since only small populations developed under anaerobic conditions, yet when oxygen was admitted to the anaerobic test plates many additional colonies appeared.

It must be admitted that negative results are never as convincing as positive ones, and that even the numerous media tested may not have provided favourable conditions for growth of cellulolytic bacteria. Perhaps the enriching nutrients, even in a mash of silverfish intestine, are inhibitory to cellulolytic bacteria. But since so few bacteria are normally seen in the gut, and since no unequivocal evidence of cellulose digestion by gut bacteria was observed, this line of attack was discontinued as unprofitable.

Finally, it is possible that intracellular symbionts might serve to digest cellulose. Such agents have been implicated in insect digestion by Buchner (1953) and culture of some forms has been claimed (Glaser, 1930); the method available to check this possibility is to examine sections of the gut for mycetomes, mycetocytes or other structures similar in nature to those found in other insects. Examination of 10μ sections at various levels of the gut of the silverfish stained in various ways, such as haematoxylin and eosin, Gram's stain, van Gieson's stain and safranin and fast green, considered diagnostic (Brooks & Richards, 1955), revealed no such structures. The gut of the silverfish is relatively simple in structure compared to that of other insects, consisting of a thin-walled oesophagus and crop, a proventriculus with well-developed teeth, and a glandular midgut which leads into a smooth-walled hindgut and rectum. If intracellular symbionts exist they escape detection by the accepted methods. In passing it may also be pointed out here that histological study shows no 'fermentative chambers' or proctodaeal pouches where extra-cellular micro-organisms might be harboured.

DIGESTION OF CELLULOSE IN BACTERIA-FREE SILVERFISH

If the silverfish does not depend upon micro-organisms to digest its cellulose, digestion should be possible in the complete absence of micro-organisms. For this purpose it is necessary to obtain silverfish in bacteria-free culture on a synthetic medium. It proved possible to sterilize eggs and to raise the silverfish on sterile nutrients. Eggs obtained during the April and May breeding season were sterilized by washes in White's solution (1931) containing mercuric chloride and alcohol. They were picked up individually with a damp inoculating loop which had previously been flamed and cooled in sterile distilled water. They were transferred to the first depression in a sterile agglutination dish containing 0·2 ml. of White's solution. They were then successively transferred to three depressions containing a like amount of sterile distilled water, care being taken to transfer a minimum of liquid in each case so as to dilute the first solution maximally. They were then transferred to a sterile test-tube containing a piece of sterile, dried, rolled oats which had previously been soaked in a solution containing 1% yeast extract and 0·02% liver extract concentrate. The small amount of water still clinging to the eggs was absorbed by the piece of oat.

That this procedure sterilizes the eggs was demonstrated by plunging a sample of eggs treated as above into vessels containing sterile Bacto A-C medium (Difco Laboratories, 1948) which supports a comprehensive list of anaerobic and aerobic organisms and is widely used to test sterility. In no case did micro-organisms appear, and nymphs hatched out in the medium when eggs were not crushed (some eggs were crushed to test for micro-organisms which might not be able to get through the egg membrane). Also, cultures containing 4 mm. scaled juveniles which had been grown on sterile media from washed eggs showed no evidence of micro-organisms. On the other hand, media inoculated with eggs taken directly from the field or from ordinary laboratory cultures showed copious growth of

micro-organisms of a wide variety. The method may therefore be considered satisfactory.

Sterile nymphs which had been allowed to grow to 4 mm. in length on sterile oats enriched by soaking in yeast extract and liver extract were placed in the sterile Warburg vessels such as were described in the first section. In one set of experiments only ^{14}C cellulose was used, in the second ^{14}C cellulose and oats because silverfish do not live well on cellulose alone. Aseptic precautions were followed throughout the experiment. The results are given in Table 3.

Table 3. *Radioactivity of* $Ba^{14}CO_3$ *derived from the respiratory carbon dioxide of bacteria-free silverfish**

Animal no. 1, fed ^{14}C cellulose with no additional food; nos. 2 and 3, fed ^{14}C cellulose and oats soaked in yeast extract and liver extract.

Planchet no.	mg. $BaCO_3$	Counts per min.	*d*/min./mg. $BaCO_3$	*d*/min./0·1 mM $BaCO_3$
		Animal no. 1		
1	1·7	458	1480	$2{\cdot}92 \times 10^4$
2	1·2	600	2710	$5{\cdot}34 \times 10^4$
3	0·6	277	2360	$4{\cdot}65 \times 10^4$
4	0·5	379	3600	$7{\cdot}10 \times 10^4$
5 Back-ground	—	33	—	—
			Average	$5{\cdot}12 \times 10^4$
		Animal no. 2		
1	1·9	255	604	$1{\cdot}19 \times 10^4$
2	2·8	384	627	$1{\cdot}23 \times 10^4$
3	0·3	74	740	$1{\cdot}46 \times 10^4$
4 Back-ground	—	38	—	—
			Average	$1{\cdot}29 \times 10^4$
		Animal no. 3		
1	2·5	164	327	$6{\cdot}44 \times 10^3$
2	1·8	134	342	$6{\cdot}73 \times 10^3$
3	0·5	68	446	$8{\cdot}78 \times 10^3$
4	0·3	52	456	$8{\cdot}97 \times 10^3$
5 Back-ground	—	29	—	—
			Average	$7{\cdot}73 \times 10^3$

* The animals weighed less than 1 mg. each.

The data clearly indicate that bacteria-free silverfish produce as much carbon dioxide with ^{14}C as do the animals taken directly from laboratory cultures when fed cellulose alone. When fed cellulose and oats, less carbon dioxide with ^{14}C appears, undoubtedly because the animals graze more on the oats and eat less cellulose. The data offer conclusive proof that the silverfish possesses a cellulase.

CELLULOLYTIC ACTIVITY OF GUT EXTRACTS; OTHER CARBOHYDRASES IN THE GUT

Since the bacteria-free silverfish digests cellulose it should be possible to detect a cellulase from its gut. Preliminary tests were made with each of the digestive organs of the silverfish in turn: crop, salivary gland and midgut. Negative results were obtained with all but the midgut. Histological examinations indicate that of all the

digestive organs only the midgut has a secretory epithelial lining with goblet-type secretory cells. Subsequent work was therefore confined to the midgut.

The midgut of each of twenty silverfish was removed from animals narcotized with carbon dioxide, by pinching with a pair of watchmaker's forceps at the fourth or fifth abdominal segment and gently teasing the animal apart with a second pair of forceps. This exposed the gut chiefly at the point of juncture between midgut and proventriculus. The midgut was grasped behind the main lobes and teased away from the rest of the intestine. The tissue was ground in a Potter homogenizer chilled with ice water, (Dockstader & Halvorson, 1950) the tip of the grinding rod being dipped into 0·02 M-phosphate or citrate buffer and placed in the tube. As each midgut was dissected it was immediately placed in the homogenizer and ground with a gentle turning motion. Since silverfish tissues are exceptionally soft, no abrasive was necessary and microscopical examination disclosed few intact cells. After the last midgut had been ground the grinding rod was washed by dropping cold buffer on it over a 12 ml. graduated conical centrifuge tube into which the contents of the homogenizer were rinsed with several washings of cold buffer until a total of 2–3 ml. of homogenate suspension had been collected. The contents were centrifuged and only the decanted supernatant liquid was used for enzyme determinations.

To test the extract for cellulose digestion, to 0·2 ml. of the enzyme solution in buffer contained in a centrifuge tube was added a 0·1 ml. sample of regenerated cellulose suspension (Trager, 1932) in 0·02 M-phosphate buffer. A layer of toluene was added and the contents of the tube were allowed to incubate overnight at room temperature. Controls included substrate solution alone with buffer, and enzyme solution alone with buffer. An aliquot (0·1 ml.) was removed at the beginning of the experiment from each tube and the protein precipitated by adding 0·1 ml. each of the barium hydroxide and zinc sulphate solutions of Somogyi (1945); after 24 hr. similar aliquots were again removed and tested. In each case the tubes were centrifuged and the supernatant liquid was tested for reducing sugar by the method of Somogyi (1952).

For measuring sugar a standard curve was first obtained at 530mμ with known amounts of sugar using the Beckman spectrophotometer modifier for small samples with pinhole slit for micro-cuvettes. Sugar present in test samples could then be determined by directly reading the sugar concentration corresponding to the optical density on the standard curve.

The data in Table 4 indicate that the midgut extract contains a cellulase. Its activity is less than that of the cellobiase and the amylase which were tested in a similar manner except that the initial dilution of homogenate was double, and twice the amount of various reagents was added in making the tests.

The activity of the cellulase depends upon the pH, and in Fig. 1 the pH/activity curve is plotted. The experiments were performed in the same manner as described above, buffer at an appropriate pH being used in the dilutions. The activity maxima were about half a pH unit apart in two successive tests; this was not the result of a pH change, as checks indicated constancy over the period of experimentation.

Table 4. *Determination of an amylase, cellobiase and cellulase from the midgut of the silverfish*

Substrates: soluble starch, 1 %; cellobiose, 0·01 %; regenerated cellulose, 0·03 %. Phosphate buffer (0·02 M) pH 6·7 for amylase and cellobiase, and McIlvaine's buffer (0·025 M) pH 4·7 for cellulase.

Reaction mixture	Time of sampling (hr.)	Reducing sugar at time of sampling (μg.)	Increase in sugar after 24 hr. (μg.)
Midgut + buffer	0	1·0	—
Midgut + buffer	24	1·5	0·5
Starch + buffer	0	1·0	—
Starch + buffer	24	2·0	1·0
Midgut + starch	0	22·0*	—
Midgut + starch	24	300·0	278·0
Cellobiose + buffer	0	20·0	—
Cellobiose + buffer	24	20·0	0
Cellobiose + midgut	0	20·0	—
Cellobiose + midgut	24	95·0	75·0
Cellulose + buffer	0	Trace	—
Cellulose + buffer	24	Trace	0
Cellulose + midgut	0	Trace	—
Cellulose + midgut	24	15·0	15·0

* This large quantity of sugar produced at 'zero hours' is attributable to hydrolysis occurring in the period of time (*c.* 15 min.) which elapsed after addition of substrate to enzyme mixture before protein precipitation.

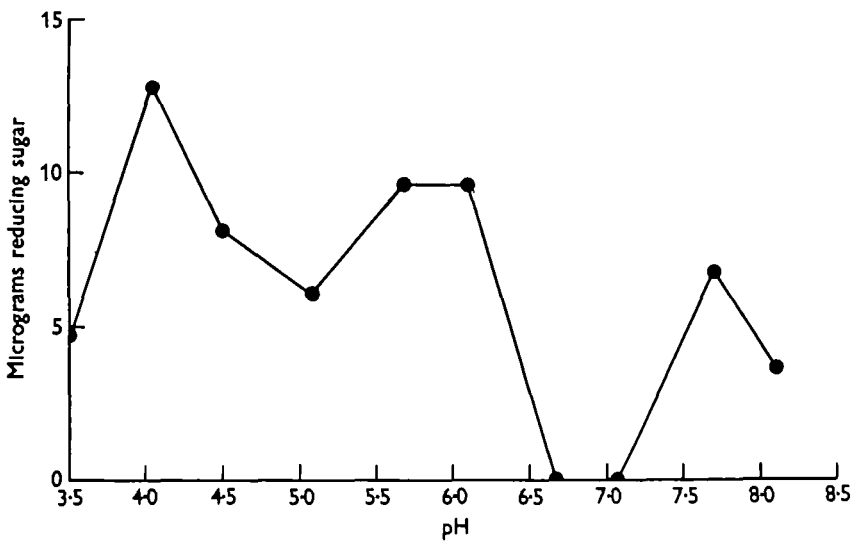

Fig. 1. The pH activity curve of silverfish cellulase.

The pH/activity curve for cellobiase was also determined and is given in Fig. 2. This enzyme attacks the cellobiose molecules which are formed by the action of cellulase upon the cellulose and is therefore of interest in this connexion.

In the final series of experiments the cellulase was further characterized by determining its precipitation with ammonium sulphate. For these experiments twice as many midguts were used as in the above experiments, and the centrifugate was reground to extract as much of the enzyme as possible. The initial solution of

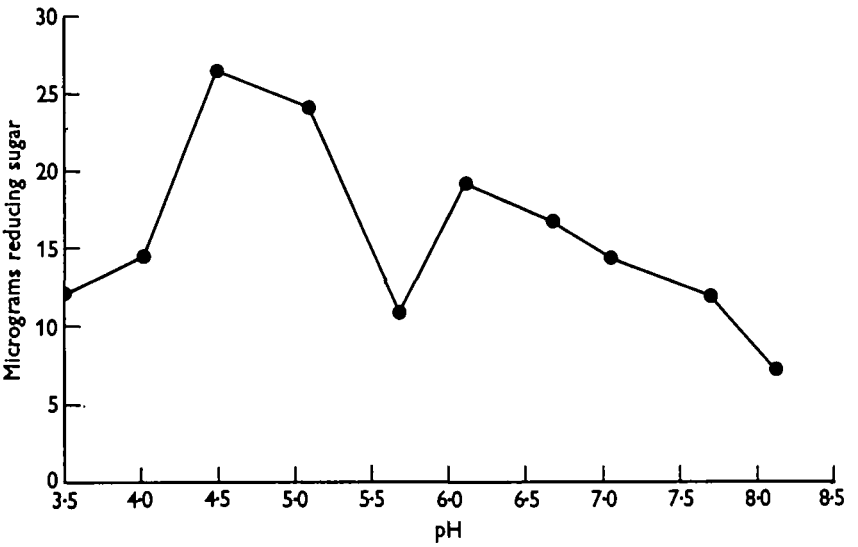

Fig. 2. The pH activity curve of silverfish cellobiase.

enzyme from forty silverfish amounted to 3 ml. To this was added the appropriate volume of saturated ammonium sulphate (which had been brought to pH 6·7 by dropwise addition of 10% potassium hydroxide), until 50–60% saturation had been reached. After this had been centrifuged, more saturated ammonium sulphate was added to the decanted supernatant fluid to bring it to 80% saturation and the procedure was repeated. For 100% saturation, crystals of ammonium sulphate were added. The high-speed Servall angle centrifuge was used to centrifuge the sample, except in initial experiments. In one series of experiments ammonium sulphate was added to test 10% intervals. No precipitate formed until 50% saturation was reached; at 50% heavy precipitation occurred and 60, 70 and 80% produced precipitates. Each centrifugate was dissolved in distilled water and washed separately into individual tubes of Visking dialysis tubing ($\frac{8}{32}$ in. diameter) in which it was dialysed against distilled water overnight with frequent changes of distilled water.

To test the activity, each sample was first dissolved in distilled water, dialysed, then buffer was added to give an optimal pH for cellulase and cellobiase activity. The data are given in Table 5. Cellulase activity is concentrated in the 60, 70 and some cases the 80% fractions.

Table 5. *Separation of cellulase activity of soluble extracts of silverfish midgut into fractions by ammonium sulphate precipitation*

Exp. no.	Fraction (%)	μg. reducing sugar produced per 1 ml. of enzyme solution per 24 hr. period
1	50–60	39·5 Adams centrifugate
	80	7·2 Adams centrifugate
	100	25·2 Servall centrifugate
2	50–60	15·8 Servall centrifugate
	80	17·3 Servall centrifugate
3	50	Trace Servall centrifugate
	60	20·0 Servall centrifugate
	70	30·0 Servall centrifugate
	80	Trace Servall centrifugate

DISCUSSION

The experiments described above clearly show that the silverfish digests cellulose and that it does so by virtue of its own enzymes, not by a cellulolytic flora or fauna. Other animals have been considered capable of cellulose digestion, notably some protozoans (Hungate, 1942, 1946), *Helix* (Holden & Tracey, 1950), *Teredo* (Greenfield & Lane, 1953), and some insects (Ripper, 1930; Mansour & Mansour-Bek, 1934). At the present time some question exists concerning animal cellulases because bacteria-free animals were never used. Some of these experiments should be repeated using tracer techniques on bacteria-free animals.

Perhaps the most successful animals which use cellulose as food depend upon a gut microflora, like the ruminants (Hungate, 1950) or upon a microfauna like many of the termites (Cleveland, 1924). This suggests that Nature's experiments with animals producing their own cellulases have been less successful during the course of evolution than her experiments with cellulolytic symbionts. In the case of ruminants, the capacity of bacteria to incorporate inorganic nitrogen in synthesizing protein, a process foreign to animal tissue, gives the symbiotic method considerable advantage over simple digestion of cellulose. The advantage in the termite, where this does not occur, is less clear. The silverfish has achieved a unique niche by virtue of its ability to utilize cellulose, since this removes it from active competition with more aggressive and highly differentiated insects. Experiments clearly indicate that silverfish cannot live on cellulose alone, since they invariably die in about a month on such a diet. However, growth occurs when a nitrogen source such as oat is supplied. This subject is the topic of a second study which will be reported separately.

SUMMARY

1. The silverfish, *Ctenolepisma lineata*, on a diet of cellulose alone shows a respiratory quotient of close to unity, indicating utilization of carbohydrate, presumably derived from cellulose.

2. The silverfish may gain weight temporarily on a diet of cellulose alone although the diet is not satisfactory for prolonged feeding.

3. The silverfish digests part of the cellulose ingested, the utilization efficiency being comparable to that of the dairy cow.

4. Silverfish fed cellulose uniformly marked with ^{14}C respire $^{14}CO_2$, indicating that cellulose is metabolized and therefore must have been digested.

5. The gut of the silverfish contains many micro-organisms, but none of the bacteria grown in favourable culture media are capable of digesting cellulose. A few moulds do, but they are never seen growing in the gut and are presumably developed from spores grazed from wood by the silverfish.

6. Bacteria-free silverfish were obtained by washing eggs in a solution of mercuric chloride and ethanol and raising the nymphs on rolled oats and vitamins under aseptic conditions.

7. Bacteria-free silverfish fed cellulose uniformly marked with ^{14}C respire $^{14}CO_2$, indicating that even in the absence of micro-organisms, *C. lineata* metabolizes cellulose and therefore must have digested it.

8. A cellulase was demonstrated in extracts of the midgut. A cellobiase and an amylase were also shown to be present. The pH optima for the cellulase are 4·0 and 6·0, with a smaller peak occasionally showing at 7·7. For cellobiase the optima were 4·5 and 6·5.

9. The cellulase was isolated in the 60 and 70% ammonium sulphate saturated fractions of the soluble proteins from the midgut.

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