THE HYDROLYTIC ENZYMES OF INVERTEBRATES

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The object of this research was to test for hydrolytic enzymes in the invertebrate kingdom, and to investigate the conditions of optimum activity of any proteoclastic enzymes found.

The number of papers dealing with the digestive processes of invertebrates is so great, and as they mostly deal with qualitative measurements, it is beyond the scope of this paper to review the literature¹ except where there are points to which it is especially necessary to refer.

METHODS

The enzymes were prepared by treating the minced tissue, of which it was desired to test the enzymic activity, with an equal weight of glycerine. This was allowed to stand for several weeks, and then the solid portions were removed by straining through cotton. In later experiments an attempt to purify these extracts was made by precipitating the glycerine extract with alcohol, and after filtering off the precipitate it was extracted with water containing a little toluol.

In all the experiments the action of the enzyme was controlled by an identical mixture, using some of the enzyme extract in which the activity had been destroyed by boiling. The ratio of the enzyme extract to the volume of the digestion mixture was 1 : 10.

Most of the extracts were made during the month of April in the years 1907 and 1908.

1. For the literature up to 1903 see O. v. Furth, *Vergleichende chemische Physiologie der niederen Tiere* (Jena, Gustav Fischer).

CARBOHYDRATE HYDROLYSIS

Table I shows the results of the action of fresh glycerine extracts on carbohydrates. Two per cent. solutions of the different carbohydrates were taken, and to each 9 c.c. 1 c.c. of enzyme extract was added. This gave an initial concentration of 1·8 per cent. of the carbohydrate. At the end of the experiment a known volume was heated with excess of Fehling's solution, and the cuprous oxide was filtered on to a Gooch crucible, washed, oxydised, and weighed as cupric oxide. The figures give the percentage of glucose formed. In the case of maltose the amount of glucose is determined from the difference in reducing power between the active enzyme and the control. In the other experiments on starch, glycogen, and saccharose, the controls were, of course, negative. The digestion was allowed to proceed for a period of forty-eight hours at 25° C.

TABLE I

Source of Enzyme	Starch	Glycogen	Saccharose	Maltose
Digestive gland of <i>Cancer pagurus</i> (edible crab)	0·51	0·43	0·30	0·48
'Liver' of <i>Patella vulgata</i> (limpet)	0·72	0·59	0·98	0·42
Intestine (oesophagus and inferior spiral) of <i>Echinus</i> <i>esculentus</i> (sea urchin)	0·98	0·54	1·25	0·63
'Disc' of <i>Ophiocoma nigra</i> (brittle star)	0·31	0·04	0·83	—

The experiments recorded in Table II were performed with the enzymes prepared by precipitation of the glycerine extracts with alcohol. Owing to length of time required to show the presence of enzymes capable of hydrolysing maltose and lactose it was found impossible to conduct a long series of experiments by the usual methods, but by using Barfoed's reagent the change from di-saccharide to mono-saccharide can be easily detected.¹ The hydrolysis of polysaccharides was proved by the disappearance of the colour given with iodine, and by reduction of copper in alkaline solution. The decomposition of saccharose into mono-saccharides was found by the appearance of reduction on heating with an alkaline copper solution.

The table shows the lengths of time before appreciable hydrolysis occurred; the temperature at which the experiments were conducted was 27° C.

1. Roaf, *Bio-Chem. Journ.*, Vol. III, p. 182, 1908.

TABLE II

SOURCE OF ENZYME	TIME REQUIRED TO SHOW APPRECIABLE HYDROLYSIS				
	STARCH	GLYCOGEN	SACCHAROSE	LACTOSE	MALTOSE
Digestive gland of <i>Cancer pagurus</i> (edible crab)	 22 hours	22 hours	6 days	8 days	22 hours
'Liver' of <i>Patella vulgata</i> (limpet)	 16 "	16 "	4 "	3 "	16 "
'Liver' of <i>Pecten opercularis</i> (scallop)	 18 "	18 "	42 hours	18 hours	18 "
Intestine (oesophagus and inferior spiral) of <i>Echinus esculentus</i> (sea urchin)	20 "	20 "	No hydrolysis	6 days	68 "
Mesenteric filaments of <i>Tedlia crassicornis</i> (sea anemone)	 66 "	No hydrolysis	No hydrolysis	No hydrolysis	39 "
Mesenteric filaments of <i>Actinia mesembryanthemum</i> (sea anemone)	No hydrolysis	No hydrolysis	No hydrolysis	No hydrolysis	96 "
'Disc' of <i>Opbiocoma nigra</i> (brittle star)	 —	No hydrolysis	24 hours	6 days	—

In addition it was found that the extract of the whole colony of *Cellaria fistulosa* (polyzoon) hydrolyses starch, and the extract of the 'pyloric caeca' of *Asterias rubens* (starfish) also acts on starch although the 'stomach' extract of the same organism is without action.

TABLE III

SOURCE OF ENZYME	HYDROCHLORIC ACID			SODIUM CARBONATE			EFFECT OF TEMPERATURE	
	n	n	n	NEUTRAL	n	n	Reaction	11°C. 25°C. 35°C.
Digestive gland of <i>Cancer pagurus</i> (edible crab)	20	25	40	80	40	25	20	11°C. 25°C. 35°C.
'Liver' of <i>Patella vulgata</i> (limpet)	 2.5	3.8	5.4	4.3	0.5	—	1.8	4.2 3.8 4.3
Intestine (oesophagus and inferior spiral) of <i>Echinus esculentus</i> (sea urchin)	1.0	1.1	2.4	0.5	0.1	—	—	3.7 5.5 6.3
'Disc' of <i>Opbiocoma nigra</i> (brittle star)	 0.5	1.5	1.4	0.4	—	—	—	1.6 2.4 2.1
Mesenteric filaments of <i>Tedlia crassicornis</i> (sea anemone)	 1.4	0.6	0.1	4.8	—	4.8	5.5	—

Protein hydrolysis was found to be present in both acid and alkali using the extract of the 'pyloric caeca' of *Asterias rubens* (starfish), but the stomach extract was without effect in either reaction. Extracts of the whole colonies of *Alcyonium digitatum* (dead man's toes), *Clema celata* (boring sponge) and *Cellaria fistulosa* (polyzoon) showed protein digestion in acid reaction but not in alkaline media, and the extract of the mesenteric filaments of *Actinia mesembryanthemum* (sea anemone) in 1% HCl showed increasing action with temperatures up to 35°C.

PROTEIN HYDROLYSIS

Protein hydrolysis was investigated, using fibrin as a substrate. The first series of experiments was made by keeping the digestive mixtures for forty-eight hours at 25° C., and at the end of that time 10 c.c. of 10 per cent. tri-chloroacetic acid was added to each 20 c.c. This was then filtered, and the nitrogen determined in 10 c.c. of the filtrate by the method of Kjeldahl. The control figures were subtracted from those given by the active enzymes, and the differences in cubic centimetres of decinormal acid are given in Table III. The first portion of the table shows the effect of digestion at 25° C. for forty-eight hours, with varying reaction, and the second portion shows the effect of varying the temperature when the reaction is kept constant.

In later experiments the rate of digestion was estimated by the coloration produced by the solution of congo red fibrin.¹ In order to test if this method gave the maxima of digestion in the same strengths of acid and alkali as the usually accepted methods, an experiment was carried out with ordinary pepsin and trypsin at 40° C. The results showed that the maximum occurs with pepsin in, at least, $\frac{n}{20}$ HCl (0.18 per cent.), and with trypsin in $\frac{n}{5}$ Na₂CO₃ (1.06 per cent.).

The congo red fibrin method was then applied to a series of extracts (Table IV). It was thought that perhaps the protein present would interfere with the digestion of fibrin, so in all except two experiments the enzyme was prepared by precipitation of the glycerine extract with alcohol, and extraction of the alcohol precipitate with toluol water. It was found that the enzyme in the case of certain extracts was apparently altered so that digestion now occurred better in sodium carbonate than in hydrochloric acid (compare Tables III and IV).

1. Roaf, *Bio-Chem. Journ.*, Vol. III, p. 188, 1908.

TABLE IV

SOURCE OF ENZYME	HYDROCHLORIC ACID						SODIUM CARBONATE						Duration and Temperature			
	n		n		Neutral		n		n		n					
	20	25	50	100	100	100	20	40	20	10	5					
Digestive gland of <i>Cancer pagurus</i> (edible crab)	{	...	...	...	...	...	20	35	60 ¹	60	20 ²	4 hours at 40° C.				
		...	...	...	...	...	40	40	10	10	10	4 hours at 37° C.				
'Liver' of <i>Patella vulgata</i> (limpet)	{	...	...	...	...	...	10	30	—	—	— ³	6 hours at 40° C.				
		...	...	...	...	...	—	10	30	30	25	65 hours at 27° C.				
'Liver' of <i>Pecten opercularis</i> (scallop)	...	...	...	...	...	...	10	15	10	—	15	48 hours at 40° C.				
		...	...	...	...	...	—	10	25	30	40	42 hours at 27° C.				
Intestine (oesophagus and inferior spiral) of <i>Echinus esculentus</i> (sea urchin)...	...	...	...	...	...	...	—	—	—	40	10	12	—	48 hours at 27° C.		
Mesenteric filaments of <i>Tedalia crassicornis</i> (sea anemone)	...	...	...	...	...	...	—	—	—	—	15	10	—	66 hours at 27° C.		
Mesenteric filaments of <i>Actinia mesembryanthemum</i> (sea anemone)...	...	...	...	...	...	...	—	—	—	—	10	30	15	10	—	72 hours at 27° C.

The figures show the amount to which the digestion mixture must be diluted in order to give solutions of equal depth of colour ; where the digestion was absent or so slight as to give no appreciable coloration the space is left blank.

1. With $\frac{n}{20}$ Na_2CO_3 the digestion at 15° = 10 c.c. to match which a similar digestion at 40° had to be diluted to 80 c.c.

2. These experiments were performed with the fresh glycerine extract.

FAT HYDROLYSIS AND COAGULATING ENZYMES

The action of the enzymes on boiled milk containing litmus was also investigated. Some experiments were performed with the fresh glycerine extract, and some with the extract further prepared as already described. In nearly all cases coagulation occurred,¹ but in order to notice this result it was often necessary to observe carefully as the coagulum became rapidly digested. Coagulation failed in only one case, and there it was quite evident that the caseinogen had become hydrolysed into simple bodies. The purified enzyme in these cases caused a precipitate, and not a solid coagulum. This was apparently due to the conditions of the experiment, as the enzyme from the same source, before preparation, caused a coagulum. In all cases except one the litmus was turned red, showing the production of acid. In that case the enzyme was one which had been precipitated with alcohol, and the fresh enzyme from the same source caused the development of acidity. The coagulation was not merely due to the development of acid.

The fresh glycerine extracts were tested on oxalated blood plasma. In several cases a distinct coagulation occurred, and in others a curdy precipitate not unlike fibrin appeared. As the observations were complicated by digestion of fibrin, it was difficult to determine whether the coagulation was absent or only masked by digestion of the resulting fibrin. At present no explanation of this result can be given, but it is recorded here in the hope that it may be of some use, and the effect is being investigated further.

The results of the action on litmus milk and blood plasma are given in Table V :—

1. A rennin-like enzyme has been found in *Suberites domuncula* (Colte, *Soc. biol.*, Vol. LIII, p. 95, 1901); in *Cancer pagurus* and *Maia squinado* (Sellier, *Soc. biol.*, Vol. LXI, p. 449, 1906, and Vol. LXIII, p. 703, 1907); in *Aphrodite aculeata* (Sellier, *Soc. biol.*, Vol. LXII, p. 693, 1907); and in *Cambarus* (Bradley, *Proc. Amer. Soc. Biol. Chem.*, p. 36, *Journ. Biol. Chem.*, Vol. IV, 1908).

TABLE V

Source of Enzyme	Blue litmus milk		Oxalated blood plasma	
	Coagulated	Turned red ¹	Not clotted	
Digestive gland of <i>Cancer pagurus</i> (edible crab)...	...	...		
'Liver' of <i>Patella vulgata</i> (limpet) ...	Coagulated	Turned red ¹	Jelly-like coagulum	
'Liver' of <i>Pecten opercularis</i> (scallop) ...	Coagulated	Turned red ¹		
	Precipitated	Turned red ²		
	Precipitated	Turned red ²		
Crystalline style of <i>Pecten opercularis</i> (scallop) ...	...	...	Curdy precipitate-like fibrin	
'Liver' of <i>Pupura lapillus</i> (dog whelk) ...	...	...	Jelly-like clot	
'Liver' of <i>Trochus xizyphinus</i> (top shell) ...	...	...	Curdy precipitate-like fibrin	
'Liver' of <i>Buccinum undatum</i> (whelk) ...	...	...	Slight precipitate	
	...	...	Slight precipitate	
Intestine of <i>Echinus esculentus</i> (sea urchin) ...	Coagulated	Turned red ¹		
	Coagulated	Turned red ²	Jelly-like coagulum	
'Disc' of <i>Ophiocoma nigra</i> (brittle star) ...	Coagulated	Turned red ¹	Slightly jelly-like	
Pyloric caeca of <i>Asterias rubens</i> (starfish) ...	Digested	Turned red ¹	Curdy precipitate-like fibrin	
	Coagulated and then rapidly digested	Turned red ¹	Coagulated	
Mesenteric filaments of <i>Tealia crassicornis</i> (sea anemone) ...	Precipitated and then digested	Not turned red ²		
	Coagulated	Turned red ¹	Curdy precipitate-like fibrin	
Mesenteric filaments of <i>Actinia mesembryanthemum</i> (sea anemone) ...	...	...	Coagulated	
<i>Alcyonium digitatum</i> (dead man's toes) ...	...	...	Jelly-like coagulum	
Benger's liquor pancreaticus ...	...	...		

1. Fresh glycerine extract.

2. Extract prepared from alcohol precipitate of glycerine extract.

DISCUSSION OF RESULTS

In considering the results of experiments on enzymes a positive result is definite, but a negative result only shows the absence of the particular enzyme sought under the conditions of experiment. A different method of extracting the tissue, or altered experimental procedure, may show an enzyme where none had previously been found. However, in the experiments here recorded it is seen that glycerine can extract all the enzymes from certain organisms, so that absence in other cases may be construed as showing absence of the missing enzyme.

In the digestion of carbohydrates it is seen that the fresh glycerine extracts (Table I) show, where any enzyme is present, all the various actions—except in the case of *Ophiocoma*, where the glycogen-splitting enzyme is practically absent. It is also seen that although the pyloric caeca of *Asterias* digests starch, the stomach is without action. With the enzymes prepared by alcohol precipitation (Table II), it is seen that *Ophiocoma* again fails to hydrolyse glycogen. The only difference from the fresh enzymes is that *Echinus* fails to act on saccharose. *Tealia* acts only on starch and maltose, whilst the allied species *Actinia mesembryanthemum* only hydrolyses maltose. It is interesting to note that Giaja¹ has found a large number of actions on glucosides and carbohydrates from various marine crustacea, and thus one is led to suppose that the number of enzymes must be infinite, or else that one enzyme can perform a variety of actions limited only to a certain extent so that one action is less marked or negative according to the stereochemical relationships of the substrate.

The question of proteoclastic activity is much more complicated, as not only the presence of an enzyme but also the optimum conditions of action must be determined. In some cases this has been fairly well determined, but in others, partly owing to difficulty in obtaining a sufficient supply of active enzyme, only a few orientating points have been determined. Thus in *Alcyonium*, *Cliona*, and *Cellaria* an enzyme active in acid has been found; in *Asterias* activity in both

1. *Soc. Biol.*, Vol. LXIII, p. 508, 1907.

acid and alkali has been shown with the pyloric caeca, but the stomach (as with starch) has been found inactive.¹ These measurements were qualitative, but not quantitative, whilst for the other enzymes quantitative estimations have been made.

Effect of Temperature.—Increase of temperature up to 35° C. causes a more rapid hydrolytic action. This has been previously observed by several workers, and is simply the usual increase of velocity with increasing temperature. The maximum is reached when the temperature is high enough to cause sufficient destruction of enzyme to overbalance the increasing rate of reaction.

Effect of Reaction.—Krukenberg in various instances states that there are two proteoclastic enzymes present, one acting in acid, and the other in alkali. In several extracts this has been confirmed (Patella, Ophiocoma, Tealia), but it is usual to find that one action is much stronger than the other. Precipitation with alcohol, by removing protein, lowers the strength of acid or alkali requisite for optimum activity.²

With Patella, alcohol precipitation changes the more effective reaction from acid to alkali, and with Echinus the action in acid disappears; but, unfortunately, the effect in alkali had not been tested before precipitation.³

The most efficient strength is much nearer the neutral point than with mammalian pepsin or trypsin. The maxima of acidity and alkalinity would depend on the temperature, as rise of temperature would lead to increased ionisation, and hence greater effective acidity or alkalinity, and possibly greater destruction of enzymes.

The presence of a rennin-like enzyme has been observed in various invertebrata by several workers, and lipase has also been found to occur widely distributed in plants and animals. The

1. Previous workers also find the pyloric caeca active and stomach inactive, v. Furth, *loc. cit.*, p. 168.

2. To cause the congo red fibrin to turn blue requires from $\frac{n}{50}$ — $\frac{n}{20}$ HCl, whilst with congo red in distilled water $\frac{n}{1000}$ HCl is sufficient, thus showing the inhibiting action of fibrin (and other proteins) to change of reaction.

3. Whether this is due to two enzymes which are differently affected by the method of preparation, as shown by Hedin for proteases of spleen (*Journ. Physiol.*, Vol. XXX, p. 155, 1904), must be determined by further investigation.

coagulation of oxalate plasma requires further investigation, but the action is not likely to be due to a kinase, as, in the absence of calcium salts, the kinase would not be able to produce thrombin from thrombogen. Hence it appears as if there were already present in the extracts some substance capable of acting to a certain extent like thrombin.

DEVELOPMENT OF SELECTIVE ACTION

The action of enzymes on stereochemically allied substances suggests that one enzyme is responsible for more than one action. The range of substances acted upon by an enzyme would depend on the degree of specialisation to which that enzyme had attained. Thus there are several explanations as to the relationship between pepsin and rennin. There may be two separate enzymes, or one enzyme with two different active groups; or the rennin action may be only the first stage of the hydrolytic action of pepsin. In certain cases the last explanation seems most applicable, because the presence of a rennin-like enzyme in plants and non-mammalian animals cannot be normally used for coagulating milk, but must be associated with some other function. That a purely hydrolytic action can clot milk can be shown by the action of alkali. Quite dilute sodium hydrate (sufficient to make the added alkali up to 0.16 per cent.) causes a coagulation of caseinogen, and with slightly stronger solutions the caseinogen is further hydrolysed, and re-dissolves. The duration of these processes depends on the strength of alkali and the temperature. All stages, from simple coagulation to a coagulation followed by rapid solution, have been observed.

The principle of evolution, when applied in its broadest sense, should show a gradual transition of chemical composition accompanying the changes in structure. As applied to enzymes one should expect that the original forms of enzymes would be less specialised, acting somewhat like an acid or an alkali, and that as the animal (or plant) became specialised its enzymes would become differentiated, so that some of them could only act on certain definite substrates. This would be accompanied by development of some special molecular

group, so that finally the solubility and other properties of the specialised enzyme might differ greatly from the original form in which the enzyme existed.

In such a scheme we would expect to find, in connection with proteoclastic enzymes, a series of enzymes ranging from those capable of acting equally in weakly acid or alkaline media, through those which act better in one reaction than another, to the more specialised ones occurring in the mammalian body, such as pepsin, trypsin, rennin, erepsin, amylopsin, thrombin, etc. On this view the question of the identity of pepsin and rennin might depend for its answer on what particular organism was selected for investigation. In one animal the two might have become differentiated, whilst in another their two actions might be bound up together. Likewise, the enzymes found in any one species would depend on the nature of the food material to which that species was accustomed, lesser action on substances allied to its food, and the less specific the enzymes were the wider, though probably less intense, their action. Some such reason must explain the presence of rennin, lactose, and possibly thrombin in organisms which normally have no need for those actions.¹ The presence of proteoclysis in both acid and alkali may not be due to two enzymes, but to one, and by various treatment one or other of these actions may be diminished, thus giving rise to some of the changes in action noted in these experiments after precipitation by alcohol.

1. Krukenberg (*Vergleich-physiologische Vorträge*, p. 72, 1886) stated that digestion in all animals is potentially one and the same.