IV. A CONTRIBUTION TO THE STUDY OF A PROTEOLYTIC ORGANISM.

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During a recent investigation of the bacterial population of a number of sewage sludges, the author isolated an organism which possessed the property of causing the rapid liquefaction of gelatin.

It was thought that a more detailed examination of this organism and its mode of action might prove of interest. Accordingly a pure culture on gelatin peptone bouillon was obtained in the usual way by the method of repeated "plating" and the characteristics of the organism were investigated.

On gelatin plates slightly greyish colonies develop in from 24 to 36 hours. These colonies are round with whitish opaque centres and the plate is rapidly liquefied.

Gelatin peptone bouillon (G.P.B.). A cup of liquefied medium appears within 48 hours and a funnel-shaped growth can be observed along the track of the needle. Liquefaction continues downwards, finally yielding an amber coloured viscous liquid which in old cultures darkens in colour and becomes more mobile. A dense white deposit forms at the bottom of the tube leaving the supernatant liquid transparent. A putrefactive odour is noticed which disappears as the age of the culture increases.

Glucose gelatin peptone bouillon. In a shake culture, gas production occurs and later liquefaction sets in on the surface of the gelatin.

Nitrate peptone bouillon. Nitrate is reduced to nitrite.

Ammonia is produced in peptone cultures.

Potato. A brownish growth with little tendency to spread.

Agar peptone bouillon. A white fern-shaped growth spreading along the track of the needle.

Milk. Coagulation followed by alkalinity. The characteristic reduction of neutral red and gas evolution occur. The organism is, therefore, a member

of the *Proteus* or intestinal group and the laboratory number D4 was assigned to it. Microscopically the organism is a short broad bacillus of dimensions $1.4 \times 0.6 \mu$. Its length, however, varies somewhat. It can exist singly but generally forms chains often of considerable length. It is motile, does not form spores and in old cultures usually takes up an involution form.

To ascertain whether the proteolytic action of the organism was due to the presence of an extracellular enzyme the following experiments were carried out. A short stab culture was made in nutrient gelatin and the organism allowed to develop until about one-fourth of the gelatin was liquefied. To this was added in one case toluene and in another a few drops of water saturated with thymol, and the tubes incubated at 21° . Liquefaction continued, and since the action of most organisms is inhibited by thymol and toluene it follows that a proteolytic enzyme was probably secreted by the bacillus.

In other experiments a large quantity of nutrient gelatin was liquefied by inoculation with the bacillus and the products submitted to filtration through a Chamberland filter candle. By this means an absolutely sterile filtrate was obtained. This was checked by plating: no colonies developed and microscopical examination of the filtrate failed to show the presence of any bacteria.

With this filtrate experiments according to the method described by Fuhrmann were made.

100 grams of gelatin were dissolved in 1 litre of water saturated with thymol. A little very finely powdered cinnabar was then added and the mixture thoroughly shaken.

15 to 20 cc. of this mixture were poured into each of a number of tubes, which were placed in a sloping position and a jet of cold water allowed to flow over them until the gelatin began to set. They were then kept in an upright position and the gelatin allowed to solidify completely; the cinnabar becomes evenly distributed through the gelatin.

The sterile filtrate was then poured into these tubes in sufficient quantity to cover the top of the gelatin slope, a little thymol water being added to prevent the growth of moulds etc.

Liquefaction set in readily and its progress could be followed by the gradual disappearance of the red gelatin slope, the cinnabar as liberated collecting at the junction of the enzyme solution and the solid gelatin. To a portion of the sterile filtrate absolute alcohol was added and the precipitate obtained filtered off.

After drying *in vacuo* at 40° to remove alcohol, this precipitate was

redissolved in sterile water containing thymol and the above experiments repeated with similar results.

Two tubes of gelatin peptone bouillon were next taken and the medium melted. To one was added a little of the precipitate obtained as above and a few drops of thymol water, while the second tube was kept without additions, to act as a check. Both tubes were incubated at 37° for 24 hours and then placed in ice when the check tube of gelatin at once solidified while that containing the precipitate remained liquid after standing in ice for over one hour.

Thus the precipitate contained the proteolytic enzyme, and while its action was rapid at 37° and less so at temperatures below this, no optimum could be obtained since the gelatin was liquid at 27° and consequently no rate of action could be determined above that temperature.

These experiments, therefore, prove that the power of causing the liquefaction of gelatin, exhibited by the organism, is due to the presence of an extracellular proteolytic enzyme.

It had been observed in a previous experiment that upon standing in the presence of the organism, the enzyme contained in the products of liquefaction of nutrient gelatin was destroyed, whereas the sterile enzyme solution retained its activity, at any rate for several days. It was proposed to endeavour to determine when the enzyme first makes its appearance and when it is destroyed and a number of experiments were made with this end in view.

Several tubes of the following description were made. A piece of narrow glass tubing about 2 inches long and $\frac{1}{16}$ inch internal diameter was fused to a thin glass rod 4 inches in length. The tube portion was graduated in millimetres and filled completely with thymol gelatin containing a small quantity of cinnabar. Before each tube was filled it was gauged by inserting a wooden peg in the open end. By this means it was ensured that the same surface area of gelatin would be in contact with the solution in every case and thus comparable results be obtained.

The rod attached to this tube was passed through a cork of such a size as to fit a number of culture tubes. Cultures were made in peptone water, inoculation being made by loop from an old culture of D4 in gelatin peptone bouillon, and after 24 hours' incubation at 37° toluene was added to one tube, thoroughly shaken and one of the above described tubes introduced so that about 2 millimetres of the gelatin tube were below the surface of the peptone water. 72 hours after inoculation a second tube was treated in a similar manner and also other tubes, 192 hours and 888 hours respectively after inoculation.

Any liquefaction of the gelatin that might occur would be due to the presence of the enzyme and the rate would be proportional to the amount of enzyme present.

The limit to which liquefaction had proceeded could easily be read on the graduated scale, a sharp line being discernible between the solid and liquid gelatin; the former being red and opaque when viewed by reflected light and the latter clear and colourless. The results of one set of experiments are given below.

(i)	24	hours	culture,	liquefaction	after	20	days=	0.0	mm.
(ii)	72	"	"	"	"	"	=	8.8	"
(iii)	192	"	"	"	"	"	=	22.5	"
(iv)	888	"	"	"	"	"	=	16.4	"

The enzyme was not secreted at once but increased in amount as the age of the culture increased up to a certain point after which it was slowly destroyed.

This disappearance of the enzyme also occurred when the organism was cultured in peptone bouillon.

(v)	48	hours	culture,	liquefaction	after	30	days=	44.0	mm.
(vi)	49	days	"	"	"	"	=	6.8	"

From the foregoing experiments it is seen that the enzyme was secreted when the organism was grown upon nutrient gelatin, peptone bouillon and peptone water. It is therefore conceivable that the enzyme is produced in the ordinary metabolism of the bacillus and regardless of the medium upon which it is cultivated. To test the accuracy of this hypothesis cultures were made in sterile egg albumin in water and also in gelatin containing a few inorganic salts. In the latter no liquefaction occurred and in the experiment with egg albumin no enzyme was found to be present (as measured by the thymol gelatin method), although when examined microscopically a number of the bacilli were found in a normal condition, and when cultured on nutrient gelatin rapidly liquefied the medium. It therefore appears that the presence of a peptone is essential to the production of the enzyme, which may be a combination of a substance secreted by the organism with a peptone. This enzyme thus formed then attacks the protein present, peptonising it and so converting it into a form suitable for the production of still more enzyme, thereby increasing the action.

If this is the case it might be expected that the higher the protein present the more enzyme would be produced provided the action could be started.

The following results corroborate this, the figures given being the amount of liquefaction which occurred after 30 days.

(a) Peptone water	= 18 mm.
(b) Peptone broth	= 44 „
(c) Nutrient gelatin G.P.B.	= 93.6 „

Experiments were made to determine whether the enzyme possessed a pepsin or trypsin character and also to ascertain whether coagulated egg albumin was attacked by the sterile filtrate obtained from a culture in egg albumin although it had no action upon gelatin.

The tubes described below were therefore made up, a strip of coagulated egg albumin being introduced into each.

1. 2 cc. sterile filtrate from G.B.P. culture + 3 cc. thymol water.
2. 2 cc. „ „ „ „ + 2 cc. „ „ + 1 cc. 1 % sodium carbonate solution.
3. 2 cc. sterile filtrate from G.B.P. culture + 2 cc. thymol water + 1 cc. 0.2 % hydrochloric acid solution.
4. 4 cc. sterile filtrate from egg albumin culture + 2 cc. thymol water.
5. 4 cc. „ „ „ „ „ + 1 cc. „ „ + 1 cc. 1 % sodium carbonate solution.
6. 4 cc. sterile filtrate from egg albumin culture + 1 cc. thymol water + 1 cc. 0.2 % hydrochloric acid solution.

After several weeks considerable decomposition had occurred in 1, 2 and 3, and to a similar extent in each case, but no change in the albumin could be detected in 4, 5 or 6.

Therefore no enzyme is secreted when the organism is grown upon egg albumin and the enzyme from nutrient gelatin cultures can act in both acid and alkaline solutions.

A number of experiments were made in order to obtain data characteristic of the enzyme which could be used for purposes of identification.

Thus the rate of liquefaction of gelatin by the enzyme (obtained from a culture in nutrient gelatin) was determined in presence of six typical acids, the amount of each present in an individual tube varying from 0.1 % to 1 % of the total contents.

Certain precautions were taken to ensure strictly comparable results and a number of curves were obtained.

It was found that the rate of liquefaction was inhibited by every acid under observation but that the retardation varied both with the acid present and the concentration. A rise in concentration of the foreign substance invariably produced a decrease in the rate at which liquefaction proceeded, but for a given rise in concentration the amount of retardation was not necessarily the same for every compound; e.g. an increase in concentration of

acetic acid from 0.1 % to 0.3 % merely slowed down the rate whereas a similar increase in concentration of hydrochloric acid was sufficient to arrest liquefaction altogether.

That the constitution of the acid present produces an effect upon the rate of liquefaction is shown by the fact that acetic and lactic acids produced the least and an approximately equal decrease in the rate of action. Tartaric and citric occupy an intermediate position while oxalic and hydrochloric acids exercised the greatest action. In the observations under discussion 0.6 % of oxalic acid arrested all liquefaction while 0.2 % of hydrochloric acid reduced the rate of liquefaction by 80 % and 0.3 % was entirely prohibitive.

It is probable also that the degree of dissociation of the acids has a considerable effect on the proteolytic activity of the enzyme, an increase in the concentration of the hydrogen ion exercising a prohibitive action.

This view is borne out by the position of the acids when placed in the order of the effect they have upon the rate of liquefaction, viz. hydrochloric, oxalic, citric, tartaric, acetic and lactic.

It is hoped that further work may be done on this subject, more especially with regard to the action of the bacillus upon blood serum and other albumins.

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