75. STUDIES ON DIFFUSING FACTORS

6. THE ACTION OF TESTICULAR EXTRACTS ON THE VISCOSITY OF VITREOUS HUMOUR PREPARATIONS

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(Received 29 February 1940)

CHAIN & DUTHIE [1939] have recently shown that testicular extracts contain an enzyme capable of hydrolysing the mucins of synovial fluid and vitreous humour. Mucinases of a similar type have been obtained from pneumococci [Dubos, 1937; Meyer *et al.* 1937] and from haemolytic streptococci [Meyer, 1938]. Since the first visible effect of the testicular enzyme on the mucins examined was to reduce their viscosity and since there is histological evidence for the existence of a mucin-like interfibrillar substance in skin [Bensley, 1934; Sylven, 1938], Chain & Duthie suggested that the diffusing factor and the mucinase in testicular extracts might be identical. This view is in accordance with their similarity in properties and their common occurrence in testicle and some bacterial filtrates. If we are to consider the characteristic effect produced by diffusing factors as being due to enzyme action in the skin it is reasonable to assume that the increased spread of injected fluids is due to the ease with which they diffuse through tissues rendered less viscous by the action of the enzyme. Apart from mucinases other enzymes which might affect the viscosity of skin constituents are the proteases which can liquefy gelatin; it has, however, been shown [Madinaveitia, 1940] that in *Cl. Welchii* filtrates at least, the proteases can be separated from the diffusing factor.

It is not yet known whether testicular extracts contain a single mucolytic enzyme or a polysaccharase and an oligosaccharase. In the latter case the increase in the reducing properties of the substrate brought about by the extract would be a measure of the combined effect of the enzymes; the decrease in viscosity would be a measure of the polysaccharase activity only and, incidentally, a measure of the diffusing factor activity should it prove that the diffusing factor is indeed identical with the mucinase. The decrease in viscosity of a mucin by the action of a mucinase is a catalytic process and the disappearance of the viscous material should obey the laws of a unimolecular reaction. It has been experimentally confirmed, using testicular mucinase, that the reaction constant is independent of the substrate concentration (Table 1) and that the half-life time of a given amount of viscous substrate is inversely proportional to the concentration of the enzyme with which it reacts (Table 2). The substrate used was an acetone powder of bovine vitreous humour. The viscosity of its aqueous solutions is greatly affected by salts. Addition of small amounts of salts causes the viscosity to fall to a minimum; on further addition the viscosity again rises, the increase being parallel to that produced by adding salts to water alone. Based on these observations a viscosimetric method has been developed for the determination of mucinases. A 1% solution of vitreous humour in a buffered *M* NaCl solution is allowed to react with a measured amount of enzyme solution. The time required to reduce the specific viscosity of the reacting mixture to half its original value is inversely proportional to the amount of enzyme used.

Determination of the optimum pH for the action of testicular mucinase indicates that it is about pH 4.7. This is somewhat lower than the optimum found by Meyer *et al.* [1937] for the mucinase of pneumococcus; the difference might well be due to differences in the substrate or in the technique employed for estimating the enzyme activity. Since the enzyme has maximum stability in the neighbourhood of pH 4.7 estimation of mucinase potency is always carried out in a citrate buffer at this pH .

A variety of solvents have been used for extracting the enzyme from dried testicle powder. $M/10$ acetic acid is much better for this purpose than water; autolysis of the testicle powder offers no advantage over direct acetic acid extraction. From an acetic acid extract (pH 4.2) the mucinase precipitates at between 30 and 70 % of saturation with $(NH_4)_2SO_4$. From the solution obtained by redissolving this precipitate in water the active constituents are very difficult to adsorb on kaolin and, once adsorbed, they are almost impossible to elute. If, on the other hand, the solution be dialysed before treatment with kaolin adsorption is easily effected and the mucinase is readily eluted by a phosphate buffer solution. From the eluate obtained in this way the enzyme can be readily re-adsorbed on alumina $C\gamma$ and again eluted with phosphate buffer solution. Purified in this way the enzyme solutions show diffusing activity of an intensity similar to that of a crude testicular extract with the same mucinase activity.

It is so far impossible to state whether the mucinase and diffusing factor are identical although the point is clearly of great importance. Whether rigid identification will be possible must await further work.

EXPERIMENTAL

Substrate. The vitreous humour of 120 cattle eyes (1.2 l.) was thoroughly stirred and then filtered with suction through three layers of muslin. 1.1 l. of filtrate were run in a small jet into 10 l. of mechanically stirred acetone cooled to 0° . After standing at room temperature overnight the asbestos-like precipitate was collected by filtration, washed with a further litre of acetone and subsequently with 1 l. ether. During the whole process the precipitate was kept covered with solvent. After washing with ether the precipitate was dried and powdered. 4.5 g. of a very light white powder were obtained. This preparation is not completely soluble in water or in $NaCl$ solutions. It is impossible to clarify the solutions by filtration through paper since the viscous material is retained by the filter; centrifuging on the other hand yields clear viscous solutions. The concentration of substrate will be given as percentage of the acetone powder in the clarified solution.

The viscosity of solutions of the substrate is an easily reproducible figure. Weighed amounts of substrate were extracted in a mortar with 100 times the weight of water. The water was added in small portions, no further addition being made until the contents of the mortar were homogeneous. When all the solvent had been added the solutions were centrifuged and the supernatants decanted through glass wool. Four different samples of solutions thus prepared required 336.5, 338.8, 330.8 and 336.2 sec. respectively to flow through an Ostwald viscosimeter of about 4.5 ml. capacity. Water tested under the same conditions required 112.5 sec.

Effect of $NaCl$ on the viscosity of the substrate. 5 ml. samples of a 0.5 % solution of substrate in distilled water were mixed with 5 ml. of $NaCl$ solutions of different concentrations, the concentration of substrate being thus reduced to 0.25 %. Another series in which the final concentration of substrate was 0.8 % was

prepared in a similar way. The flow time of each of the solutions at 25° is recorded in Fig. 1 as well as the flow time of NaCl solutions of different strength. Between 3 and 7 % the concentration of NaCl has very little effect on the flow time.

Fig. 1. Effect of NaCl on the viscosity of the substrate.

Fig. 2. Effect of pH on mucinase activity.

Variation of the viscosity of the substrate with pH and its influence on the action of testicular extract. To 4 ml. samples of 1 % substrate in *M* NaCl 1 ml. *M*/10 buffer solution was added. The great buffering capacity of the substrate between pH 6 and 7 makes the pH of the resulting mixtures different from that of the buffer used. The flow time of these solutions at 25° is recorded in Fig. 2.

The testicular extract used was prepared by grinding 1 g. dry testicle powder with 10 ml. water in a mortar. The turbid supernatant obtained by centrifuging was diluted with 9 vol. of water. To 4 ml. of the 1 % substrate solution above mentioned 1 ml. *M*/10 buffer and 1 ml. diluted testicular extract were added. In Fig. 2 the flow times at different intervals are plotted. The whole experiment was carried out at 25° with the precautions and corrections described elsewhere (p. 628). It will be seen that the enzyme action is most rapid at about pH 4.5-4.7.

Stability of mucinase at different pH. The centrifuged extract of 1 g. dry testicle powder with 10 ml. water was adjusted to pH 4.2 with *M* acetic acid.

Fig. 3. Stability of mucinase.

1 ml. portions of the clear solution were made up to 10 ml. with suitable *M*/50 buffer solutions. After 18 hr. at 38° (thermostat) the mucinase content of the different samples was estimated (Fig. 3).

Effect of the concentration of substrate on the rate of reaction. 4 vol. of a 4% substrate solution in *M* NaCl and 1 vol. *M* Na citrate buffer pH 4.7 were mixed. Consecutive twofold dilutions of this solution up to 1/16 were made, using as a diluent a mixture of 4 vol. *M* NaCl and 1 vol. *M* Na citrate buffer pH 4.7. The source of enzyme used was a centrifuged extract of 1 g. dry testicle powder with 10 ml. *M* acetic acid diluted with 10 vol. water. 4 ml. portions of the different dilutions of substrate were allowed to react with 1 ml. enzyme solution at 25°. The viscosity of the reacting mixture was measured at different intervals. The flow time was plotted against reaction time and the initial flow time of the enzyme-substrate mixture (f_0) extrapolated. When the action of the enzyme on the substrate had proceeded long enough the flow time was reduced to that of the solvent in which the reaction takes place ($f_\infty = 127$ sec.). $\frac{1}{2}(f_0 + f_\infty)$ has been taken as the flow time when half of the substrate has been rendered non-viscous. The time at which the flow time reaches this value is the half-life time of the substrate (τ). The reaction constant (k) was calculated avoiding the use of the initial flow time (f_0) which is an assumed value. Table 1 records the

Table 1

<i>c</i>	Concentration of substrate (in arbitrary units)	4	2	1	0.5	0.25
f_0	Initial flow time (in sec.) (extrapolated)	532	290	192	158	142
$\frac{1}{2}(f_0 + f_\infty)$	Flow time at half concentration of viscous material (in sec.)	360	209	160	142	134.5
τ	Half-life time of the viscous material (in min.)	29.5	29	30	31	29
$k \cdot 10^{-2} \text{ min.}^{-1}$	Reaction constant $\times 10^{-2} \text{ min.}^{-1}$	9.25	9.44	9.92	8.90	—

results thus obtained. The reaction constant and the half-life time of the viscous substrate are tolerably constant, particularly at the higher concentrations of substrate where the experimental errors are smallest.

Effect of the enzyme concentration on the rate of reaction. 45 ml. 1% substrate solution in *M* NaCl were mixed with 15 ml. *M* acetate buffer pH 4.6. The enzyme used was the centrifuged extract of 1 g. dry testicle powder with 10 ml. of water. 1 ml. portions of different dilutions of enzyme were allowed to react with 4 ml. of the buffered substrate and the flow time determined at different intervals. The half-life time of the viscous substrate was estimated as above indicated. The results are recorded in Table 2. It will be observed that the concentration of enzyme is inversely proportional to the half-life time since the product is constant.

Table 2

Concentration of enzyme	50	33	20	10	3.3
Half-life time (min.)	8	14	20	42	130
$c \times \tau$	400	455	400	420	425

Method used for the comparison of the relative mucinase contents of testicular extracts. The half-life time of the substrate being inversely proportional to the concentration of enzyme its magnitude gives a convenient measure for the mucinase content of a given preparation. It has been shown that the half-life time is independent of the concentration of substrate. However, in order to

avoid experimental errors, the solutions of substrate used had a flow time between $\frac{1}{3}$ and $\frac{1}{15}$ of the half-life time. Their flow time was about 1.3–1.7 times that of the solvent. 5 ml. of a 1% solution in *M* NaCl of the acetone powder of bovine vitreous humour mixed with 1/5 vol. of *M* Na citrate buffer and 1/5 vol. of water had a flow time of about 200 sec. (f_0) in an Ostwald viscosimeter of about 4.5 ml. capacity in which the flow time of water measured under the same conditions was 112 sec. and the flow time of a mixture of 4 parts of *M* NaCl, 1 part of *M* Na citrate buffer and 1 part of water was 127 sec. (f_∞). All measurements were made in a thermostat at $25 \pm 0.05^\circ$ and the total volume of fluid placed in the viscosimeter was 6 ml. The value of f_0 was determined for each batch of the substrate solution.

5 ml. of a mixture of 4 vol. of a 1% solution of substrate in *M* NaCl and 1 vol. *M* Na citrate buffer were placed in the viscosimeter which was kept in the thermostat at 25° . After the temperature had equilibrated (about 5 min.), 1 ml. of the enzyme solution under test (at 25°) was added. A stop watch (*A*) was started, the contents of the viscosimeter thoroughly mixed and blown into the bulb. When the meniscus reached the top mark of the viscosimeter a second stop watch (*B*) was started and the time in stop watch *A* read ± 5 sec. (*t*). When the meniscus reached the lower mark stop watch *B* was stopped, the time read (f_t), the fluid again made to fill the bulb and the procedure repeated; $t + \frac{1}{2}f_t$ is considered to be the time required for the reacting mixture to have the flow time f_t . $t + \frac{1}{2}f_t$ was plotted against f_t and the point at which the curve cuts the line $\frac{1}{2}(f_0 + f_\infty)$ was taken as the half-life time of the viscous substrate or as the inverse of the concentration. If the half-life time of the substrate is between 10 and 40 min. it can be determined with an error of less than ± 1 min., the error of the method being therefore about $\pm 5\%$.

Extraction of the mucinase from dried testicle powder. 1 g. of the powder mixed with 2 g. of sand was ground in a mortar for 10 min. with 10 ml. of water. After centrifuging, 5 ml. supernatant were obtained. The residue was ground with a further 10 ml. water and centrifuged again (7.5 ml. supernatant). The residue was extracted a third time with 10 ml. water (8.5 ml. supernatant). The supernatants were precipitated by adjusting them to pH 4.1 with *M* acetic acid and the precipitates removed by centrifuging. The third supernatant had 13% of the mucinase content of the first one. Thus most of the mucinase was removed in the first extraction. Addition of acetic acid (pH 4.1) to the aqueous extracts produces a copious precipitate of inactive material. Extraction of 1 g. of dried testicle powder with 10 ml. *M*/10 acetic acid under the conditions described above yielded a solution having 1.5 times more mucinase per ml. than when water was used.

1 g. of testicle powder ground with 2 g. of sand and 10 ml. *M*/10 Na_2HPO_4 was allowed to autolyse at 38° for 24 hr. with 1 ml. toluene. The precipitate was centrifuged and the supernatant precipitated with acetic acid. The clear solution finally obtained had 1.7 times the mucinase content of a non-autolysed aqueous extract. Autolysis has therefore no great advantage over direct acetic acid extraction.

Ammonium sulphate fractionation. 50 g. dried testicle powder, 100 g. sand and 1 l. *M*/10 acetic acid were ground in a mortar for 1 hr. In 750 ml. of the clear extract (pH 4.2) 166 g. $(\text{NH}_4)_2\text{SO}_4$ were dissolved (30% of saturation). The inactive precipitate was removed and to the filtrate a further amount of 239 g. $(\text{NH}_4)_2\text{SO}_4$ was added, the resulting solution being 70% saturated with $(\text{NH}_4)_2\text{SO}_4$ (20°). The precipitate was collected, washed with 50 ml. 70% saturated $(\text{NH}_4)_2\text{SO}_4$ and dissolved in 250 ml. of water. In this final solution 80% of the mucinase

present in the acetic acid extract was found. The solution (*pH* 4.5) loses about 15 % of its mucinase content per day when kept at +5°.

Adsorption and elution of the mucinase. Two 10 ml. portions of the redissolved $(\text{NH}_4)_2\text{SO}_4$ precipitate (*pH* 4.3) were adsorbed with 0.5 and 2 g. kaolin respectively. 30 % of the mucinase was adsorbed by 2 g. kaolin but none by 0.5 g. 180 ml. of redissolved $(\text{NH}_4)_2\text{SO}_4$ precipitate were shaken with 49 g. kaolin which adsorbed 30 % of the nitrogen but none of the mucinase present. 10 ml. samples of the solution thus purified were adsorbed with 2 g. kaolin which removed more than 90 % of the mucinase and 61 % of the nitrogen. No mucinase was eluted from the adsorbates by shaking them with 10 ml. portions of *M*/10 acetic acid, water, *M*/15 KH_2PO_4 , 2 % pyridine or 0.8 % NH_3 . 10 ml. *M*/10 borate buffer (*pH* 9.2) or 10 ml. *M*/5 Na_2HPO_4 (*pH* 8.3) only elute about 10 % of the adsorbed enzyme.

100 ml. of the redissolved $(\text{NH}_4)_2\text{SO}_4$ precipitate were dialysed in a cellophane bag against running tap water during 36 hr. the volume increasing to 250 ml. After removing the inactive precipitate 10 ml. samples of the dialysed solution (*pH* 5.6) were adsorbed by shaking with different amounts of kaolin.

g. kaolin	0.2	0.5	0.7	1.0	1.5	2.0
% mucinase adsorbed	25	85	96.5	—	—	—
% N adsorbed	14	35	—	57	77.5	87.5

10 ml. samples of dialysed $(\text{NH}_4)_2\text{SO}_4$ precipitate were adsorbed with 0.5 g. kaolin. The adsorbates were eluted with 10 ml. *M*/10 phosphate buffers of different *pH*.

<i>pH</i> of buffer	4.38	7.37	6.97	6.68
% of adsorbed mucinase eluted	45	45	34.5	16

100 ml. of dialysed $(\text{NH}_4)_2\text{SO}_4$ precipitate were shaken with 5 g. kaolin for 30 min. The adsorbate was eluted with two 50 ml. lots of *M*/10 phosphate buffer *pH* 7.3. The combined eluates contained 55 % of the adsorbed mucinase. Dialysis in cellophane bag against running tap water for 18 hr. yielded 200 ml. of solution. Two 10 ml. portions of the dialysed kaolin eluate were adsorbed with 0.2 and 0.5 ml. alumina C- γ (19 mg. Al_2O_3 /ml.) the volume being made up to 15 ml. in each case. The amounts of mucinase adsorbed were 15 and 77 % of that of the original solution. Elution with 15 ml. *M*/30 phosphate buffer *pH* 7.3 of the adsorbate with 0.5 ml. alumina recovered 85 % of the adsorbed mucinase.

The diffusing factor content of the alumina eluate was compared with that of a 1/20 dilution of an extract of 1 g. dry testicle powder with 10 ml. *M*/10 acetic acid. Double determinations were made in two rabbits by the modified technique recently described [Madinaveitia, 1939].

	Mucinase	Increase of the area of spread cm^2
Crude testicular extract	1.35	5.54
Purified mucinase	1.00	5.10

SUMMARY

A viscosimetric method is described whereby testicular mucinase may be estimated with an error considerably less than 5 %. In the preliminary concentration of the enzyme from testicle powder mucinase activity seems to run roughly parallel with diffusing activity. No definite evidence has been obtained that the mucinase and diffusing factor are identical.

The authors' thanks are due to Prof. A. R. Todd for his continued interest and advice. They are also grateful to Imperial Chemical Industries, Ltd., for grants.

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