Studies on β -Glucuronidase

4. THE PURIFICATION AND PROPERTIES OF OX-LIVER β -GLUCURONIDASE

By EVELYN E. B. SMITH* AND G. T. MILLS

Biochemistry Department, University of Glasgow

(Received 8 July 1952)

While a fairly extensive characterization of the preparation and properties of ox-spleen β -glucuronidase has been recorded (Fishman, 1939*a, b*; Graham, 1946; Mills, 1948; Mills, Paul & Smith, 1949; Bernfeld & Fishman, 1950*a*; Mills, Paul & Smith, 1953), the available data for liver glucuronidase is confined to the work of Sarkar & Sumner (1950). These authors, using low-temperature dioxan fractionation for the purification of ox-liver β -glucuronidase, achieved a 6000-fold purification of the enzyme. Their method of expressing purity, however, is based on an initial expression of activity per gram of fresh tissue, this being compared with a final activity in terms of mg. dry weight of enzyme. It is more accurate to enumerate both values in terms of a common factor, namely protein nitrogen, in which case a 900-fold purification was achieved. By a combination of metallo-protein interactions, organic solvent precipitation and ammonium sulphate fractionation it has been found possible, in the present work, to achieve a purification of ox-liver β -glucuronidase equivalent to that obtained by Sarkar & Sumner (1950). A study of the properties of the purified material does not support the observation of Sarkar & Sumner (1950) that the β -glucuronidase of ox liver is a single enzyme with a single pH optimum at pH 4.5 in acetate buffer, and data are presented to show that the liver enzyme is complex and analogous in many respects to that reported by Mills (1948) and Mills *et al.* (1953) for ox-spleen β -glucuronidase. Preliminary reports of this work have already appeared (Smith, 1951; Mills & Smith, 1952).

METHODS

Assay of β -glucuronidase preparations. Phenolphthalein β -D-glucuronide, prepared biosynthetically by the method of Talalay, Fishman & Huggins (1946), was used as substrate, and the method of assay was that described by Mills *et al.* (1953). The unit of enzyme activity is that which liberates 1 μ g. phenolphthalein in 1 hr. from 1 mM-phenolphthalein glucuronide at pH 4.5 and 38°.

When studying inhibitory substances, the enzyme digests were made up to contain 0.5 ml. buffer solution, 0.2 ml. enzyme solution, 0.1 ml. water or inhibitor solution and 0.2 ml. substrate solution.

* Carnegie Research Scholar.

Unless otherwise stated, the following buffers were employed: for the pH range 3.4–6.0, 0.2M-acetate buffers, and for the pH range 6.0–7.0, the veronal-acetate buffers of Michaelis (1931).

Filter-paper electrophoresis. This was carried out by the method of Mills & Smith (1951).

pH measurements. These were made with a glass electrode and either a Muirhead, Tinsley or Marconi pH meter.

Protein N. This was estimated by the method of Ma & Zuazaga (1942), using the Markham (1942) distillation unit.

EXPERIMENTAL AND RESULTS

A recent publication of Cohn *et al.* (1950) on the use of the interactions of different proteins with bivalent cations to separate the protein components of human plasma, suggested the possibility of using such metallo-protein complexes in the purification of β -glucuronidase. The following purification scheme has been devised, taking as starting material the stage C fraction described by Mills (1948) in studies on ox-spleen β -glucuronidase.

Finely minced ox liver was extracted twice with 2 vol. acetone and the filter cake dried in air at room temperature. The powder so obtained was extracted with water, the extract adjusted to pH 5.0 with acetic acid, buffered with acetate buffer and autolysed at 38° for 4 hr. The voluminous precipitate so formed was removed in the Sharples centrifuge and solid $(\text{NH}_4)_2\text{SO}_4$ stirred into the solution to 60% saturation, followed by equilibration at 0° for 18 hr. The precipitate was then removed by filtration, using Standard Super-Cel (Celite 519A, Johns-Manville Corporation), the filter cake extracted with water and the extract dialysed, this extract constituting the stage C of Mills's (1948) original purification.

Further purification can be conveniently divided into three stages.

Stage 1. Initial treatment of the Stage C solution with zinc acetate solution results in the precipitation of a large percentage of inactive protein. If this is removed by centrifugation, glucuronidase can be precipitated completely from the supernatant by the addition of acetone to a final concentration of 20%. This is in contrast to the higher value of 45% acetone required for precipitation of the enzyme in the absence of Zn^{2+} . Elution of the precipitated material with a 15% acetone solution containing Ba^{2+} results in a selective extraction of β -glucuronidase yielding the so-called 'barium eluate'. The procedure is represented in Fig. 1. The percentage distribution of the enzyme obtained by the above fractionation is shown in Table 1.

The success of stage 1 is dependent on a number of factors, control of which is important for reproducibility of results. These are: (1) The initial ratio of enzyme activity to protein concentration in the stage C solution is important, and the solution should be adjusted to a value of 2000–4000 units of activity/mg. protein N to avoid undue loss of enzyme in the initial zinc precipitation. (2) Strict temperature control is essential throughout as small variations in temperature may lead to critical disturbances in the system. A temperature of

$(\text{NH}_4)_2\text{SO}_4$ to 60% saturation. Celite 519 A (2%, w/v), was added to adsorb the enzyme which, after filtration, was eluted from the Celite with four 25 ml. portions of 1.8M- $(\text{NH}_4)_2\text{SO}_4$ solution. The filter cake was further extracted with similar portions of 1.6, 1.4 and 1.2M- $(\text{NH}_4)_2\text{SO}_4$ solutions. The combined eluates of each individual fraction were adjusted to a final concentration of 3M with solid $(\text{NH}_4)_2\text{SO}_4$, the precipitate spun off at 0° and dissolved in the minimum volume of water (generally 3 ml.).

Solution A: 5.48 g. zinc acetate dihydrate per 100 ml. water.

Solution B: per 100 ml. water: 0.26 g. barium acetate, 2 ml. M-sodium acetate, 0.73 ml. M-acetic acid, 15 ml. acetone. pH 5.5–5.6.

Fig. 1. Scheme of stage 1 of purification of ox-liver β -glucuronidase.

0° was employed throughout stage 1 of purification. (3) The presence of Ba^{2+} allows the precipitated enzyme to be eluted within the acetone range of 15–20% to give the barium eluate. A complete recovery of glucuronidase was not effected, some 17% remaining in the residue. This was dependent on the time period of elution. Extraction for 1 hr. allows the maximum separation of enzyme relative to the amount of inactive protein simultaneously eluted. With longer extraction periods almost all of the enzyme can be recovered but with a decrease in purity.

Table 1. Percentage distribution of ox-liver β -glucuronidase during purification

	Distribution of enzyme (%)
Stage 1	
Barium eluate	60
Precipitate (A)	15
Supernatant (B)	0
Residue (C)	17
Stage 2 (from barium eluate)	
1.8M- $(\text{NH}_4)_2\text{SO}_4$ fraction	43
1.6M- $(\text{NH}_4)_2\text{SO}_4$ fraction	30
1.4M- $(\text{NH}_4)_2\text{SO}_4$ fraction	15
1.2M- $(\text{NH}_4)_2\text{SO}_4$ fraction	2

Stage 2. Following dialysis of the barium eluate at 0°, glucuronidase was precipitated from solution by adding solid

90% of the total glucuronidase present in the barium eluate can be recovered by the above procedure with the percentage distribution shown in Table 1.

Most of the enzyme activity was associated with the 1.8M eluate, but this was discarded in view of its high content of inactive protein. The 1.6 and 1.4M fractions were highly active (80000–100000 units of activity per ml.), and were associated with low protein contents.

Stage 3. A large percentage of β -glucuronidase was precipitated from solution on dialysis of the combined 1.6 and 1.4M- $(\text{NH}_4)_2\text{SO}_4$ fractions obtained in stage 2. An investigation of the effects of controlled dialysis on the purification of the enzyme was considered and two techniques were employed.

(a) A fairly exhaustive dialysis was required to precipitate the enzyme from solution, and inactive material could be removed initially by a short dialysis period (24 hr.). A more prolonged dialysis of the supernatant (3–4 days) against several changes of distilled water precipitated the enzyme which, after centrifugation and elution with 3 ml. 1% NaCl solution, yielded a glucuronidase preparation containing 200000 units of activity per mg. protein N.

(b) Alternatively, the glucuronidase was first precipitated from solution by a 3- to 4-day dialysis against distilled water, followed by extraction of the centrifuged precipitate with 6–8 ml. of a 1% NaCl solution. Dialysis of the extract reprecipitated the enzyme, which on a further elution with 3 ml. of 1% NaCl solution gave an enzyme preparation equivalent in purity to that obtained by method (a).

Table 2. *Purification of ox-liver β -glucuronidase*

	Units*/ml.	Volume (ml.)	Total units*	Purity†	Yield (%)
Liver mince	6 000	—	—	240	—
Stage C	20 000	100	2×10^6	2 500	100
Barium eluate	12 000	100	1.2×10^6	14 000	60
1.6M-(NH ₄) ₂ SO ₄ fraction	80 000	4	32×10^4	—	16
1.4M-(NH ₄) ₂ SO ₄ fraction	60 000	3	18×10^4	—	9
Stage 3 purified material	20 000	5	10×10^4	200 000	5

* Unit of activity = 1 μ g. phenolphthalein liberated/hr. under the conditions specified in Methods section.

† Purity expressed in terms of activity/mg. protein N.

A temperature of 0° or less must be maintained throughout dialysis. When dialysis was carried out against running water (12°), rapid denaturation occurred with an overall loss in enzyme activity of 35% over a period of 8 hr., whereas losses were negligible at 0°. A short period of extraction with 1% sodium chloride solution (15 min.) preferentially elutes the enzyme from the precipitate obtained on dialysis; with longer extraction periods, higher yields of enzyme are obtained at the expense of purity. Quantitative details of the purification are shown in Table 2. Starting from minced ox liver containing 240 units of activity per mg. protein N, an overall purification of 800-fold is obtained with 5% recovery of the enzyme.

Properties of the purified material

The purified β -glucuronidase is a pale, straw-coloured solution. Solutions containing 200 000 units of activity per mg. protein N appeared to be relatively unstable, and when stored at 0° for 2 days a 25% loss in activity was apparent. After this there was no further loss in activity for at least 2–3 weeks at 0°, and the properties of the enzyme were accordingly obtained from this more stable material (150 000 units per mg. protein N).

Isoelectric point of ox-liver β -glucuronidase. The isoelectric point of purified ox-liver β -glucuronidase was determined by estimating the mobility of the enzyme on filter-paper strips at various pH values from 4.0 to 8.0. Crystalline bovine serum albumin (Armour) was examined at the same time to determine the accuracy of the method. The results of this experiment are shown in Fig. 2, which shows that the isoelectric point of β -glucuronidase is at pH 4.85 and that for crystalline bovine serum albumin is at pH 4.65. This latter value corresponds well with the accepted value of pH 4.7.

Amino-acid constitution of purified ox-liver β -glucuronidase. To 2 ml. enzyme solution containing 0.22 mg. protein N (purity = 150 000 enzyme units/mg. protein N) concentrated hydrochloric acid was added to a final concentration of 6N. The enzyme was hydrolysed by heating for 18 hr. at 110° in a sealed tube. Hydrochloric acid was evaporated off *in vacuo* over solid potassium hydroxide and the

residue dissolved in 0.2 ml. distilled water. A two-dimensional chromatogram was obtained with 50 μ l. of the extract on Whatman no. 1 paper, using as solvents: (a) *n*-butanol-acetic acid (40% *n*-butanol, 10% glacial acetic acid and 50% water by volume) and (b) phenol. The result showed the presence of seventeen amino-acids in the purified β -glucuronidase preparation with no unusual features.

Fig. 2. Electrophoretic migration of purified ox-liver β -glucuronidase (●—●) and crystalline bovine serum albumin (Armour) (○—○) on filter-paper strips. Acetate buffers, pH 4–5, and phosphate buffers, pH 6–8. $I = 0.05$.

pH-Activity curve. A typical pH-activity curve for the purified enzyme is shown in Fig. 3. While the enzyme appears to be stable over a wide pH range, the presence of a number of well-defined optima in a preparation of such high purity is suggestive of the complexity of the system. The pH-activity curve in the region of pH 5.5–7.0 was re-investigated using phosphate-citrate buffer and the presence of an optimum at pH 6.3 confirmed. Although the

activity curve presented may be considered representative of the purified enzyme, different preparations showed individual variation in the proportionality of activity at the pH maxima. It is interesting to note that all highly purified enzyme

β -glucuronidase assayed at different substrate concentrations. These changes in pH-activity curve with varying substrate concentration, which are analogous to those already reported for ox-spleen β -glucuronidase (Mills *et al.* 1953), indicate that in all probability we are again dealing with a mixture of β -glucuronidases with different pH optima and differing substrate affinity.

The effect of deoxyribonucleic acid (DNA) and other compounds on the activity of ox-liver β -glucuronidase

Bernfeld & Fishman (1950*b*) claim to have shown that calf-spleen β -glucuronidase preparations contain a thermostable coenzyme which dissociates from the parent molecule on dilution. While the nature of this coenzyme is still unidentified, it has been shown by the above authors that the action of this coenzyme may be duplicated by adding deoxyribonucleic acid (DNA) to the system.

Fig. 3. (●—●), pH-activity curve of purified ox-liver β -glucuronidase. Purity = 150 000 units per mg. protein N. (○—○), pH-activity curve of above preparation after standing at 0° for 3 weeks. These curves are not to be related quantitatively.

Fig. 4. pH-Activity curves of purified ox-liver β -glucuronidase at various concentrations of phenolphthalein glucuronide. A, 0.25 mM; B, 0.5 mM; C, 1.0 mM; D, 4.0 mM.

preparations when allowed to stand for any length of time (3 weeks at 0°) showed a gradual change in the pH-activity curve from that of well defined optima to the plateau-shaped curve shown in Fig. 3. The quantitative relationship between these two curves has not been investigated.

Fig. 4 shows the variation in shape of the pH-activity curve of a preparation of purified ox-liver

Fig. 5. (a) Effect of dilution on the enzyme activity to protein ratio (E/P) in the absence (●—●) and the presence (○—○) of 0.06% DNA. Enzyme assayed at pH 4.5. (b) Effect of dilution on the enzyme activity to protein ratio (E/P): A, in the absence and B, in the presence of 0.2% starch; C, 0.1% gum acacia and D, 0.05% crystalline bovine serum albumin (Armour). Enzyme assayed at pH 4.5.

Studies on purified ox-liver β -glucuronidase in the present work, however, do not indicate that DNA functions as a coenzyme-like substance. Enzyme activity was determined at varying dilutions in the presence and absence of DNA (final concentration, 0.06%; prepared from calf thymus by the method of Mirsky & Pollister, 1946). If glucuronidase

activity is dependent on a coenzyme dissociating on dilution, the ratio of enzyme activity to protein concentration (E/P) will drop greatly with dilution as observed by Bernfeld & Fishman (1950*b*) for calf-spleen β -glucuronidase, and a plot of the E/P ratio against protein concentration should take the form of an S-shaped curve in accordance with the dissociation expression:

$$\frac{(P-E)(C-E)}{E} = K,$$

where P = total protein, C = total coenzyme, E = active enzyme, P/C constant and K a constant. In the presence of DNA, the activity of β -glucuronidase should be proportional to the protein concentration. Such a plot is presented in Fig. 5*a* for purified liver β -glucuronidase assayed at pH 4.5. Fig. 5*a* shows that a plot of enzyme activity to protein concentration is linear at all dilutions down to a protein concentration of 0.05 μ g. protein N per ml. Such results provide no indication of a coenzyme dissociating from the parent molecule at high dilutions.

Added DNA is an effective activator of ox-liver β -glucuronidase at pH 4.5 (Fig. 5*a*). It was considered that this activating effect of DNA need not be specific and a number of other high molecular weight compounds were investigated in this respect. Albumin, gum acacia and starch were arbitrarily chosen, and their effects on the assay at pH 4.5 of purified liver β -glucuronidase at varying dilutions are shown in Fig. 5*b*.

Inhibition of ox-liver β -glucuronidase by DNA, suramin and alginic acid

While DNA is an effective activator of ox-liver β -glucuronidase at pH 4.5 (acetate buffer), a phenomenon of equal interest is the inhibitory action of DNA at lower pH values. A typical pH-activity curve in the presence of 0.06% DNA is shown in Fig. 6. Sharp inhibition was apparent below pH 4.2 and activation above this pH. The shape of the curve altered to give a single optimum at pH 4.5–5 with a rise in activity in this region.

An interesting comparison may be made between the effect of added DNA and the action of suramin on certain enzymes. Wills & Wormall (1950) and Wills (1952) have shown that suramin, a polysulphonic acid, inhibits many enzymes on the acid side of their isoelectric points. When percentage inhibition was plotted against pH in such instances, a sharp drop was evident in the inhibition-pH curve, 50% inhibition occurring at or about the isoelectric point of the enzyme. A similar sharp drop is evident in the inhibition-pH curve of liver glucuronidase in the presence of DNA, and Fig. 7 compares the effect of both DNA and suramin on liver glucuronidase activity in terms of an inhibition-pH graph. Although DNA and suramin

are dissimilar in the pH range over which inhibition occurs, their effects are none the less comparable in many respects, and suramin has also been shown to exert a similar activating effect to that of DNA in the higher pH range (Fig. 6).

Fig. 6. Effect of 0.06% DNA, 10^{-3} M-suramin and 0.1M-phthalate buffers on the pH-activity curve of purified ox-liver β -glucuronidase: A, control in 0.1M-acetate buffer; B, plus 0.06% DNA; C, plus 10^{-3} M-suramin; D, assays in 0.1M-phthalate buffers.

Fig. 7. Effect of pH on the inhibition of ox-liver β -glucuronidase by: A, 0.06% DNA; B, 0.06% alginic acid and C, 10^{-3} M-suramin.

Alginic acid is another high molecular weight compound whose inhibition of glucuronidase depends on pH, and the inhibition-pH curve for this compound is also shown in Fig. 7.

Effects of organic acids on ox-liver β -glucuronidase activity

Morrow, Carroll & Greenspan (1951), in a study of the glucuronidase activity of inbred mice, referred to the single pH optimum obtained in the assay of mouse-kidney homogenates. A most surprising feature of the activity curve was the total absence of activity below pH 3.8, the curve rising sharply from

Table 3. *The effect of structurally related organic acids on ox-liver β -glucuronidase activity*

(All compounds assayed at a final concentration of 10^{-3} M. Activity determined at pH 3.4, 4.5 and 5.2 (0.2M-acetate buffers). Enzyme purity = 170000 units activity/mg. protein N.)

Compound	K_a^*	Activation (+) or inhibition (-) (%)		
		pH 3.4	pH 4.5	pH 5.2
Benzoic acid	6.5×10^{-5}	+37	+6	+17
2-Hydroxybenzoic acid	—	-100	-4	+9
3:5-Dihydroxybenzoic acid	9.1×10^{-5}	-9	0	0
2:6-Dihydroxybenzoic acid	5.0×10^{-2}	-100	+5	+26
3:4-Dihydroxybenzoic acid	3.3×10^{-5}	0	0	0
2:5-Dihydroxybenzoic acid	1.3×10^{-3}	-100	-85	-76
3:4:5-Trihydroxybenzoic acid	—	-29	-40	-19
Phthalic acid	1st. H 1.3×10^{-3}	-100	+50	+47
isoPhthalic acid		-23	+50	+45
4:6-Dihydroxyisophthalic acid	—	-89	+48	+40
2:5-Dihydroxyterephthalic acid	—	-100	+57	+41
Benzenesulphonic acid	—	+56	+25	+28
Benzoic acid 3:5-disulphonic acid	—	-100	+55	+93
Salicylsulphonic acid	—	-100	-4	+8
Resorcinol	—	0	0	0
Alanine	—	0	0	0
Phenylalanine	—	+62	+14	-70
Tyrosine	—	+47	+10	-100
2:5-Dihydroxyphenylalanine	—	-100	-13	+19

* From *Dictionary of Organic Compounds* (1943). Ed. by Heilbron, I. H. and Bunbury, H. M. London: Eyre and Spottiswoode.

zero activity at this pH to the optimum around pH 4.5. It was noted that phthalate-hydrochloric acid and phthalate-sodium hydroxide buffers were used by Morrow *et al.* (1951), and it was considered that the lack of activity in the lower pH range was probably due to specific inhibition of enzyme

The inhibition of ox-liver β -glucuronidase activity by a number of structurally related organic compounds was studied. Inhibition was determined at pH 3.4, 4.5 and 5.2 in acetate buffer. All acids were neutralized prior to assay with N-NaOH. The results are summarized in Table 3.

In Fig. 8 is shown a comparison of the pH-inhibition curves of glucuronidase for a number of the compounds listed in Table 3.

DISCUSSION

While Sarkar & Sumner (1950) have suggested that ox-liver β -glucuronidase is a single enzyme with a single pH optimum at 4.5, the type of pH-activity curve obtained in the present work using a highly purified enzyme preparation is suggestive of the complexity of the enzyme system. The most interesting feature in this respect is the observation that all highly purified enzyme preparations when allowed to stand for any length of time (3 weeks) showed a plateau-shaped curve similar to that illustrated by Sarkar & Sumner (1950) for their purified β -glucuronidase preparation. The pH-activity curve of an enzyme is the resultant of a number of factors, including enzyme stability, and it might be assumed that the latter factor plays a predominant part in determining the activity curve of purified glucuronidase preparations. Such preparations have low-protein contents, and the enzyme may be susceptible to pH changes in the absence of the protective protein. This possibility receives support from the observation that less pure

Fig. 8. Effect of pH on the inhibition of ox-liver β -glucuronidase by: A, 10^{-3} M-benzoic acid 3:5-disulphonic acid; B, 10^{-3} M-2:5-dihydroxyterephthalic acid; C, 10^{-3} M-phthalic acid; D, 2×10^{-3} M-2-naphthol-3:6-disulphonic acid; E, 10^{-3} M-suramin.

activity by phthalic acid. This was confirmed by assaying the purified enzyme in both phthalate and acetate buffers. Reference to Fig. 6 shows the marked inhibitory effect of phthalate in the lower pH range and the considerable activation above pH 4.0. Phthalate increased the activity at the optima at pH 4.5 and 5.2.

enzyme preparations, characterized by a lower activity/protein ratio, maintain the well defined optima on standing for periods of several weeks.

No evidence is forthcoming from the present work to show that ox-liver β -glucuronidase contains a coenzyme dissociating from the molecule on dilution. The discrepancy between the present findings and those of Bernfeld & Fishman (1950*b*) for calf-spleen β -glucuronidase may be due either to the widely differing methods of purification employed, or to the effect of dilution on the stability of the enzyme prepared by Bernfeld & Fishman (1950*b*). Our investigation of the effects of added DNA on liver glucuronidase activity emphasize the fact that this compound does not act as a coenzyme-like substance. While DNA is an effective activator of liver β -glucuronidase at pH 4.5, as observed by Bernfeld & Fishman (1950*b*), the activating effect at this pH can be reproduced by a variety of other agents such as albumin, gum acacia and starch. Since the completion of this work, Beyler & Szego (1952) have also reported the activating effect of albumin on glucuronidase activity.

Wills & Wormall (1950) and Wills (1952) have shown that many enzymes are inhibited by suramin in acid solution, but not in neutral or alkaline solution, the inhibition falling sharply at or about the isoelectric point of the enzyme. It was suggested by the above authors that suramin does not combine directly with the active centres of such enzymes, but that inactivation involves the combination with, or association of, the sulphonic acid groups of suramin with basic groups lying near, or perhaps on opposite sides of the active centre, with a resultant 'bridge' formation protecting the latter. While ox-liver β -glucuronidase falls into the group A enzyme classification of Wills & Wormall (1950), where a slight change of pH, itself without effect on the activity, has a profound effect on the degree of inhibition exerted by suramin, it is obvious that the bridging mechanism proposed for suramin action on such group A enzymes would not appear to be applicable in the case of glucuronidase. In the latter instance, small molecular weight acidic compounds can cause marked inhibition of enzyme activity.

The marked dependence on pH of inhibition by DNA, alginic acid, phthalic acid, 2:5-dihydroxyterephthalic acid, benzoic acid 3:5-disulphonic acid and 2-naphthol-3:6-disulphonic acid is compared with the suramin inhibition of β -glucuronidase in Figs. 7 and 8, and the fact that the curves fall sharply from 100% to zero inhibition over a very narrow pH range in all cases suggests that certain reservations should be made when applying the suramin inhibition method to a determination of the isoelectric point of certain enzymes.

The effect on β -glucuronidase activity of aromatic acids appears to conform to certain well-defined

principles, namely: (1) The presence of one acid group on the benzene ring causes activation at all pH values studied. The sulphonic acid group appears to be more effective in this respect than the less strongly ionized carboxyl group. (2) Introduction of another acid group into the molecule invariably results in inhibition of enzyme activity at pH 3.4 concurrent with activation at pH 4.5 and 5.2. (3) Introduction of hydroxyl groups into the mono-acidic aromatic molecule is specific in effect. Provided the position ortho to the acid group is unsubstituted, the activating effect of the acid decreases progressively with hydroxyl substitution. 3:4-Dihydroxybenzoic acid is without effect on enzyme activity, while 3:4:5-trihydroxybenzoic acid functions as an inhibitor at all pH values. (4) In all instances, introduction of an hydroxyl constituent ortho to an acidic component results in complete inhibition of enzyme activity at pH 3.4 at the concentration of inhibitors studied (10^{-2} M). Direct attachment of the acid group to the benzene nucleus is not an essential feature of this inhibition since 2:5-dihydroxyphenylalanine, where the hydroxyl group is positioned ortho to a side chain containing a terminal acidic grouping, acts in a similar manner.

Inhibition by phenylalanine and tyrosine is of interest in view of their occurrence *in vivo*. The relative concentrations of both amino-acids required for inhibition are high and, while tyrosine is the more effective in this respect, a concentration of 10^{-3} M is required to effect 10% inhibition.

From a consideration of the available dissociation constants of the compounds listed in Table 3, it would appear that the degree of dissociation of any one compound has little relevance to the measure of inhibition or activation at any one pH, and that such effects are dependent more on the specific configuration of the molecule.

SUMMARY

1. A method has been devised for the purification of ox-liver β -glucuronidase, using reactions with metallic ions and organic solvents. An 800-fold purification has been achieved by such means, with a 5% recovery of the enzyme.

2. The pH-activity curve of the purified material shows optima at 4.5, 5.2 and 6.3, and the possible complexity of the enzyme is indicated.

3. The isoelectric point of purified ox-liver β -glucuronidase by filter-paper electrophoresis was found to be pH 4.85.

4. No evidence is forthcoming from the present work to indicate that ox-liver β -glucuronidase contains a coenzyme dissociating from the molecule on dilution.

5. Details are given of the inhibition and activation of ox-liver β -glucuronidase by deoxyribonucleic

acid, suramin, alginic acid and a number of other organic acids, the exact effect in each case varying with pH.

The expenses of this research were defrayed in part from the Rankin Fund of the University of Glasgow and by a

grant from the Carnegie Trust for the Universities of Scotland, which are acknowledged with grateful thanks.

We wish to thank Dr J. Reid and Dr P. Ongley for some of the organic compounds tested, and Dr D. J. Bell for the sample of sodium alginate. We are also indebted to Dr G. Leaf for assistance with the amino-acid chromatography.

REFERENCES

- Bernfeld, P. & Fishman, W. H. (1950*a*). *Arch. Biochem.* **27**, 475.
 Bernfeld, P. & Fishman, W. H. (1950*b*). *Science*, **112**, 653.
 Beyler, A. L. & Szego, C. M. (1952). *Fed. Proc.* **11**, 13.
 Cohn, E. J., Gurd, R. F. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, R. K., Gillespie, J. M., Kahnt, F. W., Liu, C. H., Mittleman, D., Mouton, R. F., Schmidt, K. & Uroma, E. (1950). *J. Amer. chem. Soc.* **72**, 465.
 Fishman, W. H. (1939*a*). *J. biol. Chem.* **127**, 367.
 Fishman, W. H. (1939*b*). *J. biol. Chem.* **131**, 225.
 Graham, A. F. (1946). *Biochem. J.* **40**, 603.
 Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.
 Markham, R. (1942). *Biochem. J.* **36**, 790.
 Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
 Mills, G. T. (1948). *Biochem. J.* **43**, 125.
 Mills, G. T., Paul, J. & Smith, E. E. B. (1949). *1st Int. Congr. Biochem. Abstr.* p. 597.
 Mills, G. T., Paul, J. & Smith, E. E. B. (1953). *Biochem. J.* **53**, 232.
 Mills, G. T. & Smith, E. E. B. (1951). *Biochem. J.* **49**, vi.
 Mills, G. T. & Smith, E. E. B. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 236.
 Mirsky, A. E. & Pollister, A. W. (1946). *J. gen. Physiol.* **30**, 117.
 Morrow, A. G., Carroll, D. M. & Greenspan, E. M. (1951). *J. nat. Cancer Inst.* **11**, 663.
 Sarkar, N. K. & Sumner, J. B. (1950). *Arch. Biochem.* **27**, 453.
 Smith, E. E. B. (1951). *Biochem. J.* **50**, viii.
 Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.
 Wills, E. D. (1952). *Biochem. J.* **50**, 421.
 Wills, E. D. & Wormall, A. (1950). *Biochem. J.* **47**, 158.

Unimolecular Films of Polymyxins A, B, D and E at the Air-water Interface

BY A. V. FEW AND J. H. SCHULMAN

Department of Colloid Science, University of Cambridge

(Received 14 October 1952)

The polymyxins consist of a group of five water-soluble basic polypeptide antibiotics possessing a specific action against Gram-negative bacteria; they are produced by many strains of the organism *Bacillus polymyxa*. Benedict & Langlykke (1947) first reported the antibacterial properties of cultures of this organism.

Chemical analysis of the hydrolysates of the antibiotics showed that a fatty acid, identified by Wilkinson (1949) as D-6-methyloctan-1-oic acid, was present in all the polymyxins. Amino-acid analyses demonstrated that polymyxin A contained L-threonine, L- α -diaminobutyric acid and D-leucine (Catch, Jones & Wilkinson, 1948); polymyxin B contained the above amino-acids with D-phenylalanine in addition (Catch, Jones & Wilkinson 1949); polymyxin C differed from polymyxin B in not containing D-leucine; polymyxin D possessed the same amino-acids as polymyxin A but in addition D-serine (Jones, 1948); and polymyxin E had the same amino-acid composition as polymyxin A,

but the intact antibiotics could be readily differentiated by paper chromatography (Jones, 1948).

The chemotherapeutic and pharmacological properties of the five polypeptides were studied by Brownlee, Bushby & Short (1952) and showed that the polymyxins A, C and D all caused severe proteinuria when administered parenterally in animals and man, this occurring even with the purest samples of the antibiotics. In this respect polymyxins B and E differed considerably since they exhibited far less nephrotoxic action.

Relatively little information has accumulated concerning the physical properties of the antibiotics. Jones (1948, 1949) has carried out chromatographic analyses of the antibiotics and has also studied their electrometric titration curves. Bell *et al.* (1949) have determined the molecular weight of polymyxin D by an osmometric method.

In the present communication, the investigation is described of the properties of unimolecular films of four of the polymyxins when spread at the air-water