Studies on Sulphatases

4. ARYLSULPHATASE AND β -GLUCURONIDASE CONCENTRATES FROM LIMPETS

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The application of β -glucuronidase concentrates to metabolic studies and biochemical assay is largely dependent on the ease of the hydrolysis of conjugated glucuronides by this agency. Chemical methods of hydrolysis involve boiling the glucuronide with concentrated acid for long periods and degradation of the aglycones under these conditions is not infrequent. It is claimed that greater yields of liberated aglycones are obtained by enzymic than by acid hydrolysis (cf. Samuels & Reich, 1952). Furthermore, the enzymic method is specific for the β -glucuronide linkage whereas acid also liberates hydroxy compounds from the ethereal sulphate conjugates which may also be formed as metabolites. Arylsulphatases are specific for the ester linkage between sulphuric acid and phenolic hydroxyl groups while glucuronidase hydrolyses both aliphatic and aromatic glucuronides. Less attention has been given to the preparation and use of arylsulphatase concentrates than of those of β -glucuronidase. However, commercial fungal preparations possessing weak arylsulphatase activity have proved useful, e.g. Butenandt & Hoffstetter (1939) cite the hydrolysis of oestrone sulphate by Taka diastase as evidence of the presence of an ester sulphate grouping in this compound. Mylase P was employed by Clarke, Akawie, Pógrund & Geissman (1950) (private communication) to demonstrate that the *laevo*-adrenaline conjugate isolated from rabbit urine was not a sulphate ester. Enzymic hydrolysis of pregnant mare's urine with mylase P gave a yield of

oestrogens 20% greater than that obtained after acid hydrolysis (Cohen & Bates, 1949, 1950). The method distinguished between oestrogens conjugated with sulphuric acid through phenolic groups and those conjugated with glucuronic acid or with sulphuric acid through aliphatic hydroxyl groups.

The potential value of arylsulphatases in metabolic studies suggested the need for stable, highly active concentrates of the enzyme. Since such concentrates might be used for investigating the metabolism of phenols which are conjugated with both glucuronic and sulphuric acids, it was necessary to prepare arylsulphatase free from β -glucuronidase and this has been the main criterion of purification in the present investigation. A highly active β -glucuronidase preparation contaminated with arylsulphatase has also been obtained. Selective inhibition of the latter enzyme provided a β -glucuronidase concentrate devoid of arylsulphatase activity.

There are four main sources of arylsulphatase, mammalian, molluscan, fungal, and bacterial; of these, molluscan sources yield by far the most active preparations (Table 1). Common molluscs are readily available either commercially or from almost any coast and they invariably possess strong β -glucuronidase activity (Dodgson, Lewis & Spencer, 1953a). Of the common molluscs the limpet, *Patella vulgata*, was chosen for study on the grounds of its availability, large size and the ease of access to the visceral hump.

Table 1. *The activity of arylsulphatases from various sources*

(The units of activity are expressed as μ g. *p*-hydroxyacetophenone liberated from potassium *p*-acetylphenylsulphate in 1 hr. under the standard conditions (Dodgson *et al.* 1953a). The last value quoted represents μ g. phenolphthalein liberated by 1 ml. enzyme extract acting on potassium phenolphthalein disulphate under the optimal conditions in 6 hr.)

Type	Source	Activity (units/g.)	Reference
Mammalian	Rat liver	1500-4500	Dodgson <i>et al.</i> (1953b)
	Rat kidney	600-900	
Molluscan	Visceral hump of <i>Patella vulgata</i> and <i>Littorina littorea</i>	7000-140 000	Dodgson, Lewis & Spencer, 1952
Fungal	Taka diastase	1300	Dodgson <i>et al.</i> (1952)
Bacterial	Extract of <i>Mycobacterium piscium</i>	59	Whitehead, Morrison & Young, 1952

EXPERIMENTAL AND RESULTS

Arylsulphatase concentrates have already been prepared by Soda & Egami (1941) from the digestive glands of *Charonia lampas* (*Tritonium nodiferum*), a large tropical marine mollusc, but it is not stated whether separation from β -glucuronidase was achieved. These workers autolysed the macerated material for several days but this was considered inadvisable in the present experiments since buffered suspensions (pH 5.5) of ground *Patella* viscera rapidly lose arylsulphatase activity at 37.5° (Dodgson *et al.* 1953*a*).

In order to obtain a stable final concentrate in powder form all fractions have been acetone-dried. Although this results in some loss of the enzyme it has the advantage of convenience of procedure and ease of repetition with simple equipment.

Preparation of acetone-dried limpet viscera. Inactivation of the enzyme during concentration was kept as low as possible by keeping the acetone and all solutions at 0°. Acetone precipitations of the soluble enzyme were carried out by the slow addition of acetone (60%, v/v, final concentration) to the rapidly stirred solution. The mixtures were then left at 0° for 15 min. before the precipitates were collected.

Limpets from Sully, Glamorgan, were starved for 5–6 days in aerated sea water. This procedure is advisable in order to remove the main bulk of the food material from the viscera. After being washed quickly in water, 100 limpets (906 g.) were removed from their shells and the visceral humps dissected out. The pooled viscera (122 g.) were macerated in 800 ml. of acetone using a chilled Townson and Mercer macerator. The macerate was quickly filtered by suction, the debris transferred to a further 600 ml. of acetone and the maceration and filtration repeated. The debris was washed three times with 50 ml. of acetone, sucked free from liquid and dried overnight *in vacuo*. The light-grey powder (powder A, 22.5 g.) was assayed for arylsulphatase and β -glucuronidase activity using potassium *p*-acetylphenylsulphate and *p*-chlorophenylglucuronide respectively as substrates (Dodgson *et al.* 1953*a*). It was advantageous to suspend the powder in buffer by means of a glass homogenizer with a smooth-ended pestle. The activities were: arylsulphatase 375 000 units/g., β -glucuronidase 200 000 units/g. (A unit of activity is that which liberates 1 μ g. of the phenol from the substrate in 1 hr. under optimal conditions.)

The values quoted were obtained from limpets collected in June and it should be emphasized that the enzyme activities

of acetone-dried preparations vary, depending on the month of collection, the length of starvation and on other unknown factors. The powder was stable for several months when kept in an air-tight bottle at 0°.

Purification of the acetone-dried viscera. Centrifugal fractionation of aqueous suspensions of powder A showed that about 95% of the arylsulphatase was associated with the supernatant whilst the bulky residue was almost devoid of activity. Powder A (60 g.) was suspended in 150 ml. of water using a pestle and mortar. (Shaking or the use of macerators and glass homogenizers led to troublesome frothing.) The suspension was centrifuged and the protein of the clear supernatant precipitated with acetone. The main bulk of this supernatant was decanted, the remainder filtered and the residue, while still wet, was washed well with acetone and the latter finally removed *in vacuo*. (The filter-cake must not be allowed to crack until it has been thoroughly washed and should be water-free before the vacuum treatment in order to avoid the formation of gums.) The buff-coloured powder obtained (powder B, 8.67 g.) had an arylsulphatase activity of 1 810 000 units/g. This represents a concentration of 4.82 times with a retention of 70% of the initial activity of powder A. Glucuronidase activity was 690 000 units/g. (concentration 3.45 times; 50% of initial activity).

Removal of β -glucuronidase. Soda & Egami (1941) used tryptaflavine (or euflavine; 2:8-diaminomethylacridinium chloride) for the purification of the sulphatases of *Charonia lampas* but they did not study its action on glucuronidase. Similar treatment of powder B with this flavine concentrated both the arylsulphatase and the glucuronidase (Table 2). Further investigation of the action of flavines (substituted acridines) was undertaken in the hope that a method of removal of β -glucuronidase would be found. Powder B (1 g.) was ground in a mortar with 35 ml. of ice-cold water and 0.65 g. of flavine. The precipitated material and undissolved flavine were spun down and the supernatant solution precipitated with acetone. This precipitate was collected by centrifuging and suspended in 20 ml. of water. The solution was centrifuged to remove undissolved material, precipitated with acetone and dried *in vacuo*. Unless otherwise stated, the pH of the original mixture was controlled by the excess of flavine present.

The results obtained with the different flavines are summarized in Table 2. Treatment with proflavine sulphate (2:8-diaminoacridine disulphate) at pH 2.9 removed almost all the glucuronidase and concentrated the arylsulphatase.

Table 2. *The enzyme activity of powder B after treatment with flavines*
(Powder B (1 g.) was treated with flavine (0.65 g.) or the pH adjusted as described in text.)

Flavine	pH of mixture	Yield (g.)	Arylsulphatase activity		Glucuronidase activity	
			(Units/g.)	(% of initial activity)	(Units/g.)	(% of initial activity)
Acridine	1.8	0.170	430 000	4.0	0	0
None	1.8*	0.178	3 760 000	37.0	0	0
Proflavine sulphate	2.9	0.128	5 660 000	39.6	78 000	1.4
Proflavine hemisulphate	2.9*	0.201	3 880 000	43.0	28 500	0.8
Euflavine	2.9*	0.212	4 010 000	47.0	40 700	1.3
None	2.9*	0.139	4 580 000	35.2	65 000	1.3
Proflavine hemisulphate	6.5	0.250	3 330 000	42.2	1 984 000	48.7
Euflavine	6.5	0.200	2 900 000	32.1	1 300 000	26.0
None	6.5*	0.292	2 570 000	41.6	688 000	37.9

* The pH of the mixture was adjusted to this pH.

Proflavine hemisulphate and euflavine (pH 6.5) concentrated both enzymes whilst a commercial specimen of acriflavine (2:8-diaminoacridine hydrochloride containing 2:8-diaminomethylacridinium chloride hydrochloride) at pH 1.8 removed all the glucuronidase and almost all the arylsulphatase. These results were mainly due to pH effects since the results obtained with any flavine at its natural pH could be achieved with other flavines if the pH of the flavine-powder *B* aqueous mixture was suitably adjusted. Furthermore, similar treatment of aqueous suspensions of powder *B*, involving pH adjustment with dilute H_2SO_4 in the absence of flavine, resulted in powders having almost identical properties with those prepared using the respective flavines (Table 2). Only in the case of acriflavine was any difference in enzyme activity shown between powders prepared in the presence and absence of flavines.

Fig. 1. The effect of pH on the arylsulphatase and β -glucuronidase of an aqueous solution of powder *B*. Portions of the solution were adjusted to various pH's, centrifuged and the precipitates and supernatant solutions assayed. At all pH's the activity of the precipitate was negligible. The β -glucuronidase ($\times$ — $\times$) and arylsulphatase ($\odot$ — $\odot$) activities of the supernatant solutions are shown.

The effect of pH on the enzymes of powder *B* was further investigated. Aqueous suspensions of powder *B* were adjusted to various pH's with dilute HCl, centrifuged, the precipitates washed with water adjusted to the same pH, centrifuged again and the residue and combined supernatants assayed. All solutions were kept ice-cold during manipulation before incubation with substrate. Fig. 1 shows that the glucuronidase and arylsulphatase activities of the supernatant solution declined sharply at low pH. There was no corresponding increase in the activities of the precipitate. It must be assumed that irreversible inactivation of the enzymes takes place at acid pH even at 0°. Combination of the supernatant and residue before assay did not increase the recovery. The inactivation of both enzymes was not progressive with time since there was no further decline in activity after the first sample had been taken (about 1 min. after pH adjustment). It is important, therefore, in adjusting limpet viscera extracts to acid pH, that the required pH is not overshoot on the acid side. The β -glucuronidase of powder *B* is inactivated at higher pH than the arylsulphatase and at pH 2.25 it is entirely removed whilst about 95% of the arylsulphatase remains.

Purification of powder *B* (1 g.), using the method employed with the flavines but adjusting to pH 2.25 and omitting the flavine yielded 0.188 g. of a stable glucuronidase-free powder (powder *C*) having an arylsulphatase activity of 3 650 000 units/g. (37.9% of the initial activity).

Simplified method. For routine purposes the preparation of glucuronidase-free arylsulphatase concentrates from acetone-dried limpet viscera was simplified. Powder *A* (25 g.) was ground in a mortar with water (200 ml.). The suspension was adjusted to pH 2.25 and the undissolved and precipitated material removed by centrifuging. Acetone was added to the supernatant and the precipitate formed collected by centrifugation. This was suspended in water (100 ml.), undissolved material was spun down, and the arylsulphatase, free from glucuronidase, was precipitated from the solution by acetone. The precipitate was collected, washed with acetone on the vacuum filter and finally dried *in vacuo*. Yield, 1.15 g.; arylsulphatase activity, 3 680 000 units/g.; concentration, 9.7-fold with a retention of 46.4% of the initial activity. The white powder (powder *D*) dissolved easily in water or 0.5M-acetate buffer, pH 5.5.

Characteristics of the purified preparation. The optimal conditions for the arylsulphatase of powder *D* were substantially the same as those determined in crude suspensions of limpet viscera (Dodgson *et al.* 1953a). The optimum pH (5.5) was unchanged but the optimum substrate concentration was 0.055M instead of 0.065M. Incubation of the purified preparation without substrate at 37.5° for 1 hr. did not inactivate the enzyme. The graph of enzyme concentration against activity was a straight line but the activity-time curve showed that the initial hydrolysis rate declined during the first 45 min. to a steady value which was maintained for at least 2 hr.

The activity of arylsulphatase concentrates in urine and the effect of various ions

A possible use of arylsulphatase concentrates is for the hydrolysis of urinary ethereal sulphates. The effect of urine on the activity of purified glucuronidase-free arylsulphatase preparations was therefore investigated. The enzyme was inhibited strongly. In a typical experiment rabbit urine caused 91.5% inhibition and human urine 87.8%. In another experiment a mixture of rabbit urine (10 ml.) at pH 5.5 and 0.5M-acetate buffer (10 ml.) at the same pH containing 70.6 mg. of potassium *p*-acetylphenylsulphate was incubated with powder having an activity of 20 000 units. After 1 hr. there was 8.5% hydrolysis and after 16 hr. only 20%. In the absence of urine, hydrolysis was complete in 11.5 hr. The cause of the inhibition of arylsulphatase was traced by testing the effect of several common urinary constituents on the enzyme (Table 3). Urea appeared to have no influence on arylsulphatase action and the various chlorides had a slight activating effect. The inhibition by urine could be attributed, however, to the presence of sulphate and

phosphate ions, both of which were strong arylsulphatase inhibitors. The inhibition by phosphate was not due to a shift in pH maximum of the enzyme from that of 5.5 in acetate buffer since the purified limpet preparations were entirely inactive in 0.2M-phosphate buffer at pH's 5-9. Both Taka diastase and rat-liver arylsulphatase have different pH optima in phosphate and in acetate buffers (Dodgson, Spencer & Thomas, 1953b).

Table 3. *The effect of various compounds on the arylsulphatase activity of limpet viscera concentrates*

(Substrate, 0.055M-potassium *p*-acetylphenylsulphate in 0.5M-acetate buffer, pH 5.5, incubated 1 hr. at 37.5°.)

Compound	Molarity	Arylsulphatase activity (%)
None	—	100
Urea	0.02	98.6
NaCl	0.01	109.0
	0.02	110.3
KCl	0.02	111.3
K ₂ SO ₄	0.01	56.3
	0.02	56.2
KH ₂ PO ₄	0.02	0
Phenylhydrazine hydrochloride	0.00005	103.3
	0.0005	124.0
MgCl ₂	0.02	109.3
BaCl ₂	0.1	107.5
Magnesium acetate	0.02	97.8

Table 4. *The activity of the arylsulphatase of limpet viscera concentrates in the presence of treated urines*

(Enzyme in 0.6 ml. M-acetate buffer was incubated with substrate dissolved in 0.6 ml. of the solutions listed.)

Solution	Activity (%)
Water	100.0
0.2M-BaCl ₂	110.0
Urine	20.3
0.2M-BaCl ₂ in urine	20.1
Urine precipitated at pH 5.5 with 0.2M-BaCl ₂ and the precipitate removed by centrifugation	18.9
Urine precipitated at pH 11.5 with 0.2M-BaCl ₂ and the precipitate removed by centrifugation	106.0

Removal of inhibiting substances from urine. The inhibitory effect of urine is sufficient to reduce the arylsulphatase activity of limpet concentrates to an inefficient level, and it is necessary to remove the sulphate and phosphate ions before the use of the concentrates in urine is practicable. Both ions may be removed by precipitation with barium chloride at pH 11.5 and urine treated in this manner is no longer inhibitory (Table 4). Precipitation of the sulphate ions only by barium chloride at pH 5.5 does not reduce the inhibitory effect of urine even after removal of the precipitated barium sulphate by

centrifugation. Phosphate ions must, therefore, be regarded as the main cause of the inhibition. The sulphate- and phosphate-free urine has a slight activating effect which may be attributed to the increased concentration of chloride ions added as barium chloride.

The following experiments show that arylsulphates present in urine are unaffected by the barium chloride treatment and that subsequently they can be hydrolysed to completion by the enzyme concentrates.

Rabbit urine (25 ml.) containing potassium *p*-acetylphenylsulphate (100 mg.) was adjusted to pH 11.5 with concentrated KOH and solid BaCl₂ (0.52 g.) added. The precipitated barium salts were removed by centrifugation and

Fig. 2. The hydrolysis of potassium *p*-nitrophenylsulphate (x-x) dissolved in treated human urine and potassium *p*-acetylphenylsulphate (O-O) dissolved in treated rabbit urine by purified limpet arylsulphatase powder D at 37.5°. The sulphate and phosphate ions of both urines were removed as barium salts at pH 11.5 and the urines were then adjusted to pH 5.5.

15 ml. of the clear supernatant were adjusted to pH 5.5 with conc. HCl and made up to 25 ml. with 0.5M-acetate buffer at the same pH. To this solution was added purified limpet arylsulphatase powder (73 600 units) and the mixture incubated at 37.5°. Portions were removed at intervals, enzyme action was stopped with 0.2N-NaOH and the liberated *p*-hydroxyacetophenone determined. A similar experiment was carried out with potassium *p*-nitrophenylsulphate (100 mg.) dissolved in human urine (25 ml.). Liberated *p*-nitrophenol was measured spectrophotometrically at 400 mμ. (Dodgson & Spencer, 1953). The results are shown graphically in Fig. 2. The sulphates of *p*-nitrophenol and *p*-hydroxyacetophenone were hydrolysed completely in 148 and 160 min. respectively.

It may be concluded that the use of limpet arylsulphatase concentrates for the hydrolysis of arylsulphates in urine is efficient provided that inhibiting ions are previously removed.

Suppression of arylsulphatase activity of concentrates containing β -glucuronidase

The complete inhibition of limpet arylsulphatase by 0.1M-phosphate and the failure of this concentration of phosphate to affect limpet glucuronidase activity suggested that powder *B* could be used as a β -glucuronidase concentrate, free from arylsulphatase activity, without actually removing the arylsulphatase.

Sodium dihydrogen phosphate was added to human urine to give a 0.2M solution which was adjusted with HCl to pH 4.0, the optimum pH of limpet viscera glucuronidase, and then diluted with an equal volume of 0.5M-acetate buffer at the same pH. To one portion of this solution potassium *p*-acetylphenylsulphate (1 mg./ml.) was added and to another portion *p*-chlorophenylglucuronic acid (1 mg./ml.). Powder *B* (1 mg./ml.) was added to each portion which was then incubated at 37.5°, and 1.2 ml. samples removed at intervals and deproteinized with 4.8 ml. ethanol. After centrifugation, 5 ml. of the clear supernatants were each added to 1 ml. *N*-NaOH and the liberated phenols in the resultant mixtures determined. Complete hydrolysis of *p*-chlorophenylglucuronic acid occurred after 2 hr. No *p*-hydroxyacetophenone was liberated from its sulphate even after 24 hr. Powder *B* can therefore be used in urine as an arylsulphatase-free β -glucuronidase preparation provided that phosphate ions (0.1M) are present.

DISCUSSION

The high arylsulphatase and β -glucuronidase contents of limpet viscera permit the preparation of concentrates of these enzymes which compare favourably in activity and ease of preparation with those derived from other sources. Powder *B* can be used as a mixture of the two enzymes or, in the presence of 0.1M-phosphate, as a β -glucuronidase concentrate free from arylsulphatase activity. Powders *C* and *D* contain glucuronidase-free arylsulphatase. These powders can be used to release phenols from their glucuronide and sulphate conjugates either simultaneously or separately and may, therefore, prove useful for the hydrolysis of urinary steroid conjugates as a preliminary to steroid analysis. In urine the use of the concentrates is limited if precipitation of phosphate and sulphate ions by barium hydroxide results in the removal of the arylsulphate under study. However, none of the common arylsulphates encountered by the authors is precipitated under these conditions.

The adoption of the term 'arylsulphatase' for the sulphatase of limpet viscera is based on its ability to hydrolyse certain arylsulphates. The term implies that the hydrolytic activity of the enzyme is confined to the ester linkage between sulphuric acid and hydroxyl groups of phenolic character. This assumption of specificity has not yet been rigorously tested but is reasonable if the specificities of the

arylsulphatases of mammals, fungi and, especially, tropical marine molluscs are borne in mind. It should be noted that the absence of β -glucuronidase from the arylsulphatase concentrates is the only step towards enzymic purity here claimed. Other enzymes are undoubtedly present and, in certain circumstances, these may limit the usefulness of the concentrates.

Limpet arylsulphatase is strongly inhibited by urine whilst the enzyme of Taka diastase and other fungal preparations is only weakly affected (Noguchi, 1923; Dzialoszynski, 1947). This inhibition is due to phosphate and sulphate ions, since at 0.02M concentration these inhibit the limpet enzyme 100 and 43.7% respectively, and the arylsulphatase of fungal origin only 4-19 and 0% respectively (Robinson, Smith, Spencer & Williams, 1952). Dzialoszynski attributes the inhibitory effect of urine on the sulphatase of Clarase to urea which inhibited the enzyme in pure buffer solution. Urea (0.02M) had negligible action on limpet arylsulphatase. The effects of other ions have revealed further differences between the arylsulphatases from various sources. Chloride ions activate the limpet enzyme (Table 3) and rat-liver arylsulphatase (Dodgson *et al.* 1953*b*) but have no effect on the arylsulphatase of Taka diastase (Robinson *et al.* 1952). Seligman, Chauncey & Nachlas (1951) state that magnesium chloride activates rat-liver arylsulphatase by 50%, but this action is much less with the limpet enzyme. The activation of limpet concentrates by magnesium chloride can probably be attributed to chloride ions since magnesium acetate is almost without effect. Dzialoszynski (1950) found that 0.00001M-phenylhydrazine completely inhibited the arylsulphatase of Clarase, whereas phenylhydrazine hydrochloride activated limpet viscera concentrates.

The effect of inorganic ions upon arylsulphatases from various sources combined with the different kinetic behaviour of the enzymes towards the same substrate (Dodgson *et al.* 1953*a*) suggests that there are basic differences between arylsulphatases. It must not be assumed, therefore, as it has been assumed in the past, that conclusions based on the study of an arylsulphatase from one source are directly applicable to the enzymes from other sources.

SUMMARY

1. Acetone-dried preparations of limpet viscera were fractionated to give stable powders containing both arylsulphatase and β -glucuronidase.

2. Irreversible inactivation of the β -glucuronidase of these powders at pH 2.25 yielded a stable arylsulphatase preparation free from β -glucuronidase.

3. The inhibition of limpet arylsulphatase by

urine can be removed by precipitation of the urine with barium chloride and may be attributed to phosphate and sulphate ions.

4. Total inhibition of limpet arylsulphatase by 0.2M-phosphate allows the use of the crude viscera

concentrates as β -glucuronidase preparations free from arylsulphatase.

5. The dissimilarity in the characteristics of arylsulphatases from various sources is commented on.

REFERENCES

- Butenandt, A. & Hoffstetter, H. (1939). *Hoppe-Seyl. Z.* **259**, 222.
- Clarke, W. G., Akawie, R. J., Pógrund, R. S. & Geissman, T. A. (1950). *18th Int. Congr. Physiol. Abstr.* p. 159.
- Cohen, H. & Bates, R. W. (1949). *Endocrinology*, **44**, 317.
- Cohen, H. & Bates, R. W. (1950). *Endocrinology*, **45**, 86.
- Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1952). *Biochem. J.* **51**, xlii.
- Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1953a). *Biochem. J.* **55**, 254.
- Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **53**, 444.
- Dodgson, K. S., Spencer, B. & Thomas, J. (1953b). *Biochem. J.* **53**, 452.
- Dzialeszynski, L. M. (1947). *Nature, Lond.*, **160**, 464.
- Dzialeszynski, L. M. (1950). *Nature, Lond.*, **166**, 157.
- Noguchi, J. (1923). *Biochem. Z.* **143**, 186.
- Robinson, D., Smith, J. N., Spencer, B. & Williams, R. T. (1952). *Biochem. J.* **51**, 202.
- Samuels, L. T. & Reich, H. (1952). *Annu. Rev. Biochem.* **21**, 129.
- Seligman, A. M., Chauncey, H. H. & Nachlas, M. M. (1951). *Stain Tech.* **26**, 19.
- Soda, T. & Egami, F. (1941). *J. chem. Soc., Japan*, **62**, 256.
- Whitehead, J. E. M., Morrison, A. R. & Young, L. (1952). *Biochem. J.* **51**, 585.

The Oxidation of Glycerol by Mycobacteria

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Nocard & Roux (1887) discovered that the growth of *Mycobacterium tuberculosis* var. *hominis* was stimulated by addition of glycerol to culture media, but Hammerschlag (1891) was the first to demonstrate utilization of glycerol. Several investigators have since shown that glycerol is a favourable source of carbon for the cultivation of many species of mycobacteria. Baudran (1910) drew attention to the simultaneous disappearance of glycerol and phosphoric acid from culture media, and suggested that glycerophosphoric acid might be formed. Merrill (1930, 1931a, b) studied the fate of glycerol in broth cultures and in synthetic media, and concluded that glycerol was completely oxidized without the accumulation of breakdown products, a view which was shared by Dingle & Weinzierl (1932), Wedum (1936) and Friedemann & Seibert (1939).

The investigation of washed cell suspensions has furnished additional information (see Edson, 1951). Nakamura (1938) reported that glycerol increased the oxygen consumption of *Myco. tuberculosis* var. *hominis*; but no direct relationship between respiration and the growth-promoting ability of glycerol could be found by Bloch, Matter & Suter (1947). Although it is clear that many acid-fast organisms oxidize glycerol, there has been little advance in knowledge of the intermediate metabolism. This

paper shows that the use of washed-suspension techniques and cell-free extracts has provided means of elucidating the mechanism of glycerol degradation.

METHODS

Organisms. The organisms used were *Myco. stercoris* (no. 3820), *Myco. smegmatis* (no. 523) and *Myco. butyricum* (no. 337). These strains were obtained from the National Collection of Type Cultures, Lister Institute, London. Stock cultures were maintained on glycerinated Dorset's egg medium, and cultivated on meat infusion broth containing 1% (w/v) peptonum B.P.C. (British Drug Houses) and 5% (v/v) glycerol. On the fifth or sixth day of incubation at 38°, the bacteria were washed and suspended in distilled water by methods described earlier (Edson & Hunter, 1943).

Bacterial extracts. Extracts were prepared by disrupting the organisms either in the wet crushing mill of Booth & Green (1938) or in the Mickle disintegrator (H. Mickle, Hampton, Middlesex). In the Mickle apparatus 4 g. of washed cells, 4 g. of no. 12 ballotini glass spheres (0.18–0.25 mm.) and 4 ml. of phosphate buffer (0.1M, pH 7.4) were mixed, precooled in each container and shaken at maximum amplitude for 30 min. in a cold room (2–3°). The suspension of disintegrated organisms and glass beads was diluted with 4 ml. of buffer per tube, the extracts clarified by centrifugation and the supernatant dialysed for 24–48 hr. against distilled water at 2–3°.