Actinomycete Metabolism: α-Phenylmannosidase of *Streptomyces griseus*

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(*Received* 23 *September* 1953)

Langlykke & Perlman (1950) have reported that *Streptomyces griseus* forms an enzyme capable of converting mannosidostreptomycin into the aglycone, streptomycin, and have described the dependence of its activity upon oxidation potential and pH value and its inhibition by heavy metals. During the course of our work it was found that synthetic phenyl α-D-mannoside was also split by the organism. Accordingly we have used this synthetic substrate to provide such general information on α-mannosidase activity as might later be used in work on the natural substrate. Although in this work the term 'mannosidase', which is used for convenience, implies a simple hydrolysis of mannosidostreptomycin or α-phenylmannoside into mannose and streptomycin or phenol, we do not exclude the possibility that the enzymic cleavage of either substrate is achieved by a more complex mechanism.

METHODS AND MATERIALS

Organism. Streptomyces griseus mutant (Dulaney, Z38) was maintained and grown on soya bean medium, as in example I of the patent describing it (Dulaney, 1951). Growth was always carried out at 28·5° on a rotary shaker (160 rev./min.) in quantities of 40 ml. medium in 250 ml. conical flasks.

Determination of enzyme activity. The substrate was phenyl α-D-mannoside, prepared by the method of Helferich & Winkler (1933) with the modification that deacetylation was carried out as by Zemplen & Kunz (1923). The determinations were based on measurement of the amount of phenol liberated.

In our early experiments we removed the phenol by distillation, using a method similar to that described by Volterra (1942) but the pH was adjusted to 6·4 with 0·1 M-KH_2PO_4, and CO_2 was also bubbled through the boiling liquid to prevent frothing. The phenol content of the distillate was determined colorimetrically (Folin & Ciocalteu, 1927). This method not only proved tedious, but also needed large samples, and simpler ones were tried. Extraction with chlorinated solvents (cf. Ellinger, Ruchhoft & Lishka, 1951) was difficult on account of persistent emulsion formation. A search was then made for reagents for estimating phenol which were not subject to interference by broth constituents. The procedure of Gottlieb & Marsh (1946), based on colour development with 4-aminoantipyrin, was found suitable in sensitivity, specificity and stability of colour formed. It was found convenient to clear the samples by addition of ethanol, which did not interfere with the test. Other methods of colorimetric estimation were rejected, that of Folin & Ciocalteu (1927) because of inconsistent blank values, that with diazotized sulphanilic acid (Schmidt, 1949) because of the instability of the colour and that involving the use of halogenated quinone chlorimides to form coloured indophenols (Singer & Stern, 1951) because of the slow rate of attaining maximum colour.

Enzyme activity was defined as being unity when 1 μm-mole of phenol is liberated per ml. in 30 min. at the temperature of incubation. The activity varies with temperature and unless otherwise stated all determinations recorded here were carried out at 28·5°. The enzyme is sensitive to aeration and therefore a standard set of conditions is laid down for the operation of the test, as below. The reagents used were: *A*, glycine buffer, pH 10·4, prepared by mixing 54 vol. of an aqueous solution containing 4·51 g. glycine and 3·48 g. NaCl in 600 ml. and 46 vol. of 0·1 N-NaOH; this is adjusted to pH 10·4 with the appropriate constituent solutions; *B*, 1% (w/v) aqueous 4-aminoantipyrin; *C*, 3% (w/v) aqueous potassium ferricyanide A.R.; *D*, 1·0% (w/v) aqueous α-phenylmannoside.

Incubation unit. A 6 × 1 in. boiling tube with a 'B24' socket to fit a gas wash bottle head (MF 28/3, Quickfit and Quartz, Ltd. London), whose inlet tube reaches to within 8 mm. of the bottom was immersed in a temperature-controlled water bath and connected in series to other units and an air supply delivering 700–900 ml./min. A vibrator-type aquarium aerator was found most suitable for a constant-rate air supply.

Enzyme activity. 15 ml. of enzyme solution (e.g. culture fluid) were transferred into the incubation unit, which was allowed to equilibrate to the required temperature for 5 min. To this was added 1 ml. of *D*; the aeration train was then connected and incubation was carried out for exactly 30 min. Immediately after this, 14 ml. of 96% ethanol (industrial methylated spirits) were added with good mixing and the solution was then filtered through a Whatman no. 42 fluted filter.

To 1 ml. of filtrate were added 10 ml. *A*, 1 ml. *B* and 3 ml. *C*, with good mixing. The colour developed was measured in a Spekker Absorptiometer (Ilford no. 605 filters; $\lambda_{max.}$, 545 mμ.) against an enzyme blank consisting of 1 ml. of filtrate from a mixture of 15 ml. of enzyme solution and 15 ml. of 96% ethanol. The readings must be taken within 10–40 min. after the reagent additions.

Standard calibration graphs were constructed by adding 1 ml. *B* and 3 ml. *C* to the following mixtures:

glycine buffer *A* (ml.)	10	9	8	7	6
5×10^{-5} M phenol (ml.)	1	2	3	4	5
to give units of enzyme activity	100	200	300	400	500

The developed colours were read against a water blank. The phenol solutions were checked by bromimetric or iodimetric analysis.

The method was checked to show the reproducibility of the phenol assay by determination of added phenol (Table 1), and the reproducibility of enzyme activity for the same broth samples (Table 2). Agreement was found to be excellent. We also investigated the effect of storage at $-10°$ on the enzyme in whole cultures and found that after 4 days no deterioration in activity had occurred.

It has also been found that the requisite aeration may be obtained by shaking 15 ml. of enzyme preparation in a 250 ml. conical flask on a rotary shaker, as described above, at 28·5°. This method was used for many of the determinations quoted below, as it was useful for carrying out a number of investigations at the same time, when comparative rather than absolute values were important. The units of activity, which differ little from those obtained in tubes (units (*T*)/ml.), will be given as units (*S*)/ml.

Whole cultures were used as enzyme sources. Very little work has yet been carried out on the purification of the enzyme.

RESULTS

Effect of time and substrate concentration

Under the conditions of aeration, temperature and pH described above, the amount of phenol liberated by the enzyme increased linearly with time (Fig. 1). In experiments in which enzyme action was allowed to continue beyond a point at which the amount of liberated phenol was about 200 μg./ml., some slowing down was observed. This was first thought to be due to an inhibitory effect of phenol, but was found not to be so, as the addition of phenol (200 μg./ml.) caused no retardation of enzyme activity. On the other hand, mannose (380 μg./ml.) did cause some inhibition.

For accurate enzyme estimations it is necessary to work on the portion of the time curve that is linear, i.e. up to about 2 μmole of liberated phenol/ml. broth.

Determination of the Michaelis constant

Though the Michaelis constant for a pure enzyme and the substrate concentration at which the enzyme shows one half its maximum activity in culture broth are not necessarily identical, the constant is still of some use in characterizing the enzyme. A number of determinations were made with 10 ml. portions of broth to which was added 1 ml. of aqueous substrate solution containing sufficient α-phenylmannoside to give concentrations of 136, 55, 27 and 11 μg./ml. of incubation mixture. Incubation with shaking was carried out for 10 min. and the liberated phenol estimated in the usual way. A typical set of figures is shown in Table 3.

In the range, 136–55 μg./ml., the liberation of phenol was, within experimental error, the same. Below this level, the hydrolysis of α-phenylmannoside was complete at the end of the incubation

Table 1. *Recovery of added phenol from non-incubated whole cultures*

For details of the estimation of phenol see text. *A* and *B* represent duplicate determinations on the same fortified broth culture sample; *a* and *b* represent duplicate colorimetric determinations on the same ethanolic filtrates.

Sample	Added phenol (μg./ml.)	Phenol recovered (μg./ml.)		Recovery (%)	
		a	*b*	*a*	*b*
1*A*	16·9	17·9	17·5	105·5	103·5
1*B*	16·9	16·7	16·7	98·8	98·8
2*A*	33·1	32·1	32·5	97·1	98·2
2*B*	33·1	32·9	32·9	99·4	99·4
3*A*	53·7	52·5	53·3	97·8	99·3
3*B*	53·7	54·2	53·8	101·0	100·1
4*A*	63·7	61·9	61·9	97·2	97·2
4*B*	63·7	63·7	63·7	100·0	100·0
		Average recovery		99·6	99·6

Table 2. *Reproducibility of enzyme activity determinations under standard conditions*

For details of the estimation and definition of enzyme activity see text. Standard deviation of individual determination, 1·4%.

Culture sample	Enzyme activity (units (*T*)/ml.)		
A	294	289	289
B	187	188	195
C	564	552	554
D	952	962	953
E	277	276	276
F	366	365	365

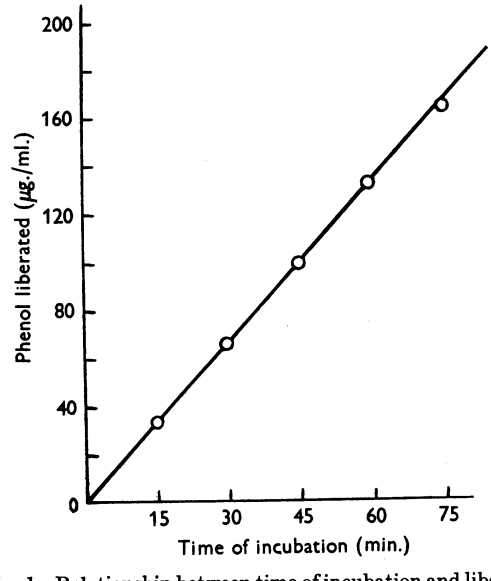

Fig. 1. Relationship between time of incubation and liberation of phenol from α-phenylmannoside by whole cultures of *Streptomyces griseus*. For conditions see text.

Table 3. *The effect of substrate concentration on enzyme activity*

10 ml. portions of culture broth were incubated with varying amounts of α-phenylmannoside in 1 ml. water. For further details see text.

Substrate concentration (μg./ml.)	Phenol liberated (μg./ml.) In experiment	Phenol liberated (μg./ml.) Theoretically possible
136	14·2	50·0
55	13·7	20·0
27	10·3	10·0
11	4·4	4·0

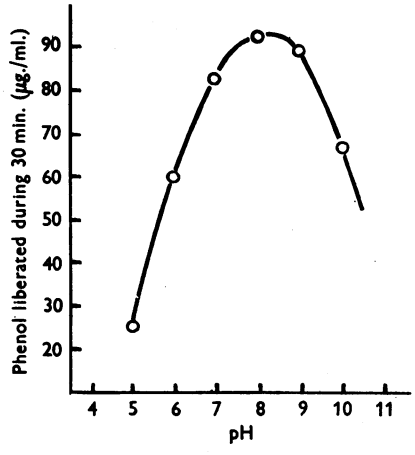

Fig. 2. The effect of pH on the hydrolysis of α-phenylmannoside by whole cultures of *Streptomyces griseus*. For conditions see text.

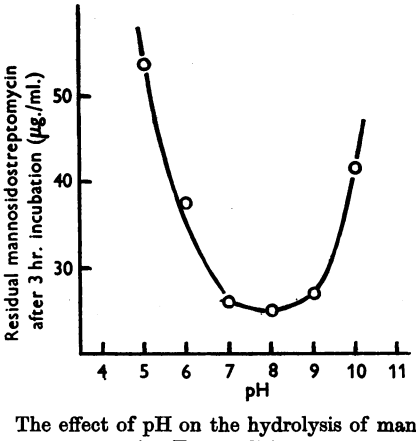

Fig. 3. The effect of pH on the hydrolysis of mannosidostreptomycin. For conditions see text.

period and the liberated phenol gives no indication of the reaction velocities. In order to measure the initial velocities, shorter incubation periods would have to be chosen in such a way that the liberated phenol could be measured before total hydrolysis occurs, but the amount of liberated phenol would then be too small for accurate estimation. All that can be deduced from the present experiments is that a Michaelis constant in excess of 10 μg./ml., i.e. approx. 4×10^{-5} M, would not be consistent with the experimental findings.

Effect of hydrogen-ion concentration

Samples of a standard 6-day culture were adjusted to various pH values with N-HCl or N-NaOH and brought to equal volumes by small compensating additions of water. The activity (units (*S*)/ml.) was determined for each under otherwise standard conditions. Fig. 2 indicates that optimum activity was shown at about pH 8·0, but that there was less than a 10 % fall from this value at either pH 7·0 or 9·0. This showed that, as active cultures without exception had pH values between 7·0 and 9·0, there was only need to adjust the pH value of the enzyme system in the most exacting experiments. As a rule, therefore, this was not done. Beyond these limits activity declined rapidly to 30 % of maximum at pH 5·0 and 70 % of maximum at pH 10·0.

A similar experiment with mannosidostreptomycin as substrate (Fig. 3) indicated that the optimum of the culture for cleavage of this substrate was again about pH 8. In this experiment 15 ml. portions of culture broth (containing a negligible amount of mannosidostreptomycin) were adjusted to various pH levels as in the above experiment. Solid mannosidostreptomycin hydrochloride was added to give a concentration of 100 μg./ml. The mixtures were incubated with shaking for 3 hr. (trial experiments had shown that the cleavage of mannosidostreptomycin was much slower than that of α-phenylmannoside). Residual mannosidostreptomycin was then estimated by the method of Emery & Walker (1949).

Effect of aeration upon enzyme activity

Enzyme—substrate mixtures were made as for the standard shake-flask test, but were distributed in various quantities in smaller (100 ml.) conical flasks, in order to vary the degree of aeration. Incubation with shaking of 15, 45 and 75 ml. portions of digest for 15 min. gave enzyme activities of 740, 600 and 220 units (*S*)/ml., respectively.

A more precise experiment was carried out under the conditions of the tube test, which was set up in the standard way except that the rate of passage of air was varied (Fig. 4). It is clear that the rate of aeration must be fixed; for the standard test, as mentioned above, it was set at 700–800 ml./min. The apparent decrease in enzyme activity with very high aeration is due to loss of phenol from the incubation unit, as was proved by passing the exit air through alkali and detecting phenol in the alkali.

Inhibition by anaerobiosis was reversible, shown by incubating broth anaerobically in presence of substrate and subsequently under aerobic conditions. Three 10 ml. portions of an enzyme-substrate digest were treated in the following way. The first was incubated anaerobically for 30 min., the second aerobically for 30 min. and the third portion aerobically and then anaerobically for the same length of time; 12, 49 and 56 μg./ml. phenol were liberated in the respective digests. This experiment was repeated and the same effect obtained after 16 hr. of anaerobiosis.

A similar dependence on aeration has been found when mannosidostreptomycin was used as substrate. In a typical experiment 20, 30, 40 and 50 ml. of culture broth to which 620 μg./ml. mannosidostreptomycin had been added were placed into 50 ml. conical flasks, which were then plugged with cotton wool. A fifth flask was filled completely with a similar mixture and closed with a rubber bung. Mannosidostreptomycin was determined after incubating with shaking for 3 hr.; 90, 85, 100, 320 and 550 μg./ml. of mannosidostreptomycin were found in the respective flasks.

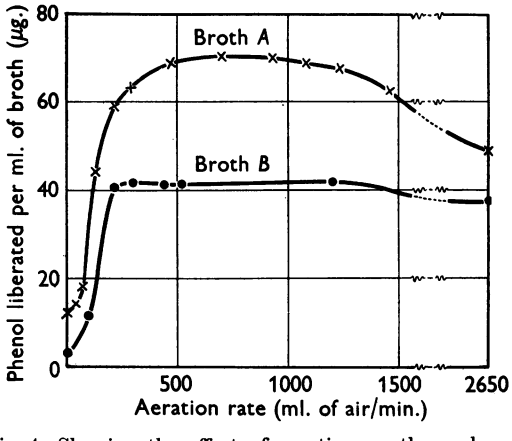

Fig. 4. Showing the effect of aeration on the α-phenylmannosidase activities of two broths (*A* and *B*) of *Streptomyces griseus*. For conditions see text.

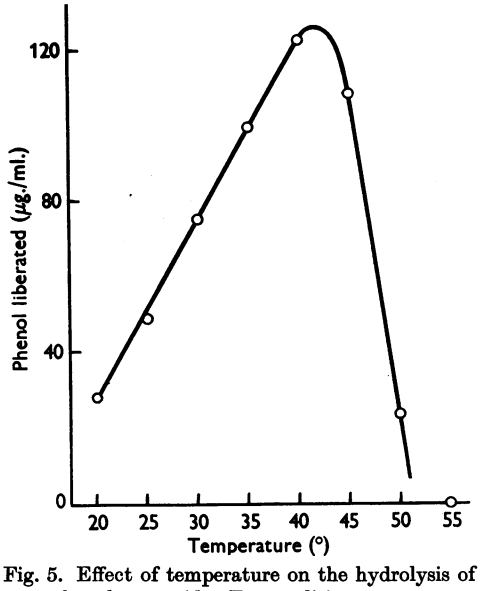

Fig. 5. Effect of temperature on the hydrolysis of α-phenylmannoside. For conditions see text.

Effect of temperature upon enzyme action

Under standard shaking conditions the optimum temperature for enzyme action was found to be about 40°. The enzyme appears to be very heat labile and is completely inactivated at 55° (Fig. 5).

The effect of phosphate on enzyme activity

In order to decide whether the cleavage of α-phenylmannoside was hydrolytic or phosphorolytic, incubation was carried out on the shaker in the presence of 0·02 M sodium phosphate of the same pH as the broth. To the control broth the same amount of sodium (as sodium chloride) was added, to eliminate any effect due to sodium. The results are shown in Fig. 6. A similar experiment with 0·05 M phosphate again showed that phosphate did not increase the rate of hydrolysis; it is therefore unlikely that α-mannoside is split phosphorolytically. (The concentration of inorganic phosphate of a normal 6-day old culture filtrate at the time of determination of enzyme activity was about 0·0003 M.)

Effect of inhibitors

Inhibition due to sugars. A number of sugars were added to enzyme preparations at 0·05 M concentration and their effect on the liberation of phenol from α-phenylmannoside over a period of 30 min. (with shaking) was ascertained. The results from a number of experiments on different broths are shown in Table 4.

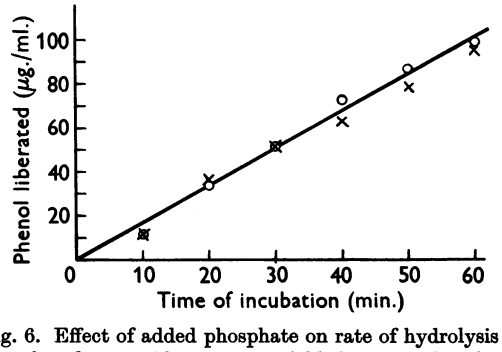

Fig. 6. Effect of added phosphate on rate of hydrolysis of α-phenylmannoside. ×—×, Added 0·02 M phosphate; ○—○, control. For conditions see text.

Effect of metal ions. The enzyme activity was reduced only by relatively high concentrations of Cu^{2+} and Fe^{2+} (as sulphates). In a number of culture broths these metals at a concentration of 0·001 M had no effect on enzyme activity, whereas a concentration of 0·01 M invariably produced strong enzyme inhibition. In some experiments, however, there was also some inhibition with 0·001 M metal salts. K^+, Mg^{2+}, Ca^{2+} and Ba^{2+} (all 0·01 M) had no effect on enzyme activity. The enzyme activities in these experiments were determined after incubation on the shaker.

Effect of sulphite, iodoacetate, arsenate and cyanide. Solutions of the sodium salts of these substances, adjusted to the pH of the culture broth with sodium hydroxide or hydrochloric acid, were added to give a concentration of 0·01 M. Incubation with shaking for 30 min. in the presence of substrate showed that sulphite and cyanide caused 62 and 90 % inhibition, respectively, arsenate stimulated enzyme activity by 40 % and iodoacetate had no effect. (These figures represent average values from several experiments.)

Table 4. *Effect of carbohydrates on enzyme activity*

Duplicate figures for the inhibition by individual sugars were obtained from separate digests, not from samples of the same digest. For other details see text.

Sugar (0·05 M)	Approx. % inhibition
Glucose	5, 2, 7
Fructose	7, 7, 0
D-Arabinose	7, 13
D-Ribose	2, 6
D-Xylose	11
Rhamnose	0, 0
Sucrose	13, 3, 2, 12
Mannitol	4, 0
α-Methylglucoside	9, 20, 12
Maltose	66, 81
Cellobiose	80, 84
Mannose	87, 87, 80, 79
α-Methylmannoside	98, 93, 96, 96

Correlation of mannosidostreptomycin level with appearance of phenyl α-D-mannosidase activity

In a number of experimental cultures the amount of mannosidostreptomycin was determined by the method of Emery & Walker (1949) and compared with the α-mannosidase activity over a number of days. It was found that high mannosidase activity was paralleled by a low level of mannosidostreptomycin. When there was no fall in mannosidostreptomycin level, little or no enzyme activity was manifest (Table 5).

DISCUSSION

The sensitivity of the enzyme to aeration conditions is noteworthy. A similar phenomenon for hydrolytic enzymes was described recently (Yamagita & Nishi, 1952), when it was shown that proteolytic hydrolysis by trypsin or chymotrypsin and the autocatalytic conversion of trypsinogen into trypsin were inhibited under anaerobic conditions. Since cell lysis starts soon after the disappearance of glucose from the medium and is followed by the appearance of α-mannosidase activity, it would appear possible that the enzyme might be intracellularly present earlier in the fermentation, and that lysis might activate it by bringing it into contact with a higher oxygen tension.

The affinity of the enzyme for α-phenylmannoside was found to be higher than for its natural substrate mannosidostreptomycin. The Michaelis constant for the latter has been found in our laboratories to be $1·72 \times 10^{-8}$ M (P. B. Dickenson & J. S. Hughes, personal communication). Owing to the difficulty of determining phenol at the necessary low levels, we were not able to estimate the Michaelis constant for the synthetic substrate. It seems, however, to be appreciably lower than that found for the natural substrate. Moreover, the rate of hydrolysis of mannosidostreptomycin was only

Table 5. *Relation between mannosidostreptomycin content and α-phenylmannosidase activity of cultures*

%*M*, mannosidostreptomycin as molar % of total streptomycin. *E*, α-phenylmannosidase activity (units (S)/ml.; in *A*, *B*, *C*, *D* and units (T)/ml.; in *E*, *F*, *G*, *H*, *I*).

	Duration of fermentation									
	Day 3		Day 4		Day 5		Day 6		Day 7	
Culture sample	%*M*	*E*	%*M*	*E*	%*M*	*E*	%*M*	*E*	%*M*	*E*
A	—	—	46	0	50	0	60	40	—	0
B	—	—	39	0	8	1490	6	940	5	600
C	51	0	49	0	46	54	36	20	—	—
D	34	0	41	0	5	1930	8	1750	6	1120
E	—	—	—	—	33	0	32	0	29	0
F	31	0	33	35	43	45	29	175	—	—
G	31	0	30	35	25	205	3	995	—	—
H	56	0	32	245	8	1605	7	1235	—	—
I	40	0	43	0	19	905	7	340	—	—

about one-sixth of that of α-phenylmannoside. A similar finding was made by Cohn & Monod (1951), who showed that the affinity of an *Escherichia coli* β-galactosidase for synthetic *o*-nitrophenyl β-galactoside was greater than for lactose.

When breakdown to streptomycin occurs, the pH of a culture broth is usually just on the alkaline side, i.e. at the optimum pH for the action of the enzyme on either substrate. This optimum is in marked contrast to the optimum pH for the action of the α-mannosidases of almond emulsin and lucerne seeds (pH 3·3–5·5; Hill, 1934) or coffee emulsin (pH 4·5–5·5; Helferich & Vorsatz, 1935).

In view of the lack of effect of inorganic phosphate on the rate of hydrolysis of phenyl α-D-mannoside, which implies non-phosphorolytic breakdown, it is strange that arsenate should so markedly increase the reaction velocity.

Only four of the sugars tested, mannose, α-methylmannoside, maltose and cellobiose, were inhibitory. The first two probably acted as competitive inhibitors. It is not clear why the last two, an α-glucoside and a β-glucoside, should inhibit. It is possible that an irreversible enzyme-glucoside complex is formed. α-Phenylglucoside was tried as an enzyme substrate but was not hydrolysed.

The inhibition of the enzyme by the heavy metals copper and iron was presumably of a general nature. Although some changes in the activity of similar microbial glucosidases have been reported with K^+, Ca^{2+}, Mg^{2+} and Ba^{2+}, such an effect was not demonstrated on our enzyme. The failure to observe it may have been due to the use of a cell suspension instead of a purified enzyme.

Of general inhibitors, only sulphite, cyanide and iodoacetate were examined. The two former inhibited strongly, whereas iodoacetate had no effect. It appears, therefore, that the enzymic action of mannosidostreptomycinase is unlikely to depend on the presence of SH groups.

The late appearance in normal cultures of an enzyme releasing mannose from mannosidostreptomycin (mannose, as the phenylhydrazone, was isolated from such cultures by Perlman & Langlykke, 1948) makes it likely that the latter is a carbohydrate storage material for the organism (cf. Frey-Wyssling, 1942) and that release of carbohydrate is brought about when the glucose is depleted. The action of the mould on phenyl α-D-mannoside parallels this and would tend to support the hypothesis that cleavage of both substrates is effected by the same enzyme. The similarity of the pH activity curves found with the two substrates, the dependence of enzyme action for each substrate on aeration and the close correlation between mannosidostreptomycin level and α-mannosidase activity all suggest such an identity.

SUMMARY

1. It has been demonstrated that cultures of *Streptomyces griseus* possess an enzyme capable of liberating phenol from phenyl α-D-mannoside.

2. The optimum pH for the activity of the enzyme is approximately 8·0.

3. The enzyme is very sensitive to conditions of aeration. Decreased aeration causes a marked decrease of enzyme activity.

4. Mannose, methyl α-D-mannoside, maltose and cellobiose strongly inhibit the enzyme. A number of other sugars have little or no effect.

5. Ferrous and cupric ions have some inhibitory effect on the enzyme. K^+, Ca^{2+}, Mg^{2+} and Ba^{2+} have no effect.

6. The rate of enzymic cleavage of substrate is not influenced by inorganic phosphate. Arsenate increases the rate.

7. Cyanide and sulphite inhibit the enzyme, iodoacetate has no effect.

8. The Michaelis constant of the enzyme with respect to phenyl α-D-mannoside is less than 10^{-4} M.

9. It is probable that the same enzyme is responsible for the conversion of mannosidostreptomycin into streptomycin and the hydrolysis of phenyl α-D-mannoside.

The authors wish to acknowledge the valuable technical assistance of Mr I. McCalla, Mr A. Rees and Miss P. Jackson.

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