583. *The Effect of Streptomycin on the Enzymic Synthesis and Degradation of Carbohydrates.*

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Streptomycin has been found to decrease the production of fructose from glucose 1-phosphate by *E. coli* and from sucrose by a cell-free extract of *A. niger*. The actions of a transglucosylase and a transgalactosylase of *A. niger* were also retarded. Other effects of streptomycin are also reported.

In a preliminary communication [1] we summarised the effect of streptomycin on several enzymes concerned with the synthesis and degradation of carbohydrates. We now report more fully the quantitative investigations of these and other enzyme systems.

In a study of resting cells of *Escherichia coli* (Gratia strain), Umbreit [2] showed that the terminal respiration process apparently involved a pyruvate–oxaloacetate condensation and he believed that it was close to this reaction that streptomycin exerted its activity. Later [3] he showed that formation of 4-carboxy-4-hydroxy-2-phosphoadipic acid, a product from the oxaloacetate–pyruvate condensation, is markedly inhibited by streptomycin. Using an enzyme extract from *E. coli* (Monod strain) we have shown (Table 1) that streptomycin also affects the metabolic process involving the ultimate production of fructose from glucose 1-phosphate (and maltose). This conversion is believed [4] to involve phosphoglucomutase, phosphohexoisomerase, etc. Streptomycin did not appear to have an appreciable effect on *E. coli* amylomaltase, the enzyme producing the homologous α-1 : 4-linked glucosaccharides and glucose from maltose.

Michalska [5] reported that streptomycin (up to 5000 μg./c.c.) had no effect on the amount of citric acid produced by four strains of *Aspergillus niger* grown on a synthetic medium containing sucrose, and did not affect their growth. Although the yield of mycelium in the presence and absence of streptomycin was not profoundly affected (Table 4), we found that the nigeran content of *A. niger* " 152 " mycelia was greatly increased when grown in the presence of streptomycin. Subculturing in the presence of streptomycin (15%) initially increased the ability of *A. niger* " 152 " to produce nigeran (after 7 subcultures) but 5 months later, after a further 4 subcultures in the presence of

Table 1. *Effect of streptomycin on fructose production by an* E. coli *enzyme extract.*

		Monosaccharides in digest (5 c.c.)		
Substrate	Streptomycin (%)	Total reducing monosaccharides (mg.)	Fructose (mg.)	Fructose (as % of total monosaccharide)
Maltose	15	144	12	8
,,	15	149	9·5	7
,,	1	215	42	20
,,	0·1	217	41	19
,,	Nil	196	43	22
,,	Nil	206	48	23
G-1-P	15	142	40	28
,,	1	121	49	40
,,	Nil	113	53	47

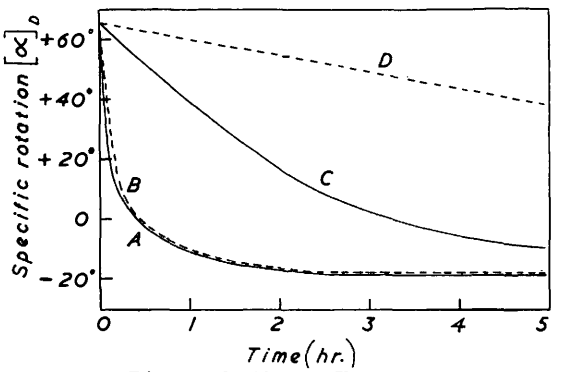
streptomycin, little or no nigeran could be detected when the mould was grown on sucrose in the absence of streptomycin. Under these conditions of very low nigeran production, a starch-like polysaccharide could be extracted from the mycelium. The starch character was shown by acid hydrolysis to glucose, its characteristic iodine absorption curve, and its

[1] Barker, Bourne, Stacey, and Ward, *Nature*, 1955, **175**, 203.
[2] Umbreit, *J. Biol. Chem.*, 1949, **177**, 703.
[3] *Idem*, *J. Bact.*, 1953, **66**, 74.
[4] Doudoroff, Hassid, Putman, Potter, and Lederberg, *J. Biol. Chem.*, 1949, **179**, 921.
[5] Michalska, *Med. Dóswiadczalna i Mikrobiol.*, 1953, **5**, 113.

susceptibility to attack by α- and β-amylase. A similar polysaccharide was detected when *A. niger* " 152 " was grown on a zinc-deficient medium (Barker and Carrington [6]).

Several of the enzymes isolated from *A. niger* " 152 " were affected by streptomycin. The transfructosylase normally acts on sucrose to give trisaccharides [mainly *O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(1 → 2) β-D-fructofuranoside], glucose, and fructose.[7] The trisaccharide formation appeared virtually unaffected but the ratio of fructose to glucose was markedly reduced (Table 2) in the presence of streptomycin. Commercial yeast invertase normally acts on sucrose to give trisaccharides [mainly *O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(6 → 2) β-D-fructofuranoside], a disaccharide, glucose, and fructose. Streptomycin reduced the formation of the disaccharide and one of the trisaccharides but affected the production of glucose and fructose less than with *A. niger* transfructosylase. The effect was demonstrated polarimetrically (Figure).

Action of streptomycin on sucrose inversions of commercial invertase.



Digest vol., 10 c.c. Temp., 29°.

Digest	Sucrose (g.)	Streptomycin (g.)	Invertase concentrate (c.c.)
A	0·33	0	0·33
B	0·33	0·10	0·33
C	1·50	0	0·01
D	1·50	1·0	0·01

Values of $[\alpha]_D$ were calculated from the original concentration of sucrose and corrected, where necessary, for the presence of streptomycin.

The ability of a cell-free extract of *A. niger* " 152 " to synthesise β-1 : 2, β-1 : 3, β-1 : 6, etc., linkages from cellobiose [8] by transglucosylation was not seriously impaired by the presence of streptomycin (15%). Bitter-almond emulsin produced glucose, gentiobiose,

TABLE 2. *Effect of streptomycin on* A. niger *transfructosylase.*

	Monosaccharides produced in digest (5 c.c.)		
Streptomycin (%)	Total reducing monosaccharides (mg.)	Fructose (mg.)	Fructose (as % of total monosaccharide)
15	27	<1·5	<5
15	26·5	<1·5	<5
1	45	6	13
0·1	70	9	13
0·01	88	19	22
0·001	102	20	20
Nil	91	22	24

laminaribiose, etc., from cellobiose in a similar manner, and in this case the laminaribiose formation was selectively retarded in the presence of streptomycin (15%).

[6] Barker and Carrington, *J.*, 1953, 3588.
[7] Barker, Bourne, and Carrington, *J.*, 1954, 2125.
[8] Barker, Bourne, and Stacey, *Chem. and Ind.*, 1953, 1287.

Other enzymes isolated from *A. niger* " 152," the actions of which were retarded by streptomycin, were the transglucosylase which produced panose, isomaltose, and glucose from maltose, and the transgalactosylase which produced oligosaccharides from lactose. Dextransucrase from *Betacoccus arabinosaceous* (Birmingham strain) synthesised only slightly less dextran in the presence of streptomycin (1% or 15%) than in its absence. Both human salivary α-amylase and soya-bean β-amylase were only slightly affected by streptomycin (1% or 15%). Important systems affected little, if at all, were the actions of potato phosphorylase and calf intestinal phosphatase on glucose 1-phosphate, the action of calf intestinal phosphatase on α-glycerophosphate and the action of pea aldolase on fructose 1 : 6-diphosphate.

Specific inhibition of enzymes by streptomycin could be explained in a variety of ways (*e.g.*, as an antimetabolite or a chemical poison) and one possibility is that it removes essential metabolites or co-enzymes by complex formation. We have shown, for example, that streptomycin sulphate precipitates heparin, chondroitin sulphate, and dextran sulphate from solutions of low ionic strength. It has also been reported that streptomycin will precipitate nucleic acids under similar conditions (Korzybski and Kurylowicz [9]). It is of interest that the concentrations of streptomycin required to produce marked effects on the isolated enzyme systems described here are appreciably greater than those in which it is employed as an antibiotic, but in the latter case higher concentrations could well be reached in localised regions of the micro-organisms although it is more likely that the antibiotic activity is exerted through enzyme systems other than those reported here.

Streptomycin is closely related in structure to a trisaccharide and might be expected to serve as a receptor molecule for suitable transglycosylases, to yield glycosyl-streptomycins. Indeed the naturally occurring mannosyl-streptomycin probably arises from such a process. It was hoped that the above studies would provide routes to new analogues of mannosyl-streptomycin, but none of the transglycosylases examined was able to utilise streptomycin as a receptor.

Experimental

Streptomycin.—The antibiotic was used as sulphate (from Messrs. Glaxo), of the medicinal grade, and appeared pure on ionophoresis and paper chromatography. Its isolation had included preparation of and recovery from the calcium chloride complex, and use of this complex in place of the sulphate gave identical results in our work (calcium chloride itself had no such effects).

General Methods.—(a) *Paper chromatography.* Sugars were separated on paper by using the organic phase of a butanol–ethanol–water–ammonia mixture (40 : 10 : 49 : 1) and detected with aniline hydrogen phthalate [10] or naphtharesorcinol.[11] Alternatively the sugars were separated as their benzylamine derivatives and detected with ninhydrin.[12]

(b) *Paper electrophoresis.* Streptomycin, or products derived therefrom, was separated by paper electrophoresis in 0·2N-acetate buffer, pH 5·0 at 15 v/cm. for 4 hr. Papers were developed severally with aniline hydrogen phthalate,[10] naphtharesorcinol,[11] a modified Elson–Morgan reagent, and alkaline diacetyl-α-naphthol.[13]

(c) *Quantitative separation and determination of reducing monosaccharides from enzyme digests.* Corbett's method [14] was adapted to separate 10—15 mg. of sugars. The columns (length, 6 cm.; diam. 1·5 cm.) were prepared from equal volumes of charcoal and " Celite 545 " which had been washed with concentrated hydrochloric acid, water, and ethanol, dialysed, and then left in a partial vacuum for 2 hr. to remove dissolved gases. This removal of gases was effected on all the liquids passing into the columns. Each column was packed as an aqueous slurry and suction applied at the base of the column to maintain the rate of elution at *ca.* 1 c.c./min. An aqueous solution (10 c.c.) of the components to be separated was passed into the column which was then washed with water. The monosaccharides, eluted in the first

[9] Korzybski and Kurylowicz, *Med. Dóswiadczalna i Mikrobiol.*, 1953, **5**, 378.
[10] Partridge, *Nature*, 1949, **164**, 443.
[11] Forsyth, *ibid.*, 1948, **161**, 239.
[12] Bayly and Bourne, *ibid.*, 1953, **171**, 385.
[13] Foster and Ashton, *ibid.*, **172**, 958.
[14] Corbett, *Chem. and Ind.*, 1953, 1285.

100 c.c., were concentrated by freeze-drying in the presence of 0·2M-acetate buffer, pH 7·0 (1 c.c.) to prevent mechanical losses during the final stages of freeze-drying. The characteristics of the column were found by elution of solutions containing known amounts of glucose and fructose in the presence of maltose, sucrose, glucose 1-phosphate, and streptomycin A sulphate (Table 3).

The total reducing sugars in the monosaccharide fraction were determined by Shaffer and Hartmann's method.[15] The fructose was estimated in the same manner after oxidation of reducing aldoses by alkaline iodine.[16] The glucose was measured by the difference in these two amounts.

Effect of Streptomycin on Various Enzymes.—(a) *Amylomaltase of* E. coli. *E. coli* (Monod strain) was grown, collected, and freeze-dried (Monod and Torriani,[17] Barker and Bourne [18]). The cells (1·5 g.) were shaken at <20° in water with glass beads for 45 min. in a Mickle tissue disintegrator. The suspension was diluted to 90 c.c., and centrifuged at 5000 r.p.m. to eliminate cell debris. Staining confirmed the absence of whole cells. Enzyme extract (5 c.c.) and maltose (0·75 g.) were incorporated in a digest adjusted to pH 7, incubated at 30°, and analysed by paper chromatography. A homologous series of oligosaccharides (maltotriose, maltotetraose, etc.) was formed together with glucose and fructose. A similar digest containing added streptomycin A sulphate (0·75 g.) produced the amylosaccharides and glucose as before, but the amount of fructose was markedly reduced.

Analysis of a similar digest containing glucose (0·75 g.) in place of maltose, showed glucose only. When dipotassium α-D-glucose 1-phosphate dihydrate (0·5 g.) replaced maltose, both glucose and fructose were produced, but no oligosaccharides were formed. The presence of streptomycin (0·75 g.) again caused a marked reduction in the amount of fructose formed.

The monosaccharides formed in the above digests were determined quantitatively on charcoal columns. The results of the analysis of 0·1 c.c. portions of the digest, removed in

TABLE 3. *Calibration of charcoal columns.*

Analysis mixture			Sugars estimated	
Glucose (mg.)	Fructose (mg.)	Other components	Glucose (mg.)	Fructose (mg.)
3	3	Sucrose, 3 mg. + Streptomycin, 9 mg.	3·12	2·99
3	3	Sucrose, 9 mg. + Streptomycin, 9 mg.	3·10	2·98
3	3	Maltose, 3 mg. + Streptomycin, 9 mg.	2·83	3·13
3	3	Maltose, 9 mg. + Streptomycin, 9 mg.	2·97	2·97
3	3	Glucose 1-phosphate, 6 mg. + Streptomycin, 9 mg.	2·97	3·02

10 c.c. of solution by serial dilution, are shown in Table 1. Electrophoretic examination of all digests did not reveal any modification of the streptomycin.

(b) *Nigeran-producing system of* Aspergillus niger "152." The mould was grown for 7 days at 30° on a series of synthetic media [6] containing sucrose (10%) and various amounts (0—10%) of streptomycin A sulphate. The mycelia were washed, shredded, freeze-dried, and weighed. Each mycelium was extracted four times with boiling water (100 c.c.) for 30 min. and the nigeran obtained by centrifuging the cold extracts. The purity of each fraction was checked by (i) its infrared spectrum,[19] (ii) the product of total hydrolysis, and (iii) its specific rotation.

The experiments were carried out with two types of *A. niger* "152": type I, the normal type, which was subcultured on sucrose–agar at intervals of 1 month; and type II, an abnormal type which was subcultured on sucrose–streptomycin–agar at intervals of 1 month. The results are given in Table 4. In all cases where the yield of nigeran was very low, a soluble starch-type polysaccharide could be detected in the aqueous extracts. It was stained blue with iodine, was attacked by α- and β-amylase, and gave glucose on acid hydrolysis.

[15] Shaffer and Hartmann, *J. Biol. Chem.*, 1920, **45**, 365.
[16] Van der Plank, *Biochem. J.*, 1936, **30**, 457.
[17] Monod and Torriani, *Compt. rend.*, 1948, **227**, 240.
[18] Barker and Bourne, *J.*, 1952, 209.
[19] Barker, Bourne, Stacey, and Whiffen, *ibid.*, 1954, 171.

TABLE 4. *The effect of streptomycin on nigeran synthesis.*

Streptomycin absent			Streptomycin present		
Culture type	Mycelium (as % of sucrose)	Nigeran (as % of mycelium)	Concn. (%)	Mycelium (as % of sucrose)	Nigeran (as % of mycelium)
I	35·3	0·6	10·0	34·5	4·9
II	30·2	5·1	10·0	29·7	16·9
5 months later					
I	26·3	0·6	10·0	33·0	2·8
			1·0	45·6	1·0
			0·1	35·7	0·5
II	58·2	<0·1	10·0	32·7	2·4
			1·0	55·3	1·7
			0·1	31·2	0·2

(c) *Transfructosylase and invertase system(s) of* A. niger " 152 ". The mould was grown on a synthetic medium containing sucrose,[6] washed, shredded, and freeze-dried. A cell-free extract was prepared, as in (*a*) above, from 3 g. of cells and diluted to 100 c.c. Digests containing this enzyme extract (5 c.c.) and sucrose (0·75 g.) were adjusted to pH 7 with sodium hydroxide and incubated at 30°. Paper chromatography showed the formation of glucose, fructose, and two trisaccharides. In a similar digest containing streptomycin A sulphate (0·75 g.), the formation of fructose was markedly reduced while that of glucose and trisaccharide was less affected. Correspondingly smaller effects were observed in the presence of amounts of streptomycin down to 0·005 g.

Quantitative measurements of the monosaccharides (Table 2) were made with the small-column technique described above. Similar digests were examined containing glucose (0·75 g.) or fructose (0·75 g.) in place of sucrose. No detectable change occurred in either the presence or the absence of streptomycin. Electrophoretic examination of all the digests failed to show any modification of the streptomycin molecule.

(d) *Other enzyme systems affected by streptomycin.* (i) The transglucosylase of *Aspergillus niger* " 152 ". Portions (5 c.c.) of a cell-free extract of *A. niger* " 152 ", grown on sucrose, were incubated with maltose (0·75 g.) at 30° in the presence of various quantities of streptomycin A sulphate (0 to 0·75 g.), the pH being adjusted in each case to 6·5—7·0 with sodium hydroxide. In the absence of streptomycin, chromatography showed the formation of panose, isomaltose, and glucose. In the presence of streptomycin (>0·005 g., 0·1%), production of these three sugars was slower. No modification of the streptomycin could be detected.

(ii) Commercial yeast invertase concentrate (B.D.H.). Two digests (pH 6·5—7·0) were prepared containing sucrose (0·75 g.), diluted invertase concentrate (5 c.c., 0·1% v/v concentrate in water), and, in one digest, streptomycin A sulphate (0·75 g.). After incubation at 25°, chromatography showed the formation of fructose, glucose, a disaccharide, and two trisaccharides in the absence of streptomycin. In the second digest the disaccharide and one of the trisaccharides could not be detected. Electrophoresis of the digests did not show any modification of the streptomycin.

Polarimetric examination of the reaction occurring in two solutions (10 c.c.) containing enzyme concentrate (0·01 c.c.), sucrose (1·5 g.), and, in one case, streptomycin A sulphate (1·0 g.) is shown in the Figure. Another pair of digests (10 c.c.) containing enzyme concentrate (0·33 c.c.), sucrose (0·33 g.), and, in one case, streptomycin (0·1 g.) were also examined (Figure). The reactions were carried out at 29°.

(iii) Transgalactosylase from *A. niger* " 152 ". The mould was grown on a synthetic medium containing lactose (10%) as the sole carbon source, the mycelium obtained was washed, shredded, and freeze-dried, and a cell-free extract isolated as previously described. Two digests (pH 6·5—7·0) were prepared containing lactose (0·75 g.), cell-free extract (5 c.c.), and, in one case, streptomycin A sulphate (0·75 g.) and incubated at 30°. Chromatography showed the formation of a trisaccharide, galactose, and glucose in the streptomycin-free digest, while in the presence of streptomycin no sugars other than lactose were detectable. Electrophoresis showed that the streptomycin had not been modified.

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