statistically different from the corresponding values obtained with normal mice.

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Actinomycete Metabolism

INCORPORATION OF ¹⁴C-LABELLED COMPOUNDS INTO STREPTOMYCIN

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Recent work (Numerof, Gordon, Virgona & O'Brien, 1954) has indicated that streptomycin can be elaborated by *Streptomyces griseus* with D-glucose as sole carbon source. The radioactive labels of added [¹⁴C]glycine and acetate were significantly incorporated only in the guanidine side chains. As these carbon atoms arise largely, if not entirely, from carbon dioxide (Hunter, Herbert & Hockenhull, 1954), atoms from the substrate are presumably incorporated only after these have been completely metabolized by the organism. Karow, Peck,

Rosenblum & Woodbury (1952) have demonstrated the incorporation of the labelled carbon of [¹⁴C]-glucose into streptomycin. They did not, however, submit their product to chemical degradation to determine the distribution of the radioactivity within the molecule. Their results have been considerably extended in our work, and we have shown that glucose is incorporated equally into all three monosaccharide residues in streptomycin. In an endeavour to discover some of the intermediate pathways involved in this process, a study has also been made of the incorporation of [¹⁴C]streptamine and N-methyl-L-glucosamine into the antibiotic by *Strep. griseus*.

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METHODS

The organism, *Streptomyces griseus*, *albus mutant* (Dulaney Z. 38), was grown on a soybean medium as described previously (Hockenhull, Fantes, Herbert & Whitehead, 1954; Hunter *et al.* 1954). Unless otherwise stated, cultures were harvested 7 days after inoculation. The techniques used in the isolation of crystalline salts of streptomycin from the broth, and in assaying radioactive products, were described in the latter paper.

Chemical degradation of streptomycin. Under acid conditions such as those specified below, streptomycin is decomposed; the glycoside link binding streptidine to the rest of the molecule is broken. The residual streptobiosamine derivative contains streptose joined by a glycosidic linkage to *N*-methyl-L-glucosamine. Vigorous acid hydrolysis of this material leads to extensive decomposition, and although some *N*-methyl-L-glucosamine may be isolated the streptose is completely destroyed.

Streptomycin sulphate was dissolved in the minimum quantity of $N-H_2SO_4$ and allowed to stand for 2 days at 37°. An equal volume of methanol was added, and the mixture allowed to stand for a further day, when addition of 1 vol. acetone completed the precipitation of streptidine sulphate. After a few hours at 0°, the colourless crystals were filtered off and recrystallized from a small volume of water. The subsequent degradation of the streptidine sulphate was by the method of Hunter *et al.* (1954).

N-Methyl-L-glucosamine hydrochloride was isolated from the mother liquors by removing first the organic solvents *in vacuo* and then the sulphuric acid by passage down a column of weakly basic anion-exchange resin in the OH^- form (Amberlite IR-4B). After evaporating the eluate and aqueous washings from the column almost to dryness *in vacuo* (or freeze-drying), hydrolysis of the methyl streptobiosaminide dimethyl acetal to *N*-methyl-L-glucosamine was effected by refluxing for 2 hr. with conc. HCl. The hydrolysate was filtered, evaporated to dryness *in vacuo* and then converted into the pentaacetyl derivative, as described for the analogous D compound (Kuehl, Flynn, Holly, Mzingo & Folkers, 1947). The crude acetyl derivative was purified by one crystallization from methanol and then by chromatography on acid-washed alumina (Peter Spence and Co. Widnes, Grade H) prepared by washing with dilute H_2SO_4 to pH 4.0–4.5 and then washing free from sulphate and drying overnight at 160° to reactivate. The substance was added in benzene solution and elution carried out with benzene-chloroform (1:1, v/v) after first washing out impurities with benzene. The solvent was removed from the eluate, whereupon the pentaacetyl-*N*-methyl-L-glucosamine formed beautiful colourless needles from chloroform-ether. The yield was 10–20%, based on streptomycin sulphate. The pentaacetyl derivative could be hydrolysed to *N*-methyl-L-glucosamine hydrochloride by refluxing for 1 hr. with 2.5 *N*-HCl, cautious evaporation *in vacuo* to dryness and crystallization from ethanol.

Materials. Uniformly labelled [^{14}C]glucose was supplied by the Radiochemical Centre, Amersham, Bucks. Subsequently we used material made in our laboratories from [^{14}C]starch which was prepared by the photosynthetic method of Porter & Martin (1952) using excised tobacco leaves (variety White Burley).

[^{14}C]Streptamine sulphate and [^{14}C]*N*-methyl-L-glucosamine hydrochloride were made by degrading [^{14}C]strepto-

mycin obtained biosynthetically from [^{14}C]glucose, as described below.

myo-Inosose was prepared from inositol by oxidation with *Acetobacter suboxydans* American Collection of Type Culture no. 621 (Posternák, 1952).

Seyllitol was made by sodium amalgam reduction of *myo*-inosose (Posternák, 1941).

RESULTS

Incorporation of [^{14}C]glucose into streptomycin.

Karow *et al.* (1952) obtained a 1% incorporation of [^{14}C]glucose into streptomycin by including the labelled sugar in the initial medium. We have found that the incorporation of isotope may be increased by 5% by delaying the addition of radioactive material until growth had proceeded for 60 hr., by which time the mycelium had almost attained its maximum dry weight. Although these experiments do not prove conclusively that the improved isotope yield is due principally to this delayed addition, the similarity of the cultural conditions is such as to render the assumption reasonable. In our experiment, 8.9×10^{-2} m-mole streptomycin with a specific activity of 29 μC /m-mole was produced after fermentation in a medium (40 ml.) to which 50 μC of D-[^{14}C]glucose (5.5×10^{-3} m-mole) was added after 60 hr. incubation. However, owing to losses during extraction, etc., only two-thirds of the streptomycin was isolated as the crystalline sulphate.

Degradation of the streptomycin revealed the distribution of the ^{14}C in the various parts of the molecule. Detailed results are shown in Table 1, where the radioactivity of the streptomycin not found in either the streptidine or in the *N*-methyl-L-glucosamine is assumed to have been present in the streptose moiety. It can be seen that, within experimental error, the radioactivity is equally distributed in all parts of the molecule with the exception of the carbons of the guanidine side chains.

Incorporation of N-methyl-L-[^{14}C]glucosamine into streptomycin. *Strep. griseus* was again allowed to grow for 60 hr. after inoculation on to the soybean medium (40 ml.). *N*-Methyl-L-[^{14}C]glucosamine hydrochloride (0.139 m-mole) with a specific activity of 23.8×10^{-2} μC /m-mole was now added, dissolved in a little water. Growth was allowed to continue until the normal harvest time, when it was found that 5.5×10^{-2} m-mole of streptomycin had been produced with a specific activity of 8.7×10^{-2} μC /m-mole. This represents a 15.5% incorporation of the radioactive label into the antibiotic molecule. Chemical degradation (see Table 2) showed that the labelling was principally located in the *N*-methyl-L-glucosamine part of the molecule, although there was a not inconsiderable amount of radioactivity also present in the streptidine moiety.

Incorporation of [^{14}C]streptamine into streptomycin. Streptamine sulphate (0.181 m-mole) with a specific activity of $20.9 \times 10^{-2} \mu\text{C}/\text{m-mole}$ was added at 60 hr., and at harvest 7.1×10^{-2} m-mole of streptomycin had been produced with a specific activity of $1.64 \times 10^{-2} \mu\text{C}/\text{m-mole}$. Here the incorporation of ^{14}C was only 3.9% , less than with glucose. Chemical degradation showed that the label was distributed throughout the molecule, although the largest amount was again found in the *N*-methyl-L-glucosamine portion.

Table 1. *Distribution of the radioactivity in [^{14}C]streptomycin obtained after growing Strep. griseus in a medium containing [^{14}C]glucose*

Organism grown in soybean medium; 60 hr. after inoculation 5.5 m-mole [^{14}C]glucose (sp. activity $9.1 \mu\text{C}/\text{m-mole}$) added; culture harvested 7 days after inoculation.

Substance	Specific activity	
	($\mu\text{C}/\text{m-mole}$)	($\mu\text{C}/\text{mg. carbon}$)
Streptomycin	29	11.6×10^{-2}
Streptidine	9.9	10.4×10^{-2}
Streptamine	8.8	12.3×10^{-2}
Barium carbonate (from guanidine carbons)	0.55	4.5×10^{-2}
<i>N</i> -Methyl-L-glucosamine	10.1	12.2×10^{-2}
Streptose (by difference)	9.2	12.8×10^{-2}

Table 2. *Distribution of the radioactivity in [^{14}C]streptomycin obtained after growing Strep. griseus in a medium containing *N*-methyl-L- ^{14}C glucosamine*

Organism grown in soybean medium; 60 hr. after inoculation 0.139 m-mole *N*-methyl-L- ^{14}C glucosamine (specific activity $23.8 \times 10^{-2} \mu\text{C}/\text{m-mole}$) added; cultures harvested 7 days after inoculation.

Substance	Specific activity ($\mu\text{C}/\text{m-mole}$)
Streptomycin	8.7×10^{-2}
Streptidine	1.42×10^{-2}
<i>N</i> -Methyl-L-glucosamine	6.4×10^{-2}
Streptose (by difference)	0.92×10^{-2}

Table 3. *Distribution of the radioactivity in [^{14}C]streptomycin obtained after growing Strep. griseus in a medium containing [^{14}C]streptamine*

Organism grown on soybean medium; 60 hr. after inoculation 0.181 m-mole [^{14}C]streptamine (specific activity $20.9 \times 10^{-2} \mu\text{C}/\text{m-mole}$) added; cultures harvested 7 days after inoculation.

Substance	Specific activity ($\mu\text{C}/\text{m-mole}$)
Streptomycin	1.64×10^{-2}
Streptidine	0.45×10^{-2}
<i>N</i> -Methyl-L-glucosamine	0.83×10^{-2}
Streptose (by difference)	0.36×10^{-2}

Table 4. *Depression of streptomycin yield by myo-inosose and its reversal by scyllitol*

Cultures grown 144 hr. in 250 ml. flasks (40 ml./flask) on rotary shakers (140 rev./min.) at 28.5° . Medium and inoculum according to Dulaney (1949). Streptomycin was determined as maltol (Boxer, Jelinek & Leghorn, 1947).

Expt.	Time of addition to medium (hr.)		Streptomycin titre (mean of four flasks) (units/ml.)
	<i>myo</i> -Inosose (1 mg./ml.)	Scyllitol (1 mg./ml.)	
1	*—	—	1470
1	48	—	1040
1	72	—	970
1	96	—	1290
2	—	—	1530
2	—	0	1670
2	—	24	1500
2	—	48	1420
2	—	72	1640
2	0	—	750
2	0	48	1550

* —; No addition.

Effect of myo-inosose and scyllitol on streptomycin formation

Supplementary experiments with the hexitol corresponding to streptamine, scyllitol, and with its parent ketone, *myo*-inosose, were carried out in an attempt to show that by their effect upon yield they might be intermediates in the biosynthesis.

myo-Inosose (1 mg./ml.), while having no apparent effect on the growth of the organism, had a consistent inhibitory effect on the yield of streptomycin. Table 4 shows the results of a typical experiment. It will be noted that *myo*-inosose had little effect when added at a late stage in the fermentation, but had the greatest effect at the time when streptomycin production was beginning. Several other experiments gave closely similar results. Scyllitol (1 mg./ml.), although it did not significantly increase streptomycin yield, appeared to reverse the inhibition caused by *myo*-inosose.

DISCUSSION

From the experiment involving [^{14}C]glucose, combined with the results of Numerof *et al.* (1954), it appears likely that glucose alone can provide *Strep. griseus* with all the carbon atoms it requires for elaborating the streptomycin molecule. The work of Hunter *et al.* (1954) has shown that carbon dioxide for the carbon atoms of the guanidine side chains may be supplied by free carbon dioxide in the gaseous atmosphere and not necessarily by carbon dioxide endogenously produced from glucose or other constituents of the medium. That is to say that free carbon dioxide is, or is in equilibrium with, the form incorporated: in a carbon dioxide-free

atmosphere the guanidine carbon atoms must arise solely from constituents of the medium.

The much greater efficiency of conversion of [^{14}C]glucose into streptomycin obtained by allowing growth to proceed for 60 hr. before adding radioactive material indicates that in the first 2 days of growth under our conditions the glucose is mainly a source of energy. Intermediates are presumably either oxidized completely or used largely for synthesis of mycelium, instead of being accumulated for streptomycin synthesis.

Streptamine does not appear to lie on a direct pathway from glucose to streptomycin, and the incorporation that we did obtain is probably preceded by degradation (possibly deamination to scyllitol or a similar compound. The final proof of the *scyllo* configuration in streptamine is given by Straube-Rieke, Lardy & Anderson, 1953). It is, however, possible that a phosphorylated or other derivative of streptamine occurs as an intermediate in the course of the biosynthesis. Such a compound might arise, for example, by formation of a new $\text{C}_1\text{-C}_6$ bond in D-glucosamine 6-phosphate as shown, followed by amination.

The strikingly efficient incorporation of *N*-methyl-L-glucosamine into streptomycin makes it likely that this compound, or a near derivative, is normally produced by *Strep. griseus* before the formation of the glycoside link with the rest of the streptomycin molecule.

The degradative evidence also revealed that *N*-methyl-L-glucosamine serves as a more efficient intermediate than glucose for the formation of the streptidine portion of the molecule. This gives rise to conjecture as to the mode of formation of L sugars by the organism. If *N*-methyl-L-glucosamine can undergo ring closure to yield a hexitol derivative, one can also visualize the reverse reaction taking place. Indeed, scyllitol and some of its derivatives are *meso* compounds, and on a purely 'theoretical' basis might be expected to split to both D and L products. Thus, we may postulate the existence of such a biosynthetic chain in *Strep. griseus* as

It is possible that some of the derivatives taking part in such a sequence will be the phosphate esters. It is also possible that the derivatives implicated throughout may be the amines (*scyllo*-inosamine, and L- and D-glucosamines).

The precise nature of the substances involved must await the outcome of further work. We do suggest, however, that the inversion of the D to the L sugar is effected by way of a hexitol intermediate.

An alternative suggestion has been made that the D-glucose $\rightarrow$ L-glucose transformation may come about by the action of a 'Waldenase' such as converts glucose 1-phosphate into galactose 1-phosphate by inversion at C_4 (Caputto, Leloir, Trucco, Cardini & Paladini, 1949). For this it would be necessary either to start with L sugars or D-idose, or to perform more than one inversion. For reasons of elegance and economy, as it accounts for the formation of both hexitols and L-glucose, we believe that our theory is to be preferred.

The failure of the mould to incorporate streptamine directly into the molecule indicates that it is not on the direct route to streptomycin. Although the experiment to test this has not yet been carried out, it is unlikely that streptidine is formed directly by guanidine transfer without the intermediacy of a diamino moiety. It is more likely that partially synthesized streptamine in the form of a modified scyllitol is first incorporated in the large molecule and that the final stages of synthesis are then carried out.

It is too early to explain the inhibition of streptomycin synthesis by inosose and the reversal of this effect by scyllitol. At least two possibilities do, however, exist: (a) interference with ring closure or ring fission in the D-glucose $\rightarrow$ cyclitol $\rightarrow$ L-glucose type of synthesis; (b) interference with the incorporation of the cyclized moiety into the streptomycin molecule.

SUMMARY

1. A 5% incorporation of D-[^{14}C]glucose into streptomycin by *Streptomyces griseus* has been obtained. This is 5 times the previous maximum (Karow *et al.* 1952) and is thought to be due mainly to delaying the addition of labelled material until the organism had almost attained its maximum bulk (60 hr. after inoculation).

2. Chemical degradation has shown that, within the limits of experimental error, the ^{14}C labels from uniformly labelled [^{14}C]glucose are distributed equally between the streptamine, streptose and *N*-methyl-L-glucosamine parts of the molecule. The carbons of the guanidine side chains contain much less radioactivity than the average of the other carbon atoms, which is consistent with the hypothesis that these carbons are incorporated only secondarily by way of carbon dioxide.

3. Streptomycin is not converted into streptomycin as efficiently as is glucose.

4. *N*-Methyl-L-[¹⁴C]glucosamine, added after the organism had grown for 60 hr., was incorporated into streptomycin in over 15% yield. The radioactivity was located largely in the corresponding part of the antibiotic molecule, but the L sugar was also converted into the streptidine moiety more efficiently than D-glucose.

5. It is suggested that the inversion of D-glucose to give L sugars proceeds by way of a hexitol-type intermediate.

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Creatine and Carbohydrate Metabolism

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In a study of the distribution of high energy phosphates in X-ray irradiated guinea pigs it was observed that a 24 hr. fast produced a marked rise in the liver creatine of control and irradiated animals. Although a considerable literature exists on the consequences of starvation on creatine distribution (Benedict, 1907; Hunter, 1928; Beard, 1943), so far as we are aware creatinuria is reported to occur in prolonged rather than short-term deprivation. It is generally accepted that the creatine arises as muscle protein is broken down (Mendel & Rose, 1911; Myers & Fine, 1913), but since in our experiments a significant elevation was obtained at a time when peripheral tissue breakdown is unlikely (cf. Addis, Poo & Lew, 1936*a*, *b*) we thought it worth while to investigate the mechanism of the effect.

METHODS

Animals. Guinea pigs were fed a liberal diet of cabbage and bran. Rats from the department strain of Wistar rats were fed a stock diet of rat cubes (Messrs Heygate and Son, Northampton). During starvation water was always available *ad lib*. Adrenalectomized rats were maintained on 1% saline. The completeness of removal of adrenals was checked at death. Thyroid deficiency was produced by giving 0.1% thiouracil in the drinking water for 6 weeks before testing. The glands were sectioned and examined *post mortem* by Dr R. V. Coxon and histological changes

associated with chronic thiouracil-induced hypothyroidism were found to be present.

Hind-limb perfusion. The rats were anaesthetized with ether. The viscera and testes were removed and the descending aorta was cannulated above the bifurcation. The animal was transected and the hind limbs were connected to the perfusion apparatus. The carcass was supported on a muslin ring in a glass funnel; this served to filter and collect the venous return. The funnel was covered with a Perspex lid. The perfusion fluid was warmed to 38° and the limbs were kept at about 38° by an infrared lamp. Perfusion fluid (60–100 ml.) was oxygenated with a Hooker oxygenator and recirculated by means of a pump designed by Dr E. H. Schuster for use in the Department of Pharmacology, Oxford. The perfusion fluid was blood diluted with an equal volume of Ringer-phosphate and contained 10 mg. heparin/100 ml. The blood was obtained from donor rats by cardiac puncture under ether. The perfusion fluid could be sampled and the pressure and flow rate measured as required.

Liver extract. The animals were killed by cervical fracture, the abdomen was opened and the liver dropped immediately into ice-cold 10% (w/v) trichloroacetic acid (TCA) and dispersed in a high-speed homogenizer. The residue after centrifuging was extracted twice with 6% (w/v) TCA and the combined extracts were made up to volume.

Blood extract. For creatine estimations the animals were decapitated, the blood was collected in 10% TCA and the precipitated protein re-extracted with TCA as above. For assay of glucose, blood was deproteinized in the cadmium sulphate reagent of Fugita & Iwataka (1931).