The Effects of Isoniazid on the Carbohydrates of *Mycobacterium tuberculosis* BCG

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1. *Mycobacterium tuberculosis* BCG was usually grown in glycerol–asparagine–casein hydrolysate medium. A soluble fraction was obtained from the cells with aq. 50% ethanol; unbound lipids were then removed and the cells were treated with dilute alkali to give, after acidification, an alkali-extractable fraction and an insoluble fraction. On occasion, lipopolysaccharides were obtained by extracting with phenol or dimethyl sulphoxide instead of alkali. The soluble fraction contained, particularly after long extraction, polysaccharide containing mainly glucose, in addition to trehalose and monosaccharides and their derivatives. The alkali-extractable fraction contained polysaccharides containing mannose, glucose, arabinose, galactose and 6-*O*-methylglucose. These could be resolved into three fractions of markedly different molecular size. It is argued that the high-molecular-weight materials originated from the outside of the cell envelope and the medium-molecular-weight materials from a middle layer of the envelope. 2. Exposure of the growing cells to isoniazid, usually at 1 or 10 $\mu\text{g/ml}$ for 6–12h, increased the total cell carbohydrate, mainly due to an increase in trehalose and in insoluble glucan. It also facilitated the extraction of polysaccharide into the medium and the soluble fraction. This produced about a 25% decrease in the amount of carbohydrate in the alkaline-extractable fraction, mainly due to a fall in glucose, arabinose and 6-*O*-methylglucose. The decrease was confined to polysaccharides of large and medium molecular weight. When intact lipopolysaccharides were extracted, their amount was also decreased by isoniazid. 3. Substitution of ammonium sulphate for asparagine and casein hydrolysate in the medium, so that glycerol was the sole carbon source, decreased the carbohydrate accumulation brought about by isoniazid but did not alter its effect on polysaccharide extraction. 4. Growth with ^{14}C -labelled substrates showed that glycerol provided two to four times as much of the cell carbon as did asparagine, when both were present. Under these conditions isoniazid inhibited the incorporation of carbon atoms from asparagine into the cells, but had little effect on the total incorporation from glycerol. These experiments also showed that the effect of isoniazid on alkali-extractable polysaccharides was due to their loss to the soluble fraction and external medium. 5. It is suggested that isoniazid inhibits a pathway (probably the synthesis of mycolic acid) involved in the formation of the cell envelope, and that this inhibition results in some re-channelling of intermediates into carbohydrate synthesis and in some loss of polysaccharides through damage to the envelope.

It has been shown that an early effect of isonicotinic acid hydrazide (isoniazid) on sensitive *Mycobacterium tuberculosis* BCG is the accumulation of soluble carbohydrates and their derivatives (Winder, Brennan & McDonnell, 1967). Further investigations of this phenomenon, which have revealed that the accumulation is complex in origin, that it is complicated by effects of isoniazid on ease of extraction of components from the cells, and that it is accompanied by changes in the carbo-

hydrates of other fractions of the organisms, are reported below. Some of these results have been published in preliminary form (Winder & Rooney, 1968a).

MATERIALS AND METHODS

Media. The glycerol–asparagine–casein hydrolysate medium, which was employed in most of the work, was the modified Sauton medium of Youatt (1958), except

that the glycerol concentration was 75.5 g/l. The glycerol-asparagine medium was identical except that the casein hydrolysate was omitted. In the glycerol-ammonium sulphate medium the asparagine also was omitted and replaced by 8 g of $(\text{NH}_4)_2\text{SO}_4$ /l.

Growth of bacteria and exposure to drugs. *M. tuberculosis* strain BCG (Glaxo Laboratories Ltd., Greenford, Middx., U.K.) was grown as described by Winder *et al.* (1967) except that the period of shaken growth was varied according to the medium. In the asparagine-casein hydrolysate medium the period was 4–5 days, in the asparagine medium it was 6 days and in the ammonium sulphate medium it was 7 days. Isoniazid or other drug was then added, and control and treated flasks were harvested, after the required incubation period, by centrifugation at 4°C and washed twice with 0.9% (w/v) NaCl.

Incorporation of ^{14}C from glycerol and asparagine. In these experiments the bacteria were grown in unlabelled medium for the usual period. In some experiments [^{14}C]glycerol of high specific radioactivity was then added to each flask at the same time as the drug, to render this substrate radioactive, and drug-treated and control flasks were harvested as usual after appropriate periods, except that they were washed four or five times. However, in most experiments, the cells from a number of flasks were obtained by centrifugation at room temperature, pooled, resuspended in the original volume of the fresh medium to which either [^{14}C]glycerol or [^{14}C]asparagine had been added, redistributed between the original number of flasks, care being taken to keep the bacteria in uniform suspension during the transfer, grown for further periods in the presence or absence of isoniazid, then harvested and washed four or five times.

Loss of ^{14}C from cells. In these experiments the growth medium contained [^{14}C]glycerol. The cells were harvested, pooled, washed four times with fresh unlabelled medium, resuspended at about their original concentration in the fresh unlabelled medium redistributed between the original number of flasks, and grown for further periods in the presence and absence of isoniazid. The cells were harvested by centrifugation and the medium was further clarified by filtration.

Extraction procedures. For analytical purposes cells from three flasks (about 12 mg of insoluble N) were normally combined to provide each sample and such samples were duplicated for each treatment. The volumes of reagents given below are for such 12 mg samples. For preparative purposes, sample size was increased severalfold and the volumes of extracting agents were increased proportionately. Except in experiments in which methods of extraction of the soluble pool were being examined, the cells were extracted at 4°C with 5 ml of 50% (v/v) ethanol for 18 h, and then with 3 × 5 ml of 50% ethanol for 1 h. The pooled extracts gave the 50% ethanol-soluble fraction. In some experiments, the cells were then extracted at 4°C with 2 × 5 ml of 5% (w/v) trichloroacetic acid for 2 h and washed with 5 ml of 50% ethanol. These extracts were combined with the 50% ethanol extracts to give the 'soluble fraction'.

Free lipids were removed from the cells by extraction with 10 ml of ethanol at room temperature and then with 3 × 10 ml of ethanol-diethyl ether (3:1, v/v) at 60°C for 5 min. The residue was then incubated with 8 ml of *m*-KOH at 25°C for 18 h, after which the volume was

adjusted to 10 ml with water. Samples (2 × 1 ml) were removed for determination of total N ('insoluble N'). The remainder was made 0.4 M with respect to HClO_4 , left at 4°C for 2 h, centrifuged, and the residue washed with 5 ml of 0.4 M- HClO_4 . The supernatants were combined to give the 'alkali-extractable fraction'. The residue is referred to as the 'insoluble fraction'.

In the extraction of lipopolysaccharides, samples, each consisting of the cells from eight flasks, were freed of the soluble and lipid fractions as described above. In the phenol method, the samples were incubated with 50 ml of aq. 45% (w/v) phenol at 65°C for 30 min. After centrifugation the upper (aqueous) layer was removed, 25 ml of water was added to the lower layer and the incubation was repeated. The upper layers were combined to provide the lipopolysaccharides. In the dimethyl sulphoxide method, the samples were treated with 50 ml of dimethyl sulphoxide at 60°C for 4 h. After centrifugation, the dimethyl sulphoxide was removed and the residue was re-extracted with 50 ml of this agent and then with 3 × 50 ml of water at 90°C for 30 min. The aqueous solutions were concentrated and combined with the dimethyl sulphoxide extracts.

Column chromatography of carbohydrates. Chromatography on Sephadex G-25 (particle diameter 20–80 μm) was carried out in a column (70 cm × 1.1 cm diameter) in water. The void volume, determined by noting the effluent position of a solution of Blue Dextran, was 30 ml and the flow rate was 20 ml/h. Sephadex G-100 (particle diameter 40–120 μm) was used in a column (63 cm × 3.5 cm diameter), in 0.1 M-acetic acid. The void volume was 200 ml and the flow rate 30 ml/h. Fractions (5 ml) were collected.

Separation of monosaccharides into two groups was carried out on a column (125 cm × 2.2 cm) of 200–400 mesh Dowex 50 W (K^+ form; X 4) by elution with water by the method of Saunders (1968). The flow rate was 25 ml/h and 5 ml fractions were collected.

Paper chromatography of carbohydrates. Descending chromatography was carried out on Whatman no. 1 or 3MM paper in either ethyl acetate-pyridine-water (10:4:3, by vol.) (solvent A) or ethyl acetate-acetic acid-90% (w/w) formic acid-water (16:3:1:4, by vol.) (solvent B). Sugars were detected on the chromatograms by a modification of the method of Anet & Reynolds (1954). The dried paper was dipped in a 0.5% solution of AgNO_3 in acetone (clarified by the addition of a few drops of water) and allowed to dry for a few minutes. It was then dipped in ethanolic NaOH (55.5 ml of a saturated solution of NaOH plus 1955 ml of ethanol), held in steam until the spots appeared, dipped in photographic fixer, washed and dried.

Quantitative chromatography was carried out on 3MM paper that had been washed with solvent and with water. The positions of the carbohydrate bands were determined by staining control strips, and the appropriate portions of the unstained paper were cut out and the sugars chromatographed off with water in a closed tank overnight. Recoveries of 96–106% were obtained with standards.

Hydrolysis conditions. Hydrolysis of polysaccharides was carried out with either 3 M-HCl in a sealed tube at 100°C for 2 h or with 4 M-HCl in a covered flask at 90–95°C for 4–5 h. HCl was removed under partial vacuum and

finally over KOH *in vacuo*. The hydrolysate was also treated with a mixed-bed ion-exchange resin (Amberlite MB-1). When the cell residue was to be analysed for components other than carbohydrates, hydrolysis was carried out in a sealed tube with 6M-HCl at 100–105°C for 16–18 h.

Analytical methods. Total carbohydrate was determined by an anthrone method (Winder *et al.* 1967) or by a phenol method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Usually, glucose was used as standard and the results are given in glucose equivalents ('glucose') but, where individual sugars had been separated by chromatography, the appropriate standards were used. Amino acids were determined by the method of Moore & Stein (1954), glucosamine and muramic acid by the method of Stewart-Tull (1968) and glycerol by the method of Renkonen (1962). Combined phosphate is the difference between total phosphate and orthophosphate, which were determined as described by Winder & O'Hara (1962), as were total N, RNA and DNA.

Determination of radioactivity. Samples (0.5 ml) were added to 10 ml of scintillation fluid [600 ml of toluene, 400 ml of 2-ethoxyethanol, 4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene]. When suspensions were to be counted, Cab-O-Sil (3%, w/v) was included in the scintillation fluid. A Packard 3380 liquid-scintillation counter was used. Quench corrections were made by internal standardization.

Materials. Sephadex materials, Blue Dextran and Yellow Dextran were obtained from Pharmacia, Uppsala, Sweden. Pancreatic casein hydrolysate was from E. Merck, A.-G., Darmstadt, W. Germany. Radioactive compounds were from The Radiochemical Centre, Amersham, Bucks., U.K. B1594 and B1575 were synthesized in the Laboratories of the Medical Research Council of Ireland, Trinity College, Dublin. B1594 has the formula 2-methylglyoxal-1-thiosemicarbazone-2-*N*⁴-methylthiosemicarbazone and B1575 has the formula 2-methylglyoxal-1-*N*⁴-methylthiosemicarbazone-2-thiosemicarbazone (Barry, Byrne, Conalty & O'Sullivan, 1967). 6-O-Methylglucose was a gift from Dr P. J. Brennan of the Department of Biochemistry, Trinity College.

RESULTS

The experiments described in this paper, unless otherwise stated, employed *M. tuberculosis* BCG grown in shaken culture in glycerol-asparagine-casein hydrolysate medium until the yield was about 40 µg of insoluble N/ml of medium, which occurred after 8–9 days' growth. The growth rate was slowly decelerating, from a generation time of about 24 h after 6 days to 48 h after 12 days, and the mean generation time during the experiments was about 35 h. The deceleration was associated with a slow increase in the size of the bacterial clumps and was probably caused by it, since the medium was capable of supporting growth for several further generations and since oxygen in the bulk of the medium was not limiting.

Under the conditions of these experiments, the minimal concentration of isoniazid that gave a

90% inhibition of growth over a period of 70 h was about 0.1 µg/ml. In most of the experiments reported here, the much higher concentration of 10 µg/ml was employed, because it had been used for most of the previous work on the phenomena investigated (Winder *et al.* 1967). There was no appreciable difference between the effects of isoniazid at 10 µg/ml and 0.5 µg/ml on the phenomena reported here. When the concentration was lowered to the region of 0.1 µg/ml similar effects were observed but they took longer, perhaps because of the need for more time to accumulate a critical concentration of drug in the cells.

As in the previous work (Winder *et al.* 1967), there was no evidence that isoniazid inhibited the growth of the bacteria during the first 6 h exposure: after this period of exposure to 10 µg/ml the yield of cells, in terms of insoluble N, was 99 ± 4% (14 experiments) of that in the control cultures. This value did not differ significantly from 100%, but it did differ significantly from 89% ($P < 0.05$, *t* test), the value calculated on the assumption of complete inhibition of growth (35 h generation time) from the start of the treatment. However, contrary to the previous experiments, there was a suggestion of inhibition of growth thereafter: after 12 h exposure to 10 µg/ml the yield of cells was 91 ± 4% (eight experiments) of that in the controls. The difference between this value and 100% approached significance ($0.1 > P > 0.05$), but there was little evidence of a difference between it and 89%, the value that should apply if no inhibition occurred during the first 6 h and complete inhibition occurred thereafter.

Comparison of anthrone and phenol procedures for carbohydrate assay. In most of the previous work on this topic the anthrone procedure was employed for the measurement of total carbohydrate. However, in much of the present work the phenol procedure was employed, since it gives more nearly the same molar extinction values with different hexoses and it also measures pentoses. Hence, results for the two procedures for some fractions of *M. tuberculosis* BCG are compared in Table 1, which shows that the two methods gave much the same result for the soluble fraction, which consists mainly of glucose derivatives, but not for the other two fractions, which contain substantial amounts of arabinose, galactose and mannose.

The effects of isoniazid determined by the two procedures were similar.

Carbohydrates in the soluble fraction. In the experiments reported in the present paper aq. 50% ethanol was used, in place of the 30% previously employed, to decrease the possibility of enzymic action during the extraction. Four successive extractions with 50% ethanol, each for 1 h under the conditions described in the Materials and Methods section, removed about 2500 µmol of 'glucose'/g of

Table 1. Carbohydrate content of some fractions of *M. tuberculosis* BCG as assayed by the anthrone and phenol procedures

Results for control cells are expressed as μmol of 'glucose'/g of insoluble N, and those for the isoniazid-treated cells are given as fractions of the control values.

Treatment	Assay method	Fraction		
		50% Ethanol-soluble	Alkali-extractable	Insoluble
Control	Anthrone	3230	1800	6480
	Phenol	3470	3020	10250
Isoniazid (10 $\mu\text{g}/\text{ml}$ for 6 h)	Anthrone	1.46	0.71	1.19
	Phenol	1.42	0.83	1.16

insoluble N in a typical experiment. After this about 200 μmol of 'glucose'/g of insoluble N were removed by three successive 1 h extractions with 5% trichloroacetic acid, and only traces thereafter. When the first extraction period with 50% ethanol was extended as described in the Materials and Methods section, effectively the entire soluble pool was removed by this agent, and this method was normally employed to obtain this pool.

During these investigations into the extraction of the soluble pool with aq. ethanol, it was noticed that a much larger proportion of the total soluble carbohydrate was extracted during the first one or two 1 h periods of extraction for the cells that had been treated with isoniazid for 6 h than for the control cells, indicating that treatment with isoniazid not only increased the amount of soluble carbohydrates in the cell but also rendered them more easily extractable.

When the aq.-ethanol extracts were fractionated on Sephadex G-25, two peaks were obtained (Figs. 1 and 2). The second peak was mainly trehalose and paper chromatography showed that only small amounts of glucose, galactose, arabinose, mannose and glycerol were present in it. The material in the first peak was eluted at and immediately after the void volume and thus included material of molecular weight up to at least 5000. Hydrolysis of the material from the first peak, followed by chromatography in solvents A and B, showed that it consisted almost entirely of glucose, with traces of mannose and 6-*O*-methylglucose. The last sugar was confined to the faster-moving portion of the peak, whereas the other two were distributed uniformly through it.

Fig. 1 shows that the first extract with aq. ethanol from control cells contained almost entirely low-molecular-weight material and Fig. 2 shows that subsequent extracts contained a much higher proportion of polysaccharide. Figs. 1 and 2 also show that isoniazid increased the amount of polysaccharide to a greater extent than it increased the amount of disaccharide, particularly with the first extract.

Fig. 1. Chromatography on Sephadex G-25 of the extracts obtained by treatment with aq. 50% ethanol for 1 h at 4°C of *M. tuberculosis* BCG; ●, control; ▲, exposed to 10 μg of isoniazid/ml for 6 h. Carbohydrate in the eluates was measured by the anthrone method. Abbreviations: V_0 , void volume; G, glucose.

When the carbohydrates in the combined aq.-ethanol extracts were fractionated by paper chromatography, eluted and assayed, the results in Table 2 were obtained. The deionization treatment employed removed sugar phosphates and phosphorylated oligosaccharides, which occur in these extracts (Winder *et al.* 1967), so that only the neutral carbohydrates were determined. The results confirmed that, although trehalose was the major component in the control cells, there was also a substantial amount of material that had the chromatographic properties of a polysaccharide. Isoniazid had a more marked effect on the amount of this polysaccharide than on the amount of trehalose. The measurement of the monosaccharides was probably not very reliable, as a consequence of the much smaller amounts present.

Carbohydrates of the alkali-extractable fraction. The alkali-extractable fraction was obtained from

the cells after removal of the soluble pool and free lipids. It was observed over a large number of experiments that exposure of the bacteria to isoniazid in the medium normally employed for 6–12 h lowered the amount of carbohydrate in this fraction to 70–85% of the control values (Tables 1, 5, 6 and 10).

When this fraction was hydrolysed and the component sugars were chromatographed in solvents A and B, mannose, glucose, arabinose, ribose, galactose and 6-*O*-methylglucose were found, in order of diminishing amount. The ribose could be accounted for entirely as being derived from RNA. Small traces of glucosamine and muramic acid were found in this fraction in some experiments, but the great bulk of these amino sugars remained in the cell residue.

When the hydrolysate of this fraction was applied to a column of Dowex 50, the sugars were resolved into two peaks. The first contained glucose

and 6-*O*-methylglucose, totalling 840 μmol of 'glucose'/g of insoluble cell N (phenol assay) for control cells in a typical experiment, whereas the second contained mannose, arabinose, ribose and galactose, totalling 1670 μmol of 'glucose'/g of insoluble cell N for control cells. The total recovery of carbohydrate after hydrolysis and application to the column was 60%. Treatment of the cells with 10 μg of isoniazid/ml for 6 h lowered the amount of carbohydrate in the first peak to 60–70% of the control values, but had little effect on the carbohydrate in the second peak. When the sugars from the two peaks from Dowex were separated by paper chromatography, eluted and assayed, the results in Table 3 were obtained. These confirm that the major monosaccharides present in this fraction were mannose, glucose and arabinose. They also show that exposure to isoniazid decreased the amounts of glucose and 6-*O*-methylglucose and, to a lesser extent, of arabinose, but had little effect on mannose or galactose.

When the unhydrolysed alkali-extractable material was chromatographed on Sephadex G-25 it was found that most of the carbohydrate in this fraction was excluded from this gel. When it was chromatographed on Sephadex G-100 the patterns illustrated in Fig. 3 were obtained. The extracted carbohydrates obviously fell into four size classes, with little overlap except between the last two (3 and 3a). The elution volume of peak 1 was rather less than that of Yellow Dextran, indicating a molecular weight above 20 000. From its elution volume, the highest point of peak 2 probably corresponded to a molecular weight of about 7000, and similarly the highest point of peak 3 probably corresponded to a molecular weight of 1000–2000. Peak 3a contained all the ribonucleotides and, hence, any low-molecular-weight products.

The material from these peaks was hydrolysed and subjected to paper chromatography in solvents A and B. Peak 1 contained arabinose, rather smaller amounts of glucose and mannose, and a little galactose. Mannose was the major sugar in peak 2; arabinose, glucose and some galactose were also present, and 6-*O*-methylglucose was also

Fig. 2. Chromatography on Sephadex G-25 of the combined second, third and fourth extracts obtained by successive treatments with aq. 50% ethanol for 1 h at 4°C of *M. tuberculosis* BCG; ●, control; ▲, exposed to 10 μg of isoniazid/ml for 6 h. Carbohydrate in the eluates was measured by the anthrone method. Abbreviations are as in Fig. 1.

Table 2. Carbohydrates of the 50% ethanol-soluble fraction from *M. tuberculosis* BCG (control and exposed to 10 μg of isoniazid/ml for 6 h)

	Total carbohydrate in fraction			Mannose and/or arabinose			Percentage of total carbohydrate recovered
	Trehalose	Polysaccharide	Glucose	Unknown oligosaccharide			
Control	3140	1070	65	186	16	63	
Isoniazid-treated	1.45	1.33	2.14	2.30	0.39	10.80	71

The extracts were concentrated, deionized with Amberlite MB-1 and chromatographed in solvent A. Bands were eluted and sugars determined by the phenol method. The control values are expressed as μmol of 'glucose'/g of insoluble cell N. The values for the treated cells are given as fractions of the controls.

Table 3. *Relative amounts of monosaccharides recovered from an acid hydrolysate of the alkali-extractable fraction from M. tuberculosis BCG (control and exposed to 10 µg of isoniazid/ml for 6 h)*

The sugars were separated into two fractions by chromatography on Dowex 50. These fractions were concentrated and chromatographed on paper. The bands were eluted and the sugars measured by the phenol method, employing standards of the authentic sugars except for 6-*O*-methylglucose, where a glucose standard was used. The amounts of sugar from the control cells are given as µmol/g of insoluble N, except that 6-*O*-methylglucose is given as glucose equivalents. The values for the isoniazid-treated cells are given as fractions of the control values. The values are not corrected for losses in hydrolysis and chromatography.

Fraction (from Dowex 50)	Sugar	Content of fraction	
		Control	Isoniazid-treated
1	Glucose	527	0.70
	6- <i>O</i> -Methylglucose	114	0.67
2	Galactose	115	1.04
	Mannose	692	1.01
	Arabinose	318	0.89
	Ribose	203	1.29

Fig. 3. Chromatography on Sephadex G-100 of the alkali-extractable fraction from *M. tuberculosis* BCG: ●, control; ▲, exposed to 10 µg of isoniazid/ml for 6 h. Carbohydrate in the eluates was measured by the phenol method. V_0 , Void volume.

present in the right-hand portion of the peak. Mannose was also the major sugar in peak 3, where it was accompanied by arabinose, glucose and some galactose. There was some variation in the relative amounts of these sugars in different portions of each peak. Ribose was the sole carbohydrate present in peak 3a.

As Fig. 3 shows, treatment of the cells with isoniazid markedly decreased the size of peak 1 and had a less-marked effect on peak 2. It had only small effects on peaks 3 and 3a, sometimes increasing and sometimes decreasing them slightly.

The possibility was considered that the alkali-extractable polysaccharides exist in the cells as lipopolysaccharides. Phenol and dimethyl sulph-

oxide were used to extract such lipopolysaccharides intact. The phenol method extracted 1690 µmol of 'glucose'/g of insoluble cell N (phenol assay) and dimethyl sulphoxide extracted 1780 µmol of 'glucose', amounts about half those usually obtained by the alkali method. Again, by both procedures, isoniazid-treated cells yielded only about 80% of the carbohydrate obtained from the controls. Since both the phenol and the dimethyl sulphoxide methods will extract polysaccharides in addition to lipopolysaccharides, this finding does not necessarily indicate any effect of isoniazid on lipopolysaccharides. Consequently, the extract obtained by the phenol method was saponified and the free fatty acids were extracted, weighed and determined by hydroxamate formation. In terms of weight, 286 mg of fatty acid/g of insoluble cell N was found in the fraction, whereas hydroxamate formation indicated 129 µmol/g of cell N, a result indicative of a very large molecular weight, suggesting that the material was largely mycolic acid. Recovery of fatty acids, both in terms of weight and of acyl groups, from isoniazid-treated cells was again only about 80% of that from the controls, indicating that lipopolysaccharides were among the substances in this fraction affected by isoniazid treatment.

Characterization of the insoluble fraction. The insoluble fraction consisted of protein, peptidoglycan, polysaccharide, bound lipids and DNA. Its composition with respect to some components is given in Table 4, which shows that glucosamine, arabinose and glucose were its major sugar components. Extraction of this material with hot trichloroacetic acid removed, in addition to DNA, most of the arabinose, glucose, galactose and mannose, leaving glucose as the only neutral sugar remaining in appreciable amount in the residue.

More than half of the extracted carbohydrate was eluted from columns of Sephadex G-25 at or near the void volume, indicating a fairly large molecular weight. The remainder contained appreciable amounts of free arabinose, galactose and glucose, presumably produced by hydrolysis during the acid treatment.

Isoniazid increased the amount of carbohydrate in this fraction (Tables 1, 5, 6 and 10). Proportionately the increase was larger in that portion not solubilized by hot trichloroacetic acid, where an accumulation of glucose occurred. It produced no appreciable alteration in the content of amino sugars.

Effects of the composition of the medium on the action of isoniazid. The results given so far were all obtained with the glycerol-asparagine-casein hydrolysate medium during both growth and exposure to isoniazid. Omission of casein hydrolysate during growth and exposure made little difference to the effects of isoniazid, perhaps leading to a slight decrease in the degree of carbohydrate accumulation that it caused.

When both asparagine and casein hydrolysate were replaced by ammonium sulphate as a source of nitrogen, so that glycerol was the sole carbon source (a small amount of citrate was present, but this is not utilized by *M. tuberculosis*), the control cells contained a much higher amount of carbohydrate per unit of N than normal, particularly in the soluble and insoluble fractions (Table 5). When the components of the soluble fraction were assayed after separation by paper chromatography, it was found that the polysaccharide, trehalose and glucose were each about doubled by the change in medium. Exposure to 10 µg of isoniazid/ml in this medium did not result in any appreciable change in the total cell carbohydrate after 6 h (Table 5), and this was also true after 12 h. The usual diminution in the amount of alkali-extractable carbohydrate was observed, and this was usually partly compensated for by a slight increase in soluble carbohydrate (Table 5), which appeared to be due mainly to an increase in the soluble polysaccharide.

When the glycerol content of either the asparagine-casein hydrolysate medium or the ammonium sulphate medium was lowered to one-tenth of that employed throughout the rest of this work, the total carbohydrate per unit of N in the control cells was decreased by about 20% in both cases, the decrease taking place mainly in the insoluble fraction and in the polysaccharide of the soluble fraction. However, this decreased glycerol concentration did not greatly affect the action of isoniazid on the carbohydrates of the cells in either medium, though it lessened the magnitude of the fall in alkali-extractable carbohydrate (Table 6) and also probably had slight effects on changes produced by isoniazid on individual components of the soluble fraction, but these would require further investigation.

Table 4. *Components of the insoluble fraction from M. tuberculosis BCG*

Composition	
	(µmol/g of insoluble N)
Arabinose (as 'glucose')	2413
Glucose	1329
Galactose (as 'glucose')	537
Mannose (as 'glucose')	241
Glucosamine	4500
Muramic acid	618
Glycerol	153
Amino acids (as 'leucine')	21 550

Table 5. *Comparison of the effects of exposure to isoniazid (10 µg/ml for 6 h) on the carbohydrate content of fractions from M. tuberculosis BCG grown in glycerol-asparagine-casein hydrolysate medium and in glycerol-ammonium sulphate medium*

Carbohydrates were determined by the phenol method. The results for control cells are given as µmol of 'glucose'/g of insoluble N and those for isoniazid-treated cells are given as fractions of the control values. Means $\pm$ s.e.m. are given with the number of experiments in parentheses.

Medium	Treatment	Carbohydrate content			
		Soluble fraction	Alkali-extractable fraction	Insoluble fraction	Total
Glycerol-asparagine-casein hydrolysate	Control	3395 $\pm$ 154 (12)	4306 $\pm$ 251 (11)	10 038 $\pm$ 820 (7)	18 017 $\pm$ 796 (11)
	Isoniazid	1.41 $\pm$ 0.03 (10)	0.85 $\pm$ 0.01 (10)	1.19 $\pm$ 0.04 (6)	1.12 $\pm$ 0.03 (12)
Glycerol-ammonium sulphate	Control	6498 $\pm$ 261 (8)	4919 $\pm$ 254 (5)	16 907 $\pm$ 2207 (5)	28 200 $\pm$ 2173 (5)
	Isoniazid	1.07 $\pm$ 0.02 (4)	0.77 $\pm$ 0.05 (4)	0.94 $\pm$ 0.06 (3)	0.96 $\pm$ 0.01 (4)

Table 6. *Effect of glycerol concentration on the effects of exposure to isoniazid (10 µg/ml for 6 h) on the carbohydrate content of fractions from M. tuberculosis BCG grown in glycerol-asparagine-casein hydrolysate medium and in glycerol-ammonium sulphate medium*

Carbohydrates were determined by the phenol method. The results give values measured as µmol of 'glucose'/g of insoluble N, for isoniazid-treated cells expressed as fractions of the control values.

Medium	Concn. of glycerol (g/l)	Carbohydrate content			
		Soluble fraction	Alkali-extractable fraction	Insoluble fraction	Total
Glycerol-asparagine-casein hydrolysate	75.5	1.49	0.84	1.10	1.11
	7.55	1.46	0.91	1.02	1.10
Glycerol-ammonium sulphate	75.5	1.03	0.77	1.00	0.97
	7.55	1.12	0.92	1.14	1.09

Experiments were also carried out in which bacteria, grown in the usual glycerol-asparagine-casein hydrolysate medium, were washed and transferred to the same medium, to ammonium sulphate medium, and to medium with all nitrogen omitted, all with and without 10 µg of isoniazid/ml, and incubated under the usual growth conditions for 6h. During this period there was a marked increase in the total carbohydrate per unit of insoluble N in the control cells in the ammonium sulphate and nitrogen-free media. In the presence of isoniazid there was a greater increase in total carbohydrate than in its absence in all cases and there was the usual diminution in amount of alkali-extractable carbohydrate.

Effects of isoniazid on incorporation of ^{14}C from constituents of the medium. In most of the experiments involving the incorporation of ^{14}C , including those illustrated here, the cells from a number of flasks were harvested, resuspended in fresh, radioactive growth medium at about their original concentration, and redistributed between flasks. This was done to minimize flask-to-flask variation, but it apparently shocked the cells to some extent, so that incorporation of radioactivity into control cells was not subsequently linear over the period of investigation. Further, since only a small proportion of the radioactivity in the medium could be incorporated into the cells during the periods employed, repeated washings of these were necessary during harvesting. It was discovered subsequently that this extensive washing eluted a certain amount of material from the cells, particularly the isoniazid-treated cells. As a result of these factors the increase in soluble and total carbohydrate resulting from isoniazid treatment was usually less than in other experiments (compare Table 7 with Tables 1, 2, 5, 6 and 10).

With control cells, incorporation of ^{14}C from asparagine was slightly slower during the first 6h period than in the second, but the opposite was

true of uptake of ^{14}C from glycerol, though this effect with glycerol was not usually as marked as in the experiment illustrated in Table 7. These effects were presumably due to transfer to the fresh medium. The results in Table 7 show that during the first 6h period about four times as much carbon from glycerol was incorporated into the control cells as from asparagine, and only about twice as much during the second period, though in other experiments values up to four times as much were observed during the second period. These results show that glycerol provided two or more times as much of the cell carbon as did asparagine, but that the latter did contribute an appreciable amount.

The results in Table 7 show that the total incorporation of carbon from [^{14}C]asparagine was markedly decreased by isoniazid over the 12h period, the decrease being particularly marked with the insoluble fraction. In the ammonium sulphate medium the incorporation of carbon from [^{14}C]glycerol was inhibited, though not as markedly as the incorporation of asparagine when the 12h period is considered. With the asparagine medium, the effect of isoniazid on the incorporation of carbon from glycerol was particularly complicated by adjustments taking place in the control cells over the initial period: this is shown by the fact that isoniazid had no appreciable effect on total incorporation of carbon over 12h, but appeared to have an inhibitory effect over the shorter period. In experiments in which the cells were not pooled and resuspended, so that the results were not complicated by adjustments taking place in the control cells, isoniazid slightly stimulated the total uptake of [^{14}C]glycerol from glycerol-asparagine-casein hydrolysate medium for exposure periods of up to 6h, but slightly inhibited its uptake from glycerol-ammonium sulphate medium.

These results indicate that isoniazid had an inhibitory effect, particularly from 6h onwards, on some metabolic process for which asparagine,

Table 7. Effects of isoniazid (10 µg/ml) on incorporation of ^{14}C from glycerol and asparagine into growing *M. tuberculosis* BCG

[2- ^{14}C]Glycerol had a specific radioactivity in the medium of 38.5 µCi/mol and [U- ^{14}C]asparagine had a specific radioactivity of 2063 µCi/mol. The measured d.p.m. in the fractions was divided by the d.p.m./mg-atom of total carbon in the labelled substrate to obtain the values for incorporation of carbon. Carbohydrate was determined by the phenol method.

determined by the phenol method.							
Treatment	Fraction	Carbohydrate (μmol of 'glucose'/g of insoluble N)		Incorporation of carbon from [^{14}C]glycerol (mg-atoms of carbon from glycerol/g of insoluble N)		Incorporation of carbon from [^{14}C]asparagine (mg-atoms of carbon from asparagine/g of insoluble N)	
	Time ...	6h	12h	6h	12h	6h	12h
Glycerol-asparagine medium							
Control	Soluble	3220	3060	14.7	16.9	1.61	2.40
	Lipid	—	—	22.3	32.3	6.39	13.5
	Alkali-extractable	4900	4540	15.6	17.7	3.36	5.65
	Insoluble	17800	15600	29.8	51.6	8.30	20.2
	Total	25900	23200	82.4	118.5	19.66	41.8
Fraction of control value							
Isoniazid	Soluble	1.17	1.22	1.09	1.19	0.83	0.92
	Lipid	—	—	0.91	1.40	1.07	0.60
	Alkali-extractable	0.87	0.79	0.61	0.86	0.92	0.63
	Insoluble	0.97	1.07	0.68	0.92	0.77	0.45
	Total	0.99	1.05	0.80	1.08	0.90	0.55
Glycerol-ammonium sulphate medium							
	Time ...	Carbohydrate (μmol of 'glucose'/g of insoluble N)		Incorporation of carbon from [^{14}C]glycerol (mg-atoms of carbon from glycerol/g of insoluble N)			
		6h	12h	6h	12h		
Control	Soluble	5850	4640	12.7	16.2		
	Lipid	—	—	21.2	33.3		
	Alkali-extractable	5670	5260	9.40	18.1		
	Insoluble	23630	17760	26.5	44.8		
	Total	35250	27670	69.8	112.4		
Fraction of control value							
Isoniazid	Soluble	1.04	1.10	1.20	1.16		
	Lipid	—	—	0.70	0.80		
	Alkali-extractable	0.75	0.74	0.81	0.78		
	Insoluble	0.82	1.03	0.87	0.84		
	Total	0.84	0.99	0.87	0.86		

when present, provided some of the carbon atoms. The overall utilization of glycerol, when present simultaneously with asparagine, was in contrast little affected by isoniazid over a 12 h period. When asparagine was not present glycerol must have provided that portion of the carbon otherwise provided by asparagine and, presumably, the inhibitory effect of isoniazid on glycerol incorporation under these circumstances was due to partial inhibition of this provision.

The slight increase brought about by isoniazid in the carbohydrate in the soluble fraction in the

experiment illustrated in Table 7, and the larger increase observed when the cells were not harvested and resuspended, were accompanied by increase in radioactivity derived from glycerol, showing that this was due in part to the synthesis of fresh carbohydrate from glycerol.

To account for the extent of the fall brought about by isoniazid in the carbohydrate content of the alkali-extractable fraction in terms of inhibition of synthesis of this carbohydrate would require complete inhibition of this synthesis. The results in Table 7 show that isoniazid did not inhibit

incorporation of ^{14}C into this fraction to anything like the required extent. The results can be accounted for satisfactorily by assuming that isoniazid did not markedly inhibit synthesis of the components of this fraction but rather led to a loss both of labelled and unlabelled material from it into the soluble fraction and into the medium.

In other experiments it was shown that isoniazid at $1\text{ }\mu\text{g/ml}$ had very much the same effects as those described above for $10\text{ }\mu\text{g/ml}$.

Effects of isoniazid on ^{14}C -labelled cells. One of the possibilities considered was that isoniazid might lead to a carbohydrate accumulation by affecting the utilization of endogenous material within the cells rather than by affecting uptake or utilization of fresh substrate. This was investigated by 'chase' experiments, in which the cells were grown with a labelled carbon source and subsequently washed and transferred to unlabelled medium before exposure to isoniazid. The results of one of these experiments is given in Table 8. The two other experiments gave very similar results. With the control cells there was quite a marked turnover of cell carbon: the amount of radioactive carbon decreased by about 40% in 12h, which was about one-third of the generation time applicable before the experiment and probably an even smaller fraction of the actual generation time, owing to the effects of washing and transfer on cell growth. However, it is not known whether this turnover normally occurs in growing cells or whether it was largely initiated by the washing and transfer process. Isoniazid increased the amount of radioactivity remaining in the soluble fraction, as it

increased the amount of carbohydrate in that fraction. It had little effect on the loss of total radioactivity from the cells for about 6h (the length of time depended on the concentration of isoniazid) but thereafter there was a marked decrease in loss of radioactivity. This was not due to decreased loss of radioactivity to the medium, since isoniazid always increased this. It must be assumed that it was due mainly to an effect on respiration of endogenous substrates and that the release of radioactive carbon dioxide was decreased by isoniazid at least after 6h exposure, although this was not directly measured. Since respiration, as measured by oxygen uptake in a Warburg apparatus, was found to be increased by isoniazid over the whole period of these experiments, such decreased release of carbon dioxide probably applied only to that derived from endogenous carbon.

The effects of isoniazid on the release of material into the medium from ^{14}C -labelled cells was investigated in more detail in other experiments. The results given in Table 9 were typical. These confirmed that exposure to isoniazid for 3h or longer resulted in increased release of material from the cells to the medium and had a particularly marked effect on the release of non-diffusible material. Some of the material released into the medium was carbohydrate, and isoniazid about doubled this release. If it is assumed that the carbohydrate so released had the same specific radioactivity as the glycerol in the growth medium, then the carbohydrate accounted for about one-third of the radioactivity released from the control cells and about

Table 8. *Effects of isoniazid on distribution of ^{14}C in M. tuberculosis BCG grown in media containing [^{14}C]glycerol and transferred to unlabelled media*

The growth media contained [^{14}C]glycerol at a specific radioactivity of $20.5\text{ }\mu\text{Ci/mol}$. Carbohydrate was determined by the phenol method.

Medium during growth and after transfer	Treatment in fresh medium	Period in fresh medium (h)	Carbohydrate (μmol of 'glucose'/g of insoluble N)			Radioactivity (d.p.m./mg of insoluble N)			
			Soluble fraction	Remainder	Total	Soluble fraction	Remainder	Total in cells	Medium
Glycerol-asparagine-casein hydrolysate	Control	0	2840	14000	16800	407	6170	6610	8
		3	3090	17200	20300	265	6270	6540	121
		6	2790	16300	19100	198	5260	5460	116
		12	3730	14700	18400	198	3920	4120	216
	Isoniazid ($1\text{ }\mu\text{g/ml}$)	3	4340	14500	18800	346	5880	6220	171
		6	3790	14900	18700	259	5140	5400	185
		12	4110	17700	21800	213	4860	5070	245
	Isoniazid ($10\text{ }\mu\text{g/ml}$)	3	4130	15800	20000	331	5700	6030	70
		6	5360	15400	20700	313	5820	6130	219
		12	4630	18600	24700	250	4680	4930	349
Glycerol-ammonium sulphate	Control	0	6400	—	—	830	12100	13000	81
		3	5780	—	—	634	11800	12400	—
		6	6260	—	—	588	10800	11400	69
	Isoniazid ($10\text{ }\mu\text{g/ml}$)	3	6440	—	—	652	11900	12500	180
		6	7100	—	—	621	12100	12800	231

Table 9. *Effects of isoniazid (10 µg/ml) on the release of ¹⁴C and carbohydrate from ¹⁴C-labelled M. tuberculosis BCG after transfer to unlabelled medium*

The cells were grown in glycerol-asparagine-casein hydrolysate medium containing [1-¹⁴C]glycerol at a specific radioactivity of 95 µCi/mol. After transfer the cell concentration was 0.22 mg of insoluble N/ml and cells provided a total of 4253 d.p.m./ml. Carbohydrate was determined by the phenol method.

Period after transfer (h)	¹⁴ C in medium (d.p.m./ml)		Carbohydrate in medium (nmol of 'glucose'/ml)	
	Control	Isoniazid	Control	Isoniazid
0	22.8	22.8	0	0
3	85.9	99.0	56.9	118
6	98.9	149	79.1	157
10	135	202	95.9	217
12	154	225	132	311
12 (dialysed)	8.4	42.6		

one-half of the radioactivity released from the isoniazid-treated cells.

Comparison of the effects of isoniazid with those of other anti-tuberculosis drugs. The effects of exposing growing *M. tuberculosis* BCG to four other anti-tuberculosis drugs for 12h at growth-inhibitory concentrations are compared with the effects of exposure to isoniazid in Table 10. All of the drugs led to some increase in carbohydrate per unit of insoluble N. With the two thiosemicarbazones, the increase was substantial only in the case of the soluble fraction; with streptomycin it extended to all fractions; and ethionamide, like isoniazid, caused a decrease in alkali-extractable carbohydrate. Ethionamide also resembled isoniazid in causing a marked increase in the combined phosphate (i.e. phosphate other than orthophosphate) in the soluble fraction and in the amount of substances absorbing at 260nm, effects shown to only a minor extent by the other drugs, but the extent to which the increased extinction was due to the drugs themselves or their metabolites was not determined.

DISCUSSION

The fact that the polysaccharides extracted by dilute alkali from *M. tuberculosis* BCG can be resolved into three distinct fractions of markedly different molecular size provides a clue to the probable origins of that fraction. The obvious explanation for the existence of three distinct size fractions (assumption that each fraction consists of a single heteropolysaccharide) is unlikely for several reasons: the shapes of the peaks eluted from Sephadex suggest heterogeneity, each of the peaks contains several sugars, and variation in monosaccharide composition within peaks is observed. A second possible explanation (that *M. tuberculosis* BCG tends to make all its polysaccharides in the same

three sizes) also seems very unlikely. A simple and reasonable explanation can be based on the hypothesis that the cell-envelope structure remains sufficiently intact during treatment with alkali to present a series of molecular sieves to the escape of macromolecules. In its simplest form it involves the assumption of two such barriers: an outer barrier, somewhat within the envelope surface, that prevents the escape of molecules larger than, say, 10 000 daltons and an inner barrier that cuts off at, say, 3000 daltons. This hypothesis can account for the existence of three fractions in the Sephadex eluates, but to account for the definition of the peaks and the sharpness of the separation between them, the subsidiary assumptions must be made that, after extraction of the cells with aqueous ethanol, it is mainly high-molecular-weight polysaccharides that exist outside the outer barrier and mainly high- and medium-molecular-weight polysaccharides that remain between the two barriers, and further that the potentially alkali-extractable polysaccharides of the envelope are not of a great variety of sizes.

On the basis of these considerations we suggest that alkali solubilizes from the outside surface of the cell envelope large polysaccharides containing mainly arabinose but also glucose, mannose and a little galactose. This agrees reasonably closely with the composition suggested by Imaeda, Kanetsuna & Galindo (1968) for the outermost layer of the envelope of mycobacteria on the basis of electron microscopy and fractional extraction. From a middle layer alkali extracts polysaccharides containing mainly mannose, but also glucose and arabinose together with a little galactose and 6-O-methylglucose. Either this layer is surrounded by the outer barrier discussed above, or cross-linking between non-alkali-soluble structures within the layer converts it itself into a three-dimensional barrier to escape of its own material. Again, the

Table 10. *Effects of exposure to some anti-tuberculosis drugs for 12 h in glycerol-asparagine-casein hydrolysate medium on the composition of M. tuberculosis H37Rv*

The control values, except for the yield, are expressed per g of insoluble N. All other results are given as fractions of the control values. Carbohydrate was measured by the anthrone method. The minimal concentrations of the drugs required to give 90% inhibition of growth over 70 h under the conditions of these experiments were (in $\mu\text{g/ml}$): B1594, 10; B1575, 10; streptomycin, 0.2; ethionamide, 0.3; isoniazid, 0.1.

Drug	Yield of cells (mg of insoluble N/100 ml of medium)	Carbohydrate (μmol of 'glucose')				Soluble fraction					
		Soluble fraction (7)	Alkali- extractable fraction (7)	Hot acid- soluble fraction (7)	Residue (7)	Total (7)	Substances absorbing at 260 nm (μmol of 'AMP') (7)	Amino compounds (μmol of 'leucine') (5)	Combined phosphate (μg -atoms of P) (6)	RNA (μg -atoms of P) (5) 1733 $\pm$ 331 (5)	DNA (μg -atoms of P) (6) 729 $\pm$ 21 (6)
None	4.62 $\pm$ 0.47 (24)	3388 $\pm$ 378 (7)	2059 $\pm$ 132 (7)	4946 $\pm$ 737 (7)	1633 $\pm$ 266 (7)	12 076 $\pm$ 865 (7)	235 $\pm$ 21 (7)	5561 $\pm$ 1084 (5)	1073 $\pm$ 198 (6)		
Fraction of control value											
B1594 (10 $\mu\text{g/ml}$)	0.97 $\pm$ 0.06 (7)	1.35 $\pm$ 0.08 (7)	1.08 $\pm$ 0.03 (7)	1.10 $\pm$ 0.06 (7)	1.16 $\pm$ 0.04 (6)	1.14 $\pm$ 0.03 (7)	1.29 $\pm$ 0.05 (7)	1.10 $\pm$ 0.10 (5)	1.09 $\pm$ 0.07 (6)	1.10 $\pm$ 0.10 (4)	0.97 $\pm$ 0.07 (4)
B1575 (10 $\mu\text{g/ml}$)	0.96 $\pm$ 0.06 (7)	1.45 $\pm$ 0.09 (6)	1.02 $\pm$ 0.10 (7)	1.07 $\pm$ 0.04 (6)	1.15 $\pm$ 0.10 (5)	1.12 $\pm$ 0.05 (6)	1.29 $\pm$ 0.10 (7)	1.15 $\pm$ 0.11 (5)	1.07 $\pm$ 0.06 (5)	0.98 $\pm$ 0.13 (4)	1.04 $\pm$ 0.10 (4)
Streptomycin (2 $\mu\text{g/ml}$)	0.88 $\pm$ 0.02 (6)	1.61 $\pm$ 0.08 (6)	1.44 $\pm$ 0.08 (6)	1.52 $\pm$ 0.15 (5)	1.73 $\pm$ 0.09 (5)	1.53 $\pm$ 0.09 (6)	1.12 $\pm$ 0.03 (6)	1.14 $\pm$ 0.10 (6)	1.20 $\pm$ 0.05 (5)	0.98 $\pm$ 0.04 (4)	0.93 $\pm$ 0.03 (4)
Ethionamide (1 $\mu\text{g/ml}$)	1.09 $\pm$ 0.23 (8)	1.54 $\pm$ 0.15 (7)	0.79 $\pm$ 0.04 (8)	1.08 $\pm$ 0.06 (8)	1.36 $\pm$ 0.09 (6)	1.25 $\pm$ 0.05 (6)	1.50 $\pm$ 0.10 (7)	0.87 $\pm$ 0.05 (5)	1.43 $\pm$ 0.20 (7)	1.13 $\pm$ 0.08 (7)	0.97 $\pm$ 0.07 (7)
Isoniazid (10 $\mu\text{g/ml}$)	0.91 $\pm$ 0.04 (8)	1.78 $\pm$ 0.15 (5)	0.74 $\pm$ 0.07 (5)	1.26 $\pm$ 0.16 (5)	1.55 $\pm$ 0.12 (4)	1.33 $\pm$ 0.03 (5)	1.59 $\pm$ 0.19 (5)	0.93 $\pm$ 0.04 (5)	1.49 $\pm$ 0.14 (5)	1.02 $\pm$ 0.09 (3)	0.94 $\pm$ 0.03 (3)

sugars solubilized agree reasonably well with the composition of the middle layer of the mycobacterial cell wall postulated by Imaeda *et al.* (1968). We suggest that the inner barrier is provided by the peptidoglycan that exists as the basal layer of the cell envelope (Takeya & Hisatsune, 1963; Takeya, Hisatsune & Inoue, 1963; Imaeda *et al.* 1968), perhaps assisted by remnants of the cytoplasmic membrane, and that only the small polysaccharides and substances of lower molecular weight can penetrate through this from the cell interior. This picture is not in agreement with the polysaccharide composition of isolated purified 'cell walls' of mycobacteria, which consist essentially of a peptidoglycan-arabinogalactan-mycolic acid complex sometimes containing a glucan (Cummins & Harris, 1958; Kotani, Kitaura, Hirano & Tanaka, 1959; Misaki & Yukawa, 1966; Kanetsuna, 1968). However, these preparations apparently consist only of the basal layer of the envelope (Takeya & Hisatsune, 1963; Takeya *et al.* 1963; Imaeda *et al.* 1968), the outer layers having been removed by the treatments employed in obtaining them, which include ultrasonic disintegration, trypsin treatment, and extraction with lipid solvents, phenol, dimethyl sulphoxide or ethanolic potassium hydroxide. For this reason, we use the term 'envelope' to refer to the structure as it exists around the living mycobacterial protoplast.

If this picture is correct, then the finding that isoniazid does not interfere appreciably with the synthesis of alkali-extractable polysaccharides but does lead to the disappearance from the alkali-extractable fraction of high- and medium-molecular-weight materials, particularly containing glucose, 6-O-methylglucose and arabinose, and including lipopolysaccharides, combined with the appearance of increased amounts of polysaccharide in the medium and the soluble fraction, indicates that isoniazid disorganizes the outer layers of the cell envelope in such a fashion as to facilitate elution of some materials from them. Recent evidence indicates that a very early action of isoniazid is interference with the biosynthesis of mycolic acids (Winder, Collins & Rooney, 1970). This would prevent the attachment of mycolic acids to polysaccharides to form the lipopolysaccharides of the cell envelope. This might permit the leaching of such polysaccharides from the envelope into the aqueous environment.

Of the agents tested, only ethionamide is similar to isoniazid in leading to loss of carbohydrate from the alkali-extractable fraction. The action of ethionamide on mycobacteria is also similar to that of isoniazid to the extent that both lead to fairly early loss of acid-fastness (Schaefer, 1960), which is not shown by most antimycobacterial drugs. Loss of acid-fastness is itself evidence of damage to the

cell envelope, but requires about 24h exposure to the drug, so that the changes in the alkali-extractable fraction, which are marked within 6h, and increased release of polysaccharide into the soluble fraction and the medium, which can be observed within 3h, are probably indicators of earlier stages in the same damage.

A number of other lines of evidence also indicate that changes in the cell envelope of mycobacteria occur early in isoniazid action: cell walls are more easily obtained from isoniazid-treated *M. tuberculosis* BCG than from normal organisms by ultrasonic treatment (Winder *et al.* 1970); isoniazid diminishes the incorporation of radioactivity from [^{14}C]glycerol into the wall fraction (Winder *et al.* 1970); a decrease in the hydrophobic properties of *M. tuberculosis* BCG occurs within 4h of exposure to isoniazid (Youatt, 1965); and the altered extractability of triglycerides from the isoniazid-treated organism (Winder & Rooney, 1968b) is most readily accounted for on the basis of a change in the properties of the envelope.

Cross-linking of the peptidoglycan of the cell wall has been suggested as a possible site of isoniazid action, on the basis of the fact that isoniazid can interfere with transpeptidation reactions (Lorand & Jacobsen, 1967). However, in view of the restriction of isoniazid action to mycobacteria, interference with the synthesis of one of the structures characteristic of the mycobacterial envelope is a likelier means of damage to this envelope, and recent evidence of interference by isoniazid in the formation of mycolic acid-containing lipopolysaccharides of the envelope supports this (Winder *et al.* 1970).

Other hypotheses as to the area of metabolism primarily affected by isoniazid have been reviewed in detail by Winder (1964) and Youatt (1969).

Several of the effects of isoniazid on the carbohydrates of *M. tuberculosis* BCG can be interpreted in terms of the facilitated extraction of polysaccharides from the cell envelope as discussed above. However, this does not apply to the increase in total cell carbohydrate, which includes an increase in the trehalose content of the soluble fraction and in the polysaccharide content of the final cell residue. This increase may be in part a non-specific consequence of growth inhibition, a conclusion favoured by the fact that some accumulation of carbohydrate is shown with all the inhibitory drugs tested. However, this does not seem to be the complete explanation, since isoniazid causes carbohydrate accumulation in substantially less than 6h, but inhibition of growth takes longer than this, and since isoniazid causes this accumulation to an appreciable extent only in media containing asparagine.

The radioisotope experiments have shown that, when asparagine is present, isoniazid may produce

little change in the total incorporation of carbon from glycerol into the cell, even though there is an increased rate of oxygen uptake, but that it diminishes the uptake of asparagine, at least after 6h. They have further shown that, in the absence of asparagine, the uptake of glycerol is decreased and that, under either circumstance, the mobilization of endogenous carbon is decreased after a time. The results can most easily be interpreted by assuming that isoniazid interferes with a synthetic pathway for which the carbon skeleton of glycerol, or the skeletons of both glycerol and asparagine, are utilized and that this leads to some accumulation of intermediates that can be converted into carbohydrate and can increase the oxygen-uptake rate. It must be assumed that these intermediates inhibit or repress the uptake of asparagine, the utilization of endogenous carbon stores and the uptake of glycerol when amino acids are not present in the medium. It must further be assumed that, when glycerol is not the sole carbon source in the medium, the regulatory situation as far as its uptake is concerned is somewhat different, so that no decrease in uptake follows the accumulation of intermediates. Evidence exists for regulatory control over glycerol uptake in *Escherichia coli* (Sanno, Wilson & Lin, 1968) and in *Mycobacterium phlei* (Bowles & Segal, 1965). Further analysis of the situation on the basis of present evidence would be highly speculative.

If the arguments used earlier to deduce that the high-molecular-weight components of the soluble and alkali-extractable fractions originate from the cell envelope are correct, and if we are correct in assuming that the peptidoglycan is one of the barriers to the escape of high-molecular-weight material, then the substantial amount of high-molecular-weight material extracted by hot trichloroacetic acid from the 'insoluble' fraction probably originates from the alkali-insoluble material in the envelope, since the peptidoglycan is not solubilized by the hot-acid treatment and hence should still present a barrier. This suggests that the glucan of the cell residue is the only intracellular polysaccharide present in substantial amount under the growth conditions.

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