Effects of Isoniazid on the Composition of Mycobacteria, with Particular Reference to Soluble Carbohydrates and Related Substances

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1. When growing *Mycobacterium tuberculosis* BCG was exposed to 0.5–10 $\mu\text{g.}$ of isoniazid/ml. there was intracellular accumulation of soluble carbohydrate, combined phosphate and substances absorbing at 260 $\text{m}\mu$. Yellow pigments were formed when modified Sauton medium was used, but not with Proskauer & Beck medium. These processes were apparent after 1 hr. but were more marked after about 6 hr. These effects were not found with an isoniazid-resistant strain. 2. After 6 hr. exposure of the sensitive strain to 10 $\mu\text{g.}/\text{ml.}$ there was little change in the amounts (per g. of insoluble nitrogen) of total lipid, glycolipid, RNA, DNA or of carbohydrate in the nucleic acid fractions. 3. The major accumulation was of $\alpha\alpha'$ -trehalose. There was also accumulation of glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, trehalose 6-phosphate (tentatively identified), a polysaccharide containing only glucose, and an oligosaccharide containing glucose and glucose 6-phosphate, but not of glycerol and glycerol 3-phosphate. The u.v.-absorbing materials appeared to be nucleotide sugar derivatives. 4. In *Mycobacterium smegmatis* a similar accumulation of trehalose occurred on exposure to isoniazid, but there was little accumulation of other compounds. 5. No evidence could be found that isoniazid specifically affected the oxidation of glycerol or glycerol 3-phosphate. 6. It is suggested that the primary action of isoniazid on mycobacteria may be partial inhibition of a reaction in some central area of metabolism, such as glycolysis.

Isonicotinic acid hydrazide (isoniazid) is bactericidal towards *Mycobacterium tuberculosis* at very low concentrations, although it is almost totally devoid of activity against micro-organisms from other genera. A number of hypotheses to explain its action have been formulated but none has found much experimental support (Winder, 1964a).

A line of investigation of this action was opened by the observation that the exposure of growing *M. tuberculosis* to isoniazid leads to the accumulation of intracellular soluble carbohydrates and phosphates (Winder, 1964b). This paper reports the results of further investigations into the effects of isoniazid on the composition of mycobacteria, with particular reference to the accumulation of soluble carbohydrates and related compounds, and the possible relations between the phenomena and a primary lesion caused by the drug are discussed. Some of the results have been published in preliminary form (Winder & Brennan, 1964; Brennan & Winder, 1965; Winder & Flynn, 1965).

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MATERIALS AND METHODS

Growth of bacteria. *M. tuberculosis*, strain BCG (Glaxo Laboratories Ltd.) was inoculated into 100 ml. batches of modified Sauton medium (Youatt, 1958) and was grown stationary at 37° for about 4 days and then on a rotary shaker set at 200 rev./min. (amplitude 1 in.) for a further 4 days. Isoniazid was then added to the required number of flasks and treated and control bacteria were harvested after suitable incubation periods.

The isoniazid-resistant strain was produced by transfer of a heavy inoculum on to Löwenstein-Jensen medium containing 1000 $\mu\text{g.}$ of isoniazid/ml., followed by repeated subculturing on the same medium. It was grown in the same way as the sensitive strain except that the medium contained 5% human serum.

M. smegmatis was treated similarly, except that Proskauer & Beck medium was used and the stationary and shaken growth periods were each 24 hr.

General extraction procedure. Cultures were harvested by transfer to chilled centrifuge tubes and centrifuging at about 4°. The cells were washed twice by suspension in ice-cold 0.9% (w/v) NaCl and centrifuging at 4°. The extraction procedure was based on that of Winder & O'Hara (1962). Pooled cells from two flasks were extracted successively at

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about 4° with 5 ml. of 30% (v/v) ethanol for 2–3 hr. The residues were similarly extracted with 5 ml. of 5% (w/v) trichloroacetic acid for 2–3 hr., 5 ml. of 5% trichloroacetic acid for 2–3 hr. and 10 ml. of water. The five supernatant solutions were pooled to give the 'soluble fraction'.

The residues were then extracted with 10 ml. of ethanol for 2–3 hr. at room temperature and three times successively with 10 ml. of ethanol-ether (3:1, v/v) for 4 min. at 60°. The supernatant solutions were pooled to provide the 'lipid fraction'. The solutions were reduced to small volume at 45° and dried *in vacuo* over P_2O_5 to constant weight. After weighing they were dissolved in chloroform-methanol (2:1, v/v) and samples were taken for phosphorus and carbohydrate determination.

To the residues was added 8 ml. of N-KOH and the mixtures were incubated at 25° for 18 hr., made to 10 ml. and samples were taken for protein or nitrogen determinations. The remainder was made 0.4N with respect to $HClO_4$. The mixture was left at 4° for 2 hr., centrifuged, the residue washed with 5 ml. of ice-cold 0.4N- $HClO_4$, and the supernatants were combined to give the 'RNA fraction'. The residues were extracted with 5 ml. of 5% trichloroacetic acid for 15 min. at 90° and the residue was washed with a further 5 ml. of the acid and 5 ml. of water. The extracts were combined to give the 'DNA fraction'.

Fractionation of the soluble fraction with barium acetate. This was carried out according to procedure A of LePage (1957).

Paper chromatography. Non-phosphorylated sugars were separated by chromatography, usually in a descending direction, on Whatman no. 1 or 3MM paper. Appropriate sugars were run as markers. The solvents used are listed in the appropriate places.

Sugar phosphates were chromatographed on Whatman no. 1 or 3MM paper which had been pre-washed twice with N-formic acid, then several times with distilled water, and dried. Appropriate sugar phosphates were used as markers.

Carbohydrates and related compounds were located by a permanganate-periodate spray (Lemieux & Bauer, 1954) or by dividing strips of the chromatograms into about 20 parallel bands, eluting these and determining carbohydrate in the eluates. Reducing sugars were sought by spraying with aniline-oxalate (Horrocks & Manning, 1949), aniline-phthalate (Cummins & Harris, 1956), ammoniacal silver nitrate (Partridge, 1949) and benzidine (Bacon & Edelman, 1951). Phosphate groups were detected by the molybdate spray of Hanes & Isherwood (1949).

Chemical assays. Orthophosphate, total phosphate, phospholipid, RNA, DNA and total nitrogen were determined as described by Winder & O'Hara (1962). The difference between total phosphate and orthophosphate represented the 'combined phosphate'. Protein was determined by a biuret method (Gornall, Bardawill & David, 1949) with serum albumin, fraction V, as standard. Chemical determination of glycerol was carried out by the method of Renkonen (1962).

Carbohydrate was determined by a modification of the anthrone method of Scott & Melvin (1953). Volumes (4 ml.) of 0.2% anthrone in conc. H_2SO_4 were transferred to 16 mm. x 125 mm. test tubes placed in ice. Samples (2 ml.) of suitable dilutions of the test materials were carefully layered on top. Each tube was well shaken and immediately placed in a water bath at 90° for exactly 20 min., removed and cooled rapidly. With lipid solutions samples were

evaporated to dryness in the assay tubes, 2 ml. of water was added and the tubes were chilled. Anthrone reagent was then added and the tubes were heated at 90° for 30 min. with frequent shaking and cooled. The charred lipid present after this treatment was removed by centrifugation. Appropriate blanks and glucose standards were included. Tubes were read at 650 m μ immediately after cooling.

Examination of carbohydrates and their derivatives in the soluble fraction. In a typical experiment, the total soluble fraction from the cells of *M. tuberculosis* from 11 flasks which had been exposed to 10 μ g. of isoniazid/ml. for 12 hr. and the same fraction from cells from 11 control flasks were fractionated with barium. Carbohydrate determinations were carried out on samples of each fraction. The fractions were then further examined as described below.

(a) Barium-soluble ethanol-soluble fraction. This fraction was treated with Dowex 50 (H^+ form) and was reduced to a small volume under reduced pressure at about 40°. The entire fraction was placed as a band on Whatman 3MM paper and chromatographed, descending, in ethyl acetate-pyridine-water (10:4:3, by vol.) for 21 hr. with suitable markers. A strip containing the markers and some of the unknown material was sprayed with permanganate-periodate. The first 2 cm. from the origin contained no carbohydrate, but a large amount of other material, and was discarded. A large amount of carbohydrate was present as a broad band whose centre had R_{Glc} (ratio of its R_F to that of glucose) about 0.6. There was also a band with R_{Glc} 1.5.

The band of R_{Glc} 1.5 was eluted and rechromatographed. Its R_F in several solvents was the same as that of glycerol and it behaved like glycerol with the sprays listed above. Its identity as glycerol was confirmed by assay by the chemical method of Renkonen (1962) and the specific enzymic method described later.

The portion of the original chromatogram covering the range of R_{Glc} values from 0.40 to 0.80, which contained all of the carbohydrate, was eluted, concentrated, applied as a band to a sheet of Whatman 3MM paper and chromatographed with ethyl acetate-pyridine-water in a descending direction for 40 hr. When a strip was sprayed with permanganate-periodate a main band (R_{Glc} 0.60) and two minor bands (R_{Glc} 0.46 and R_{Glc} 0.70) were noted. These bands were eluted and investigated as described below.

The position of the main band suggested that it was a disaccharide. Spectrophotometry of the reaction products with cysteine- H_2SO_4 (Dische, Shettles & Osnos, 1949) and carbazole- H_2SO_4 (Gurin & Hood, 1939) suggested that the monosaccharide component was glucose. On hydrolysis with 2N- H_2SO_4 at 100° for 3 hr., neutralization with $BaCO_3$, removal of Ba^{2+} ions with Amberlite IR-120 (H^+ form), and chromatography in butan-1-ol-acetic acid-water (11:4:10, by vol.), propan-2-ol-water (4:1, v/v) and ethyl acetate-pyridine-water (10:4:3, by vol.), only one monosaccharide was observed and it had the mobility properties of glucose in each of these solvents (R_F 0.21, 0.72 and 0.16 respectively). The identity of glucose was confirmed by the use of glucose oxidase as described later. In addition, the hydrolysate contained some of the original sugar, showing that it was moderately resistant to hydrolysis, and a sugar whose chromatographic properties suggested a trisaccharide and hence, presumably, was produced by acid-catalysed condensation of glucose with unchanged disaccharide (Peat, Whelan, Edwards & Owen, 1958). The original disaccharide was non-reducing in the arsenomolybdate reaction (Nelson,

1944) and gave no reaction on paper with benzidine, ammoniacal silver nitrate, or the aniline sprays. The slowness of its reaction with permanganate-periodate was further evidence that it was non-reducing. These findings, indicating a non-reducing disaccharide containing only glucose, strongly suggested that it was trehalose. On chromatography it behaved exactly like $\alpha\alpha'$ -trehalose in propan-1-ol-ethyl acetate-water (6:1:3, by vol.) (R_{Glc} 0.81), butan-2-one-acetic acid-water (6:1:1, by vol.) (R_{Glc} 0.45), ethyl acetate-pyridine-water (10:4:3, by vol.) (R_{Glc} 0.64), ethyl acetate-pyridine-water (8:2:1, by vol.) (R_{Glc} 0.61), ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.) (R_{Glc} 0.70), ethyl acetate-acetic acid-water (14:3:3, by vol.) (R_{Glc} 0.95), butan-1-ol-benzene-pyridine-water (5:1:3:3, by vol., top layer) (R_{Glc} 0.41) and butan-1-ol-acetic acid-water (11:4:10, by vol.) (R_{Glc} 0.95). Some of these solvents give slight separation between the trehalose isomers and so the evidence was that the isolated material was $\alpha\alpha'$ -trehalose. The absence of β configuration was further shown by incubating some of the isolated material with 5mg. of β -glucosidase (EC 3.2.1.21) in 2.5 ml. of 0.1M-tris buffer, pH 7.5, at 37° for 1 hr.: no evidence of loss of disaccharide or of appearance of glucose could be found.

The carbohydrate in the other two bands (R_{Glc} 0.46 and 0.70) referred to above was also shown by the same methods to be $\alpha\alpha'$ -trehalose. The heavy load placed on the paper, together possibly with some of the impurities present, apparently led to the formation of these multiple bands of trehalose.

To prepare a crystalline trehalose derivative, a portion of the barium-soluble ethanol-soluble fraction from isoniazid-treated *M. tuberculosis* was acetylated; the derived acetate had m.p. 73° and mixed m.p. 75° with authentic $\alpha\alpha'$ -trehalose octa-acetate (m.p. 77°). There appear to be two forms of crystalline $\alpha\alpha'$ -trehalose octa-acetate with different melting points: most values in the literature are in the region of 100°, but several authors quote values in the region of 79° (Birch, 1963).

(b) Barium-soluble ethanol-insoluble fraction. This fraction was dissolved by shaking with water and Dowex 50 (H^+ form) and then chromatographed as a band on acid-washed Whatman 3MM paper in a descending direction for 36 hr. with isobutyric acid-aq. NH_3 (sp.gr. 0.880) soln.-water (66:1:33, by vol.) as solvent. When a strip was sprayed with permanganate-periodate two main reacting areas were observed: one was strongest near the origin and the other had a centre R_f of about 0.5. The remaining strips of the paper were therefore divided into two bands: one extending from the origin to R_f 0.3 and the other extending from R_f 0.3 to the front. The two bands were eluted separately and the eluates investigated as described below.

(i) Slowly moving material: The eluted slowly moving material was concentrated and again chromatographed in isobutyric acid-aq. NH_3 soln.-water, this time for 90 hr., with glucose 6-phosphate, trehalose 6-phosphate and trehalose 6,6'-diphosphate as markers. When a strip was sprayed with permanganate-periodate two bands appeared: one of these remained very close to the origin, and the R_{Glc6P} (R_f divided by that of glucose 6-phosphate) of the other was 0.19. When the remainder of the chromatogram was divided into 8 cm. $\times$ 2 cm. bands and the anthrone reaction carried out on samples of the eluates from these bands, the carbohydrates were found to be localized in the same two

bands as reacted with the permanganate-periodate spray. Neither of these bands contained reducing sugars.

A sample of eluate from the band near the origin was hydrolysed with $\text{N-H}_2\text{SO}_4$ at 100° for 6 hr. and neutralized with BaCO_3 . Glucose only was obtained, as judged by chromatography in three solvent systems. Determination of total phosphate showed none to be present. Thus this material appeared to be a glucose-containing polysaccharide whose insolubility in ethanol led to its precipitation in the barium-soluble ethanol-insoluble fraction.

A sample of the eluate from the band whose R_{Glc6P} was 0.19 gave, after hydrolysis in the same fashion, only glucose and glucose 6-phosphate, as judged by their chromatographic properties. The unhydrolysed material had a glucose:phosphate ratio about 2.5:1, and was non-reducing. Its rate of migration in the above solvent was much slower than that of trehalose 6-phosphate (R_{Glc6P} 0.77) and even of trehalose 6,6'-diphosphate (R_{Glc6P} 0.38). These findings suggested an oligosaccharide containing from four to six glucose residues, with two of these phosphorylated in the 6-position.

(ii) Rapidly moving material: The eluate containing the more rapidly moving material was concentrated and chromatographed again in isobutyric acid-aq. NH_3 soln.-water for 36 hr. with a number of markers. On spraying a strip with permanganate-periodate five reacting bands were detected. Another strip of the paper was divided into 17 cm. $\times$ 3 cm. bands, each of which was eluted and the carbohydrate content of a sample of the eluate determined. Four peaks of sugar content were observed which corresponded to four of the reacting bands. From the remainder of the chromatogram bands were cut out corresponding to the bands which reacted with permanganate-periodate, and assays for carbohydrate, phosphate and some other components were carried out on the eluates. The following information was obtained on these bands.

The first band was weak and, in fact, was observed definitely only with material from isoniazid-treated cells. Its slow reaction with the spray suggested that it was non-reducing. It contained anthrone-reacting material. Its R_{Glc6P} (0.77) was the same as that of trehalose 6-phosphate, and it was concluded that it was probably that compound.

The second band was strong. Its quick reaction with the spray suggested that it was reducing. Prolonged acid hydrolysis of the eluate produced glucose. It had a glucose:phosphate ratio of 1 and had the same R_f as glucose 6-phosphate. Assay with glucose 6-phosphate dehydrogenase (EC 1.1.1.49) as described below gave a value in close agreement with that obtained by the anthrone method. Hence this band consisted mainly of glucose 6-phosphate.

The third band was weaker. It also had an apparent monosaccharide:phosphate ratio of 1. Its R_{Glc6P} (1.18) was similar to those of glucose 1-phosphate, fructose 1-phosphate and fructose 6-phosphate. Treatment of the eluate with phosphatase and chromatography of the products showed the presence of glucose and fructose in about equal amounts, together with a larger amount of a pentose tentatively identified as arabinose. Specific enzymic assays for glucose 1-phosphate and fructose 6-phosphate (as described below) showed both to be present, but in substantially smaller amounts than the total carbohydrate in the band. The pentose phosphate may have been an artifact, as discussed below.

The fourth band reacted strongly with the spray. It

contained phosphate but reaction with anthrone gave no evidence for the presence of sugar. Its R_{Glc6P} (1.51) agreed with that of glycerol 3-phosphate. Assay with glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) (as described below) confirmed the presence of this substance, though the amount was not as great as expected from the strength of the permanganate-periodate reaction. Phosphate in excess of glycerol 3-phosphate was also present, but this may have been due to some overlapping with the orthophosphate band.

The fifth band contained no phosphate. The eluate gave some reaction with the anthrone reagent, but the colour was not typical of a hexose. Its R_{Glc6P} (1.79) agreed with that of glycerol, and some of this substance may have been carried down as a contaminant of the barium-soluble ethanol-insoluble fraction, but some other substance seems to be present to account for the anthrone reaction.

(c) Barium-insoluble fraction. This material was not fractionated further, but various assays were carried out on it whose results are given in a later section.

Enzymic assay of carbohydrates and their derivatives in the soluble fraction. The extraction procedure was as described earlier except that 0.4N-HClO₄ was employed instead of trichloroacetic acid. This extract was neutralized with 40% (w/v) KOH and added to the 30% ethanol extract to give the 'soluble fraction'.

Glucose was estimated by the glucose oxidase (EC 1.1.3.4) method of Huggett & Nixon (1957). Glycerol was estimated by using glycerol dehydrogenase (EC 1.1.1.6) as described in leaflet 4-63 on glycerol dehydrogenase published by the Worthington Biochemical Corp., Freehold, N.J., U.S.A. D-Glycerol 3-phosphate was assayed by using glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) as described by Hohorst (1963b). Glucose 6-phosphate was determined by using glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and fructose 6-phosphate was assayed with glucose phosphate isomerase (EC 5.3.1.9) and glucose 6-phosphate dehydrogenase, both according to Hohorst (1963a). Glucose 1-phosphate was estimated by using phosphoglucomutase (EC 2.7.5.1) and glucose 6-phosphate dehydrogenase (Bergmeyer & Klotzsch, 1963). Dihydroxyacetone phosphate was estimated by using glycerol 3-phosphate dehydrogenase as described by Bücher & Hohorst (1963).

Measurement of glycerol and glycerol 3-phosphate dehydrogenase activities. *M. tuberculosis* BCG was grown and isoniazid (10 µg./ml.) was added as described earlier. After 6 hr. cells from treated and control flasks were harvested by filtration and were freeze-dried. Extracts were prepared usually by suspending 0.4 g. of dried cells in 8 ml. of buffer, exposing to 20 kc./sec. ultrasonic vibration from an MSE-Mullard 60 w generator for five successive 1 min. periods, the temperature being maintained below 10°, centrifuging at 20000g for 30 min. and discarding the sediment.

The tetrazolium assay system for dehydrogenase activity was based on the method of Lin, Koch, Chused & Jorgensen (1962). The assay mixture contained 0.3 ml. of extract (about 3 mg. of protein), 1 m-mole of phosphate buffer, pH 7.0, 100 µmoles of glycerol or glycerol 3-phosphate, 2 mg. of KCN, 0.1 mg. of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 0.1 mg. of phenazine methosulphate in a total volume of 3 ml. The reaction was timed from the addition of the phenazine and was followed for 20 min. at 550 mµ at 20°. Readings for blanks lacking substrate were subtracted from the analytical figures to

obtain dehydrogenase activities. A millimolar extinction coefficient 11 was used for the formazan.

The assay system for nicotinamide nucleotide-dependent glycerol and glycerol 3-phosphate dehydrogenase activities (EC 1.1.1.6 and EC 1.1.1.8 respectively) contained about 0.4 ml. of the extract, buffer, 200 µmoles of glycerol or glycerol 3-phosphate and 0.5 µmole of NAD⁺ or NADP⁺ in a total volume of 3 ml. When isoniazid was added its concentration varied from 30 to 300 µg./ml. The reaction was followed at 340 mµ, the temperature being 20° or 37°. Appropriate controls were done. Buffers tried in the assay system were: 0.2 M-potassium phosphate, pH 7.4; 0.03 M-sodium pyrophosphate, pH 9.0; 0.25 M-sodium carbonate-sodium bicarbonate, pH 9.5; 0.04 M-2-amino-2-methylpropane-1,3-diol, pH 9.0; 0.1 M-hydrazine buffer, pH 9.1; 0.1 M-hydrazine-0.2 M-phosphate, pH 7.4. The same buffer was usually employed in preparing the extract, which was done either by ultrasonic disintegration, as described above, or by grinding with Ballotini beads (no. 12) in a chilled mortar for 3 min.

Chemicals. These were all obtained from commercial sources, except for αα'-trehalose 6-phosphate and αα'-trehalose 6,6'-diphosphate, which were gifts from Dr D. G. Anderson, University of Wisconsin, Madison, Wis., U.S.A., and ββ'-trehalose, which was a gift from Professor S. Peat, University College of North Wales, Bangor.

RESULTS

General effects of isoniazid on the composition of mycobacteria. Table 1 shows some of the results of exposing growing *M. tuberculosis* to 10 µg. of isoniazid/ml. for various periods of time. Soon after exposure there was a marked increase in the amount (per unit of cell nitrogen) of sugar, combined phosphate and u.v.-absorbing substances in the soluble fraction. The concentrations of these substances reached after 6 hr. did not alter much over the subsequent 18 hr. A yellow colour also made its appearance in the soluble and lipid fractions, but this effect, unlike the other changes, did not occur when the medium was changed to Proskauer & Beck medium. The other cell components investigated by the methods described in this paper did not change, or changed little, relative to nitrogen within 6 hr. (Table 1). However, other fractionation techniques have revealed changes in some acid-insoluble fractions, particularly after longer periods of exposure to isoniazid.

The ratio of the cell insoluble nitrogen/ml. in cultures exposed to 10 µg. of isoniazid/ml. to that in control cultures was 0.986 ± 0.053 (s.e.m.) after 6 hr. exposure (seven experiments) and 1.007 ± 0.053 after 12 hr. (13 experiments). The 12 hr. figure indicates with 95% probability that rate of cell growth in terms of nitrogen was affected less than 25% within this period.

Reduction of the concentration of isoniazid to 0.5 µg./ml., which is not far above the minimum inhibitory value under the conditions of these

Table 1. Effect of exposure to isoniazid for various periods of time on the composition of Mycobacterium tuberculosis BCG growing in Sauton medium

Concn. of isoniazid (μg./ml.)	Period of treatment (hr.)	Soluble fraction				Lipid fraction			Nucleic acid fraction			
		Combined phosphate (μg atoms of P)	Carbo- hydrate (μmoles of glucose equiv.)	Substances absorbing at 260 mμ (μmoles of AMP equiv.)		Total lipid (g.)	Phospho- lipid (μg atoms of P)	Glyco- lipid (μmoles of glucose equiv.)	Yellow colour	RNA (μmoles of nucleotide)	DNA (μmoles of nucleotide)	Carbo- hydrate (μmoles of glucose equiv.)
		942	3540	205	Ratio of values to those of 1 hr. control	3.83	1400	1480	±	1280	1027	7000
0	1				±	0.95	1.05	1.16	±	0.99	0.98	1.06
0	6	1.01	1.07	1.05	±	0.97	1.01	1.14	+	1.02	1.06	1.14
10	0.5	1.12	1.01	1.01	±	1.00	1.22	1.23	+	1.06	1.03	1.19
10	1	1.48	1.05	1.10	+	0.95	1.24	1.26	+	1.11	1.17	1.08
10	2	1.49	1.13	1.19	+	0.95	0.96	1.06	+	1.10	1.02	1.14
10	4	1.60	1.36	1.47	+				+			
10	6	2.03	1.65	1.57	+				+			

The results in the first line (1 hr. control) are expressed per g. of insoluble cell nitrogen, and are derived from a typical experiment. The yellow colours were assessed visually and are given in arbitrary units. All other results are expressed as ratios of the values to the 1 hr. control values, and are means obtained from the results of three experiments.

experiments, did not appreciably alter its effects on the soluble fraction, except to reduce the intensity of the yellow colour.

When an isoniazid-resistant strain of BCG was used, 10 μg. of isoniazid/ml. did not have any effect on its composition within 12 hr. Even a concentration as high as 1 mg./ml. had only slight effects, and none on the concentration of soluble carbohydrate in the cell.

Growing *M. smegmatis* also responded to isoniazid by accumulating soluble carbohydrate, the rate of accumulation being similar to that in *M. tuberculosis*. Unlike the situation with *M. tuberculosis*, there was little evidence of accumulation of combined phosphate or of u.v.-absorbing substances. There was no appearance of yellow colour, but this may have been due to the use of Proskauer & Beck medium. Again, effects on the other cell components investigated were minor, except that the phospholipid content was reduced. The results were slightly less clear than for *M. tuberculosis*, because the growth rate of *M. smegmatis* was about four times as great, so that definite changes due to the slightly unbalanced growth took place in the composition of the controls during an experimental period of 6 hr.

Further examination of components of the soluble fraction. When the soluble fraction of *M. smegmatis* was fractionated with barium (Table 2) it was found that no sugar accumulation occurred in the barium-insoluble fraction, and little occurred in the barium-soluble ethanol-insoluble fraction. These findings agreed with the observation that little accumulation of combined phosphate occurred in this organism. Almost all the accumulation of sugar occurred in the barium-soluble ethanol-soluble fraction (Table 2) and this sugar was shown to be almost entirely trehalose. The small amount of free glucose which was present in this fraction was also increased by isoniazid treatment (Table 2).

In *M. tuberculosis*, also, none of the accumulated carbohydrate was barium-insoluble (Table 2). Again most of the accumulated material was barium-soluble, ethanol-soluble and only one sugar, trehalose, was detected in that fraction. However, this organism differed from *M. smegmatis* in having a substantial accumulation of sugar in the barium-soluble ethanol-insoluble fraction (Table 2). By the chromatographic methods described earlier, this material was divided into a series of further fractions, the main components of which were (i) a polysaccharide containing glucose as the only sugar, (ii) an oligosaccharide containing about four to six glucose residues, with about two of these phosphorylated in the 6-position, (iii) small amounts of a substance with properties of trehalose 6-phosphate, (iv) glucose 6-phosphate, (v) a mixture of sugar phosphates, including glucose 1-phosphate, fructose

Table 2. *Fractionation of the soluble carbohydrates from control and isoniazid-treated Mycobacterium smegmatis and Mycobacterium tuberculosis BCG*

M. tuberculosis was exposed to 10 µg. of isoniazid/ml. for 12 hr. in Sauton medium and *M. smegmatis* for 6 hr. in Proskauer & Beck medium. Fractionation with barium was carried out as described in the text. Polysaccharide and phosphorylated oligosaccharide fractions were determined by the anthrone reaction on the eluates from the first 6 cm. and from 8 to 14 cm. respectively, after two chromatographic steps as described in the text. The glucose 6-phosphate fraction and the glucose 1-phosphate fraction were measured by the anthrone reaction on the eluates from bands with $R_{\text{Glucose P}}$ 1.00 and 1.18 respectively, after two chromatographic steps as described. Trehalose was measured by the anthrone reaction on the eluate from the appropriate area of the chromatogram, after two chromatographic steps as described in the text with *M. tuberculosis* and one with *M. smegmatis*. No allowance has been made for losses in elution. Free glucose was determined by the glucose oxidase method, without chromatographic purification.

	Total carbohydrate in fraction			
	<i>M. tuberculosis</i>		<i>M. smegmatis</i>	
	Control (µmoles of glucose equiv./g. of insoluble N)	Isoniazid- treated	Control (µmoles of glucose equiv./g. of cell protein)	Isoniazid- treated
Total soluble fraction	3100	5590	778	1200
(a) Barium-insoluble fraction	169	162	13.0	9.0
(b) Barium-soluble ethanol-insoluble fraction	452	1171	65	71
(i) Polysaccharide fraction	31.0	144		
(ii) Phosphorylated oligosaccharide fraction	28.4	111		
(iii) Glucose 6-phosphate fraction	80.6	159		
(iv) Fraction containing glucose 1-phos- phate and fructose 6-phosphate	25.6	39.4		
(c) Barium-soluble ethanol-soluble fraction	2560	4310	588	1066
(i) Trehalose recovered	1215	2010	369	618
(ii) Free glucose in fraction	< 1	< 1	3.6	11.7

6-phosphate and perhaps arabinose phosphate (see the Discussion section). These fractions were increased when the bacteria had been exposed to isoniazid (Table 2). Assays by specific enzymic methods confirmed that glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate had accumulated after 3 hr. exposure to isoniazid, and probably after 1 hr. (Table 3).

The presence of glycerol and glycerol 3-phosphate was also detected. Isoniazid had no clear effect on the concentration of either of these (Table 3). The amount of dihydroxyacetone phosphate in the soluble fraction was so low that the figures obtained give only a general indication of concentration, so that the apparent effect of isoniazid must be regarded with reservation.

The increase in soluble u.v.-absorbing material which took place on exposure to isoniazid was associated with the barium-soluble ethanol-insoluble fraction. This material probably consisted at least partly of nucleoside diphosphate sugars. When an absorption spectrum of this fraction derived from isoniazid-treated cells was done with the same fraction from control cells as blank, the resulting difference spectrum was typical of a

uridine nucleotide (maximum at 257 mµ and a minimum at 239 mµ; E_{250}/E_{260} 0.92; E_{280}/E_{260} 0.42). When this fraction was chromatographed several distinct ultraviolet-absorbing peaks were obtained, all with similar spectral properties. It is possible that derivatives of isoniazid itself may account for some of the ultraviolet absorption, though it would be expected that such compounds would have extinction maxima at rather higher wavelengths than those observed.

The soluble yellow pigments which accumulated when *M. tuberculosis* growing on Sauton medium was exposed to isoniazid were precipitated in the barium-insoluble fraction.

Effect of isoniazid on the metabolism of glycerol. No evidence of more than very slight traces (less than 0.05 µmole/min./mg. of protein) of glycerol-dependent reduction of either NAD⁺ or NADP⁺ was found in extracts of control cells or of cells which had been exposed to 10 µg. of isoniazid/ml. for 6 hr. Addition of isoniazid to the assay system did not lead to increased glycerol-dependent reduction of NAD⁺ or NADP⁺, though the extinction changes due to isoniazid itself could have concealed very minor effects. A low activity of NADP⁺-dependent

Table 3. *Effect of exposure to isoniazid on concentrations of substances related to carbohydrate metabolism in Mycobacterium tuberculosis BCG as determined by enzymic methods*

Concn. of isoniazid ($\mu\text{g./ml.}$) Expt. (a)	Period of treatment (hr.)	Glycerol ($\mu\text{moles/g. of N}$)	Glycerol 3-phosphate ($\mu\text{moles/g. of N}$)	Glucose 6-phosphate ($\mu\text{moles/g. of N}$)	Glucose 1-phosphate ($\mu\text{moles/g. of N}$)	Fructose 6-phosphate ($\mu\text{moles/g. of N}$)	Dihydroxy-acetone phosphate ($\mu\text{moles/g. of N}$)
0	1	265	293	51.7		12.0	
10	1	250	243	57.6		15.9	
10	3	258	253	86.9		24.5	
Expt. (b)							
0	6	173	140	50.3	2.9	9.5	1.8
10	6	137	151	78.0	5.0	15.5	2.4

Table 4. *Effect of treating growing Mycobacterium tuberculosis BCG with 10 $\mu\text{g.}$ of isoniazid/ml. for 6 hr. on glycerol 3-phosphate dehydrogenase and glycerol dehydrogenase activities in cell-free extracts as measured by tetrazolium reduction*

The assay was carried out as described in the text. Dialysis was for 24 hr. at 4°.

Source of extract	Treatment of extract	Glycerol 3-phosphate dehydrogenase ($\mu\text{moles/min./mg. of protein}$)	Glycerol dehydrogenase ($\mu\text{mole/min./mg. of protein}$)
Control cells	—	2.32	0.01
Isoniazid-treated cells	—	2.54	0.03
Control cells	Dialysed	1.98	
Isoniazid-treated cells	Dialysed	2.18	
Control cells	Dialysed; NADP ⁺ added	2.36	
Isoniazid-treated cells	Dialysed; NADP ⁺ added	1.86	

glycerol 3-phosphate dehydrogenase (about 1.0 $\mu\text{mole/min./mg. of protein}$) could be shown under optimum assay conditions. Again isoniazid did not affect this activity.

Only a minute trace of glycerol dehydrogenase activity could be demonstrated by tetrazolium reduction applied to extracts of the control or isoniazid-treated cultures (Table 4). The extracts from control cells showed a capacity to reduce tetrazolium salts in the presence of glycerol 3-phosphate, most of which appeared to be nicotinamide nucleotide-independent (EC 1.1.99.5) (Table 4). Although treatment of the cells with isoniazid frequently resulted in extracts with this activity slightly increased, this increase also extended to some other enzymes and was possibly an indirect effect of isoniazid on enzyme extractability. Addition of isoniazid (100 $\mu\text{g./ml.}$) to the assay system had no effect on glycerol or glycerol 3-phosphate dehydrogenase activity.

DISCUSSION

Mycobacteria can vary considerably in composition from one batch of cultures to another, even when grown in the same medium and harvested at

the same point in the growth cycle. In spite of this variation between batches of cultures, young cultures invariably respond to the addition of isoniazid by accumulating soluble sugars as is described in this paper. Young cultures were employed in this work because the bacteria in them are in a state of high sensitivity to isoniazid (Mitchison & Selkon, 1956) and because they are in a state of approximately balanced growth with respect to composition, so that variation in composition within an experiment (for each of which a single batch of cultures was always employed) was usually small.

$\alpha\alpha'$ -Trehalose occurs in several esterified forms in mycobacteria (Asselineau, 1962) and the free sugar has been reported from *Mycobacterium phlei* (Pangborn & Anderson, 1933). The fact that such high concentrations of free trehalose occur in both *M. tuberculosis* and *M. smegmatis* indicates that it is an important storage carbohydrate in mycobacteria. Its biosynthesis is via trehalose 6-phosphate (Cabib & Leloir, 1958; Goldman & Lornitzo, 1962), so that the occurrence of the latter compound in *M. tuberculosis* is not unexpected. The phosphorylated oligosaccharide found, which contains about four to six glucose residues, of which about

two are phosphorylated in the 6-position, may be related to the phosphorylated polysaccharide of *M. tuberculosis* of which a sub-unit is trehalose 6,6'-diphosphate (Narumi & Tsumita, 1965). The arabinose phosphate found in the barium-soluble ethanol-insoluble fraction may be an artifact: Mandl & Neuberg (1952) have shown that fructose 6-phosphate can be converted into arabonic acid 5-phosphate in the presence of barium hydroxide and oxygen and hence it is possible that cleavage of fructose 6-phosphate to arabinose 5-phosphate occurred during the barium fractionation.

The findings with *M. smegmatis*, in which little except trehalose accumulates in the presence of isoniazid, led to the suggestion that the utilization of this sugar is specifically inhibited (Winder & Brennan, 1964). However, the subsequent finding, that in *M. tuberculosis* all the identified components of the soluble carbohydrate pool increase on exposure to isoniazid, indicates that no such simple conclusion can be reached. Presumably *M. smegmatis* possesses a greater capacity than *M. tuberculosis* to stabilize the concentrations of sugar phosphates.

It is possible that the increased endogenous respiration shown by *M. tuberculosis*, which has been exposed to isoniazid (Schaefer, 1960), which was interpreted as due to increased membrane permeability, is a consequence of an increased content of soluble carbohydrates, as would be expected from our findings.

There are three general ways in which isoniazid might bring about this accumulation of soluble sugars. First, it might inhibit the incorporation of sugar, as such, into other fractions, such as polysaccharides, glycolipids or glycoproteins. However, the results in this paper indicate that isoniazid does not necessarily reduce the carbohydrate content of the acid-insoluble fractions, which is against this hypothesis, and other investigations (Winder & Brennan, 1965, and unpublished work) lead to the same conclusion.

Secondly, it might stimulate the conversion of carbon compounds from the medium into carbohydrate. Glycerol is the main (though not the only) carbon source in the medium in this case and its main route of metabolism in mycobacteria appears to be by phosphorylation followed by dehydrogenation (Hunter, 1953; Winder & Brennan, 1966). The finding that isoniazid has little effect on the concentration of glycerol and glycerol 3-phosphate in *M. tuberculosis* suggests that isoniazid does not stimulate the uptake or phosphorylation of glycerol, and treatment with isoniazid does not appear to increase the dehydrogenase activity of the cells towards glycerol or glycerol 3-phosphate. The possibility that isoniazid might stimulate the conversion of amino acids, which also are present in

the medium, into carbohydrate has not yet been investigated.

Thirdly, isoniazid might inhibit carbohydrate catabolism or the conversion of products of this catabolism into either or both of the other major cell components, protein and lipid. The observed accumulation of soluble carbohydrate requires less than a 20% reduction in the rate of respiration or of synthesis of protein and lipid. Although most reports indicate that isoniazid has little effect on respiration unless its concentration is very high or the exposure prolonged (see Winder, 1964a), an inhibitory effect of the required size may not be excluded. The evidence on the effect of isoniazid on protein metabolism is conflicting (Gangadharam, Harold & Schaefer, 1963; Tsukamura & Tsukamura, 1963; Tsukamura, Mizuno & Tsukamura, 1964), but, with the exception of one report (Tomcsányi, 1965), it does not favour a specific effect on protein synthesis. The lack of change in the amounts of most cell components relative to total nitrogen, as reported in the present paper, also seems to be against a specific effect on protein synthesis, and could best be accounted for by assuming that isoniazid partially inhibits a reaction in some central area of metabolism, such as glycolysis, and that this leads to a fairly balanced reduction in the rate of synthesis of protein and lipid, with accumulation of the surplus carbon as soluble carbohydrate.

Results in this paper provide evidence that the changes here reported arise from the lesion which ultimately leads to the bactericidal action of isoniazid. These changes are brought about by a concentration not much above the minimal growth-inhibitory one; they are produced well before the bactericidal effect becomes apparent, which requires about 24 hr.; they, like its bactericidal effectiveness, are comparatively little affected by the drug concentration above the minimal value (Mackaness & Smith, 1953; Singh & Mitchison, 1954), and they are not observed when an isoniazid-resistant strain is used.

The finding that the accumulation of yellow pigments in *M. tuberculosis* on exposure to isoniazid is almost completely eliminated by changing the growth medium, although this does not alter the growth-inhibitory effect, argues against the relationship between the bactericidal action and the formation of these pigments which has been suggested (Youatt, 1961, 1962). The fact that the concentration of pigments is dependent on isoniazid concentration suggests that these may be derived from isoniazid itself.

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REFERENCES

- Asselineau, J. (1962). *Les Lipides Bactériens*, pp. 173-180, 232-258. Paris: Herman et Cie.
- Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
- Bergmeyer, H. U. & Klotzsch, H. (1963). In *Methods of Enzymatic Analysis*, p. 131. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Birch, G. G. (1963). *Advanc. Carbohydr. Chem.* **18**, 201.
- Bücher, T. & Hohorst, H. J. (1963). In *Methods of Enzymatic Analysis*, p. 246. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Brennan, P. & Winder, F. G. (1965). *Abstr. 2nd Meet. Fed. Europ. biochem. Soc., Vienna*, p. 75.
- Cabib, E. & Leloir, L. F. (1958). *J. biol. Chem.* **231**, 259.
- Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
- Dische, Z., Shettles, L. B. & Osnos, M. (1949). *Arch. Biochem.* **22**, 169.
- Gangadharam, P. R. J., Harold, F. M. & Schaefer, W. B. (1963). *Nature, Lond.*, **198**, 712.
- Goldman, D. S. & Lornitzo, F. A. (1962). *J. biol. Chem.* **237**, 3332.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Gurin, S. & Hood, D. B. (1939). *J. biol. Chem.* **131**, 211.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Hohorst, H. J. (1963a). In *Methods of Enzymatic Analysis*, p. 134. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Hohorst, H. J. (1963b). In *Methods of Enzymatic Analysis*, p. 215. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Horrocks, R. H. & Manning, G. B. (1949). *Lancet*, i, 1042.
- Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, ii, 368.
- Hunter, G. J. E. (1953). *Biochem. J.* **55**, 320.
- Lemieux, R. U. & Bauer, H. F. (1954). *Analyt. Chem.* **26**, 920.
- LePage, G. A. (1957). In *Manometric Techniques and Tissue Metabolism*, 3rd ed., p. 268. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
- Lin, E. C. C., Koch, J. P., Chused, T. M. & Jorgensen, S. E. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 2145.
- Mackness, G. B. & Smith, N. (1953). *Amer. Rev. Tuberc.* **67**, 322.
- Mandl, I. & Neuberg, C. (1952). *Arch. Biochem. Biophys.* **37**, 83.
- Mitchison, D. A. & Selkon, J. B. (1956). *Amer. Rev. Tuberc.* **74**, No. 2, part 2, 109.
- Narumi, K. & Tsumita, T. (1965). *J. biol. Chem.* **240**, 2271.
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Pangborn, M. C. & Anderson, R. J. (1933). *J. biol. Chem.* **101**, 105.
- Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
- Peat, S., Whelan, W. J., Edwards, T. E. & Owen, O. (1958). *J. chem. Soc.* p. 586.
- Renkonen, O. (1962). *Biochim. biophys. Acta*, **56**, 367.
- Schaefer, W. B. (1960). *J. Bact.* **79**, 236.
- Scott, T. A. & Melvin, E. H. (1953). *Analyt. Chem.* **25**, 1656.
- Singh, B. & Mitchison, D. A. (1954). *Brit. med. J.* i, 130.
- Tomcsányi, A. (1965). *Amer. Rev. resp. Dis.* **92**, 119.
- Tsukamura, M., Mizuno, S. & Tsukamura, S. (1964). *Jap. J. Microbiol.* **8**, 105.
- Tsukamura, M. & Tsukamura, S. (1963). *Jap. J. Tuberc.* **11**, 14.
- Winder, F. (1964a). In *Chemotherapy of Tuberculosis*, p. 111. Ed. by Barry, V. C. London: Butterworth and Co.
- Winder, F. (1964b). *Biochim. biophys. Acta*, **82**, 210.
- Winder, F. & Brennan, P. (1964). *Biochim. biophys. Acta*, **90**, 442.
- Winder, F. G. & Brennan, P. (1965). *Biochem. J.* **96**, 77 p.
- Winder, F. G. & Brennan, P. (1966). *J. Bact.* **92**, 1846.
- Winder, F. G. & Flynn, I. (1965). *Biochem. J.* **96**, 77 p.
- Winder, F. G. & O'Hara, C. (1962). *Biochem. J.* **82**, 98.
- Youatt, J. (1958). *Aust. J. exp. Biol. med. Sci.* **36**, 223.
- Youatt, J. (1961). *Aust. J. exp. Biol. med. Sci.* **39**, 93.
- Youatt, J. (1962). *Aust. J. exp. Biol. med. Sci.* **40**, 197.