I. J. Med. Sc.
Eighth Series. Vol. 3. No. 8
August, 1970

NOTE: This article was unfortunately printed in the June issue without the necessary tables. It is here reproduced in its entirety.

THE LIPIDS OF *MYCOBACTERIUM TUBERCULOSIS* BCG: FRACTIONATION, COMPOSITION, TURNOVER AND THE EFFECTS OF ISONIAZID

By

PATRICK J. BRENNAN SEAMUS A. ROONEY

and

FRANK G. WINDER

Department of Biochemistry and Laboratories of the Medical Research Council of Ireland, Trinity College, Dublin.

Introduction

The original purpose of the work recorded here was to confirm and elucidate certain reported effects of isoniazid on the lipids of growing mycobacteria.

Prolonged exposure of *Mycobacterium tuberculosis* to isoniazid has been found to reduce the amount of extractable lipid, particularly lipid solubilized by cold methanol[1]. It has been reported[2] that exposure of *Mycobacterium avium* to isoniazid for short periods leads to an increase in extractable lipid and to a decrease in the amount of fatty acid, with a reduction in the amounts of these fatty acids of chain length greater than C_{18} relative to the amounts of palmitic and stearic acids. There is also evidence that isoniazid produces an immediate slight inhibition of the incorporation of acetate into *M. tuberculosis* and a considerable inhibition after eight days[3], and there is also a report[4] that isoniazid inhibits the incorporation of acetate by cell-free extracts of *M. avium* into fatty acids, though this was not found with extracts of *M. tuberculosis* or *Mycobacterium smegmatis*[5].

In this paper we show that isoniazid has at least three distinct effects on the lipids of BCG. It reduces the amount of long chain fatty acids (above C_{16}) and increases the amount of shorter chain acids, which is similar to the effect reported by Ebina *et al.*[2] Isoniazid also reduces the incorporation of radioactivity into bound lipid and phospholipid. Finally, it considerably reduces the amount of triglyceride extracted with ethanol-ether in an Anderson-type fractionation[6,7], leaving an increased amount in the chloroform-soluble and bound lipid fractions. This redistribution effect is masked when a more vigorous method is used for lipid extraction. Some of these results have been published in preliminary form[8,9,10].

In the course of these investigations it became apparent that the Anderson type of lipid fractionation procedure is not ideal for quantitative studies

on changes in the lipid composition of mycobacteria. In this paper a comparison is made between such a procedure and a method based principally on that used by Brennan & Ballou[11].

Finally these investigations provided information on the rates of incorporation of radioactivity from ^{14}C-glycerol into several lipid fractions.

Experimental

Growth of the bacteria

The bacteria were grown, exposed to isoniazid and harvested, as described previously[12]. The glycerol concentration in the medium was normally 75.5 g/l.

Extraction and fractionation of lipids

Lipids were obtained from isoniazid-treated and control cells by one of the following methods.

In procedure A the cells from five flasks were pooled, harvested by centrifuging at about 2° and washed several times with 0.9% NaCl. To remove most of the water-soluble carbohydrate and glycerol which otherwise contaminated some of the lipid fractions, the cells were extracted for about 16 h with 50 ml of 30% (v/v) ethanol at about 2°. The pooled extracts provided the '30% ethanol-soluble fraction'. This treatment was found not to effect the subsequent extraction of the lipids. Lipids were then extracted by a simplified adaptation of the Aebi, Asselineau and Lederer[7] modification of the Anderson[6] procedure. The cells were treated with 50 ml of 1:1 (v/v) ethanol-ether for one week at room temperature in the dark, and then with two further volumes of 50 ml, each for 24 h.

The mixtures were separated by centrifuging. The pooled supernatants provided the 'ethanol-ether soluble lipids'. Continued extraction with this solvent under these conditions yielded negligible further lipid. The residue was extracted with 50 ml of chloroform for three days at room temperature in the dark, and then with two further volumes of 50 ml, for 48 h and 24 h. respectively. The mixtures were separated by centrifuging, and the chloroform layers were carefully pipetted from underneath the pellicles and pooled to provide the chloroform-soluble lipids. The residue was refluxed with 50 ml of 1:1 ethanol-ether containing 1% HCl for 1 h. After centrifuging and removing the supernatant the residue was extracted with 25 ml of ethanol-ether (1 : 1, v/v) and then with 25 ml of chloroform for 24 h. The pooled extracts provided the 'bound lipids'. The final cell residue was incubated with 20 ml of N KOH at 30° for 18 h and samples were taken for determination of carbohydrate, glycerol and total nitrogen.

The extract containing the ethanol-ether soluble lipids was evaporated to dryness. The residue was shaken with a mixture of 25 ml of ether and 5 ml of water. The aqueous layer was removed and combined with a further 5 ml aqueous washing of the ethereal layer to give the 'water washings'. The ethereal layer was concentrated by evaporation, two volumes of acetone were added and the mixture was left overnight at about 2°. The supernatant was separated by centrifuging, the residue was washed with ice-cold acetone, and the supernatant and washings were pooled to provide the

'neutral fat' fraction. The residue was refluxed with three portions of 25 ml of acetone, each time for 15 min and the supernatants were removed after centrifuging at 40°-50° and pooled to provide the 'wax A' fraction. The residue remaining after this treatment constituted the 'phospholipid fraction'. The chloroform-soluble lipids were not further fractionated. Usually the bound lipids were fractionated by evaporating to dryness, extracting with ether to give the ether-soluble fraction and dissolving the remainder in water to give a water-soluble fraction.

Procedure B was based on the method used by Brennan & Ballou[11]. Cells from twenty flasks were harvested by filtration, washed with 0.9% NaCl and extracted with 300 ml of chloroform-methanol-water (16:6:1, by vol.) by shaking for 24 h at room temperature. The suspension was filtered and the cell residue was re-extracted with the same solvent and finally with 300 ml of methanol. The filtrates after each extraction were pooled, dried in a rotary evaporator and washed by the method of Folch *et al.*[13] The lipids recovered are described as 'total soluble lipid'. The lipid was further fractionated into 'neutral' and 'phospholipid' fractions by the procedure of Hanahan *et al.*[14] based on the solubility of the neutral lipid in cold acetone.

The cell residue remaining after removal of total soluble lipid was refluxed with 300 ml of chloroform-methanol-water (16:6:1, by vol.) containing 1% HCl for 1 h and filtered. The residue was then re-extracted with chloroform-methanol-water (16:6:1, by vol.) and also filtered. The dried pooled filtrates were washed by the method of Folch *et al.*[13] The lipid from this treatment is called 'bound lipid'. The final delipidated cell residue was incubated with N KOH and samples removed for total nitrogen determination as described above.

Analytical methods

Lipids were deacylated by the method of Ballou, Vilkas and Lederer[15]. Total phosphorus was determined by the method described by Le Page[16], carbohydrate by an anthrone procedure[17], glycerol and triglyceride by the method of Renkonen[18], acyl groups by the method of Stern & Shapiro[19], and total nitrogen by a Kjeldahl method[20]. For determination of radioactivity samples were plated, dried, weighed and then counted for 10,000 counts using a Nuclear Chicago gas flow counter. In the determination of the dry weight of lipids the volume of solutions was reduced in a rotary evaporator, they were transferred to tared test tubes, reduced to near dryness at 45° and then dried *in vacuo* over P_2O_5 to constant weight.

Acid hydrolysis of lipids for qualitative determination of their composition was carried out in 2N HCl at 100° for 3 h. Hydrolysis products were chromatographed on sheets of Whatman 3 MM paper in ethyl acetate-pyridine-water (5:3:2, by vol.) (solvent A) or in ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.) (solvent B). Deacylated lipids were chromatographed in isopropanol-ammonia (2:1, by vol.) (solvent C). Products of lipid degradation were detected on paper chromatograms with a silver nitrate-sodium hydroxide dip reagent[21] or with an aniline-phthalate spray[22].

Thin layer chromotography was carried out on plates of silica gel—(0.5

mm thick) in the following solvents: — light petroleum-diethyl ether-acetic acid (85:15:2, by vol.) (solvent D), chloroform-methanol-7N ammonia (90:10:1, by vol.) (solvent E), chloroform-methanol-acetic acid-water (30:15:4:2, by vol.) (solvent F), light petroleum-diethyl ether (9:1, v/v) (solvent G). Triglycerides were also chromatographed on plates of silica gel H impregnated with 3% silver nitrate and developed in chloroform-benzene (9:1, v/v) (solvent H). Phospholipids were located with the molybdenum blue reagent[23], glycolipids with phenol-phosphoric acid[11] and phosphatidylethanolamine with ninhydrin. All other lipids were located with iodine, or in the case of preparative chromatograms with Rhodamine 6G or a water spray[24]. Lipids were recovered from silica gel by removing the zones and extracting with chloroform-methanol (1:2, v/v) and chloroform-methanol (2:1, v/v). The extracts were then dried and washed by the procedure of Folch *et al.*[13]

Lipids were saponified with ethanolic KOH[5]. Methyl esters of the resulting fatty acids were prepared with methanolic HCl[11], diazomethane[25], or by the methods of Metcalf & Schmitz[26]. These were analysed by gas-liquid chromatography on columns of polyethylene glycol adipate on 100-200 mesh Celite. Assignments for each peak were obtained from a semi-logarithmetic plot of relative retention times against chain length and degree of unsaturation of standard mixtures of fatty acid methyl esters. Peak areas were estimated by a disc integrator or by weighing the peak tracings.

Materials

Phosphatidylinositol was isolated from yeast[27]. Dimannophospho-inositide A and B, phosphatidylethanolamine and diphosphatidyl-glycerol were obtained from *Mycobacterium phlei*[11], the glycerylphosphoryl derivatives of these lipids were obtained by deacylation and preparative paper chromatography[28]. Methyl mycolate, glycerol monomycolate, wax D and cord factor from mycobacteria were gifts from Dr. J. Asselineau, University of Toulouse, France. Tri- and diglycerides were obtained from Sigma Chemical Company, London.

Results

The lipids of rapidly growing BCG, using an Anderson-type procedure

In all the work described in this paper the bacteria were grown under shaken conditions and were harvested at an early stage of the culture, when growth was approximately exponential. When the lipids were extracted from such cells by procedure A, the various fractions were obtained in the yields illustrated in Table 1. By far the largest single category of lipids was the bound lipids, with moderately large amounts of neutral fat and wax A fractions. The yield of chloroform-soluble lipids varied greatly from experiment to experiment. The material obtained in the water washings of the ethanol-ether extract was probably the residue of the water-soluble pool from the cells: when this pool was not removed by prior treatment with 30% or 50% ethanol, much more material was contained in the water washings.

TABLE 1

The weights, and glycerol, carbohydrate and phosphate contents, of the lipid fractions obtained from BCG by Procedure A, and the effects of exposure to 10 μg of isoniazid/ml thereon.

Isoniazid-treated and control cells were harvested after 3, 6 and 12 h and the lipid fractions isolated by Procedure A. Where the results are not given as percentages or ratios, they are expressed per g of total nitrogen in the cell residue. The weight of cell residue is an approximate figure derived from another experiment. The control values given are means derived from the 3, 6 and 12 h controls, but in obtaining the ratios the values for isoniazid-treated cells were divided by the values for the coresponding controls.

	Mean values for fractions from control cells						Ratios of content of fraction from isoniazid-treated cells to that from control cells											
		Glycerol		Carbohydrate		Phosphate	Weight			Glycerol			Carbohydrate			Phosphate		
							(h of exposure to isoniazid)											
Fraction	Weight mg/g of N	Total content (μmoles/g of N)	Percent by weight of the fraction	Total content (μmoles/g of N)	Percent by weight of the fraction	Total content (μmoles/g of N)	3	6	12	3	6	12	3	6	12	3	6	12
Ethanol-ether-soluble lipid																		
(i) water-washings	189	251	12.2	685	65.3	29	0.76	0.82	0.46	0.76	0.61	0.47	0.77	0.78	0.52			
(ii) neutral fat	832	760	8.4	57	1.2		0.89	0.87	1.03	0.88	0.86	1.08	2.73	0.86	0.34			
(iii) phospholipid	259	181	6.4	141	9.8	206	0.86	1.00	0.66	0.95	0.71	0.90	1.21	0.62	0.35	0.81	0.66	0.83
(iv) wax A	969	778	7.4	35	0.6	33	0.78	0.58	0.30	0.78	0.53	0.22	0.90	0.64	0.30	0.71	0.67	0.39
Chloroform-soluble lipid	508	460	8.4	18	0.6		1.38	1.85	1.56	1.44	1.98	1.27	0.60	0.75	1.50			
Bound lipid	3,747	1,351	3.3	2,660	12.8		1.01	0.88	1.00	1.09	1.10	1.42	0.95	0.66	0.85			
Total lipid	6,504	3,781	5.4	3,596	10.0		0.99	0.90	0.93	1.00	0.97	0.97	0.95	0.69	0.74			
30% Ethanol soluble	2,002			1,248	11.2	218	1.17	1.03	1.18				1.43	2.10	2.16	3.47	2.45	2.86
Cell residue	*c* 10,000	1,073	*c* 1.0	9,521	*c* 17.0					1.06	0.95	0.93	0.88	0.88	0.82			
Total of all fractions	*c* 18,500			14,365	*c* 14.0								0.96	0.93	0.91			

Table 1 also shows the glycerol and carbohydrate content of these fractions. The glycerol content was fairly constant at about 8% in most lipid fractions but was markedly lower in the bound lipids. The apparent presence of a small percentage of glycerol in the cell residue may have been due to interference in the glycerol assay by the large amount of carbohydrate present in this fraction.

The carbohydrate content was very low in the chloroform-soluble, wax A and neutral fat fractions, but high in the bound lipids and phospholipids and very high in the water washings. The fractions were hydrolyzed, the monosaccharides were separated by paper chromatography, located with aniline-phthalate and their amounts roughly estimated by comparison of the spots with a series of standard spots. In the water washings glucose was present, with very much smaller amounts of mannose and galactose. In the neutral fat and phospholipid fractions glucose and about one-fifth its amount of mannose were present. Traces of glucose were detected in the wax A fraction. Glucose and slightly smaller amounts of galactose and arabinose were found in the chloroform-soluble fraction. Arabinose was the dominant sugar in the bound lipid fraction, with slightly less galactose and about half as much mannose, and the final cell residue was similar in these respects. The residue contained also glucose when the cells had not been pre-extracted with 30% ethanol.

The neutral fat, wax A and chloroform-soluble lipid fractions obtained by procedure A were all found by thin-layer chromatography in solvent D to contain triglycerides as the major single component, while the bound lipid fraction contained only a trace of these. Free fatty acids and mycolic acids were found by the same means to be present in all of these fractions. Fatty acid esters, similar in properties to ethyl esters, were present in the neutral fat and bound lipid fractions, perhaps as artefacts formed during the extraction of these fractions. A considerable proportion of the lipid in all of these fractions remained at the origin during chromatography with solvent D. By the use of the more polar solvent E, this material was found

TABLE 2

The content of triglyceride in the lipid fractions from BCG, and the effects of exposure to isoniazid (10 μg/ml for 18 h) thereon.

Lipid fraction	Control cells mg of triglyceride/g of N	Isoniazid-treated cells Ratio of triglyceride content to that of control
Neutral fat	353	0.63
Wax A	177	0.28
Chloroform-soluble	255	0.78
Bound lipid	45	3.80

Each lipid fraction was chromatographed in solvent A. The triglyceride was located with iodine, eluted, hydrolysed and the glycerol estimated. Triglyceride was calculated on the basis of a molecular weight of 860.

TABLE 3

Fatty acid composition of the triglyceride from the lipid fractions of BCG and the effects of exposure to isoniazid (10 μg/ml for 18 h) thereon.

Cells	Lipid Fraction	Percentage by weight of fatty acids								Total medium chain fatty acids	Total long chain fatty acids
		$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{20:0}$	Others*	$C_{12:0}-C_{16:1)}$ †	$C_{17:0}-C_{26}$
Control	Neutral fat	0.4	3.7	30.5	5.3	17.9	31.7	6.5	4.1	45±3	55±3
Isoniazid		1.4	5.0	36.0	5.9	17.0	25.6	5.6	3.4	52±2	48±2
Control	Wax A	0.4	2.3	26.6	4.0	27.2	27.5	6.7	5.5	36±1	65±1
Isoniazid		1.3	4.7	30.2	12.4	19.6	25.0	2.6	4.1	52±1	49±1
Control	Chloroform-soluble lipid	0.2	2.4	27.8	3.5	36.0	13.1	10.5	6.4	38±3	63±3
Isoniazid		0.3	3.6	34.9	4.9	25.1	16.6	10.2	4.5	52±7	49±7
Control	Bound lipid	—	3.0	29.0	7.2	21.6	30.4	2.6	6.2	36±5	64±5
Isoniazid		—	3.4	33.3	7.5	22.3	22.6	6.0	4.8	42±4	59±4
Control	Total soluble lipid (Procedure B)	—	—	—	—	—	—	—	--	43	57
Isoniazid		—	—	—	—	—	—	—	--	57	43

* This consisted of two fatty acids. The major one of these, in the case of all fractions, had a retention time suggesting $C_{16:2}$ or $C_{17:0}$. The retention time of the minor component suggested $C_{14:2}$ or $C_{15:0}$.

† The mean and standard deviation for two separate experiments.

to include glycerol monomycolate (R_f 0.40) in the case of the neutral fat, wax A and chloroform-soluble fractions; while cord factor and/or wax D (R_f 0.18) and an unidentified compound (R_f 0.33) were also found to be prominent in the chloroform-soluble fraction.

The amount of triglyceride in these fractions was determined (Table 2). The results showed that the neutral fat fraction contained the largest amount of triglyceride but that substantial amounts were present in the wax A and chloroform-soluble fractions, confirming the impression given by qualitative chromatography.

The triglycerides isolated chromatographically from the various fractions were also saponified and their fatty acids were converted to methyl esters and analyzed by gas chromotography, using a system which would separate fatty acids in the range C_{10} to C_{26}. The results are summarized in Table 3. All the triglycerides seemed grossly similar in that $C_{16:0}$, $C_{18:0}$ and $C_{18:0}$ constituted the great bulk of these acids in each case. However, certain distinguishing features were observable. The ratio of $C_{18:0}$ to $C_{18:1}$ in the triglycerides of the neutral fat, wax A, chloroform-soluble and bound lipid fractions was 0.56, 0.99, 2.75 and 0.71 respectively. The ratio of the saturated fatty acids to unsaturated fatty acids was 1.60, 2.02, 4.64 and 1.50 in the same order. The triglyceride of the neutral fat fraction had the highest content of fatty acids below C_{17}, while that in the chloroform-soluble fraction had the highest content of $C_{20:0}$. These findings suggest that the distribution of the triglycerides between the different fractions, with the exception of the bound lipids, could be accounted for, in part as least, by the assumption that increased chain length and increased saturation decrease the extractability of triglycerides into ethanol-ether and into acetone. Obviously, the difficulty in extracting from the bacteria the triglycerides which appear in the bound lipid fraction cannot be explained in this fashion.

When the triglycerides from the various fractions were chromatographed on silver nitrate-impregnated silica gel plates in solvent H, which is reported[29] to separate triglycerides according to degree of unsaturation, each fraction yield two spots only. One of these had chromatographic properties similar to that of tripalmitin, and hence presumably contained saturated triglycerides only, while the other travelled between tripalmitin and triolein, suggesting triglycerides containing saturated and unsaturated acids.

Phosphate determination carried out on the various fractions derived from the ethanol-ether extract from the bacteria showed that most of the phosphate was present in the phospholipid fraction. Samples of the phospholipid were subjected to thin layer chromatography in solvent F. The pattern obtained was similar to that reported previously for *M. phlei*[11] and to that obtained for the phospholipids from procedure B, described below.

The lipids of rapidly growing BCG, using chloroform-methanol extraction

When the lipids were extracted by procedure B the results in Table 4 were obtained. This procedure gave a markedly higher yield of total soluble lipids than procedure A. The main cause of this difference appeared to be a more than ten-fold higher yield of phospholipids by procedure B than

TABLE 4

Yield of lipid fractions isolated from BCG by Procedure B and the effect of exposure to isoniazid (10 μg/ml for 18 h) thereon.

Treatment of the cells	Wet weight of cells (g)	Insoluble nitrogen (g)	Lipid				
			Water-soluble* (mg/g N)	Chloroform-methanol-soluble* (mg/g N)	Neutral lipid (mg/g N)	Phospholipid (mg/g N)	Bound lipid (mg/g N)
None	12.1	150.4	4,110	5,060	1,100	4,230 (41.8 mg P/mg N)	1,720
Isoniazid	11.6	169.6	4,060	5,070	1,160	4,050 (41.9 mg P/mg N)	1,240

* Material solubilized by extraction with chloroform-methanol-water (16 : 6 : 1, by vol.). The water-soluble material was contained in the aqueous layer of the Folch wash, while the chloroform-methanol-soluble material was in the lower organic phase.

procedure A. The total yield of neutral lipids was similar to the combined yields of the neutral fat, wax A and chloroform-soluble fractions by procedure A. The amount of bound lipids remaining after the extraction of soluble lipids by procedure B was substantially less than that after removal of soluble lipids by procedure A . Hence it appears that the great bulk of the phospholipids in this organism is not extracted into the 'phospholipid' fraction by procedure A, but remains with the bound lipids.

The neutral lipid fraction was examined further. Deacylation and paper chromatography (solvent B) showed that glycerol was the only prominent water-soluble product. Preparative thin-layer chromatography in solvent D and location of the constituent lipids with iodine, Rhodamine 6G or water showed several bands. A band with a R_f of 0.85 had mobility properties identical with those of the methyl esters of fatty acids. The major band had a R_f of 0.60, similar to that of a trigyceride such as tripalmitin. Minor bands of R_f values similar to those of free fatty acids (0.32) and diglyceride (0.20) were also present.

When the triglyceride was eluted from these plates, deacylated and subject to paper chromatography (solvent A), glycerol was the only water-soluble product. Analyses for acyl groups and for glycerol showed the presence of three of the former to one of glycerol. The total yield of triglyceride by this procedure was 654 mg per g of N and was similar to the total amount of triglyceride from the soluble lipids of procedure A (Table 2). Thin layer chromatography (solvent G) of the methyl esters of the triglyceride fatty acids showed the complete absence of methyl mycolate (R_f 0.43). The R_f obtained (0.78) was identical to the methyl stearate, indicating straight chain fatty acids. When these were separated by GLC the resulting picture was similar to that obtained with the triglycerides from the total soluble lipids of procedure A.

Hydrolysis of the phospholipid fraction and paper chromatography (solvent B) showed the presence of inositol, mannose, and glycerol. Unlike the phospholipid from procedure A it yielded no glucose. Thin layer chromatography of this material in solvents E and F, comparison of the R_f values of the resulting spots with those of markers, and examination of their staining reactions with Rhodamine 6G, molybdenum blue and ninhydrin showed the presence of cardiolipin, phosphatidylethanolamine, phosphatidylinositol and two dimannophosphoinositides[11]. Paper chromatography of the deacylated phospholipids (solvent C)and comparison with standards showed the presence of bis (glycerylphosphoryl) glycerol, glycerylphosphoryl ethanolamine, glycerylphosphoryl inositol, glycerylphosphorylinositol dimannoside, and small amounts of glycerylphosphorylinositol pentamannoside. The components of this fraction were in approximately the same proportions as they were in the phospholipid fraction from procedure A, although all in much larger amounts.

When the bound lipid fraction obtained by this procedure was chromatographed in solvent D, no triglyceride and only small amounts of free fatty acids were observed. However, most of the lipid remained at the origin in this solvent. Hydrolysis of the lipid and chromatography in solvents A and B showed only arabinose, while glycerol was entirely absent. Deacylation of the lipid and paper chromatography in solvents B and C. again showed mostly arabinose with small amounts of a slower moving compound, probably an arabinose-containing disaccharide[30].

The incorporation of ^{14}C from glycerol into lipids

In order to gain some information on the rate of synthesis of lipids in this organism, cultures were grown in glycerol-containing media in the usual fashion, a small amount of ^{14}C-glycerol of high specific activity was added while the cultures were still in rapid growth, and flasks were harvested after various periods.

The results of two such experiments are given in Table 5. The rate of uptake of radioactivity was lower in experiment 2. This was presumably due to the lower glycerol content in the medium, which may have resulted in decreased utilization of glycerol relative to the other carbon sources and in a slight decrease in the metabolic rate.

The generation time of the bacteria, in the usual medium at the stage of growth of the cultures used, is rather over 24 h. If a figure of 24 h be taken for convenience, than a fraction whose carbon content is the same as that of glycerol, which is derived directly from glycerol in the medium, and which does not turn over, would in 3 h have a specific activity 4.6% of that of the glycerol in the medium, in 6 h 9.5% of that of the glycerol, and in 12 h 20% of that of the glycerol. A higher carbon content and the existence of turnover would both tend to increase the specific activity over these values; while a lower carbon content, origin from the glycerol in the medium via a pool of intermediates, origin of part of the carbon from the asparagine or caesin hydrolyzate in the medium, and a longer generation time would all tend to reduce the specific activity.

The specific activity of the water washings of the ethanol-ether fraction indicated that the components of these washings turned over rapidly. This is in keeping with their representing a residue of the soluble pool (see below), as already mentioned.

The increase in specific activity of the neutral fat fraction was very rapid. This indicated a high rate turnover, particularly if the fatty acids were derived in part from sources other than glycerol. The increase in specific activity of this fraction continued in a linear fashion up to the stage when it had reached a value about 80% of that of the glycerol source.

The increase in the specific activities of the wax A and chloroform-soluble fractions was rather slower, but still indicative of a fair degree of turnover. The labelling of the chloroform-soluble fraction relative to the other fractions varied markedly between the two experiments, being relatively slower in the first experiment. The increase in specific activity of the phospholipids and the ether-soluble fraction of the bound lipids tended to be slightly slower again, while that of the bulk of bound lipids was slower still. However, even in the last case there was some evidence of turnover, even when allowance is made for a high carbon content and even if it be assumed that all of the carbon in the fraction originates from glycerol, which may very well not be the case.

The total radioactivity in the total lipids is compared at the bottom of Table 5 with that in the soluble fraction and cell residue. It can be seen that the total assimilation of glycerol was about equally divided between the lipids and the residue. The specific activity of the residue was not determined, but it usually contained at least 50% more dry weight than the total lipid, so that its specific activity must have been lower. The specific

TABLE 5

Incorporation of ^{14}C-glycerol into the lipids of BCG and the effects of 10 μg of isoniazid/ml thereon. The medium in Experiment 1 contained 75.5 g of glycerol/litre, while that in Experiment 2 contained 7.55 g/litre. ^{14}C-glycerol was added at the same time as the isoniazid, to give the glycerol in the medium a specific activity of 22.4 cpm/mg in Experiment 1 and 64 cpm/mg in Experiment 2.

Fraction	Mean size of fraction		Radioactivity in fraction Experiment 1						Radioactivity in fraction Experiment 2					
			Control			Isoniazid-treated			Control			Isoniazid-treated		
	Exp. 1	Exp. 2	1 h	3 h	6 h	1 h	3 h	6 h	3 h	6 h	12 h	3 h	6 h	12 h
	(mg/g N)		† Specific activity as a percentage of that of the glycerol in the medium											
Ethanol-ether soluble lipids														
(i) water washings	164	207	27	70	78	38	65	91	30	57	86	40	52	77
(ii) neutral lipid	656	909	18	38	77	23	50	88	18	34	77	19	37	75
(iii) phospholipid	269	437	6	13	34	3	6	17	9.3	19	43	8	13	33
(iv) wax A	1,234	1,258	10	26	57	13	29	49	10	22	49	9	20	43
Chloroform-soluble lipid	104	67	5	18	47	10	14	46	17	33	85	31	36	86
Bound lipid		3,550							7	12	26	6	10	17
(i) ether soluble	733	838	10	20	46	6	11	28	8	16	39	3	6	22
(ii) remainder	2,373		8	16	30	7	15	24	—	—	—	—	—	—
			total activity in fraction (cpm/mg of N)											
Total lipid	5,533	6,418							36.5	86.6	173.1	38.5	77.3	142.5
	μmoles glucose/g N													
Soluble fraction*	677	1,594	3.0	3.6	5.6	3.6	7.2	8.9	29.8	53.0	64.0	35.0	46.1	111.9
Cell residue			17.3	28.5	61.8	16.1	38.3	50.5	42.9	95.9	155.0	42.1	82.4	133.0

* The size of the 30% ethanol soluble fraction was based on the amount of soluble carbohydrate, determined as described previousiy[12].

† The specific activity of each fraction (cpm/mg of fraction) is expressed as a percentage of the specific activity of the glycerol in the medium (cpm/mg of glycerol).

activity of the soluble fraction is not given, since the presence of salts etc. in this fraction deprives such a value of much of its meaning. The uptake of total radioactivity into it showed saturation kinetics, indicative of an expected marked turnover. This was confirmed in the case of its major component, trehalose, through its isolation by paper chromatography[12] and direct determination of its specific activity. In experiment 1 this trehalose reached a specific activity of 86% of that of the glycerol in the medium in 3 h, proving rapid turnover.

The effects of isoniazid on the yield of total lipids and of lipid fractions from BCG

Exposure of growing cultures of BCG to 10 μg of isonazid/ml for periods of up to 18 h produced no marked alteration in the yield of total lipids obtained by either of the extraction procedures employed (Tables 1, 4 and 5). Further, when procedure B was used there was no marked alteration in the amounts of the individual fractions, with the exception of the bound lipids (Table 4).

When the lipids were fractionated by procedure A the results were rather variable but showed certain constant features which are referred to below. The results given in Table I were typical.

Exposure to isoniazid for a period of 6 h or more and sometimes in even shorter periods, produced a reduction in the yield of total ethanol-ether soluble material, with an approximately compensating increase in the yield of chloroform-soluble material. The total amount of bound lipid was relatively little affected, which, as is explained below, was the result of two opposing changes. The fall in the ethanol-ether soluble material was invariably accounted for mainly by a fall in wax A, with a smaller contribution from the water washings. A decrease was usually also shown by the phospholipid fraction, but the neutral fat fraction was affected much more erratically and was sometimes even increased.

The effect of isoniazid on the amount of carbohydrate in the lipid fraction was determined (Table 1). The percentage of carbohydrate in the wax A, chloroform-soluble and neutral fat fractions was so low that the assays probably were not reliable and little attention need be paid to apparent changes in these fractions. Isoniazid fairly consistently reduced the carbohydrate content of those fractions in which it could be reliably determined, leading to a reduced content in the total lipids. The fall in carbohydrate content was obviously responsible for the bulk of the decrease in weight of the water washings, and would have tended to reduce the content of bound lipids and phospholipids, but was obviously not responsible for the apparent redistribution of material between the wax A and chloroform-soluble fractions.

The effect of isoniazid on the glycerol content of the lipid fractions was also determined (Table 1). The changes that isoniazid produced in the amounts of glycerol in the lipid fractions closely paralelled its effect on the weight of these fractions. The amount of glycerol in the wax A fraction was markedly reduced and the amounts in neutral fat and phospholipid were affected less markedly and more erratically, while the amounts in the chloroform-soluble and bound lipid fraction were increased. The total

amount of glycerol in the combined lipid fractions was scarcely affected by exposure of the cells to isoniazid for up to 12 h.

The effects of isoniazid on the triglycerides of BCG

In order to extend the conclusions derived from the study of the effects of isoniazid on the yields and gross composition of the lipid fractions, these fractions were subjected to thin-layer chromatography and visual comparison was made between the patterns observed for the control cells and those for the isoniazid-treated cells. When the cells had been exposed to isoniazid for 6 h or more, there was a visually obvious decrease in the triglyceride content of the wax A fraction and, to a slight extent after longer periods of exposure, of neutral fat fraction. There was a corresponding increase in the amount of triglyceride in the chloroform-soluble and bound lipid fractions. This was particularly dramatic in the case of the bound lipid fraction, which normally contained only a trace of triglyceride. All of these effects increased up to 18 h, which was the longest period of exposure tried.

The conclusion arrived at from a visual inspection of these chromatograms were confirmed by a further experiment in which the triglycerides were eluted and determined (Table 2). The results show a fall in triglyceride in the wax A and neutral fat fractions and an increase in the triglyceride in the bound lipids, but this experiment was rather unusual in not showing an increase in the chloroform-soluble fraction.

No changes in other components of most of the fractions were obvious by this procedure except that, after the longer periods of exposure, slight changes in the amounts of fatty acids and fatty acid esters were found. In the neutral fat fraction there was slightly more of these components in the isoniazid-treated cells than in the control cells, while in the wax A fraction, which contained no fatty acid esters, there was a similar increase in the amount of free fatty acids in the isoniazid-treated cells. The bound lipid fraction from isoniazid-treated cells contained more fatty acid and less fatty acid esters than the fraction from control cells. Any effect that isoniazid had on the amount of the phospholipid fraction involved all components about equally, as judged by examination of chromatograms of the intact phospholipids or of their deacylation products.

In order to determine whether the effect of isoniazid in bringing about a redistribution of triglycerides between the fractions obtained by procedure A was due to its causing an alteration in the type of fatty acids incorporated into them, the fatty acid methyl esters of the triglycerides from all fractions were prepared and analyzed by gas-liquid chromatography. The results are shown in Table 3. Exposure to isonazid was found clearly to increase the total proportion of fatty acids from $C_{12:0}$ to $C_{16:1}$ relative to the total of the long chain fatty acids. With very few exceptions, each individual acid within these classes behaved in the same way as the total.

Thus, isoniazid did prove to have an effect on the fatty acid composition of the triglycerides, but this was in fact the opposite of the type of effect which would be required to account for the redistribution of the triglycerides in purely physical terms.

This effect of isoniazid on the fatty acid composition of triglycerides was confirmed with the triglycerides from the total soluble lipids prepared by

procedure B (Table 4). Further, it was not confined to the triglycerides: a similar effect was also found when the total fatty acids obtained by saponification of each of the crude fractions from procedure A were examined. Hence, treatment with isoniazid appears to result in a marked reduction in formation of fatty acids longer than C_{16}.

Effects of isoniazid on the incorporation of ^{14}C from glycerol into the lipids of BCG

When isoniazid was added at the same time as ^{14}C-glycerol to growing cultures of BCG, it had the effects which are illustrated in Table 5. The rate of increase in specific activity of the ether-soluble bound lipids was reduced by isoniazid within 1 h by nearly 50%, while its effect on the increase in specific activity of the phospholipid was about equally rapid. Isoniazid slightly reduced the specific activity of the remainder of the bound lipids and of the wax A after about 6 h. It caused initially a slight increase in the specific activity of the neutral fat, chloroform-soluble fraction and the water washings, which disappeared after a time.

When the total radioactivity in the lipids is considered it may be seen that isoniazid caused a slight increase in this for about 3 h. This was followed by a fall in the total radioactivity which was brought about partly by a slight reduction in total yield of lipids which occurred after 6 h and longer, as referred to earlier, and partly by the reduction in specific activities of the fractions referred to above. This may be compared with the situation in the cell residue, where some diminution in radioactivity occurred within 1 h, but was not appreciable until about 6 h had elapsed. In all cases there was an increase in the amount of radioactivity in the soluble fraction. The nett effect of these changes was that isoniazid caused an increase in the total radioactivity in the cells for about the first 3 h, but thereafter the situation was rather variable: in most experiments, as in experiment 1, a nett decline set in by about 6 h, but in some, such as experiment 2, very little nett change occurred for 12 h.

Discussion

There have been several publications on the lipids of BCG as obtained by Anderson-type procedures[31,32], although only Asselineau[34] has included young cultures and none has dealt with shaken cultures. The composition of our cultures as determined by a procedure of this type is in agreement with those described in these papers in that the bulk of the free lipid is found in the neutral fat and wax A fractions. A value for the bound lipid is quoted only in one publication[32] and corresponds fairly well with our figure for ether-soluble bound lipid.

However, up to ten-fold variations occur in published figures for phospholipids, while there are still larger variations in yield of chloroform-soluble lipids and even larger variations in sub-fractions of that fraction. Some of the variation is accountable for in terms of varying age and conditions of growth[32,34], but there is greater variation between the results of different workers using apparently similar experimental material[31,33]. We have noticed similarly large variations in the yield of the chloroform-sol-

uble fractions and to a less extent of the phospholipid fraction, between apparently identical cultures extracted in the same fashion. Further, the amount of phospholipid recovered by the Anderson-type procedure, although similar to the amount obtained by Asselineau[32], is only about one-tenth of the recovery obtained by using procedure B. This phenomenon is also obvious from the work of Akamatsu & Nojima[35]. In addition, as has previously been noted[36], several fractions contain common classes of components such as triglycerides and glycerol monomycolate.

Hence, although the extraction and fractionation procedures of Anderson[6] together with subsequent modifications[7] have proved of great value in structural investigations on the lipids of mycobacteria, they are far from ideal for analytical purposes. For these purposes chloroform-methanol extraction procedures[11,13], such as procedure B in the present paper, followed by chromatographic fractionation, are more suitable. However, since the Anderson-type fractionation uncovered some interesting phenomena, some of our results by this method have been reported in this paper.

Chloroform-methanol extraction shows that the major portion of the free lipids in cultures of BCG grown under our conditions are phospholipids and that these consist mainly of cardiolipin, phosphatidyl ethanolamine, phosphatidyl inositol and two of the dimannophosphoinositides[11].

Next to the phospholipids in amount, and much more prominent in most fractions obtained by the Anderson-type procedure, are triglycerides. With the Anderson-type procedure these are distributed between several fractions in a fashion which is related to their fatty acid composition and which may be in conformity with solubility properties, except for the small amount of triglycerides in the bound lipid fraction which have a composition in terms of fatty acids resolved by our gas liquid chromatography technique closely resembling that in the neutral fat fraction, suggesting that these 'bound' triglycerides may be retained by a permeability barrier. This is in keeping with the observations of Kotani *et al.*[37] who found a decrease in bound lipids and an increase in ethanol-ether soluble lipids after rupture of the cells by ultrasonic means.

It is generally reported that about one third of the non-hydroxylated fatty acids from human and bovine strains consist of fatty acids having 16 or less carbon atoms, one third having 18 or 19 carbon atoms, and one third having 20 or more[38,39,40]. Our results for the triglycerides may be compared with those of Agre & Cason[39] for the ethanol-ether soluble lipids of human strains, apparently from mature cultures. In both cases C_{16} fatty acids represent about one third. Our value of about one further third each for $C_{18:0}$ and $C_{18:1}$ is substantially higher than their 12% for the two combined, while their 22% for C_{19} straight and branched combined greatly exceeds the traces which we found. These differences may reflect differences between the fatty acid composition of triglycerides and that of other lipids of *M. tuberculosis*. For example, 10-methylstearic acid has been definitely identified only in the phosphoinositides of mycobacteria and appears to be mostly absent from other phosphoglycerides[11]. However, probably at least part of the differences arise from the fact that we used younger cultures for our studies than did most workers: Lennarz *et al.*[41] reported that in *Mycobacterium phlei* $C_{18:0}$ decreases with age of culture, while $C_{18:1}$ is replaced by 10-methylstearic acid as cultures age.

The bound lipids obtained after removal of the free lipids with chloroform-methanol-water contain arabinose as the only water-soluble component in appreciable amount and, thus, probably consist mainly of arabinose mycolate, which has been isolated from the bound lipids of *M. tuberculosis*[42,43] and which probably arises by partial hydrolysis of lipopolysaccharides of the cell envelope[37,44,45]. The galactose which accompanies arabinose in the bound lipids obtained by the other procedure is also likely to have originated in part from phospholipids not extracted by ethanol-ether but may also have originated in part from envelope polysaccharides[47,48].

The general impression conveyed by these results, indicating that a high proportion of the unbound lipids of these young cultures consist of triglycerides and phospholipids, is that several of the types of lipid which are regarded as characteristically present in large amount in mycobacteria tend to accumulate only in older cultures, at least in the case of BCG. This is illustrated by the low content of wax D found by ourselves and by other workers[32] using young cultures of BCG.

We suggest, on the basis of findings and of investigations into the cell walls of mycobacteria[37,44,47,48], that a substantial proportion of the total lipid of BCG consists of fatty acids, mainly mycolic acids, esterified to polysaccharides of the cell envelope. Fragments of these lipopolysacchardes are released by acid hydrolysis to constitute the 'bound lipid' fraction. It is suggested that the differences between the carbohydrate composition of the bound lipid fractions obtained by the two extraction procedures are due in part to the removal of some of the lipopolysaccharide from the cells in procedure B by chloroform-methanol-water. It is further suggested that in old cultures autolytic action and other changes convert part of the envelope lipopolysacchardes to wax D and other such materials.

The most striking result of the radioisotope experiments was the very rapid incorporation of ^{14}C from glycerol into the neutral fat fraction and the still rapid, though rather slower, incorporation into the wax A and chloroform-soluble fractions. In view of the high triglyceride content of these fractions, the result is a strong indication of a high rate of turnover of triglycerides, so that these cannot be thought of as a metabolically stable storage compounds, but may have a definite role as intermediary metabolites. The high rate of labelling of triglyceride by *Chlorella vulgaris* has been noted[49]. The turnover rate of at least the carbon of phospholipids is lower.

Isoniazid appears to have three distinct effects on the lipids of BCG, apart from its effect on the carbohydrate content of some of the lipid fractions which is probably related to its effects on other carbohydrate fractions from BCG[12,48]. First, it interferes with the incorporation of ^{14}C from glycerol into the 'ether-soluble bound' lipid and into the phospholipid fraction obtained by procedure A, suggesting that it interferes with the synthesis of major components of these fractions. The effect on incorporation into bound lipid is likely to be due at least in part to inhibition of the synthesis of mycolic acid which has recently been demonstrated[50]. Secondly, it decreases the ratio of fatty acids of chain lengths from C_{18} to C_{26} to those of chain lengths C_{12} to C_{16} in the triglycerides and probably also in the total lipids of BCG. This may be due to interference with an elongation mechanism, which may also be involved in mycolic acid synthesis. Thirdly,

it reduces the amount of triglyceride extracted with ethanol-ether in the Anderson-type procedure, leaving an increased amount in the chloroform-soluble and bound lipid fractions. This effect does not appear to be a consequence of the change in fatty acid composition of the triglycerides, since this change would be expected, if anything, to increase the triglyceride solubility in ethanol-ether. It may be a consequence of a change in properties of the cell envelope resulting from interference with mycolic acid synthesis.

Whether the above suggestions as to possible interrelationships between the three observed effects of isoniazid on the lipids of BCG are correct or not, it is unlikely that they are entirely independent of each other, since all appear after relatively short periods of exposure to isoniazid at a fairly low concentration. Similarly, it is likely that there is a common explanation for these effects and for the changes in soluble carbohydrates[12], certain polysaccharide fractions[48] and the cell content of nicotinamide nucleotides[21] which also result from isoniazid action. It is possible that all are related to changes in the cell envelope[48,50] and that these changes in the long run lead to loss of acid fastness and cell death.

Summary

1. The lipids in *M. tuberculosis* BCG derived from young shaken cultures in a glycerol-asparagine-casein hydrolyzate medium were extracted and fractionated by a modified Anderson method (procedure A) and by extraction with chloroform-methanol, followed by washing of the extracted lipids and their separation into neutral lipids and phospholipids (procedure B). In both cases bound lipids were subsequently extracted after refluxing with acidic solvents.

2. By procedure A, the washed ethanol-ether fraction was 2.0—2.6 g (per g of cell nitrogen), of which only about 0.25—0.45 g was phosphollipid. The chloroform-soluble fraction varied from 0.07 to 0.5 g, while the bound lipid fraction was 3.1—3.7 g, of which about 0.7—0.8 g was ether-soluble. Triglycerides were the major components of the neutral fat, wax A and chloroform-soluble fractions, and about 0.8 g of purified triglyceride could be recovered from these. The carbohydrate content of these three fractions was 1% or less.

3. Evidence was obtained that fractionation procedures based on the Anderson method are not satisfactory for analytical purposes.

4. By procedure B neutral lipid was about 1.1 g, phospholipid about 4 g and and bound lipid about 1.7 g. The composition of the phospholipid fraction obtained by both procedures was similar to that from *M. phlei.*

5. The fatty acids up to C_{26} from the triglycerides from the various fractions were separated by GLC. Although $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ were the major fatty acids in all cases, there were substantial differences between the fractions, and the distribution of triglycerides between the fractions in procedure A may be explicable largely in terms of fatty acid composition.

6. The rates of incorporation of ^{14}C from glycerol into the fractions obtained by procedure A indicated some degree of turnover in all fractions

and that it was particularly high in fractions with a high content of triglycerides, suggesting that these lipids have a role as metabolic intermediates.

7. Exposure of the cells to isoniazid had three effects on the lipids. It interfered with the incorporation of ^{14}C from glycerol into bound lipid and into the phospholipid fraction obtained by procedure A. It decreased the ratio of $C_{18}-C_{26}$ acids to $C_{12}-C_{16}$ acids. It reduces the amount of triglyceride which could be extracted with ethanol-ether, increasing the amount subsequently extracted with other solvents. Possible relationships between these effects, and between them and the effect of isoniazid on mycolic acid synthesis, are discussed.

Acknowledgments

We thank Miss Helen Gorman and Miss Alice Harrison for technical assistance. This work was supported by grants (AI-02842 and AI-07254) from the U.S. Public Health Service and by Arthur Guinness Son and Co. (Dublin) Ltd.

References

1. H. P. Russe and W. R. Barclay, Amer. Rev, Tuberc., 72 (1955) 713.
2. T. Ebina, M. Motomiya, T. Munakata and G. Kobuya, C. R. Soc. Biol., Paris, 155 (1961) 1176.
3. D. Koch-Weser, W. R. Barclay and R. H. Ebert, Amer. Rev. Tuberc., 71 (1955) 556.
4. T. Ebina, T. Munakata and. M Motomiya, C. R. Soc. Biol., Paris, 155 (1961) 1190.
5. F. G Winder, P. Brennan and C. Ratledge, Biochem. J., 93 (1964) 635.
6. R. J. Anderson, Fortschr. Chem. Org. Naturst., 3 (1939) 145.
7. A. Aebi, J. Asselineau and E. Lederer, Bull. Soc. Chim. Biol., 35 (1953) 661.
8. F. G. Winder and P. Brennan, Biochem, J., 96 (1965) 77P.
9. F. G. Winder and S. A. Rooney, Biochem. J., 103 (1967) 58P.
10. F. G. Winder and S. A. Rooney, Amer. Rev. Resp. Dis., 97 (1968) 938.
11. P. Brennan and C. E. Ballou, J. Biol. Chem., 242 (1967) 3046.
12. F. G. Winder, P. J. Brennan and I. McDonnell, Biochem. J., 104 (1967) 385.
13. J. Folch, M. Lees and G. H. Sloane-Stanley, J. Biol. Chem. 226 (1957) 497.
14. D. J. Hanahan, J. C. Dittmer and E. Warashina, J. Biol. Chem., 228 (1957) 685.
15. C. E. Ballou, E. Vilkas and E. Lederer, J. Biol. Chem., 238 (1963) 69.
16. G. A. Le Page, in W. W. Umbreit, R. H. Burris and J. F. Stauffer, Manometric Techniques, Burgess Publishing Co., Minneapolis, 2nd Ed., 1949, p. 185.
17. T. A. Scott and E. H. Melvin, Anal. Chem., 25 (1953) 1656.
18. O. Renkonen, Biochim. Biophys. Acta, 56 (1962) 367.
19. I. Stern and B. Shapiro, J. Clin. Pathol., 6 (1953) 158.
20. F. G. Winder and C. O'Hara, Biochem. J., 82 (1962) 98.
21. E. F. Anet and T. M. Reynolds, Nature, 174 (1954) 930.
22. C. S. Cummins and H. Harris, J. Gen. Microbiol., 14 (1956) 583.
23. J. C. Dittmer and R. L. Lester, J. Lipid Res., 5 (1964) 126.

24. M. E. Tate and C. T. Bishop, Can. J. Chem., 40 (1962) 1043.
25. S. R. Lipsky and R. A. Landowne, in S. P. Colowick and N. O. Kaplan, Methods in Enzymology, vol. VI, Academic Press, New York, 1963, p. 513.
26. L. D. Metcalf and A. A. Schmitz, Anal. Chem., 33 (1961) 363.
27. W. E. Trevelyan, J. Lipid Res., 7 (1966) 445.
28. Y. C. Lee and C. E. Ballou, Biochemistry, 4 (1965) 1395.
29. L. H. Morris, New Biochemical Separations, Van Nostrand, London, 1964, p. 295.
30. I. Azuma and Y. Yamamura, J. Biochem., 52 (1962) 200.
31. E. Chargaff, Hopp Seylers Z. Physiol. Chem., 217 (1933) 115.
32. J. Asselineau, These, Univ. Paris, 1950.
33. V. Portelance and M. Panisset, Rev. Canad. Biol., 16 (1957) 112.
34. J. Asselineau, Ann. Inst. Pasteur, 81 (1951) 306.
35. Y. Akamatsu and S. Nojima, J. Biochem., 57 (1965) 430.
36. J. Asselineau, The Bacterial Lipids, Holden-Day, San Francisco, 1966, p. 24.
37. S. Kotani, T. Kitaura, T. Hirano and A. Tanaka, Birken's J., 2 (1959) 129.
38. C. F. Allen and J. Cason, J. Biol. Chem., 220 (1956) 407.
39. C. L. Agre and J. Cason, J. Biol. Chem., 234 (1959) 2555.
40. J. Asselineau and E. Lederer in V. C. Barry, Chemotherapy of Tuberculosis, Butterworths, London, 1964, p. 10.
41. W. J. Lennarz, G. Scheuerbrandt and K. Bloch, J. Biol. Chem., 237 (1964) 664.
42. I. Azuma and Y. Yamamura, J. Biochem., 53 (1963) 275.
43. N.P.V. Acharya, M. Senn and E. Lederer, Compt. Rend., 264 (1967) 2173.
44. F. Kanetsuna, Biochem. Biophys. Acta, 158 (1968) 130.
45. A. Misaki and S. Yukawa, J. Biochem., 59 (1966) 511.
46. I. Azuma, Y. Yamamura and K. Fukushi, J. Bact., 96 (1968) 1885.
47. T. Imaeda, F. Kanetsuna and B. Galindo, J. Ulstrastruct. Res., 25 (1968) 46.
48. F. G. Winder and S. A. Rooney, Biochem J., 117 (1970) 355.
49. B. W. Nichols, A. T. James and J. Brueur, Biochem. J., 104 (1967) 486.
50. F. G. Winder, P. Collins and S. A. Rooney, Biochem. J., 117 (1970) 27 P.
51. F. G. Winder and P. Collins, Am. Rev. Resp. Dis., 100 (1969) 101.