CONSTITUENTS OF A "TOXIC-LIPID" OBTAINED FROM *MYCOBACTERIUM TUBERCULOSIS*

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In 1950 one of us[1] described the extraction from virulent tubercle bacilli of a lipid fraction which was toxic for mice upon repeated intraperitoneal injections. In view of the fact that no other constituents of the tubercle bacillus were known to have a similar degree of toxicity and because with the original method of surface extraction with petroleum ether used this fraction was obtained from only such bacterial strains as were able to multiply in a susceptible host organism, a great effort was made during the subsequent five years to analyse the chemical nature of the toxic fraction. The work was conducted as a co-operative project in which three different laboratories collaborated at one time or another. The results of this investigation have been published in a series of papers[2–7].

The toxic material had been termed "cord factor" because it was obtained from the surface of bacteria from cord-forming strains by superficial extraction with petroleum ether. This treatment disrupted the bacterial cords without killing the bacteria[1] and it was suggested that the toxic lipid freed from the bacterial surface was also responsible for holding the bacteria in a parallel arrangement in tight bundles. Cord formation had been described by MIDDLEBROOK, DUBOS AND PIERCE as a characteristic feature of virulent bacteria as contrasted with avirulent bacteria which did not form cords[8].

The chemical investigation of cord factor showed that the toxic lipid was identical with trehalose-6,6′-dimycolate[7]. This structure has recently been confirmed by synthesis[9,10]. In the course of this investigation more than 300 different lipid fractions were tested for toxicity. Separation of cord factor from other lipid components is sometimes a difficult process. Many fractions were found to contain traces of trehalose-6,6′-dimycolate but unless the compound was present in a fraction none of the many lipids obtained from at least a dozen mycobacterial strains had any toxicity at all.

It was therefore surprising when SPITZNAGEL AND DUBOS[11] described the isolation of a toxic lipid from tubercle bacilli which, according to them, was clearly different from cord factor. We were naturally interested to see whether we could have missed in our previous work a compound of such striking properties as that described by these authors and we proceeded to prepare a batch of "toxic lipid" following as precisely as possible SPITZNAGEL AND DUBOS' method.

The present paper describes these experiments. A product was obtained which

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according to all available information was identical with the material described by SPITZNAGEL AND DUBOS. This fraction was analyzed chemically. It was separated into six components, the chemical structure of which has been studied. One of the six components was trehalose-6,6'-dimycolate (cord factor). It was the only toxic constituent present in the fraction corresponding to SPITZNAGEL AND DUBOS' "toxic lipid".

EXPERIMENTAL

Bacterial cultures. The bacteria used in these experiments were of the original BCG strain used at the Pasteur Institute in Paris for the production of BCG vaccine*. They were harvested from Sauton surface cultures after four weeks growth and then dried over P_2O_5 *in vacuo* as described by SPITZNAGEL AND DUBOS[11].

Extraction and fractionation of the "toxic lipid". The dried BCG bacilli were extracted with monochlorobenzene as described by SPITZNAGEL AND DUBOS[11]; fractionation of the extract with petroleum ether following the directions of these authors gave a wax fraction ("fraction 7" of SPITZNAGEL AND DUBOS), m.p. 45–47°**, $[\alpha]_D = +14° \pm 2°$ ($CHCl_3$; $c = 3.20$), insoluble at +4° in petroleum ether in 1% yield; SPITZNAGEL AND DUBOS[11] give the following data for their "fraction 7": m.p. 49–51°, yield: 1%.

Chromatography of the "toxic lipid" on magnesium silicate. 7.508 g of "fraction 7" dissolved in 10 ml petroleum ether were chromatographed on 100 g magnesium silicate (Siegfried, Zofingen, Switzerland) mixed with 50 g Celite; for each elution 1000 ml solvent were used (Table I).

Fraction I. Elutions 3 to 6 were pooled and crystallized from petroleum ether. A white microcrystalline powder, m.p. 52–55°; $[\alpha]_D = +2° (\pm 1°)$ ($CHCl_3$,$c = 2.20$), was obtained; the following data establish that the substance is a triglyceride of two molecules of hexacosanoic acid and one molecule of palmitic or similar acid.

	% C	% H	
found	78.34	12.79	mol.wt. (Rast) 950
	77.93	12.83	
calculated for $C_{71}H_{138}O_6$***	78.39	12.78	mol.wt. 1087.83

The infrared spectrum shows the absence of hydroxyl groups, an ester band at 1720 cm^{-1} and bands at 1250 (w), 1175 (s) and 1105 (m) characteristic of triglycerides (SHREVE *et al.*[12]).

The substance, m.p. 52–55°, is neutral; saponification gave a product consisting for 92.5% of an acid fraction (calc. 91.6%) and a hydrosoluble portion devoid of sugars, containing 7.5% glycerol as determined by the method of HARVEY AND HIGHBY[13] (calc. 8.4%).

The acid was separated into two distinct fractions by dissolution in a small volume of ether and precipitation with 3 volumes methanol. The precipitate (65% of the acid fraction, corresponding to two molecules of hexacosanoic acid) was recrystallized from benzene and obtained as colourless crystals melting at 76°. The methyl ester, m.p. 60°, (methyl hexacosanoate, m.p. 62°) was analysed:

	% C	% H	% $(O)CH_3$
found	79.32	13.11	4.02
calculated for $C_{27}H_{54}O_2$	78.96	13.25	3.62

The methanol-soluble acid fraction melted at 47–48° (palmitic acid, m.p. 62°) and had a titration equivalent of 270 (palmitic acid 256); it gave a methyl ester melting at 25–26° (methyl palmitate 30.5°).

	% C	% H
found	76.62	12.72
	76.47	12.47
calculated for $C_{17}H_{34}O_2$	75.50	12.67

It seems that this fraction still contains a certain amount of hexacosanoic acid.

Fraction II. Eluate 10 was crystallized from petroleum ether and was obtained as a white

* We wish to thank Drs. J. TRÉFOUËL AND J. BRETEY of the Pasteur Institute for the bacterial cultures.

** All melting points were taken on the Kofler block and are corrected.

*** Triglyceride containing two molecules of hexacosanoic and one molecule of palmitic acid.

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microcrystalline powder melting at 52–54°, $[\alpha]_D = +3° \pm 1°$ ($CHCl_3$, $c = 3.06$). This substance was neutral.

	% C	% H	
found	76.73	12.21	mol. wt. (Rast) 884, 876
	77.08	12.62	
calculated for $C_{51}H_{89}O_6$*	75.87	12.24	mol. wt. 807

The infrared spectrum shows the absence of hydroxyl groups, the presence of an ester band at 1720 cm^{-1} and of the triglyceride bands mentioned above.

Saponification of this substance gave 95% acid, the greatest part of which (85%) was soluble in methanol and melted after recrystallization from benzene at 47–48°.

	% C	% H	
found	75.23	12.63	mol. wt. (titration) 270
calculated for $C_{16}H_{32}O_2$	74.94	12.58	mol. wt. 257

These data indicate that this acid is a mixture of homologs with a mean molecular weight somewhat higher than that of palmitic acid.

The hydrosoluble portion contained 9.2% glycerol, as estimated by the method of HARVEY AND HIGHBY[13]. Fraction II seems to be essentially a triglyceride containing palmitic and higher acids.

Fraction III. Eluates 11 and 12 were pooled and recrystallized in cold ether; we thus obtained a microcrystalline powder with a diffuse melting point: 54–62°, $[\alpha]_D = +5° \pm 1°$ ($CHCl_3$; $c = 1.93$). This substance was also neutral.

	% C	% H
found	77.38	12.40
	77.29	12.18
calculated for $C_{55}H_{108}O_5$**	77.58	13.02

The infrared spectrum shows an –OH band at 3400 cm^{-1} and an ester band at 1720 cm^{-1}.

The product of saponification was 91% acid and this acid fraction consisted for two-thirds of a methanol-insoluble acid, melting at 75° (titration equivalent 420, calculated for $C_{26}H_{52}O_2$: 396.7) and resembling the corresponding acid of fraction I.

	% C	% H
found	79.20	13.01
calculated for $C_{26}H_{52}O_2$	78.72	13.21

The methanol-soluble acid melted again at 47–48°, as above. The estimation of glycerol in the hydrosoluble portion gave 11.9%. (Theoretical value for a diglyceride of hexacosanoic acid: 10.8%.) This fraction thus seems to be a mixture of diglycerides containing principally hexacosanoic acid.

Fraction IV. Eluates 17 and 18 were pooled and crystallized from cold petroleum ether; we thus obtained a microcrystalline powder melting at 50–53°, $[\alpha]_D = +8° \pm 2°$ ($CHCl_3$, $c = 2.13$). The substance was neutral.

	% C	% H
found	79.04	12.94
	79.33	13.17
calculated for $C_{107}H_{212}O_7$***	79.78	13.22

The infrared spectrum of this substance shows an –OH band at 3400 cm^{-1} and ester bands at 1720 cm^{-1}.

The product of saponification was 94% acid and this acid fraction consisted for about 75% of a methanol-insoluble acid melting at 54–55°, which was identified with mycolic acid by its titration equivalent (found 1,100, calculated 1,298) and infrared spectrum.

	% C	% H
found	81.22	13.32
calculated for $C_{88}H_{176}O_4$	81.40	13.66

The hydrosoluble portion contained 6.9% glycerol, as estimated by the method of HARVEY AND HIGHBY[13] (calculated 5.7%).

* Tripalmitine, m.p. 65°.

** Diglyceride of hexacosanoic acid.

*** Diglyceride containing one molecule of mycolic acid $C_{88}H_{176}O_4$ and one molecule palmitic acid.

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These results show that fraction IV is essentially a diglyceride (or a mixture of diglycerides) containing one molecule of mycolic acid and one of a lower molecular weight fatty acid.

Fraction V. Eluate 19 was crystallized from petroleum ether at 0° and was obtained as a white microcrystalline powder melting at 45–46°, $[\alpha]_D = +10° \pm 1.5°$ ($CHCl_3$ or benzene; $c = 2.20$); the following data establish its structure as an α-monoglyceride of mycolic acid.

	% C	% H
found	79.84	13.22
	79.49	12.92
calculated for $C_{91}H_{182}O_6$	79.63	13.36

The infrared spectrum of this substance shows an –OH band at 3330 cm^{-1}, an ester band at 1680 cm^{-1} and C–O bands at 1170 (m), 1100 (w) and 1050 (m) typical for monoglycerides[12].

Oxidation with lead tetraacetate shows the presence of a 1,2 glycol. 42 mg glycerol monomycolate dissolved in 1 ml benzene were added to 2 ml glacial acetic acid and 1 ml of a 0.08 *N* solution of lead tetraacetate. The solution was kept 2 hours at 70°. After cooling, 4 ml of a 5% solution of KI were added and the excess oxidant titrated with 0.1 *N* sodium thiosulfate. 0.03 ml hyposulfite were consumed, whereas a blank consumed 0.78 ml. The difference (0.75 ml) corresponds to 1.2 moles of oxidant (for a mol.wt. of 1,372).

Saponification of this substance gave a product consisting for 92.3% of an acid fraction (calc. 93.1%), melting after precipitation from ether-methanol at 53–55°.

	% C	% H	% $(O)CH_3$	
found	81.12	13.34	1.02	titration equiv. 1,290
calculated for $C_{88}H_{176}O_4$	81.40	13.66	1.16	mol.wt. 1,298

The infrared spectrum of this acid is characteristic of a mycolic acid. The hydrosoluble portion contained 6.0% glycerol as estimated by the method of HARVEY AND HIGHBY[13] (calc. 6.9%).

The absence of glycerides of low molecular weight fatty acids in this fraction was indicated by the negative reaction with hydroxylamine using the method of RAPPORT AND ALONZO[15] (mycolic esters of glycerol or of sugars do not give hydroxamates with this reagent).

Fraction VI. Eluate 28 was precipitated from ether-methanol and 414 mg were then chromatographed on a column of 10 g silica gel Davison; 291 mg were eluted immediately with petroleum ether; after dissolution in ether and precipitation with methanol, a white powder was obtained, m.p. 43–44°, $[\alpha]_D = +38° \pm 3°$ ($CHCl_3$; $c = 1.40$). Cord factor of BCG has a m.p. 43–45° and $[\alpha]_D = +40°$[7].

	% C	% H	% $(O)CH_3$
found	77.84	12.25	0.8
			0.6*
calculated for $C_{186}H_{366}O_{17}$	77.70	12.83	0.52

The infrared spectra of fraction VI and cord factor of BCG are shown in Fig. 1.

Saponification of fraction VI gave a product consisting for 88% (theor. value 87.5%) of a substance identified as mycolic acid by m.p. (51–53°), titration equivalent (1,290) and elementary analysis.

	% C	% H	% $(O)CH_3$
found	81.70	13.36	0.79
calculated for $C_{88}H_{176}O_4$	81.40	13.66	1.16

The hydrosoluble portion (11.6%; theor. value 11.9%) had an optical rotation of $[\alpha]_D = +222° \pm 20°$ (theor. value for trehalose $+197°$).

Toxicity tests. For these experiments, both Albino CF1 mice (Carworth Farms, New City, N.Y.) and C57 Black mice (Jackson Memorial Laboratories, Bar Harbor, Me.) were used. The material to be tested was dissolved either in Bayol F (light paraffin oil) or in olive or sesame oil. We have previously used these oils for toxicity tests in mice and found that the toxicity of all toxic fractions decreases slightly with the use of the three oils in this order.

The fractions were injected either intraperitoneally or intravenously. For the intraperitoneal injections 0.05 or 0.1 ml of the clear oily solutions were injected once or repeatedly. For intravenous injections, emulsions were prepared containing the desired amounts of lipid to be tested dissolved in oil and emulsified in a solution of Tween-80 in saline. The final concentration of oil was 5%, that of Tween-80 1%. The described amount of lipid was contained in 0.1 ml of this emulsion, which was injected intravenously.

* It is to be noted that this cord factor contains one or two methoxyl groups whereas the cord actor of BCG described previously[7] was devoid of methoxyl.

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RESULTS

A. The chemical nature of the "toxic lipid"

Chromatographic separation of the "toxic lipid" ("fraction 7" of SPITZNAGEL AND DUBOS[11]) gave six distinct fractions (Table I).

The first five fractions are glycerides; the presence of glycerol in the hydrosoluble portion obtained after saponification has been checked by the colorimetric method of HARVEY AND HIGHBY[13]. The figures found are in agreement with the structures assigned to each fraction.

TABLE I

Chromatography of 7.508 g "toxic lipid" on 100 g magnesium silicate + 50 g Celite; 1000 ml solvent per elution.

Eluate No.	*Solvent*	*Weight of eluate*
1	petroleum ether	0 mg
2	petroleum ether	0 mg
3	petroleum ether-benzene 9:1	163 mg
4	petroleum ether-benzene 9:1	941 mg
5	petroleum ether-benzene 9:1	339 mg
6	petroleum ether-benzene 9:1	210 mg
7	petroleum ether-benzene 9:1	94 mg
8	petroleum ether-benzene 9:1	126 mg
9	petroleum ether-benzene 9:1	28 mg
10	petroleum ether-benzene 1:1	505 mg
11	petroleum ether-benzene 1:1	189 mg
12	petroleum ether-benzene 1:1	162 mg
13	petroleum ether-benzene 1:1	107 mg
14	petroleum ether-benzene 1:1	76 mg
15	petroleum ether-benzene 1:1	52 mg
16	petroleum ether-benzene 1:1	38 mg
17	benzene	182 mg
18	benzene	68 mg
19	benzene-ether 9:1	2509 mg
20	benzene-ether 9:1	358 mg
21	benzene-ether 9:1	142 mg
22	benzene-ether 9:1	80 mg
23	benzene-ether 9:1	45 mg
24	benzene-ether 1:1	41 mg
25	benzene-ether 1:1	13 mg
26	ether	14 mg
27	ether	7 mg
28	ether-methanol 8:2	531 mg
29	ether-methanol 8:2	50 mg
30	ether + 1% acetic acid	151 mg
31	ether + 1% acetic acid	39 mg
	Total weight	7260 mg = 96.7%

Fraction I (eluates 3–6), m.p. 52–55°, $[\alpha]_D = +2°$, is a triglyceride containing two molecules of hexacosanoic acid and one molecule of palmitic or an analogous acid; a similar triglyceride has been reported by NOLL[14] in wax C fractions of various strains of mycobacteria.

Fraction II (eluate 10), m.p. 52–54°, $[\alpha]_D = +3°$, is a triglyceride containing mostly palmitic or similar acids.

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The infrared spectra of fractions I and II are in agreement with the assigned structure [no –OH band, ester band at 1720 cm^{-1} and C–O absorption bands at 1250 (w), 1175 (s) and 1105 (m) ascribed to the triglyceride structure by SHREVE *et al.* [12].

Fraction III (eluates 11, 12), m.p. 54–62°, $[\alpha]_D = +5°$, is a mixture of diglycerides containing mostly hexacosanoic acid.

Fraction IV (eluates 17, 18), m.p. 50–53°, $[\alpha]_D = +8°$, is a diglyceride (or mixture of diglycerides) containing one molecule of mycolic acid, m.p. 54–55°, and one molecule of palmitic or analogous acid.

The infrared spectra of fractions III and IV show an –OH band at 3400 cm^{-1} and ester bands at 1720 cm^{-1}.

The more exact identification of the fatty acids present in fractions I to IV will be reported later.

Fraction V (eluate 19), m.p. 45–46°, $[\alpha]_D = +10°$, is an α-monoglyceride of mycolic acid, $C_{91}H_{182}O_6 \pm 5\ CH_2$; the mycolic acid is a 3-hydroxy, *x*-methoxy mycolanoic acid*, $C_{88}H_{176}O_4 \pm 5\ CH_2$, m.p. 53–55°, and has been identified by m.p., elementary analysis, titration equivalent (1,290) and infrared spectrum.

Fraction V is identical with fraction B_1 isolated and identified by NOLL[14] in the wax C fractions of various strains of mycobacteria. NOLL concluded from the optical rotation of this compound (his preparation had $[\alpha]_D = +6.4° \pm 2.5°$) that part of the optical activity must be due to the β-carbon of glycerol and that therefore this substance must be an α-monoglyceride. We have confirmed this experimentally by oxidation with lead tetraacetate; 1.2 molecules of the oxidant were consumed, in agreement with the assigned structure. The synthesis of an α-monoglyceride of mycolic acid identical with the natural compound is reported in a separate paper (DEFAYE AND LEDERER[17]**).

Fraction VI (eluate 28) has been identified with the cord factor of the BCG strain, described by NOLL *et al.*[7]; this identification is based on melting point (43–44°), optical rotation ($[\alpha]_D = +38 \pm 3°$), elementary analysis and infrared spectrum which is identical to that of cord factor (Fig. 1)***. This identification is confirmed by the results of the saponification and also of the toxicity tests described in the following section.

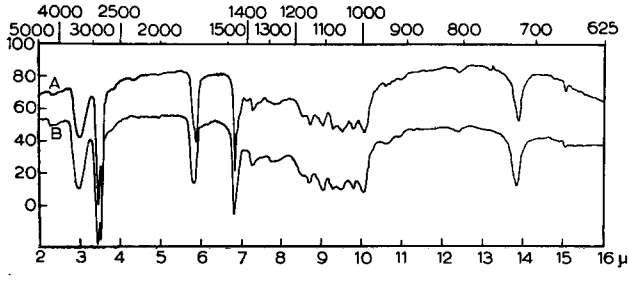

Fig. 1. Infrared spectra; substances resolidified after melting: A. fraction VI of "toxic lipid"; B. cord factor of BCG.

* For the nomenclature of mycolic acids, see ASSELINEAU AND LEDERER[16].

** The synthetic ester has $[\alpha]_D = +9° \pm 1.5°$, despite an optically inactive β-carbon of glycerol; this shows that the above argument is not valid.

*** A slight difference should be mentioned however: the mycolic acid of the previously described cord factor was methoxyl-free, whereas the mycolic acid of the above mentioned cord factor is partly methoxylated.

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TABLE II

EFFECT OF INTRAPERITONEAL AND INTRAVENOUS INJECTIONS OF CRUDE "TOXIC LIPID"

No. of mice per group	*Material injected*	*No. of injections*	*Route of injection*	*Amount injected per dose (mg)*	*Loss of weight*
10	"Toxic lipid"	1	i.p.	0.5	++
10	"Toxic lipid"	1	i.p.	0.1	++
10	"Toxic lipid"	1	i.p.	0.01	+
10	"Toxic lipid"	5	i.p.	0.5	++
10	"Toxic lipid"	5	i.p.	0.1	++
5	"Toxic lipid"	5	i.p.	0.03	+
5	"Toxic lipid"	5	i.p.	0.01	—
5	"Toxic lipid"	5	i.p.	0.003	—
10	"Toxic lipid"	1	i.v.	0.1	++
10	"Toxic lipid"	1	i.v.	0.05	++
10	"Toxic lipid"	1	i.v.	0.025	+
10	"Toxic lipid"	1	i.v.	0.012	—
10	cord factor	1	i.p.	1.0	++
10	cord factor	1	i.p.	0.1	++
10	cord factor	5	i.p.	0.1	++
10	cord factor	5	i.p.	0.01	++
10	cord factor	5	i.p.	0.005	++
10	cord factor	1	i.v.	0.1	++
10	cord factor	1	i.v.	0.01	++
10	cord factor	1	i.v.	0.005	++

B. Toxicity tests

a. *Crude "toxic lipid"*. The crude "toxic lipid" corresponding to "fraction 7" of SPITZNAGEL AND DUBOS[11] was tested for toxicity both upon single and repeated intraperitoneal injections. The mice were weighed daily. Three weeks after the first injection the experiments were discontinued. Table II summarizes the results of these tests. The weight losses are characterized by the following symbols: + if the maximum loss of weight was within 10% of the body weight before injection, ++ if between 10 and 20% of the original body weight was lost and — if no loss of weight occurred.

The results indicate that one single intraperitoneal injection of "toxic lipid" was not lethal for mice. C57 Black mice were used for the intraperitoneal, and albino mice for the intravenous injection.

The loss of weight occurring after one single injection was considerable. Depending on the dose, it took from three to seven days for the mice to recover. After repeated injections the mice died from the effects of "toxic lipid". The symptoms, of which the profuse pulmonary hemorrhages were the most conspicuous, were the same as have been observed after injections of cord factor. These observations have been repeatedly described in detail and seem to have been confirmed by SPITZNAGEL AND DUBOS[11].

Indeed, the observations with the "toxic lipid" were so similar to the ones made with cord factor that analogous experiments with this compound were carried out at the same time and are noted for comparison in Table II. These tests confirm the previously published data.

b. *Individual fractions*. The biological findings noted in Table II suggested that the toxic component of the "toxic lipid" was identical with, or very similar to

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OF CORD FACTOR IN C57 BLACK MICE

Days after first injection on which mice died	*No. of survivors after 21 days*
—	10
—	10
—	10
5, 8, 10, 10, 15	5
14. 16, 17	7
8	4
—	5
—	5
7, 9, 11	7
—	10
—	10
—	10
6	9
—	10
7, 8, 8, 10, 10, 11, 13, 13, 16, 16	0
8, 8, 9, 10, 10, 13, 15	3
9, 11, 12, 12, 13	5
7, 7, 7, 8, 8, 9, 9, 9, 10, 11	0
7, 7, 8, 8, 9, 9, 13, 13, 16	1
9, 11, 13, 16	6

trehalose-6,6′-dimycolate. As described in the experimental section of this paper, it was possible to separate the "toxic lipid" into six distinct fractions, of which fraction VI appeared to be cord factor. The toxicity tests of these six fractions are recorded in Table III. Fractions I to V were completely devoid of toxicity, whereas fraction VI had the characteristic toxicity of cord factor.

The "toxic lipid" (or for that matter cord factor) was not lethal after one single intraperitoneal injection. The few rare instances such as those listed in Tables II and III are not regularly reproducible effects. This characteristic feature of the cord factor toxicity has been stated many times before and no explanation has been found for this peculiarity. The possible reasons have been discussed previously. However, it has now been found that with the intravenous route of injection it is possible to kill mice with one injection. Death still occurs after a delay of one to two weeks. The symptoms are similar to those observed after repeated intraperitoneal injections, except of course that there is no peritonitis. Intravenous injections of the blank emulsion were always well tolerated, and so were control injections of as much as 2.0 mg of various non-toxic, chemically related lipids such as mycolic acid and certain esters of mycolic acid.

TABLE III

EFFECTS PRODUCED BY ONE INTRAVENOUS INJECTION OF "TOXIC LIPID" OR ITS CONSTITUENTS IN C_{57} BLACK MICE

Material injected	*Dose (mg)*	*Survival time of individual mice (days after injection)*	*Number of survivors*	*Loss of weight*
Crude "toxic lipid"	0.1	7, 9, 11	7/10	++
Crude "toxic lipid"	0.05	—	10/10	++
Fraction I	0.1	—	8/8	0
Fraction II	0.1	—	8/8	0
Fraction III	0.1	—	8/8	0
Fraction IV	0.1	—	8/8	0
Fraction V	0.1	—	8/8	0
Fraction VI	0.1	6, 7, 9, 9, 10, 10, 11, 11	0/8	+++
Fraction VI	0.05	7, 7, 8, 8, 10, 10, 11, 11	0/8	+++
Cord factor	0.1	6, 6, 6, 7, 7, 8, 8, 11	0/8	+++
Cord factor	0.05	6, 8, 9, 9, 9, 10, 10, 11	0/8	+++

DISCUSSION

For a long time experiments with lipids from tubercle bacilli have suffered from the fact that the extracted materials were ill-defined chemically and that it was difficult therefore to compare results obtained by various authors. With regard to the toxic

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component of the tubercle bacillus, this difficulty has been overcome by the identification of cord factor as trehalose-6,6'-dimycolate[7].

The description by SPITZNAGEL AND DUBOS[11] of the results obtained with their "toxic lipid" suggested to us that they had obtained—by different methods of extraction—a lipid-containing cord factor. We have asked Dr. DUBOS for a reference sample of his toxic lipid, but he was unfortunately unable to spare any of his material. An infrared spectrum* of a sample would have allowed us to compare our product with his and to settle the question whether or not the two toxic fractions were identical. Lacking this reference, we can only state that a careful comparison of the chemical data published by SPITZNAGEL AND DUBOS with the results reported in this present paper makes it very likely that the toxic portion of their compound was indeed cord factor.

As to the biological data, the only difference between the results published by SPITZNAGEL AND DUBOS and our own is the fact that in their experiments one single intraperitoneal injection was lethal for mice whereas in our experience, except for rare instances of occasional death occurring after intraperitoneal injections of very large amounts of cord factor (see Tables II and III), repeated injections are needed to produce lethal effects using this route of injection. As shown in this paper, the intravenous route of injection is in many respects more satisfactory because a single injection kills regularly after a delay of about 7 to 14 days.

We can offer no explanation for the variance in the results obtained by SPITZNAGEL AND DUBOS, who observed lethal effects after one single intraperitoneal injection. Since over a period of more than five years we have consistently failed to make similar observations, and since the "toxic lipid" which was obtained by the method of SPITZNAGEL AND DUBOS is a mixture of inactive glycerides and cord factor (trehalose-6,6'-dimycolate) the latter being the only toxic component, it seems difficult to reconcile our observations with those made by SPITZNAGEL AND DUBOS.

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SUMMARY

A lipid extracted by the method described by SPITZNAGEL AND DUBOS has been separated by chromatography into six distinct fractions, one of which was toxic and has been identified as cord factor (trehalose-6,6'-dimycolate).

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TRANSPORT OF ALKALI CATIONS BY KIDNEY CORTEX SLICES

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While it is clear that Na is actively transported by the renal tubular cell, since Na flux normally occurs against a concentration gradient and Na extrusion from the cell can be inhibited by factors such as cold, anoxia, CN, etc. (MUDGE[1], DAVIES[2], CORT AND KLEINZELLER[3]) such a definite statement has not yet been possible with reference to other alkali cations. The specificity of any transport process may be tested by comparing the fluxes of molecularly or atomically related substances, *i.e.* optical isomers, substituted organic compounds, etc. In the specific problem at hand, Li^+ transport has been compared with Na^+ transport, and Rb^+ with K^+, as representing the closest analogues with the same chemical reactivity.

FOULKS, MUDGE AND GILMAN[4] have infused isotonic LiCl into dogs and have observed a rise in K excretion and urine pH as compared with a control infusion of NaCl, while MUDGE[1] and ANDERSON AND MUDGE[5], on the other hand, have shown, in experiments where Li was added to the incubation medium of kidney slices, that within wide limits Li^+ behaved much like Na^+ and did not change O_2 uptake or tissue HCO^-_3 content.

It has been suggested that K^+ transport may be a purely passive phenomenon (MUDGE[1], ANDERSON AND MUDGE[5], CORT AND KLEINZELLER[3]) since the uptake of K^+ by kidney slices from media with different concentrations of K^+ shows a direct relationship to K^+ concentration in the medium, each uptake curve being exponential in time. Rb behaves so much like K that it may be administered to K-deficient, alkalotic animals and will correct the extracellular alkalosis, and it appears that it may freely enter cells without signs of toxicity (RELMAN, ROY AND SCHWARTZ[6], ANDERSON AND MUDGE[5]).

The kinetics of Li^+ and Rb^+ transport have not been precisely determined however, nor have any of the authors cited actually directly analysed for either of the

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