STUDIES ON FRACTIONS OF METHANOL EXTRACTS OF TUBERCLE BACILLI

I. Fractions Which Increase Resistance to Infection

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It is a well established fact that killed tubercle bacilli can be used to increase the resistance of laboratory animals to experimental tuberculosis. Among the earlier experiments those of Opie and Freund (1) stand out. These workers conspicuously elevated the relative resistance of rabbits by repeated injection of heat-killed tubercle bacilli. Among many other authors, Dubos, Schaefer, and Pierce (2) later demonstrated in mice the immunogenic efficacy of single doses of phenol-killed tubercle bacilli.

It is reasonable to expect, therefore, that certain substances derived from tubercle bacilli by chemical means would be active in promoting increased resistance to experimental tuberculosis. Crowle (3), in a recent review, has summarized the attempts of many authors during the recent decades to implicate the proteins and polysaccharides of culture filtrates, the "waxes," phosphatides and lipopolysaccharides of the cell bodies, and combinations of these in the immunogenic capacity of the killed tubercle bacillus. The experiments with *antigène méthylique* of Nègre and Boquet (4), Nègre (5), and Nègre and Bretey (6) were the most promising of such efforts. The enhanced resistance, elicited in rabbits and guinea pigs by these workers, was observed in mice by Weiss and Dubos (7, 8) following a single injection of a similar material extracted from phenol-killed tubercle bacilli by methanol at 55°C. The methanol extract of Weiss and Dubos was less toxic in immunogenic doses than whole killed tubercle bacilli, though no more protective on a weight for weight basis. The use of adjuvants such as typhoid polysaccharide, however, increased the level of the protective effect.

The object of the present study is the purification and characterization of the active substance or substances of methanol extract. In this respect it is our purpose to describe the preparation of fractions and subfractions of methanol extracts of virulent tubercle bacilli (strain H37Rv), and to present experiments demonstrating the value of certain of these preparations when used as chemically derived vaccines. The experiments reported were performed in 1957 and

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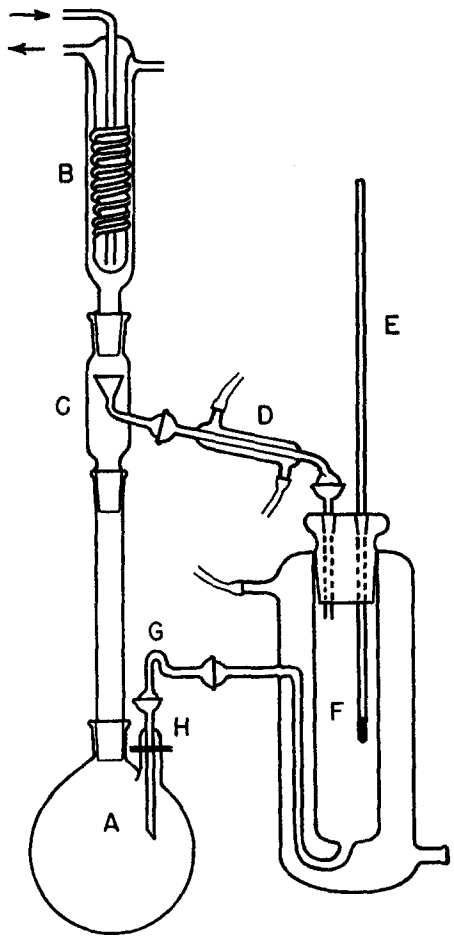

FIG. 1. Diagram of constant temperature, continuous extraction apparatus.

A, distilling flask, 2,000 ml.; ⅋ 29/42 outer joint, center; flat ground platform joint side (*c*).*

B, Friedich reflex condenser with ⅋ 29/42 inner joint.

C, distillate collector, column insert with sealed-in funnel. ⅋ 29/42 top and bottom, ball joint on collecting tube shunt.

D, jacketed connecting tube. (Liebig condenser with ball and socket joints.)

E, 8 inch insertion thermometer with ⅋ inner joint.

F, jacketed extractor for 180 × 60 mm. thimbles (*c*).

G, syphon-loop connector with ball and socket joints (*c*).

H, delivery tube adaptor with socket joint, top, and flat ground joint, bottom (*c*).

* (*c*) *indicates custom made items according to author's specifications.*

early 1958. Their publication was delayed until the preparative procedures had been repeated and standardized and until the biological properties of the new products had been determined.

Materials and Methods

Preparation of Vaccines:

The "tubercle bacilli germ mass (vacuum-dried) X-0482, Lot A and Lot B," used for the extraction of the materials to be described in this report was obtained from the research department of Parke, Davis & Co. through the generosity of R. W. Sarber. *Mycobacterium tuberculosis* var. *hominis* (H37Rv) had been grown on Long's synthetic medium for approximately 3 months. The cultures were killed by a 3 hour treatment in flowing steam and then filtered. Lot A (May, 1956) was held without preservative and vacuum-dried, yielding 165 gm. dry weight. Lot B (November, 1950) was stored under 0.5 per cent phenol until June, 1956. At this time it was filtered and vacuum-dried, yielding 1095 gm. dry weight. Upon receipt at this laboratory, Lots A and B were pooled and pulverized by blending at high speed with acetone chilled to below −50°C. The acetone powder was collected on a Buchner funnel, rinsed with anhydrous diethyl ether, and dried in a vacuum oven at 50°C.

Preparation and Fractionation of Extracts from Tubercle Bacilli.—An apparatus was designed capable of continuously extracting 100 gm. or more dry germ mass at carefully controlled temperatures. As seen in Fig. 1, the apparatus is a modified Soxhlet extractor in which the extraction chamber has been placed outside the distilling column.

The condensate is conducted to the extraction chamber by means of the distillate collector in the section below the condenser. The temperature of the extraction is controlled in two ways. First, the connecting tube from the distillate collector is jacketed so that it may be cooled or heated as desired. It is particularly important that the condensate coming from the distilling column to the extraction chamber be maintained at a fairly high temperature if the extraction is to be effected at a temperature significantly above room temperature, or cooled if to be carried out many degrees below the boiling point of the solvent. Second, the extraction chamber itself is jacketed with a relatively large volume of water circulated by a water bath. It was found that while this latter jacket will maintain a high temperature fairly well, it is not sufficient to adjust the extraction temperature to a relatively low level before the chamber automatically empties. The center of the large germ mass contained in the asbestos thimble (180 × 60 mm.) would remain close to the boiling point of the arriving solvent were the desired extraction temperature not approximated during the passage of the solvent through the shunt from the distilling column. With a more efficient temperature control in the shunt, it would be quite possible to limit fluctuation of the extraction temperature to a fraction of a degree Centigrade. The apparatus as illustrated here permitted fluctuations within a 3 to 4 degree range.

It has been found that extraction at temperatures at least as high as 55°C., as initiated by Weiss and Dubos (7) are necessary to remove from the germ mass all the methanol-soluble substance which increases the resistance of mice to experimental tuberculosis. The *antigène méthylique* of Nègre and coworkers was extracted at 37°C. Fractional extraction of the germ mass by methanol at 40°C. prior to an extraction at 60°C. provided a material (ME-40°) roughly equivalent to *antigène méthylique*, and another (ME-60°), presumed to contain what-

ever supplementary activity is extractable at higher temperature. From 100 gm. of H37Rv, 9.2 gm. was removed by acetone, 10.8 gm. by methanol at 40° in 72 hours, 2.6 gm. by methanol at 60° in 72 hours. Continued extraction with methanol at 60° for 5 more days yielded 0.6 gm. more, and another 5 days but another 0.3 gm.

Fractional extraction was abandoned as a purification technique.

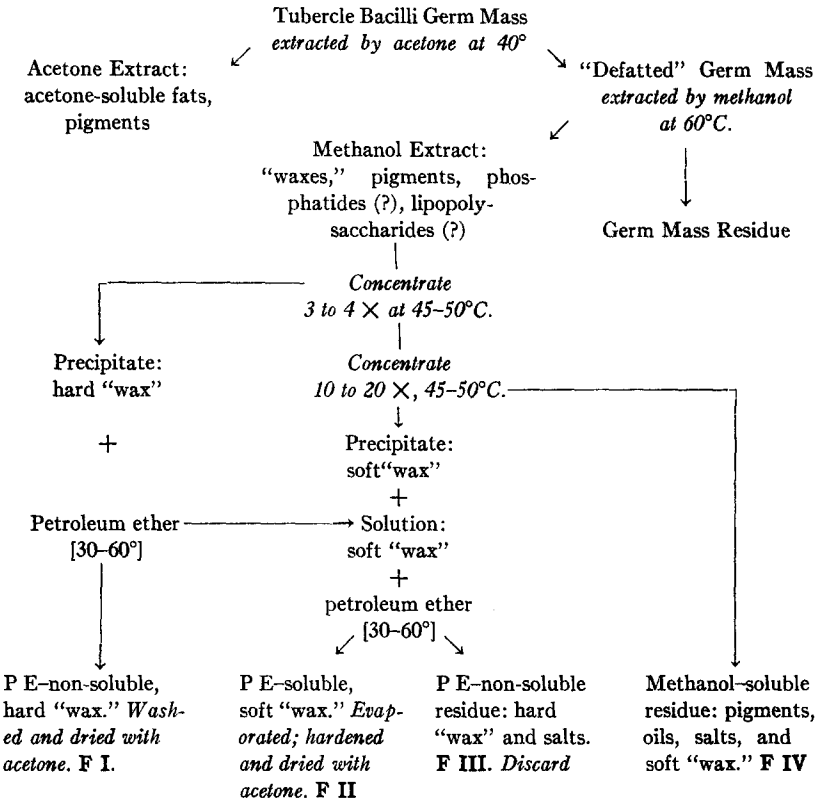

FIG. 2. Flow sheet for the extraction and fractionation of methanol extract of tubercle bacilli (H37Rv).

A typical flow sheet for the extraction and crude fractionation of the methanol extract (ME) is presented in Fig. 2.[1]

The germ mass (generally 100 gm.) is extracted with acetone at 36–40°C. for 48 hours. Between 9 and 10 per cent of the dry weight is removed by this "defatting" procedure. After washing the residue with cold methanol and allowing it to drain several times, methanol is added to the distilling flask and the extraction temperature is adjusted to 58–60°C. After

[1] All solvents used in these preparations were Merck reagents. Technical grade methanol and acetone contain compromising residues.

48 hours ME, presumably including the *antigène méthylique* of Nègre and Boquet, is removed. Another 10 per cent or more of the dry weight of the starting material is found in in this extract.

Upon cooling there is a general precipitation of material soluble in warm methanol. In an effort to control this to advantage, the methanol extract (approximately 1000 ml.) is placed in a vacuum flask and maintained between 45° and 50° by means of an electric hot-plate while a jet of air induced by vacuum is directed on the surface of the solution. In this fashion, the volume of the extract is slowly reduced until the various components reach their critical concentration for this temperature. The first fraction to precipitate out of solution is characterized by light white floccules in the first stages. Later when the volume has been reduced to between 200 and 300 ml. it becomes heavier and sticky, being contaminated with the second distinct fraction, which is a soft, yellow, wax-like substance. This latter material is collected separately between 250 and 50 ml. volume. Below that point it becomes heavily contaminated with inorganic salts (F III) and residue products soluble in cold methanol (F IV). F IV contains highly pigmented materials. Only the first two fractions, F I and F II, have been refined.

Both F I and F II can be pulverized and washed relatively free of the pigmented material characteristic of F IV in ice-cold methanol, and collected on a small Buchner funnel. They are then removed to an acetone slurry, which maintained at a low temperature permits further hardening, pulverization, and removal of fatty materials. It must be stated, however, that aside from a very small amount of yellowish residue, the fractions appear to be insoluble in acetone at even higher temperatures. Acetone, therefore, is used to replace all other organic solvents associated with the preparations of F I and F II, and is used very effectively as a drying agent.

The major component of F I is insoluble in petroleum ether [30–60°] and petroleum ether [60–70°] although slightly soluble and extractable by petroleum ether [90–100°] whereas F II is very soluble in this solvent.[2] Thus three washings of crude F I with petroleum ether [30–60°] suffices to remove any gross contamination of F II; and similarly, dissolving crude F II leaves a fine white residue of F I and F III contaminants. Petroleum ether is removed from both fractions by acetone washes, although F II must be evaporated to near dryness and repulverized in acetone.

Subfractionation.—F I can be subfractionated in several ways. However, Methods A and B, illustrated in Fig. 3, appear to be the most reproducible.

Method A was suggested by the fact that some lipopolysaccharides are slightly soluble in ethanolic salt solutions. Ethanol is added to a water suspension of F I until the solution is 33 per cent ethanol by volume. F I is noticeably more soluble in this mixture than in distilled water, but the addition of sodium chloride to 0.5 per cent of total alcoholic volume causes the precipitation of 90 per cent or more of the total fraction. For the experiments reported below, the precipitate was resuspended in water and reprecipitated in the same manner a second time. It accounted for 46 mg. of an original 50 mg., a typical sample size, carried through the subfractionation procedure. The supernatant solution was evaporated over a hot-plate to remove ethanol.

[2] A simple test indicated that unless F I, F II, and the petroleum ether are scrupulously dry, F I may be extracted into solution. One drop of water in 100 ml. of petroleum ether is sufficient to ruin the separation. Recent studies indicate that purified hydrocarbons such as methylpentane and *n*-hexane might better distinguish between F I and F II. The cyclic hydrocarbons are less satisfactory in this regard, thus a petroleum ether cut rich in such compounds might prove unsatisfactory altogether.

Method B depends on the fact that approximately 80 per cent of the fraction is precipitated from the hot methanolic solution by the dropwise addition of cupric acetate in methanol. This is apparently a stoichiometric combination of the copper with some chemical grouping associated with this class of compound.[3] Precipitation occurs up to a certain point with

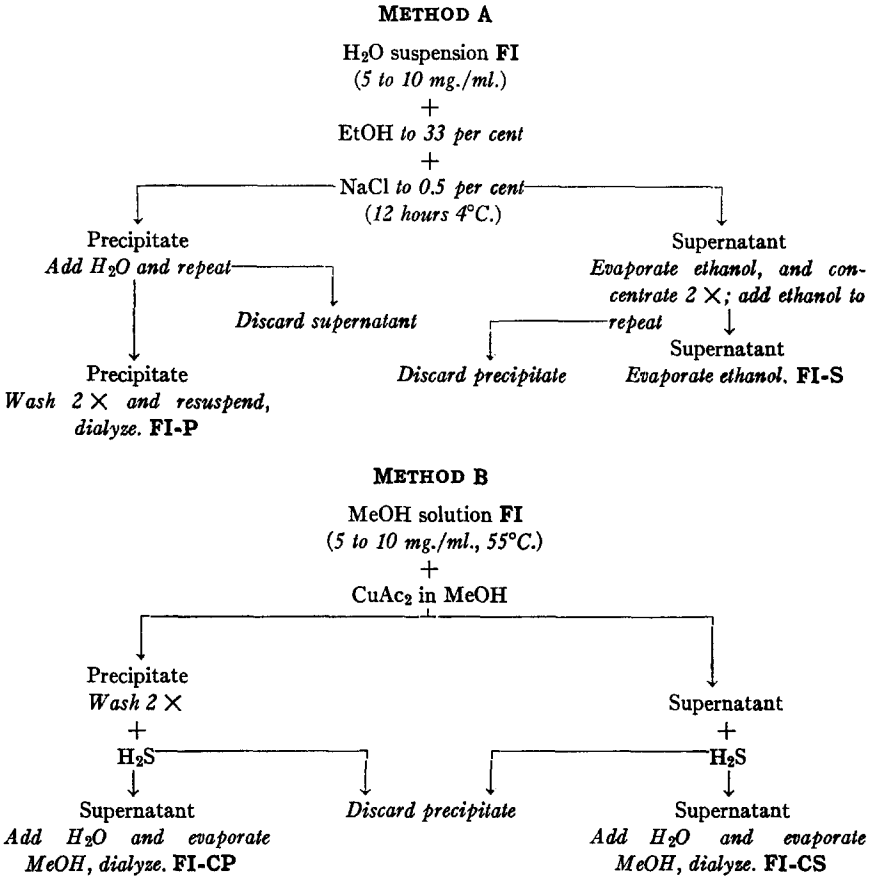

FIG. 3. Subfractionation of FI (See Fig. 4) by ethanol and sodium chloride from water suspension, Method A; and by cupric acetate from methanol solution, Method B.

absolutely no hydrogen sulfide-detectable copper in solution. Only when no further precipitation occurs will the blue color appear in the supernatant. The precipitate can be solu-

[3] Several divalent metal ions were tried in the search for a precipitant for a fraction of methanol extract. Among lead, mercury, iron, calcium, barium, and copper, only copper seemed effective and manageable without special attention to pH and temperature. Barium also complexed methanol extract substance but lacking color in solution did not seem as convenient as copper. Pangborn (9) has mentioned the use of barium in the purification of a phosphatide from tubercle bacilli as a barium salt.

bilized with Na_2-ethylene-diaminetetraacetic acid (versene), but removal of the copper from both supernatant and precipitate is most easily effected by bubbling hydrogen sulfide through the methanol suspension. The resulting methanol solutions are pipetted to an equal volume of warm water, evaporated over a hot-plate to remove most of the methanol and hydrogen sulfide, then dialyzed against water. During this procedure, it is imperative that all methanol solutions be maintained above 55°C. in order to avoid spontaneous precipitation of a part or all of the fraction.

All dialyses were performed with Visking cellophane tubing No. 18/32. This tubing is impermeable to substances of molecular weight 6,000 as determined by dialysis studies with insulin (9, 10).

Properties.—The physical and chemical properties of the fractions described above will be reported in further detail in a later publication. No attempt will be made at this time to relate these substances to the waxes, soft waxes, phosphatides, and/or lipopolysaccharides described by other authors. One of the most interesting subfractions, the supernatant (FI-S) from the treatment of F I with ethanol and sodium chloride (Method A), has not to date been analyzed because it has been available in such limited quantities.

Fractions F I and F II both contain less than 0.2 per cent Kjeldahl nitrogen. Certain preparations of F I have contained less than 0.1 per cent N. Both fractions contain phosphorus, but depending upon the preparation the content will vary from 1.7 per cent to 2.4 per cent by the standard Fiske-Subarrow titration as described by Kabat and Mayer (11). F I and F II are both Molisch-positive although F I is much the stronger in this regard. In F I this property is confined to that moiety precipitable by ethanol and sodium chloride (FI-P), which gives a bright red-violet reaction. FI-S, concentrated for the test, gives a faint brownish color. The F II reaction color is more violet than red. Semiquantitative Molisch tests (11), by comparison with known amounts of reducing sugars such as glucose, galactose, or mannose, indicate that a large portion of FI-P is carbohydrate, perhaps as much as half.

Experimental Materials:

Mycobacterium tuberculosis var. *bovis* (Vallée) MV was used to challenge mice. In all experiments described below, the bacteria were grown on Dubos-Middlebrook liquid medium for surface growth for 10 days at 37°C. (12). The pellicle germ mass was harvested by skimming the surface with a spatula, pressed dry on absorbent filter papers, and weighed into a grinding tube. A suspension suitable for intravenous injection in mice was prepared by dispersing the germ mass in 0.2 per cent tween 80 in physiological saline with a teflon grinder. This was appropriately diluted in tween-saline to deliver 0.1 mg. paper-dry tubercle bacilli per mouse in 0.2 ml.

Micrococcus pyogenes var. *aureus* (Giorgio) (13) for staphylococcal challenge was grown 18 hours in tryptone broth at 37°C. For intravenous injection 0.05 ml. of this culture was delivered to each mouse in a total volume of 0.2 ml.

Animals.—Rockefeller Swiss albino male mice were used throughout the study. The animals were received shortly after weaning at an age of approximately 4 weeks and were randomized into groups of 5 or 6 depending on the requirements of the experiment. The mice were maintained in metal boxes, fed antibiotic-free pellets (Rockland mouse diet, Rock-

land Farms, New York), and were provided with water *ad lib.* During the 1st week they received 3 per cent terramycin in their drinking water (*cf.* Dubos and Schaedler, reference 14, page 705). After being infected with tubercle bacilli, mice were removed to isolated quarters and housed two to a cage.

Statistical Methods:

Tabulation of Data in "A" Tables.—All data obtained from survival experiments with mice are tabulated in vertical columns as individual survival times expressed in days following challenge. Extreme data have been rejected with 90 per cent confidence or greater according to the Q test (15).[4] High values have been rejected with 95 per cent confidence or better. These are represented by R followed by the value obtained in parentheses.

When a group was deficient in number of animals an objectively appropriate liberty has been taken in the placement of blanks (—) in the column of data. The first such deficiency was placed in the first or last position, whichever shifted the column so as most to approximate the pattern established by the control group. Thus any shortage of data was consistently used to minimize the apparent experimental efficacy of a vaccine preparation. The second blank, if any, was recorded in the middle of the column following the first half of the group. These measures permitted comparison of all the groups at various points in the course of the experiment by scanning the tables horizontally. This is essential to the understanding and significance of B tables.

Survivors, at the time the experiment was terminated, are represented in the table by S. For the statistical analysis these had to be disregarded as no justifiable value could be assigned to them. The analysis of data in Table I A includes only the first ten survival times in each group. No analysis of Table IV A was attempted. Probability values were obtained from calculations of Student's t according to Hoel (16).

B Tables, Cumulative Average Survival Times.—Each survival time in order of occurrence of death is averaged with all the preceding survival times within its group. Such a set of figures for any one group is suitable for plotting graphically the state of resistance or susceptibility of the group to the imposed infection. The curves obtained are relatively smooth compared to what would be expected from a plot of the primary data; but the slope of any given curve is related to the variability and range of the primary data. If time is plotted along the ordinate, the elevation of the curve is an expression of the resistance or susceptibility achieved by the experimental treatment. This method of handling data for the visualization of group response in survival experiments has been introduced by Riley (17, 18).

[4] A questionable value in a small number of observations may be rejected with 90 per cent confidence if $Q_{0.90}$ exceeds the value in the table for a given number of observations, n, Q being determined by the formula,

$$Q = (x_n - x_{n-1})/w$$

in which x_n is the questionable value, x_{n-1} its nearest neighbor, and w the range including x_n.

n	$Q_{0.90}$	$Q_{0.95}$	
6	0.56	0.71	$Q_{0.95}$ values are (estimated from progression of Student's t, for 0.05 probability)
10	0.41	0.51	
11	0.39	0.48	
12	0.38	0.45	

A value for the *mean cumulative average* (MCA) was calculated from four successive cumulative averages in the middle of the set. Although this number is not the true mean of the set, it is close to the true mean when the set of values is complete. In the middle region of the set or the curve, while the values take into account the early deaths, the slope will have recovered from their effect even though moderately extreme. Although there were very few survivors in the present studies with experimental tuberculosis, there were not infrequently some rather late deaths which do not correlate with any known experimental condition. With staphylococcus infections there were not only late deaths, but there were more survivors, the latter being very difficult to deal with when the data are desired in terms of time of death. The *mean cumulative average* virtually eliminates the effect of extraordinarily late deaths and survivors. Thus, the contribution of extreme data could not lead to an impression of increased resistance.[5]

An *index of resistance* (*IR*) provides a satisfactory means for the comparison of group response from experiment to experiment. This index is nothing more than the quotient of the *mean cumulative average* from an experimental group of animals divided by the *mean cumulative average* for the control group. Thus, the control group is arbitrarily assigned an $IR = 1$. The decimal fraction in excess of 1 multiplied by 100 gives the per cent increase in mean cumulative average survival time afforded by the treatment.

EXPERIMENTS AND RESULTS

1. In Tables I A and I B are recorded the data from an experiment designed to test the relative effectiveness of subfractions of F I used as vaccine to increase the resistance of mice to tuberculosis. Vaccination consisted of a single intraperitoneal injection of 1 mg. of F I in 0.2 ml. water, or that amount of the subfraction derived from 1 mg. of F I. Thus approximately 0.9 mg. of FI-P and 0.1 mg. of FI-S were used; 0.2 mg. FI-CS and 0.8 mg. of FI-CP. The mice were challenged intravenously with 0.1 mg. (wet weight) of pellicle grown *M. tuberculosis*, strain Vallée, (MV) 47 days after vaccination; *i.e.* roughly 7 weeks.

Examination of the data and the plotted curves (Fig. 4) of the cumulative average survival times suggests that, while F I offers slight protection, its subfractions are more promising. Most notable are the supernatants of either the ethanol-NaCl method or the cupric acetate method. Indeed the *indexes of resistance* indicate that mice vaccinated with FI-S or FI-CS could be expected to survive respectively 40 per cent and 30 per cent longer than controls injected with water. The precipitates, FI-P and Cu-P, outperform the parent fraction F I by insignificant margins. The experiment further suggests some sort of non-active form of FI-S or FI-CS in the total F I fraction.

2. Another vaccination experiment was designed to compare the effectiveness of admixtures of F II and F I with F I subfractions. The results are shown in Tables II A and II B, and the cumulative averages of selected groups are plotted in Fig. 5. The interesting facts revealed by this experiment are that, while 0.1 mg. of F I is shown to be of little value, and 1.0 mg. of F II to have

[5] For this purpose Riley uses the average survival time of the first 50 per cent of the animals to die, or the *median cumulative average*.

TABLE I A

Resistance to M. tuberculosis Infection: Individual Survival Time—(Days)*

Order of death	Vaccination preparation‡					
	Water	FI 1 mg.	FI-P ~0.9 mg.	FI-S ~0.1 mg.	Cu-P ~0.8 mg.	Cu-S ~0.2 mg.
1	9	12	R(4)§	13	9	13
2	11	13	13	14	12	14
3	12	14	14	18	13	14
4	13	14	15	19	14	17
5	13	16	16	20	16	20
6	14	16	17	21	21	21
7	14	17	—	22	22	22
8	14	18	17	24	22	22
9	15	19	22	24	24	24
10	18	19	24	28	28	28
11	R(39)	20	R(53)	35	S	31
12	S	22	S	35	S	R(45)
$\bar{x}$‖	13.3	15.8	17.25	20.3	18.1	19.5
t	—	2.28	2.67	4.26	2.29	3.58
P		0.05	0.04	0.004	0.05	0.008

TABLE I B

Resistance to M. tuberculosis Infection: Cumulative Average Survival Time*

Order of death	Vaccination preparation‡					
	Water	FI 1 mg.	FI-P ~0.9 mg.	FI-S ~0.1 mg.	Cu-P ~0.8 mg.	Cu-S ~0.2 mg.
1	9.0	12.0	R§	13.0	9.0	13.0
2	10.0	12.5	13.0	13.5	10.5	13.5
3	10.7	13.0	13.5	15.0	11.3	13.7
4	11.2	13.25	14.0	16.0	12.0	14.5
5	11.6	13.8	14.5	17.0	12.8	15.6
6	12.0	14.2	15.0	17.5	14.2	16.5
7	12.3	14.6	—	18.0	15.3	17.3
8	12.5	15.0	15.3	19.0	16.1	18.0
9	12.8	15.5	16.3	19.5	17.0	18.6
10	13.3	15.8	17.2	20.3	18.1	19.5
11	R	16.2	R	21.6	S	20.5
12	S	16.7	S	22.75	S	R
MCA	12.8	14.4	14.9	17.9	14.6	16.9
IR	1.0	1.12	1.16	1.40	1.14	1.32

* Mice challenged intravenously 0.1 mg. (wet weight) Vallée MV in 0.2 ml. saline, 0.05 per cent tween 47 days after vaccination.

‡ Mice vaccinated intraperitoneally with 0.2 ml. of distilled water suspensions.

§ R, rejected value not used in statistical analysis. Actual survival time in parentheses.

‖ Statistical analysis includes only first 10 data in each group.

low grade activity at best, mixtures of these two fractions are remarkably active. Where F I and F II are combined in equal amounts, even where the total dry weight of the mixture administered to each mouse is but 0.1 mg., survival time is increased nearly 70 per cent. A total vaccination dose of 1.0 mg. lengthens survival time approximately 80 per cent. Since these are considered important observations, it is worth mentioning that this pattern has been repeated in subsequent experiments, although the *IR* values were not of the same order.

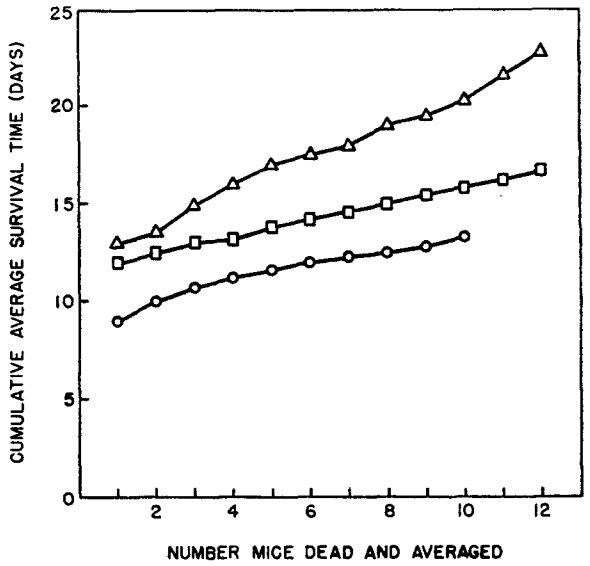

FIG. 4. The increase in cumulative average survival time of mice infected with virulent tubercle bacilli (Vallée MV) following vaccination with 1 mg. fraction FI of methanol extract (M), 0.1 mg. of subfraction FI-S (△). Water vaccinated controls (○).

As each successive animal succumbed to the infection, its survival time was averaged with those of the preceding victims of its group. Each point represents the new average survival time at the time of death of the number mice indicated on the abscissa. The slope of the curve relates to the variability and range within the group. The level on the vertical axis expresses the increase in resistance to the infection.

Except that the time elapsed between vaccination and challenge was 39 days, all known conditions were the same for this experiment as those in the experiment above. Nonetheless all groups survived more than twice the time as those previously discussed. This is possibly attributable to a variation in the state of the challenge organisms. With the across-the-board extension of the survival times, the *index of resistance* seems to increase also. Thus, the group receiving FI-S as vaccine resists 47 per cent longer than the controls even though they received only half the quantity of vaccine required to give them 40 per cent

TABLE II A

Resistance to M. tuberculosis Infection: Individual Survival Time—(Days)*

Order of death	Vaccination preparation‡						
	Water	FI 0.1 mg.	FII 1.0 mg.	FI + FII 1.0 mg.	FI + FII 0.1 mg.	FI-P ~0.4 mg.	FI-S ~0.05 mg.
1	18	14	14	—§	39	27	25
2	22	26	27	45	44	28	39
3	27	26	34	47	46	38	42
4	32	29	36	52	50	42	46
5	36	44	44	53	53	42	49
6	43	46	48	63	54	48	51
7	46	50	50	63	56	50	52
8	49	56	52	63	56	51	58
9	49	58	53	64	57	64	67
10	63	59	70	65	60	74	70
$\bar{x}$.....	38.5	40.8	42.8	57.1	51.5	46.4	49.9
t.....	—	0.34	0.65	3.52	2.64	1.23	1.87
P.....	—	0.76	0.54	0.01	0.03	0.25	0.10

TABLE II B

Resistance to M. tuberculosis Infection: Cumulative Mean Survival Time*

Order of death	Vaccination preparation‡						
	Water	FI 0.1 mg.	FII 1.0 mg.	FI + FII 1.0 mg.	FI + FII 0.1 mg.	FI-P 0.4 mg.	FI-S 0.05 mg.
1	18.0	14.0	14.0	—§	39.0	27.0	25.0
2	20.0	20.0	20.5	45.0	41.5	27.5	32.0
3	22.3	22.0	25.0	46.0	43.0	31.0	35.3
4	24.7	23.7	27.7	48.0	44.7	33.7	38.0
5	27.0	27.8	31.0	49.2	46.4	35.4	40.2
6	29.7	30.8	33.8	52.0	47.7	37.5	42.0
7	32.0	33.6	36.1	53.8	48.9	39.3	43.4
8	34.0	36.4	38.1	55.0	49.7	40.75	45.25
9	35.8	38.8	39.8	56.2	50.6	43.33	47.7
10	38.5	40.8	42.8	57.2	51.5	46.4	49.9
MCA.....	27.8	29	32.1	50.7	46.9	36.5	40.9
IR.....	1.0	1.04	1.15	1.82	1.69	1.31	1.47

* Mice challenged intravenously 0.1 mg. (wet weight) Vallée MV in 0.2 ml. saline, 0.05 per cent tween 39 days after vaccination.

‡ Mice vaccinated intraperitoneally with 0.2 ml. of distilled water suspensions.

§ Group deficient in numbers. See explanation in text.

advantage in the previous experiment. The same relationship holds for FI-P, which for the purposes of comparison can be considered equivalent or slightly better than total F I in resistance-enhancing capacity.

3. An experiment was undertaken in an attempt to establish the time which must elapse following vaccination with F I before mice show increased resistance to tuberculosis. Sixty mice were randomized into groups of five and maintained under identical conditions for 12 weeks before the proposed challenge date.

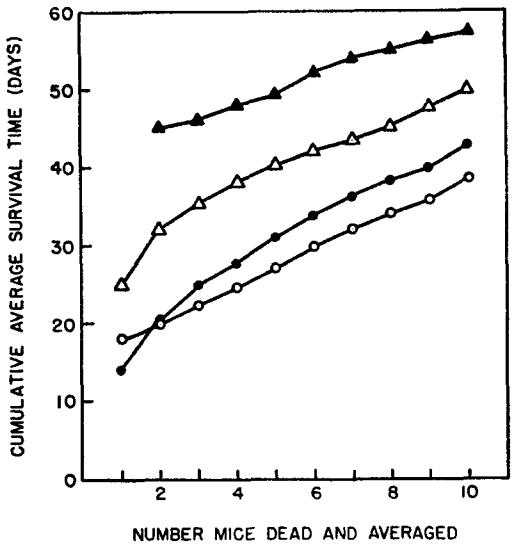

FIG. 5. The increase in cumulative average survival time of mice infected with virulent tubercle bacilli (Vallée MV) following vaccination with 1 mg. FII (●), 0.05 mg. FI-S (△) (*cf.* Fig. 4), 0.5 mg. FI plus 0.5 mg. FII (▲). Water vaccinated controls (○) (see Table II B).

For explanation of graph see legend for Fig. 4.

During this period, groups of ten were vaccinated at intervals so that by the challenge date there would be a group of ten mice representing each of five vaccination periods, 1, 2, 4, 8, and 12 weeks. Ten mice were injected with water 12 weeks before challenge. The same F I suspension was used to vaccinate all groups. The results presented in Tables III A and III B demonstrate that resistance is high at 2 weeks following vaccination with F I, but that at 4 weeks, virtually all advantage over the controls had been lost. However, mice vaccinated 8 and 12 weeks before challenge, did show a significantly high *IR*.

4. Of considerable interest is the high *IR* at 2 weeks. Studies by Dubos and coworkers (14, 28, 29) which were in course at the time these experiments were being performed, suggested the possibility that heightened levels of resistance to

TABLE III A

Resistance to M. tuberculosis Infection: Individual Survival Time—(Days)*

Order of death	Vaccination period (wks.)‡					
	Water (12)	1	2	4	8	12
1	21	22	29	21	—	28
2	24	31	34	24	21	31
3	31	34	35	30	41	41
4	34	34	45	38	42	42
5	34	38	48	41	43	43
6	34	48	48	41	—	43
7	38	48	51	42	48	43
8	38	53	51	48	48	48
9	42	56	61	49	50	48
10	48	70	62	51	50	51
$\bar{x}$.....	34.4	43.4	46.4	38.5	42.9	42.8
t.....	—	1.75	2.79	0.99	2.06	2.63
P.....	—	0.12	0.02	0.36	0.085	0.03

TABLE III B

Resistance to M. tuberculosis Infection: Cumulative Average Survival Time*

Order of death	Vaccination period (wks.)‡					
	Water (12)	1	2	4	8	12
1	21.0	22.0	29.0	21.0	—	28.0
2	22.5	26.5	31.5	22.5	21.0	34.5
3	25.3	28.8	32.7	25.0	31.0	36.7
4	27.5	30.2	35.75	29.5	34.7	38.0
5	28.8	31.8	38.2	31.0	36.75	39.0
6	29.7	34.5	39.8	32.5	—	39.7
7	30.9	36.4	41.4	33.9	39.0	40.1
8	31.75	37.5	42.6	35.6	40.5	41.1
9	32.9	40.4	44.7	37.0	41.9	41.9
10	34.4	43.4	46.4	38.5	42.9	42.8
MCA.....	29.2	33.2	38.8	31.7	37.7	38.2
IR.....	1.00	1.14	1.33	1.08	1.29	1.31

* Mice challenged intravenously 0.1 mg. (wet weight) Vallée MV in 0.2 ml. saline, 0.05% Tween.

‡ Mice vaccinated intraperitoneally with 1 mg. FI in 0.2 ml. distilled water.

TABLE IV A

Resistance to Staphylococcus (Giorgio) Infection: Individual Survival Time—(Days)*

Order of death	Vaccination period (wks.)‡					
	Water (12)	1	2	4	8	12
1	2	2	—	—	§	—
2	2	3	3	2		3
3	4	3	4	4		4
4	4	4	5	4		4
5	4	5	6	6		4
6	5	7	—	—		5
7	5	8	12	7		S
8	5	S	S	12		S
9	8	S	S	S		S
10	S	S	S	S		S

TABLE IV B

Resistance to Staphylococcus (Giorgio) Infection: Cumulative Average Survival Time*

Order of death	Vaccination period (wks.)‡					
	Water (12)	1	2	4	8	12
1	2.0	2.0	—	—	§	—
2	2.0	2.5	3.0	2.0		3.0
3	2.7	2.7	3.5	3.0		3.5
4	3.0	3.0	4.0	3.3		3.7
5	3.2	3.5	4.5	4.0		3.75
6	3.5	4.2	—	—		4.0
7	3.7	4.6	6.0	4.6		S
8	3.9	S	S	5.8		S
9	4.3	S	S	S		S
10	S	S	S	S		S
MCA.....	3.3	3.8	4.8	4.0		3.8
IR.....	1.00	1.15	1.45	1.21		1.15

* Mice challenged intravenously with 0.05 ml. of 18 hour broth culture diluted with 0.15 ml. saline.

‡ Mice vaccinated intraperitoneally with 1 mg. FI in 0.2 ml. distilled water.

§ Data for the 8 week period not reported owing to disparity of behavior in the two subgroups of five mice.

tuberculosis might be an expression of a non-specific increase in the activity of the general defenses of the mouse. The fact that methanol extract was of mycobacterial origin, and that resistance to tuberculosis was increased, may be coincidental and need not imply immunological specificity. To examine this possibility, sixty mice from the same weaning as those used for Experiment 3, randomized and cared for in the same way, and vaccinated at the same time with the same suspension of F I, were challenged with staphylococcus (Giorgio).

The results in Tables IV A and IV B show that there is indeed a considerable increase in resistance to staphylococcus infection at 2 weeks, which falls off to a lower level by 4 weeks.[6] The number of survivors in the 12 week group makes the group difficult to evaluate numerically. However, if survivorship has any

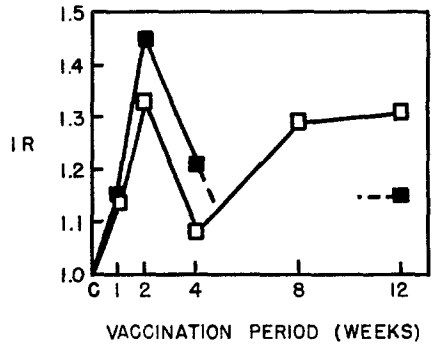

FIG. 6. Variation of *index of resistance* (*IR*) with respect to length of vaccination period. All mice received 1 mg. FI except the controls (C) which received 0.2 ml. water 12 weeks before challenge. Infection with tubercle bacilli (Vallée MV) (□). Infection with staphylococcus (Giorgio) (■). See Tables III B and IV B.

meaning in experiments of this nature, resistance at 12 weeks post vaccination should be at least as high as at 4 weeks. The actual *IR* values obtained are plotted in Fig. 6 along with those from Experiment 3.

DISCUSSION

Statistical Methods.—In most experimentation with infectious processes in animals, wide variation is a rule rather than an exception. Thus, it becomes important to have some guide to the significance of the differences observed between control and experimental groups, particularly when the number of animals in each group is small. Calculations of *P* have provided that guide. In groups in which survivorship deprives the experiment of data, or in which

[6] The data for the 8 week period had to be disregarded since the disparity of behavior of the two groups of five mice, comprising the family of ten animals, was sufficient to compromise the findings.

extreme high values would tend to exaggerate the efficacy of a protective agent, the calculation of *mean cumulative average* (*MCA*) and *index of resistance* (*IR*) constitute an attempt to reduce the group response to a justifiable single term.

From the experiments reported certain trends in these quantities become apparent. As the *mean survival time* ($\bar{x}$) of the control group is increased, variability of individual response increases, hence the probability (P) that the observed difference between two means could occur by chance. However, the *index of resistance* also increases, indicating a more pronounced difference. For example, the FI-P vaccinated group in Table I A shows $P = 0.04$, which is acceptable considering the small actual difference between the means. In Table II A a similarly vaccinated group shows $P = 0.25$, an unacceptable level, yet the *IR* in Table II B derived from these data is higher than the *IR* in Table I B, hence a greater detectable difference. These observations apply similarly to the FI-S vaccinated groups, although P increases much less drastically and does not attain an unacceptable level. From these observations, it is readily apparent that the subfraction FI-S is the most effective preparation. A single injection of a maximum dose of 0.05 mg. per mouse (*cf.* Table II B) was sufficient to increase the *mean cumulative average* survival time approximately 50 per cent.

For evaluations of the efficacy of treatment the *IR* is the quantity most heavily leaned upon. A high confidence level is desirable, of course, and in the case of the most promising fractions this was consistently 90 per cent or better ($P < 0.1$) and generally above 95 per cent ($P < 0.05$).

Preparation of Methanol Extract.—One among many preliminary experiments deserves mention at this point. The relationship between the *antigène méthylique* of Nègre and Boquet (4), which was originally obtained by extraction of tubercle bacilli by methanol at 37°, and methanol extract (ME), extracted by Weiss and Dubos (7) at 55°, was of some concern. Both were capable of bestowing some protection on experimental animals, but the relative activities on a weight for weight basis in the same species of animal were not known. Since such information would have a bearing on the preparation of active fractions of ME, the fractional extracts of H37Rv, ME-40°, and ME-60°, were compared in a mouse protection test.[7] They were found to be roughly equivalent, though inferior to a ME-60° preparation derived from BCG (Phipps). Since ME-60° represented but a quarter of the total activity extractable by methanol, the complete methanol extract was settled upon as a starting material.

[7] The preparation of the materials has been described. With them were also tested an ME-60° from BCG (Phipps) and an F I fraction from ME-40° (H37Rv). Groups of ten female mice were vaccinated with 2 mg. ME-60°, 3 mg. ME-40°, 3 mg. F I (ME-40°), and 1 mg. of ME-60° (BCG). Thirty-one days after vaccination the mice were challenged intravenously with 0.1 ml. of a dispersed growth culture of Vallée (MV) grown 14 days in tween-albumin medium. The controls showed a *mean cumulative average* survival time of about 16, the vaccinated groups about 19.5; thus, an $IR = 1.2$. Only F I (ME-40°) displayed any activity at one-third the dose mentioned above.

Mechanisms.—The question of mechanisms of resistance must be raised in discussing the effect of mixing F II and F I into the same vaccine. It is seen that the activity of F I is enhanced well beyond what would be expected from the independent contribution of F II to the level of resistance if the effect of the admixture were a mere summation. While F I alone has no value in a dose of 0.1 mg. per mouse, 0.05 mg. administered with an equal amount of F II has considerably more effect on resistance than an equal amount of FI-S. FI-S is ten times more active than F I alone on a weight for weight basis. These findings are certainly reminiscent of adjuvant-antigen systems, and the fact that F II is a wax-like material would not make the relationship impossible. White, with Bernstock, Johns, and Lederer (19), has demonstrated wax-D from H37Rv capable of acting as adjuvant for antibody production, and with Marshall (20) for allergic encephalomyelitis. Wax-D is a chloroform-soluble, methanol-insoluble glycolipid prepared according to Aebi, Asselineau, and Lederer (21). Freund and Stone (22), however, have recently published results of an excellent series of experiments which demonstrate that ten times more wax-D is needed than whole killed tubercle bacilli to induce allergic encephalomyelitis in mice and aspermatogenesis in guinea pigs. However, when either F I or F II was substituted for mycobacteria as adjuvants to elicit delayed hypersensitivity to picryl chloride, according to Chase (23), these substances proved to be ineffective in this system (24).

It must be remembered, on the other hand, that the whole tubercle bacillus is a rich packet of biological activities, all theoretically residing in substances capable of chemical definition. Combined in fractions, or associated in complex in their natural state, or recombined in test tubes, it is reasonable to suppose that the activities of such substances, revealed by suitable experiments in suitable animals, could be complementary, supplementary, masked, antagonistic, inhibitory, or combinations of these. For this reason, it should not be surprising that purification of a substance with protective activity from tubercle bacilli should effect no apparent increase in specific activity over the whole killed tubercle bacilli. The material may have been separated from its natural adjuvant or other synergistic agent. Either F I or F II could be an example of this phenomenon. Nor should a potentiation of activity cause surprise, when it need only be postulated that the material in the course of its purification was separated from competitive or inhibiting substances, or that it was released from a complex in which it is but partially free to manifest its activity. FI-S or FI-CS might illustrate such a situation.

Such might also be the fate of the adjuvant activity displayed by wax-D as employed by Freund and Stone (22). Another possibility suggested by the experiments of these authors is that fragments of bacillary bodies may account for some of the biological activities of chemical fractions of tubercle bacilli. It is certainly true that the detection of such contaminants is difficult and that

measures taken by other authors to assure their absence are not particularly convincing. This matter will be taken up in more detail in a later report (24).

Prolongation of survival time in animals vaccinated with killed tubercle bacilli, or even diminished numbers of viable bacteria in their various organs, cannot be attributed to any one known immune or non-immune mechanism of resistance. Furthermore, no immune mechanism of which the bacillary component or of which the host component is detectable, has ever been conclusively implicated in resistance to tuberculosis, experimental or otherwise. The possible exception to this is the recent work with macrophages *in vitro*, reported by Fong, Schneider, and Elberg (25, 26), demonstrating the presence in the serum of vaccinated animals of a substance which enhances the survival of "immune" macrophages infected with tubercle bacilli. This substance, however, is not specifically evoked by tubercle bacilli.

Some of the points discussed in the preceding paragraphs are pertinent to the interpretation of the experiments recorded in Tables III and IV, and represented graphically in Fig. 6. The rather high level of resistance to both tuberculosis and staphylococcus infection apparent 2 weeks after vaccination brings up the question of specificity and whether a so called immune mechanism can be imagined. If so, are we dealing with a protective antigen common to mycobacteria and staphylococci? Evidence for the existence of common antigenic substances has been presented by Lagercrantz (27) using passive hemagglutination tests; but whether or not other microorganisms have these antigens, or whether they have anything to do with resistance can be answered only by immunological information more sophisticated than survival times of vaccinated animals. Some bacterial lipopolysaccharides increase resistance against a variety of infections including staphylococcus and tuberculosis (28), *Mycobacterium fortuitum* (29), and *Salmonella typhosa, Escherichia coli, Pseudomonas aeruginosa and Proteus vulgaris* (30, 31); and although a classical immunological interpretation cannot be ruled out, none very convincing have been shown to date.

In nearly all of these "broad specificity" effects, however, there is a marked fall-off after about 3 weeks. Data reported by Dubos and Schaedler (14) indicate that even vaccination with living BCG produces by 2 weeks a peak resistance to challenge with *Mycobacterium fortuitum* (Penso) but that the level of resistance had fallen off noticeably by 25 days post vaccination. In the case of staphylococcus (Giorgio) infection of mice vaccinated with killed BCG, this lower level of resistance endures at least 10 weeks (14), which lends support to the findings in Table IV that mice still show increased resistance to staphylococcus 12 weeks after vaccination with F I.

Hypothesis.—Fig. 7 summarizes diagrammatically an hypothesis which could account for the results of Experiments 3 and 4, and which could serve as a useful model upon which to design further experiments. The bimodal curve of

resistance to experimental tuberculosis strongly suggests that at least two independent and possibly supplementary mechanisms are involved. One effect, exhibiting broad specificity, or broad spectrum resistance, appears quite soon after vaccination, reaches a maximum between 2 and 3 weeks and then declines. A second increase in resistance to tuberculosis is provided perhaps by the stimulation of a more specific and longer sustained mechanism. The period during which the animals are not protected, occurring sometime between 3 and 5 weeks, represents the failure of these two effects to overlap in time with sufficient combined influence to afford a significant prolongation of survival.

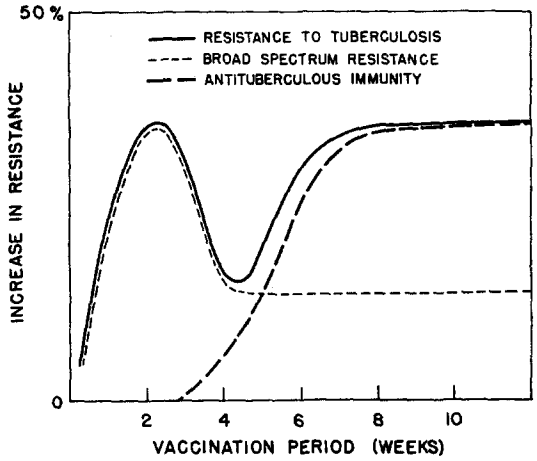

FIG. 7. Hypothetical resolution of the discontinuous pattern of resistance to tuberculosis provided by vaccination with F I.

This low resistance interval cannot be rigidly defined with either respect to time of appearance or duration. Indeed, it may not appear at all if one were to employ complex vaccines, such as whole killed tubercle bacilli or complete methanol extract, in which there may be many biologically active substances. And theoretically, if purified materials were used in large enough quantities, the stimuli to both mechanisms would be such as to provide the overlap of effects necessary to assure continuous elevation of resistance.

Experiments are in progress to distinguish these effects and to identify the subfraction of F I responsible for each.

Tuberculosis and Immunology.—Generally speaking, tuberculosis is a poor tool for the investigation of some principles of immunology pertinent to this discussion. If challenge doses intended to insure a short survival time are used, some immune mechanisms of importance at the time of challenge may be overwhelmed. At best one could say that it might not have contributed to resistance; at worst it would pass unnoticed. Were the challenge dose low enough

that all mechanisms of defense at the time of infection might be thought to contribute toward the increased survival time of the animal, the immune or resistant state, *i.e.* that condition possibly altered by vaccination, would be all but impossible to evaluate. Survival time has usually been extended well beyond that necessary for the animal to have acquired a certain immune resistance from the challenge itself, and though this may be facilitated by vaccination it may not involve the same processes and mechanisms operative in the first hours of the infection. Furthermore in the case of a slowly progressive or chronic disease, there is the possibility of a quite different general host physiology and pattern of resistance to infections.

Thus, at the present state of knowledge, experimentation with vaccine preparations against tuberculosis in animals is probably most profitably reduced to the empirical evaluation of their effectiveness. Findings of more theoretical significance might best be transferred to more easily manipulated systems for further study or extension. As for the effectiveness of a vaccine preparation, two criteria have been put forward by the proponents of BCG as minimum standards: first, survivorship or extended survival time equivalent to that afforded by BCG, and second, long-lasting protection.

Pierce and Dubos (32) have made an exhaustive study of the increased resistance in Rockefeller Swiss mice following vaccination with several strains and dosages of BCG. Analysis of their data for a group of these mice injected intravenously with approximately 10^6 viable units of BCG (Phipps), the most effective preparation employed by these authors, reveals an $IR = 1.43$, or an increase of survival time by little more than 40 per cent. This is in the range of resistance provided by vaccination with 0.05 to 0.1 mg. of FI-S. Lévy and Conge (33) report experiments using Rockefeller Swiss mice in which some groups vaccinated with living BCG achieved an $IR = 1.6$ 34 days post vaccination. It must be mentioned in this regard, however, that BCG has been reported by Bloch and Segal (34) to increase survival time roughly threefold ($IR = 2.8$ and 3.5) in CF-1 mice challenged with H37Rv 2 to 3 weeks after vaccination. These authors, comparing vaccinated and control median survival times, actually an unreliable value in small samples, report "vaccination indexes" of 3.7 and 4.8 (*i.e.* greater than fourfold increase in survival time) respectively for the groups referred to. Bloch is of the opinion, however, that the efficacy of BCG, and presumably therefore, of any vaccine, varies greatly with the strain of mice employed.[8]

From the results of experiments with FI-S presented above, nothing can be said about the duration of this effect except that the level of resistance bestowed

[8] In a personal communication, Dr. Bloch states that another worker was able to elicit only minor increases of resistance in a not highly inbred strain of Swiss mice using Dr. Bloch's strain of BCG, which is derived from the Copenhagen strain; but that later with CF-1 mice provided by Dr. Bloch was able to obtain results similar to those of Dr. Bloch.

on this strain of mice by the parent fraction, F I, remains undiminished at 12 weeks post vaccination. The advantage of FI-S or unpurified F I mixed with F II is that these preparations are non-toxic, non-allergic (*i.e.* will not elicit tuberculin sensitivity in guinea pigs), and non-infectious (24). It is strongly felt that these characteristics should be added to the criteria of an acceptable vaccine against tuberculosis.

The authors wish to express their gratitude to Miss A. Hafleigh for her assistance in the analysis of the data and the preparation of the tables.

SUMMARY

Fractionation procedures yielding partially purified vaccine preparations from a 60°C. methanol extract of tubercle bacilli have been described. Some of the preparations have the characteristics of lipopolysaccharides. Certain ones have been found capable of increasing resistance to experimental tuberculosis in albino mice of the Rockefeller Swiss strain. The levels of resistance elicited by these preparations are equivalent to those following vaccination with BCG (Phipps) in this strain of mice as reported by other authors. The admixture of two of the crude fractions in amounts as small as 0.05 mg. each per dose per mouse affords an even greater increase in resistance. Neither of these substances alone in larger doses can approach this degree of efficacy in mouse protection experiments.

The protective activity appears to involve the stimulation of two supplementary mechanisms, one providing a peak resistance between 1 and 3 weeks post vaccination but falling off to a lower level thereafter, the other not responding fully until approximately 6 weeks but continuing undiminished through a 12 week post-vaccination period. The first of these peaks corresponds to an increase in resistance against staphylococci as well as tubercle bacilli. The possibility that the term "broad specificity," rather than "non-specificity," might best describe this phenomenon permits the implication of classical immune mechanisms.

BIBLIOGRAPHY

1. Opie, E. L., and Freund, J., An experimental study of protective inoculation with heat-killed tubercle bacilli, *J. Exp. Med.*, 1937, **66**, 761.
2. Dubos, R. J., Schaefer, W. B., and Pierce, C. H., Antituberculous immunity in mice vaccinated with killed tubercle bacilli, *J. Exp. Med.*, 1953, **97**, 221.
3. Crowle, A. J., Immunizing constituents of tubercle bacillus, *Bact. Rev.*, 1958, **22**, 183.
4. Nègre, L., and Boquet, A., Essais de traitment de la tuberculose expérimentale du lapin et du cobaye par l'antigène méthylique, *Ann. Inst. Pasteur*, 1925, **39**, 101.
5. Nègre, L., Résistance antituberculeuse sans allergie, conférée par l'antigène

méthylique. Etude de sa durée et l'action préventive de cette produit associée à celle du BCG., *Ann. Inst. Pasteur*, 1952, **83**, 429.
6. Nègre, L., and Bretey, J., Nouvelle preuve expérimentale de l'action immunisante de l'antigène méthylique, *Am. Rev. Tuberc.*, 1953, **17**, 1065.
7. Weiss, D. W., and Dubos, R. J., Antituberculous immunity induced by vaccination with killed tubercle bacilli or with a soluble bacillary extract, *J. Exp. Med.*, 1955, **101**, 313.
8. Weiss, D. W., and Dubos, R. J., Antituberculous immunity induced by methanol extracts of tubercle bacilli—its enhancement by adjuvants, *J. Exp. Med.*, 1956, **103**, 73.
9. Pangborn, M. C., Lipo-proteins in serology and the role of lipid haptenes, *Discussions Faraday Soc.*, 1949, **6**, 110.
10. Craig, L. C., King, T. P., and Stracher, A., Dialysis studies. II. Some experiments dealing with the problem of selectivity, *J. Am. Chem. Soc.*, 1957, **79**, 3729.
11. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C Thomas, 1948.
12. Dubos, R. J., and Middlebrook, G., Media for tubercle bacilli, *Am. Rev. Tuberc.*, 1947, **56**, 334.
13. Smith, J. M., and Dubos, R. J., The behavior of virulent and avirulent staphylococci in the tissues of normal mice, *J. Exp. Med.*, 1956, **103**, 87.
14. Dubos, R. J., and Schaedler, R. W., Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infections. I. Protective effects, *J. Exp. Med.*, 1957, **106**, 703.
15. Dean, R. B., and Dixon, W. J., Simplified statistics for small number of observations, *Anal. Chem.*, 1951, **23**, 636.
16. Hoel, P. G., Introduction to Mathematical Statistics, ed. 2, New York, John Wiley, Inc., 2nd edition, 1954.
17. Riley, V., Demonstration of differences between normal and tumor-bearing animals, *Proc. Soc. Exp. Biol. and Med.*, 1958, **97**, 169.
18. Riley, V., The melanoma as a model in a rational chemotherapy study, in Pigment Cell Biology, New York, Academic Press, 1959, 389.
19. White, R. G., Bernstock, L., Johns, R. G. S., and Lederer, E., The influence of components of *M. tuberculosis* and other mycobacteria upon antibody production to ovalbumin, *Immunology*, 1958, **1**, 54.
20. White, R. G., and Marshall, A. H. E., The role of various chemical fractions of *M. tuberculosis* and other mycobacteria in the production of allergic encephalomyelitis, *Immunology*, 1958, **1**, 111.
21. Aebi, A., Asselineau, J., and Lederer, E., Sur les lipides de la souche humaine "Brévannes" de *Mycobacterium tuberculosis*, *Bull. Soc. chim. biol.*, 1953, **35**, 661.
22. Freund, J., and Stone, S. H., Effectiveness of tuberculo-glycolipid as an adjuvant in eliciting allergic encephalomyelitis and aspermatogenesis, *J. Immunol.*, 1959, **82**, 560.
23. Chase, M. W., Experimental sensitization with particular reference to picryl chloride, *Internat. Arch. Allergy and Appl. Immunol.*, 1954, **5**, 163.

24. Williams, C. A., Jr., Studies on fractions of methanol extracts of tubercle bacilli II. Toxic and allergenic properties of fractions employed as anti-tuberculous vaccine, *J. Exp. Med.*, 1960, **111,** in press.
25. Fong, J., Schneider, P., and Elberg, S. S., Studies on tubercle bacillus-monocyte relationship. I. Quantitative analysis of effect of serum of animals vaccinated with BCG upon bacterium-monocyte system, *J. Exp. Med.*, 1956, **104,** 455.
26. Elberg, S. S., Schneider, P., and Fong, J., Cross-immunity between *Brucella melitensis* and *Mycobacterium tuberculosis*. Intracellular behavior of *Brucella melitensis* in monocytes from vaccinated animals, *J. Exp. Med.*, 1957, **106,** 545.
27. Lagercrantz, R., Immunological studies of mycobacteria. 2. Studies of erythrocyte-adsorbable fractions from different strains, *Acta. Path. et Microbiol. Scand.*, 1956, **38,** 492.
28. Dubos, R. J., and Schaedler, R. W., Reversible changes in the susceptibility of mice to bacterial infections. I. Changes brought about by injection of pertussis vaccine or of bacterial endotoxins, *J. Exp. Med.*, 1956, **104,** 53.
29. Böhme, D., and Dubos, R. J., The effect of bacterial constituents on the resistance of mice to heterologous infection and on the activity of their reticuloendothelial system, *J. Exp. Med.*, 1958, **107,** 523.
30. Landy, M., and Pillimer, L., Increased resistance to infection and accompanying alteration in properdin levels following administration of bacterial lipopolysaccharides, *J. Exp. Med.*, 1956, **104,** 383.
31. Landy, M., Increase in resistance following administration of bacterial lipopolysaccharides, *Ann. New York Acad. Sc.*, 1956, **66,** 292.
32. Dubos, R. J., and Pierce, C. H., Differential characteristics *in vitro* and *in vivo* of several substrains of BCG. IV. Immunizing effectiveness, *Am. Rev. Tuberc.*, 1956, **74,** 655.
33. Lévy, F. M., and Conge, G., Le test de protection de la souris critère d'activité de B.C.G., *Rev. immunol.*, 1955, **19,** 210.
34. Bloch, H., and Segal, W., Viability and multiplication of vaccines in immunization against tuberculosis, *Am. Rev. Tuberc.*, 1955, **71,** 228.