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Received September 18, 1964. P.S.E.B.M., 1965, v118.

Resistance to Experimental Tuberculosis Stimulated by Fractions from Attenuated Tubercle Bacilli. (30009)

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It was reported from this laboratory that disruption of viable BCG suspended in light mineral oil led to isolation of a product, consisting predominantly of cell walls, which could be heat-sterilized with retention of immunogenic potency for mice(1). In contrast to these findings, Youmans *et al*(2-5) isolated from viable BCG or H37Ra after mechanical disruption of the cells in buffered aqueous sucrose solution cytoplasmic particles which were immunogenic for mice. This fraction was reported to lose its protective capacity following heat sterilization.

The two types of vaccines reported above cannot be directly compared from the data reported, because different criteria were chosen in evaluation of protective potency. In the test used to evaluate the oil disruption product, mice were vaccinated intravenously and later challenged with an aerosol containing a few virulent bacilli(6). After this type of challenge, tuberculosis takes a relatively mild course; the animals are sacrificed to determine the presence or absence of visible tuberculous lesions in lungs and the number of virulent tubercle bacilli in lungs and spleens. The dose of the oil disruption product needed to produce significant protection was about 100 μg (dry weight). The assay employed by Youmans to determine the immunogenicity of the particulate fraction was based on intravenous challenge of vaccinated and control mice with massive doses of virulent tubercle bacilli. This type of tuberculosis is much more severe than that induced by pulmonary challenge, and the effectiveness of the vaccine is judged by the number of mice that survive 30 days after challenge or by the median survival times. In this test, Youmans' particulate fraction was protective only at a dose of 20 mg (moist weight) administered intraperitoneally.

In view of the apparent differences between the two vaccines, it seemed advisable to compare their immunogenicity by preparing the oil disruption product and the particulate fraction from the same starting lot of cells and testing the preparations in parallel, together with Rosenthal's viable BCG vaccine, in both of the above mentioned assays.

Materials and methods. Preparation of vaccines. a) *Particulate fraction and oil disruption product from BCG.* A strain of BCG from the Pasteur Institute, which had been

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maintained and stored on Sauton's potato medium, was subcultured once in Sauton's liquid medium, grown as pellicle culture on this medium for 10 days, and harvested on a sterile gauze filter supported on a stainless steel screen. The moist harvest was divided and 25 g were used to prepare the particulate fraction(5). All procedures were performed at temperatures not higher than 4°C. The final pellet, consisting of the particulate fraction, was suspended in 0.01 M phosphate buffer (pH 7.0) and stored in the cold for one day prior to use in animal experiments. The equivalent of 27 mg moist particulate fraction was plated on Dubos' albumin agar. An average of 17.5 viable acid-fast bacilli per mg (moist weight) were detected.

The remaining 25 g of the moist cell mass were suspended in mineral oil (light liquid petrolatum N.F.) to a volume of 100 ml and ruptured in the Sorvall refrigerated pressure cell at a pressure of 40,000 psi at 5-10°C. The effluent was centrifuged in a Sorvall SS-34 rotor at 13,000 RPM (20,000 *g*) for one hour to collect the oil-insoluble products. These were washed 3 times with light mineral oil by resuspension and centrifugation, then resuspended to a final concentration of 100 mg per ml (moist weight) in saline containing 0.2% "Tween 80" and heated for 30 minutes at 65°C. Twenty-seven mg of the material gave no growth upon culture for acid-fast organisms. This preparation was termed "oil disruption product."

b) *Particulate fraction and oil disruption product from H37Ra.* The H37Ra strain used for this experiment (obtained from Dr. W. Steenken, Trudeau Institute, New York) had been maintained on Petragnani's medium. It was subcultured twice on Sauton's liquid medium and then grown as pellicle cultures on modified Proskauer and Beck medium at pH 7.0(7). The 4-week-old cultures were harvested on sterile gauze filters and washed on the filters with 0.01 M phosphate buffer, pH 7.0(5). A portion of the cell mass (80 g) was processed to prepare the particulate fraction in the same manner as described for BCG. The final particulate fraction was suspended in 0.01 M phosphate buffer (pH 7.0) and stored in the frozen state until used for animal experimentation. When 27 mg of moist particulate fraction was plated on Dubos' albumin agar, an average of 1000 viable acid-fast bacilli per mg was detected.

The breakup residue, or first sediment (5000 RPM, 3,900 $\times$ *g*), of the cells processed in the Waring blendor and the Sorvall pressure cell, which consisted predominantly of cell walls, was not discarded but suspended in saline containing 0.2% "Tween 80," heated for 30 minutes at 65°C and tested in animals. Upon culturing, 3 mg of the product was found free of viable acid-fast organisms.

The remainder of the harvested and washed cell mass (10 g) was suspended in mineral oil to give a volume of 50 ml processed to prepare the oil disruption product in the same manner as described for BCG. Upon culturing, 27 mg of the final product was found to be free of viable acid-fast organisms.

Immunity tests. Female albino Swiss mice, 22 to 25 days of age, from the colony of the Rocky Mountain Laboratory, were employed in all protection tests.

a) *Protection of mice against massive intravenous challenge with virulent* Mycobacterium tuberculosis. The procedure used by Youmans and Youmans(5) was followed. Mice in groups of 20 were inoculated intraperitoneally with 0.2 ml volumes containing graded doses of the various bacillary fractions, and challenged intravenously 4 weeks later with 1 mg (moist weight) of a suspension of virulent *M. tuberculosis,* H37Rv (strain obtained from Dr. W. Steenken). The challenge doses of H37Rv for mice immunized with the BCG products or the H37Ra products were 2.4 $\times$ 10^7 and 7.2 $\times$ 10^7 viable particles, respectively. The acquired resistance usually was measured on the basis of mice which survived for 30 days (2-5). In cases where the degree of immunity was low and more than 50% of the mice died within the first 30 or 60 days after challenge, vaccine efficacy was measured by comparing median survival times of vaccinated and control mice using the method of Litchfield(8).

b) *Protection of mice against exposure to aerosols containing virulent* M. tuberculosis. Mice in groups of 20 were vaccinated intra-

TABLE I. Protection of Mice Against Massive Intravenous Challenge with Virulent Tubercle Bacilli Given 4 Weeks After Intraperitoneal Immunization with Particulate Fraction or Oil Disruption Product from BCG, or Living BCG.

		Results after challenge with 2.4×10^7 virulent H37Rv			
		30 days		60 days	
Vaccine	Dose	Survivors	Median survival time (95% confidence limits)	Survivors	Median survival time (95% confidence limits)
	(mg*)	(%)	(days)	(%)	(days)
Particulate fraction	20.0	85	—	45	56.6 (47.6–67.4)
	5.0	60	—	35	40.0 (27.8–59.6)
	1.25	45	25.8 (21.2–31.4)	10	27.7 (20.5–37.4)
Oil disruption product, heated	20.0	100	—	95	—
	5.0	100	—	80	—
	1.25	100	—	85	—
Living BCG	.2†	80	—	60	—
Controls	None	30	20.9 (18.0–24.5)	25	20.9 (18.0–24.3)

* Moist weight, 20 mice per dose.

† 7.7×10^6 viable count.

venously with 0.2 ml volumes containing graded doses of the various bacillary fractions. Four weeks later the mice from each group, together with unvaccinated control mice, were challenged by exposure to an aerosol containing virulent *M. tuberculosis*, H37Rv, in a Middlebrook apparatus by procedures previously described(6). Four weeks after challenge, all mice were sacrificed and examined for the presence of pulmonary tubercles. The lungs of 10 mice from each group of animals vaccinated with fractions prepared from BCG and nonvaccinated controls were cultured to determine the number of virulent tubercle bacilli present per gram of homogenized lung tissue.

In both types of protection test, mice were also inoculated with 0.2 mg (moist weight) of a standardized living BCG vaccine supplied by Dr. Rosenthal, Tice Laboratory, Chicago, Ill. This dose contained 7.7×10^6 viable particles, as determined at time of inoculation.

Results. (1) *Comparison of immunogenicity of particulate fraction with that of oil disruption product.* The immunizing potencies of these fractions prepared from BCG and that of a living BCG standard vaccine, as determined by the method of Youmans, are compared in Table I. Of the mice receiving 20 mg moist particulate fraction 85% survived for the 30-day period. Since 30% of the nonimmunized control mice survived for 30 days, the immune response to the particulate fraction prepared in this laboratory was comparable to that reported by Youmans(2-5). The dose response was noteworthy: 60% and 45% of the mice given 5 mg and 1.25 mg, respectively, survived. In contrast, no mice which received the oil disruption product died, even at the lowest dose level of 1.25 mg. This protection appeared superior to that afforded by 0.2 mg moist BCG standard which contained 7.7×10^6 viable organisms. Instead of concluding the test at the end of the 30-day period, the mice were observed for an additional 30 days (Table I). At each dose level, only 5% to 20% of the mice immunized with oil disruption product had died, whereas 55% to 90% of those animals immunized with the cytoplasmic particulate fraction had died.

Wherever possible, median survival times and their 95% confidence limits(8) were also determined and are included in Table I. They are applicable only to the particulate fraction and show significant differences from the controls only in groups receiving the 2 higher doses. After 70 days, more than 50% of all groups of mice vaccinated with oil disruption product were still alive whereas those receiving even 20 mg of particulate fraction had no more survivors than the controls.

In a repeated test, which included an additional 4-fold dilution of oil disruption product, all normal control animals died and as little as 0.31 mg moist weight (equivalent to about 60 μg dry weight), still afforded protection almost equal to that of the highest dose.

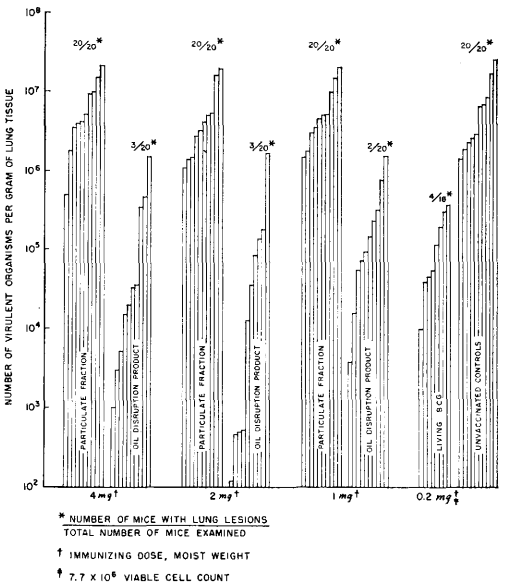

FIG. 1. Protection of mice against air-borne challenge with virulent tubercle bacilli by intravenous vaccination with the particulate fraction from BCG, oil disruption product from BCG, or living BCG. Comparison of number of virulent organisms per 100 mg of lung tissue and number of mice with lung lesions 4 wk after challenge. (Each bar represents one mouse.)

In our experience, it has not been possible to isolate sterile particulate fractions from disrupted BCG by following the centrifugation procedures outlined by Youmans(5). Thus, the effective mouse dose (20 mg, moist weight) of the particulate fraction used in the above described protection test contained approximately 350 viable cells.

Results of the protection test based on immunization by the intravenous route and challenge by exposure to aerosols containing virulent bacilli are presented in Fig. 1. The number of mice with lung lesions and the number of virulent tubercle bacilli present per gram of homogenizing lung tissue in mice vaccinated with 4, 2, or 1 mg each of particulate fraction or oil disruption product, or with 0.2 mg living BCG were determined 4 weeks after challenge. Eighty-five to 90% of the mice vaccinated with oil disruption product and about 80% of those vaccinated with living BCG were found to be free of pulmonary lesions, whereas such lesions were found in all of the mice immunized with the particulate fraction. The bar diagram shows that lungs of mice vaccinated with oil disruption product or viable BCG harbored considerably fewer virulent bacilli than those of mice vaccinated with the particulate fraction or of nonvaccinated control mice. To analyze data presented in Fig. 1, the median number of bacilli was determined for combined groups (first step of the median test) (9). When combining the results for each of the 3 groups of mice which had been given the particulate fraction with those of the control group, one-half of each group's scores were above the median and one-half were below. This test failed to show any difference between control mice and mice immunized with the particulate fraction, insofar as numbers of tubercle bacilli in the lungs were concerned. In the case of the oil disruption product, however, at each dose level, lungs of 9 out of 10 animals had a lower viable cell count than the combined median, and lungs of 9 out of 10 control animals contained more viable cells than the combined median. The Fisher exact probability test was chosen to analyze the data split at the median. The computed probability of occurrence by chance alone ($p = 0.0005$) is interpreted as evidence that significant protection had been afforded by the oil disruption product. A similar level of probability ($p = 0.0002$) was computed from the combined median of the lung culture data of the group of mice vaccinated with BCG and of the group of control mice. The same method indicated that the difference between values for mice given oil disruption product in any of the 3 doses, and for those given viable BCG was not significant.

(2) *Immunogenicity of particulate fraction compared with oil disruption product prepared from H37Ra.* We also compared the immunogenicity of a particulate fraction from H37Ra with that of oil disruption product prepared from the same culture, since the former has been reported to yield more consistent results in protection tests than the corresponding fraction isolated from BCG (3). For preparation of the particulate fraction from H37Ra, it was considered essential not only to adhere to procedures by which it was isolated from bacilli by Youmans and co-workers but also to follow their procedures for cultivating the cells. Since the heat-labile

TABLE II. Protection of Mice Immunized with Fractions from H37Ra or Living BCG and Challenged 4 Weeks Later with Virulent Tubercle Bacilli.

Vaccine	Intraperitoneal immunization, intravenous challenge with 7.2×10^7 H37Rv				Intravenous immunization, aerosol challenge with H37Rv	
	Immunizing dose	No. of mice	Survivors‡	Median survival time (95% confidence limits)	Immunizing dose	Animals without lung lesions‡
	(mg*)		(%)	(days)	(mg†)	(%)
Particulate fraction	20.0	20	60	—	4.0	0
	5.0	20	30	18.6 (17.5–19.8)	2.0	0
	1.25	20	20	20.4 (18.7–22.3)	1.0	0
Insoluble cell residue of particulate fraction, heated	20.0	20	80	—	4.0	0
	5.0	20	95	—	2.0	0
	1.25	20	45	—	1.0	0
Oil disruption product, unheated	20.0	20	80	—	4.0	90
	5.0	20	80	—	2.0	100
	1.25	18	61	—	1.0	90
Oil disruption product, heated	20.0	20	70	—	4.0	100
	5.0	17	77	—	2.0	80
	1.25	20	55	—	1.0	80
Living BCG	.2§	19	58	—	.2§	80
Controls	None	40	5	19.0 (18.6–20.0)	None	0

* Moist wt.
† " " 10 mice per dose.
‡ 30 days after challenge.
§ 7.7×10^6 viable cells.

particulate fraction is described as being of protoplasmic origin, and the heat-stable resistance-enhancing activity of oil disruption product is assumed to be a cell wall constituent, we also examined the immunogenicity of the insoluble residue from the particulate fraction. This residue, which consisted of cell walls and unbroken cells, was sterilized by treatment with heat. Finally, it was considered worthwhile to test the oil disruption product both before and after heat treatment.

The particulate fraction from H37Ra, which was subsequently prepared for this study according to the procedure outlined above, was found to contain far more viable cells than the corresponding fraction from BCG described in the first section of results (approximately 20,000 cells and 350 cells per mouse dose of 20 mg, respectively), even though the former had been filtered through a Millipore® membrane filter of 0.45 μ porosity(5). A possible explanation for this difference lies in the observation that, under these conditions, cells of H37Ra are considerably shorter than those of BCG and probably have a different sedimentation rate.

Results of the protection test based on intraperitoneal immunization and intravenous challenge with a large dose of virulent H37Rv are given in Table II. Again, as in the case of the particulate fraction from BCG reported in this work, the immune response to the 20 mg dose of the corresponding fraction from H37Ra was comparable to that reported in the literature(2-5). Although more mice of the groups given 5 mg and 1.25 mg of the particulate fraction survived for 30 days than did animals of the nonimmunized control group, median survival times of the two groups did not differ significantly. (Note the markedly small range between upper and lower 95% confidence limits.) In contrast, median survival time of mice given the smallest dose of oil disruption product (1.25 mg) was greater than 30 days, and the protection this dose afforded appeared to equal that of the living BCG standard.

The finding that heat treatment did not impair the protective ability of the oil disruption product was not surprising; however, it was rather unexpected to find that the heat treated, insoluble residue remaining after removal of the particulate fraction provided, in this test, protection superior to that of the particulate fraction itself.

Results of the protection test which consisted of intravenous immunization and challenge by aerosol containing virulent H37Rv also are listed in Table II. All of the mice immunized with the particulate fraction had pulmonary lesions, whereas only 0-20% of the mice immunized with oil disruption product and 20% of the mice given viable BCG had pulmonary lesions. Thus, as in the case of the particulate fraction from BCG, the corresponding fraction from H37Ra gave little or no protection in this test. Also without effect was the heated, insoluble residue from which the particulate fraction had been prepared and which consisted predominantly of cell walls. Since rupture of the starting organisms leading to this fraction had been performed in buffered sucrose, this finding is in agreement with results reported elsewhere (1,10) that cell walls prepared by disrupting organisms in aqueous menstrua instead of in oil provided very little, if any, protection in the test with pulmonary challenge.

(3) *Tests for delayed hypersensitivity to PPD produced by oil disruption product from BCG.* Guinea pigs in groups of 6 were vaccinated subcutaneously, intravenously, or intraperitoneally with 0.2 mg, moist weight, viable BCG or 15 mg, moist weight, of the same batch of oil disruption product from BCG used in the test for immunogenicity in mice. The viable BCG cells were administered at the standard dose level which was used in the mouse protection test, whereas the dose of oil disruption product employed in this test for sensitivity exceeded by a ratio of 15 to 1 that required to produce significant protection in mice. Subcutaneous and intravenous administration of the oil disruption product was performed in either a single dose or in 2 or 3 doses four days apart. Five weeks after the last vaccination, the animals were inoculated intradermally with serial 5-fold dilutions containing PPD in amounts ranging from 1 to 0.008 μg. The lesions which did not disappear within 48 hours after challenge with PPD were typical delayed reactions. Any lesion with induration less than 0.4 mm in thickness was considered negative. Five out of 6 animals vaccinated subcutaneously with 0.2 mg viable BCG standard reacted to 0.2 μg, whereas none of the 18 animals vaccinated with 15 mg of oil disruption product reacted to this dose of PPD and only 2 out of these 18 animals reacted to a 5-fold larger dose of PPD (1 μg). The skin reactions of the animals immunized with BCG to 0.2 μg of PPD varied in thickness from 0.4 to 1.2 mm and the area of induration from 8 $\times$ 10 to 12 $\times$ 12 mm. The lesions of the 2 animals vaccinated with oil disruption product which reacted to 1 μg of PPD were 0.7 mm thick and the areas of induration were 7 $\times$ 8 mm and 12 $\times$ 13 mm, respectively. From these results, it is evident that the oil disruption product was considerably less active in sensitizing guinea pigs against PPD than was the viable BCG vaccine. Essentially the same results were obtained when the oil disruption product was administered intravenously. Sensitivity to PPD for both BCG and oil disruption product vaccinated animals was greater when the intraperitoneal rather than the conventional subcutaneous route was used to administer the vaccines.

Discussion. The protective value of BCG has been consistently confirmed in a large number of different experimental hosts among which a varying degree of susceptibility and natural resistance is to be expected. To evaluate the effectiveness of nonviable vaccines, it is considered necessary, therefore, always to compare their effect in parallel dose response assays with the viable cell vaccine. In experimental infections for evaluation of prophylactic agents, the results observed may be strongly influenced by the choice of animal, route of inoculation, interval between vaccination and challenge, size of challenge dose and other conditions of the test. In tuberculosis, the choice of the mouse as an experimental host appears well justified(11, 12). The test system so far preferred in experimental tuberculosis has been based on massive intravenous challenge. Significantly longer survival times in vaccinated than in unvaccinated animals provided quantitation of the resistance stimulated by the vaccines. The test fails, however, to reveal the mechanism by which a vaccine exerts its effect, for example, whether the protection is specific or nonspecific. The desirability of developing a test in which the infection occurring

from the challenge takes a milder course than in animals challenged by the above method and yet permits quantitative differentiation between vaccinated and nonvaccinated animals was stressed by Bloch(12). According to this author, small infective doses would more closely duplicate conditions which prevail in the great majority of cases of human tuberculosis. The test system used in this laboratory, which is based on pulmonary challenge of mice by a few virulent tubercle bacilli, appeared to meet these requirements. Indeed, according to the results described here and elsewhere(10), we consider this test system to be the most valid now available for evaluating the relative efficacies of nonliving and living vaccines against experimental tuberculosis. In this test, nonspecific antigens such as *Bordetella pertussis* vaccine and endotoxin prepared from *Salmonella* are without value and interference does not appear to be the mechanism of protection(6). Under the conditions of the aerosol challenge test, the lungs of unprotected mice show numerous grossly visible lesions and the organs contain many tubercle bacilli. Mice given a good vaccine, such as viable BCG or H37Ra or oil disruption product, have few, if any, lung lesions and harbor relatively few of the pathogens in their tissue. Based on these criteria, heat-killed whole cells, with or without treatment with light mineral oil, stimulated only moderate resistance(10); cell walls and protoplasm isolated from BCG ruptured in aqueous suspensions(1) and Nègre-type methanol extracts(10) were without effect. As reported here, the same was true for the Youmans-type particulate fraction even when administered in 20 mg doses. At this dose level the particulate fraction stimulated considerable resistance in the massive challenge test but conferred no protection at dose levels at which the viable BCG and oil disruption product proved effective. Using massive challenge, but intravenous instead of intraperitoneal immunization, we obtained essentially similar results (not reported here). On a weight basis, therefore, oil disruption product approached or equalled live vaccine in ability to produce all of the phenomena that have so far been used in our laboratory as criteria to judge the effectiveness of experimental vaccination. Future work plans include attempts to immunize animals with aerosols prepared from oil disruption product and to gain information on the length of time during which immunization with this vaccine is effective.

In our experience, it has not been possible to prepare sterile Youmans-type particulate fractions from either disrupted BCG or H37Ra by following the centrifugation and filtration procedures outlined by Youmans *et al*(5). Although we found up to 20,000 viable acid-fast organisms per mouse dose of the particulate fraction from H37Ra, this number of viable cells has been reported to be much too small to produce a detectable immune response(2,3).

Additional experiments (not reported here) showed that treatment of the protoplasmic particulate fraction with oil did not enhance its protective potency in either the massive or aerosol challenge test. Whether the low-level protection stimulated by the particulate fraction is attributed to a substance which differs from the antigen responsible for immunity afforded by oil disruption product or whether the vaccine potency can be attributed to the same component(s), present in the particulate fraction in low concentration, is not known.

Resistance-enhancing properties of vaccine preparations described in the literature, with the exception of Nègre's methanol extract (13) and Youmans' particulate fraction(14), were always accompanied by ability to sensitize to tuberculin (*e.g.*, Smith *et al*(15)). This property is expected for vaccines consisting of living or dead bacilli or purified allergenic factors derived from them(12). The above two extracts, which did not sensitize guinea pigs to tuberculin although they afforded protection of mice against massive challenge with virulent tubercle bacilli, did not protect mice against infection by the pulmonary route. The oil disruption product was effective in relatively small nonsensitizing doses in both types of protection test. It is noteworthy that the cell walls isolated from BCG disrupted in aqueous menstrua instead of oil are highly allergenic(16-18) and afford significant protection in tests employing massive challenge, as reported by

Kotani *et al*(17) and confirmed in this work, but afford poor protection in the pulmonary challenge test(1,10). The effect of oil is not yet understood. Work in progress has shown that removal of most of the oil from an oil disruption product by extracting it with petroleum ether in a Soxhlet apparatus did not impair the resistance-enhancing potency or restore allergenicity.

Summary. The protective values against experimental tuberculosis in mice of Youmans' cytoplasmic particulate fraction and of oil disruption product, both prepared from the same lots of *Mycobacterium tuberculosis,* strains BCG and H37Ra, were tested in parallel with viable BCG vaccine. Two tests were employed, one based on intravenous challenge with large doses and the other on pulmonary challenge with a few virulent tubercle bacilli (H37Rv). Results are presented demonstrating that the vaccine efficacy of the oil disruption product approached or equalled that of similar doses of viable BCG by all criteria used to estimate the response, while its ability to sensitize guinea pigs to PPD was considerably less than that of viable BCG. The cytoplasmic particulate fraction prepared according to the method of Youmans *et al,* while effective at high dose levels in the massive challenge test, was not effective in the aerosol challenge test. In contrast to the particulate fraction, sterilization by heat did not impair the protective potency of oil disruption product.

The authors express gratitude to the Montana Tuberculosis Association for the technical services of Larry Ewalt.

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Received November 12, 1964. P.S.E.B.M., 1965, v118.

Intestinal Transport of Amino Acids and Glucose in Flounder Fish.* (30010)

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The transmucosal movement of aromatic amino acids and glucose by mammalian and amphibian intestine has been widely reported and is considered to be an active process. Little is known about the intestinal transport

* This study was supported by grants of Nat. Science Foundation (GB-2113) and Nat. Inst. of Arthritis & Metab. Dis., NIH, (AM-02217-07).
† Supported by U.S.P.H.S. training grant 5 T1 GM 461, Nat. Inst. of Gen. Med. Sci.