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VII. Active principles of tuberculin prepared from non-protein substrates.

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Diagnostic tuberculins which are used routinely at the present time, appear to be unsatisfactory because they lack certain requirements. The ideal preparation should be simple and constant in composition and should be prepared by a standard method; it should be free from substances which give non-specific reactions and these should show reasonable correlation with the extent and severity of the tuberculous infection. Because the interpretation of a tuberculin test depends chiefly upon what is seen on the skin, it is important that there be no extraneous factors modifying the capacity of the skin to react specifically. Since vasomotor excitability appears to run parallel with the intensity of the tuberculin reaction, it is evident that non-specific proteins or indeterminate irritating substances which affect vasomotor excitability should be excluded.

For these reasons the problem has been studied by using tuberculins prepared from non-protein synthetic media. Preliminary studies with such material were reported by the writer.[1, 2] These tuberculins contain only elements manufactured by the tubercle bacilli in the nutrient substrate. From this media it is possible to separate chemically a number of fractions which represent a tuberculin principle more accurately than ordinary complex tuberculins.

The present investigation includes (A) sensitization experiments in normal, non-tuberculous guinea pigs, (B) intracutaneous tests in tuberculous guinea pigs, and (C) clinical diagnostic tests with tuberculin fractions in a series of juvenile patients.

Preparation of Tuberculin Fractions. Tuberculin was prepared from non-protein media of the following composition, according to a formula of Gessard and Vaudremer:[3]

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[1] Eberson, F., PROC. SOC. EXP. BIOL. AND MED., 1925, xxii, 346.

[2] Eberson, F., *Ibid.*, 1924, xxi, 539; *Am. Rev. of Tuber.*, 1925, x, 681; *Am. J. Dis. Child.*, 1925, xxix, 29.

[3] Gessard, C., and Vaudremer, A., *Compt. rend. Soc. de biol.*, 1922, xxcvi, 1012.

Ammonium succinate	0.5	gm.
Dipotassium phosphate	0.5	gm.
Magnesium sulphate	0.25	gm.
Calcium chloride	0.125	gm.
Distilled water	100.0	cc.

To one portion of this substrate 2 per cent glycerine was added. After the tubercle bacilli had been growing on this media for five to six weeks, the tuberculin was prepared according to the usual method by filtration and concentration to one-tenth of the original volume. The growth and staining properties of tubercle bacilli were modified by this synthetic media. In the glycerinated portion the organisms grew more luxuriantly than in the portion to which no glycerine had been added. In the former, half of the organisms lost their original acid-fast characteristic, while all of them lost it in the glycerine-free media.

A weighed volume of tuberculin (100 gm.) was fractionated by adding it to 19 volumes of pure methyl alcohol to effect complete precipitation. The filtrate, after treatment with four volumes of pure ethyl ether, yielded a precipitate containing a second fraction. From the resultant filtrate a third fraction was obtained by concentrating the material at 37 degrees Centigrade to practical dryness. These substances were dissolved separately in a given volume of physiological salt solution to which 0.25 per cent ether-tricresol was added as a preservative. The alcohol-insoluble fraction represented 4.6 per cent by weight of the original tuberculin, and one cubic centimeter of the test material contained 49.8 milligrams of active substance. The ether-insoluble fraction, 2.3 per cent by weight of the original tuberculin, contained 25.3 milligrams of active substance in one cubic centimeter. The ether-soluble third fraction was exceedingly small in amount and up to the present time has not been checked with sufficient quantitative accuracy to warrant a definite figure. Roughly, it represented less than 0.5 per cent by weight of the original tuberculin and contained approximately 4.4 milligrams of active substance in one cubic centimeter. This fraction was gummy and fatty or waxy and *gave none of the tests for protein.*

Sensitization Experiments in Normal, Non-Tuberculous Guinea Pigs. Duplicate series of normal guinea pigs weighing 250-300 grams were injected weekly with gradually increasing amounts of each of the tuberculin fractions by the subcutaneous route. The number of injections totalled ten and ranged from

0.1 cubic centimeter for the first dose to 1.2 cubic centimeters of the different fractions at the time of the last injection. From the quantitative determinations it was calculated that the animals received the following total amounts of active substances in the course of treatment: Alcohol-insoluble fraction, 318.7 mg.; ether-insoluble fraction, 192.3 mg.; ether-soluble fraction, 29.5 mg.

Intracutaneous tests were made one week after the last injection. Homologous as well as heterologous fractions were injected in amounts of 0.01 cubic centimeter into the shaved and sterilized skin of the abdomen. The results were read after 24 hours and daily thereafter. The outcome of these experiments was decisive and confirmed the studies which have been alluded to already. Of added significance here is the fact that *protein-free substrates yielded a tuberculin which was active in toto and contained specific sensitizing substances that could be separated out by chemical methods.* One or more injections of derivatives or fractions from ordinary tuberculin or from a non-protein tuberculin substrate, therefore, sufficed to elicit typical skin reactions in non-tuberculous guinea pigs. These results are illustrated in Figures 1-3. Control animals were uniformly negative with the same substances.

Intracutaneous Tests with Tuberculin Fractions in Tuberculous Guinea Pigs. In the tuberculized guinea pig it was demonstrated that these same fractions would give unequivocally positive skin reactions by the intracutaneous technic. The results were obtained repeatedly in several duplicate series of animals. Such tests were made after the inguinal lymph nodes had become palpable in animals previously inoculated subcutaneously with a constant amount of human tubercle bacilli of fixed virulence (1.2 mg. dry weight). In most instances this occurred after three weeks. Each of the test substances was injected in amounts of 0.01 cubic centimeter into the skin of the abdomen, and the results were noted after 24, 48, and 72 hours. An illustration of such a reaction with the ether-insoluble fraction is shown in Figure 4.

Clinical Diagnostic Tests with Tuberculin Fractions in Children. Intracutaneous tests have been made thus far on 150 children and a smaller group of adults. Sixty juvenile patients have been tested with fractions prepared from ordinary tuberculin.

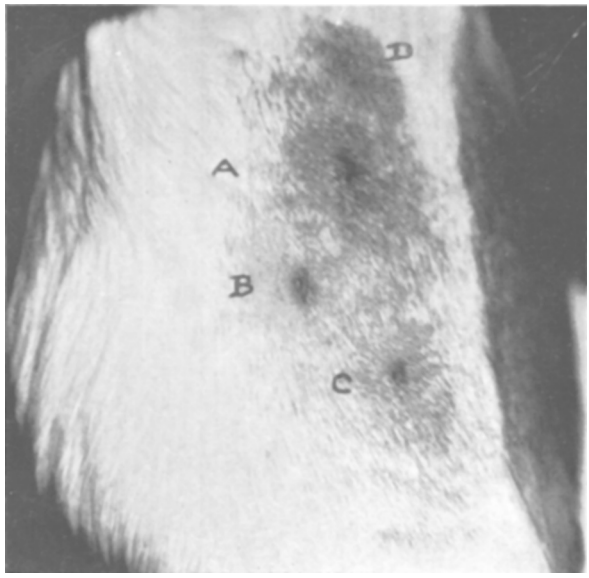

FIG. 1.

Intracutaneous tests in non-tuberculous guinea pig sensitized with ether-soluble fraction prepared from synthetic, protein-free tuberculin substrate, non-glycerinated. Reactions 24 hours old to 0.01 cc. of homologous fraction, (D); ether-soluble fraction (glycerinated substrate), 0.01 cc., (A); ether-insol. fraction (glycerinated substrate), 0.01 cc., (B); original tuberculin from protein-free, glycerinated substrate, 0.01 cc., (C).

The remainder received protein-free substrate tuberculin and its fractions. These preparations, eight in number, included a duplicate series of substances which were derived from glycerine-free tuberculin substrates. Controls were made with ordinary human and bovine tuberculins and with physiological salt solution in each patient. For the intracutaneous tests 0.1 cubic centimeter of a 1:1,000 dilution of each test substance was injected. The results were read after 24, 48, and 72 hours. The amount of "tuberculin substance" which was injected differed for each preparation. The fractions contained decidely less of the active substance in any give dose in comparison with ordinary tuberculin, yet they proved to be potent as well as specific. A few of these reactions are illustrated in Figures 5-7. The relative amount of active substance in a given test was calculated as follows: Ordinary old tuberculin, 0.1 mg.; alcohol-insoluble fraction, 0.005 mg.; ether-insoluble fraction, 0.0025 mg.; ether-soluble fraction, 0.0005 mg.

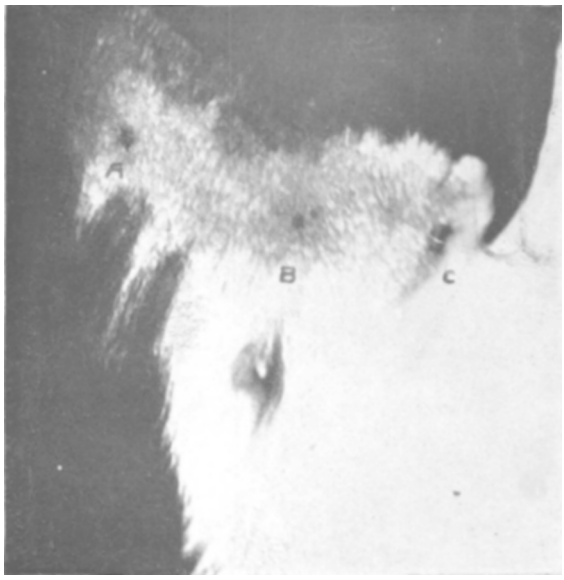

FIG. 2.

Intracutaneous tests with 0.01 cc. of tuberculin fractions in non-tuberculous guinea pig sensitized with ether-insoluble fraction prepared from synthetic, protein-free tuberculin substrate, glycerinated. Reactions 24 hours old. Homologous fraction, ether-insoluble, (A); ether-soluble fraction (glycerinated substrate), (B); original tuberculin from glycerinated substrate, (C).

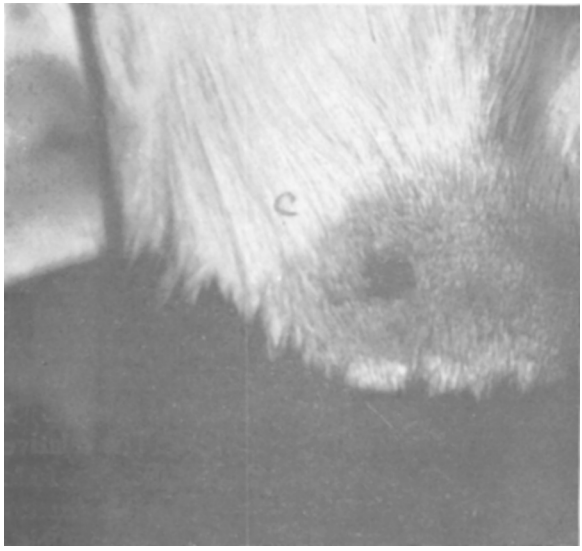

FIG. 3.

Same guinea pig as in Fig. 2, showing reaction in natural size at (C), corresponding to intracutaneous injection with 0.01 cc. of tuberculin from glycerinated non-protein substrate.

Intracutaneous tests with tuberculin fractions gave definite skin reactions which were usually less severe than those which were observed with ordinary tuberculins. The results, nevertheless,

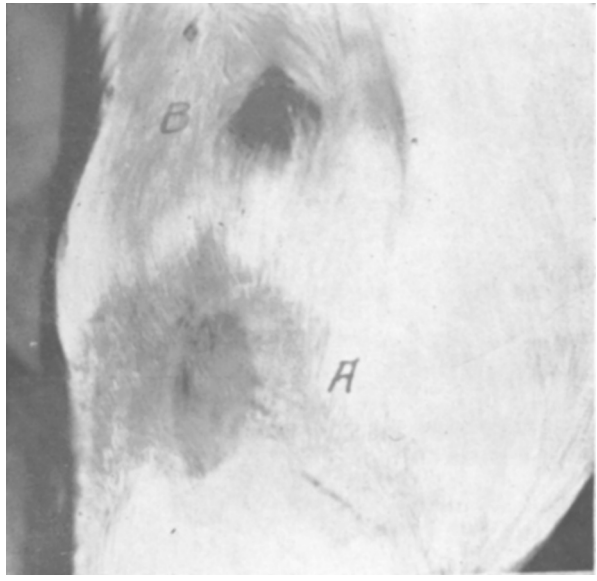

FIG. 4.

Tuberculous guinea pig, three weeks after inoculation with human tubercle bacilli. Intracutaneous test with 0.01 cc. ether-insoluble fraction derived from glycerinated non-protein tuberculin substrate. (A) drainage tuberculous abscess at (B). Skin test 24 hours old.

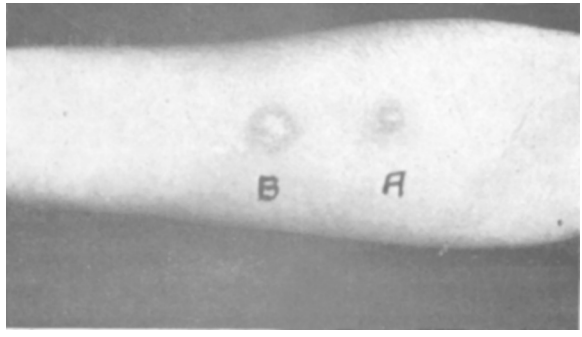

FIG. 5.

Intracutaneous tests in patient with peribronchial tuberculosis and tuberculosis of cervical lymph nodes. (A) 0.1 cc. of 1:1,000 dilution of ether-insoluble fraction fromno n-protein, glycerine-free tuberculin substrate. (B) 0.1 cc. of 1:1,000 dilution of same fraction with glycerinated substrate. Amount of active substance in fraction, 0.0025 milligram. Reaction 24 hours old.

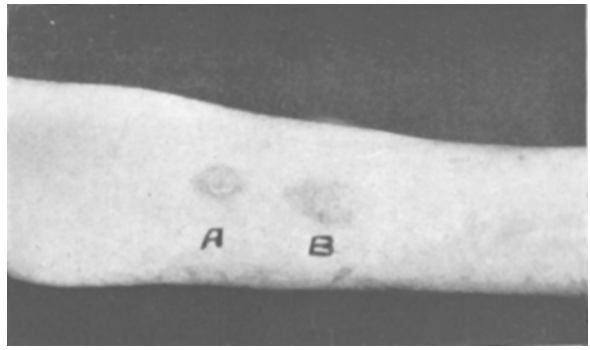

FIG. 6.
Intracutaneous tests in patient with peribronchial tuberculosis. (A) 0.1 cc. of 1:1,000 dilution of non-protein, glycerine-free tuberculin. (B) 0.1 cc. of 1:1,000 dilution of same substance, glycerinated. Amount of active tuberculin substance, 0.008 milligram. Reaction 48 hours old.

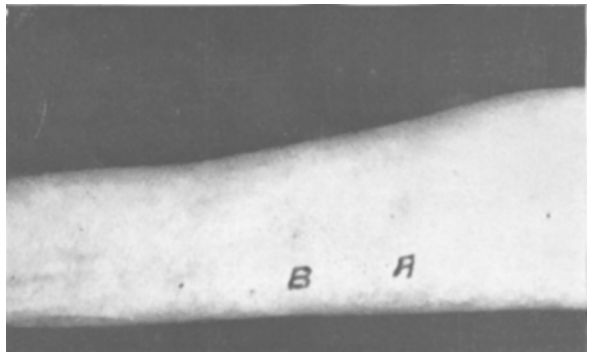

FIG. 7.
Intracutaneous tests in same patient as in Fig. 6. (A) 0.1 cc. of 1:1,000 dilution of ether-soluble fraction from non-protein, glycerine-free tuberculin substrate. (B) 0.1 cc. of 1:1,000 dilution of same fraction from glycerinated substrate. Amount of active tuberculin substance, 0.0005 milligram. Reaction 72 hours old.

could be better correlated with the clinical histories, physical findings, and with the X-ray. In certain instances the observations suggested that the reactions might be correlated with the degree of activity or of an arrested condition in a given tuberculous infection. However, more data are being analyzed in order to verify this striking observation. Of special interest was a group of contact patients in a number of whom a clinical diagnosis of tuberculosis could not be made. In these children the ordinary tuberculins gave markedly positive reactions while the fractional tuberculins gave negative reactions. This was true

also in another group of patients who had no positive clinical findings and whose X-ray films showed bronchial adenitis.

Summary of Tuberculin Tests in a Series of 150 *Children.*

	Per cent
Positive intracutaneous tests with ordinary O. T. and with tuberculin fractions	70.0
Positive tests with ordinary O. T. and negative tests with tuberculin fractions	30.0
Positive tests with ordinary O. T. in patients with negative clinical findings and negative X-ray	22.0
Positive tests with tuberculin fractions in patients with negative clinical findings and negative X-ray	00.0

SUMMARY AND CONCLUSIONS

It has been shown for the first time that small amounts of tuberculin fractions prepared from protein-free synthetic substrates are capable of sensitizing normal, non-tuberculous guinea pigs. In these animals, typical skin reactions can be elicited subsequently by intracutaneous injection of minute amounts of homologous as well as heterologous fractions and of unfractionated tuberculin from which such fractions have been prepared.

Clinical trial of these fractions in juvenile patients has demonstrated for the first time that the substances have diagnostic value. Positive skin tests were perfectly correlated with positive clinical and laboratory findings of tuberculous infection. Such was not the case for ordinary old tuberculin in over 20 per cent of young patients in a group of 150 who were studied routinely. In a number of instances the observations suggested that the reactions might be correlated with the degree of activity or with arrested tuberculous infection.

The present studies point to a method which makes an accurately standardized tuberculin available for routine clinical work. It is believed that the active principle of tuberculin can best be isolated by this or by a similar method.

The observations on sensitization suggest that these may be applied to studies of immunity, and by extension, to therapeutic studies in the experimental animal. Such work, now in its third year of progress, will be reported in the future.