A STUDY OF SENSITIVITY TO HEMOPHILUS PERTUSSIS IN LABORATORY ANIMALS

II. HEMOPHILUS PERTUSSIS ALLERGEN AND ITS ASSAY ON LABORATORY ANIMALS

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This paper deals with our attempt to isolate different fractions from *Hemophilus pertussis* organisms and to estimate their influence on the development of sensitivity. It is also concerned with methods of assaying *H. pertussis* allergen.

THE PREPARATION OF FRACTIONS FROM H. PERTUSSIS

The *H. pertussis* organisms used for this work were grown on blood agar or in liquid media; however, no difference was noted in the final products derived from organisms grown in these media. In the course of this work we prepared several fractions from *H. pertussis* organisms.

One of these fractions was a denaturated nucleoprotein which we prepared by first extracting lipoids from the organisms, following the technique of R. J. Anderson (1927*a*, 1927*b*, 1932). According to this method, the bacilli collected at the end of the growth period were first dehydrated with acetone and then extracted with a mixture consisting of equal parts of alcohol and ether containing 1 per cent hydrochloric acid. After removal of the lipoid fraction the organisms were extracted twice with water acidified to pH 4. In this way proteins soluble in slightly acid condition were eliminated. Finally, the bacilli were extracted twice with water adjusted by the addition of sodium hydroxide to an alkaline reaction (red to phenolphthalein) and preserved with merthiolate 1:10,000. The extraction was continued until neutralization of the sodium hydroxide ceased. At the end of this period the liquid separated by centrifugation was Berkefeld-filtered.

By following this procedure we were able to prepare a dilute solution of *H. pertussis* nucleoprotein, which we concentrated by salting out with 600 grams of ammonium sulfate per liter of filtrate. The resulting precipitate was removed by filtration, redissolved in a small volume of water, dialyzed, preserved with merthiolate (1:10,000), and Berkefeld-filtered.

Chemical tests performed with this material indicated a complex protein containing a nucleoprotein group. This protein complex is soluble in water at a neutral or alkaline reaction, is nondialyzable, and is insoluble in 60 per cent ammonium sulfate. It is heat-coagulable and its isoelectric point is near pH 4. In the absence of sodium chloride it can be purified by repeated precipitation with acid at pH 4. Solutions of this nucleoprotein so purified were used for the chemical tests.

The material gave positive biuret, xanthoprotein, Millon, Hopkins-Cole,

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and Sakaguchi (Weber, 1930) tests, the last one reacting positively when an amount of protein containing 0.5 mg nitrogen was used. The diphenylamine test of Dische for nucleoprotein, modified by Thomas (1931), Seibert (1940), and Dische (1944), gave a strong positive reaction with a solution of this protein containing 0.5 mg or less of nitrogen. The carbazole test described by Gurin and Hood (1939, 1941) also gave a strong positive reaction in concentrations of 0.05 mg nitrogen or less. (In our work we found it more convenient to use 0.25 per cent carbazole than 0.5 per cent.) After a portion of nucleoprotein was ashed with a mixture of sulfuric and nitric acids, the hydrolyzate reacted positively with molybdate solution and magnesia mixture, indicating phosphate.

We also performed partial hydrolysis of the nucleoprotein by heating it with 5 per cent sulfuric acid for 1 hour on a boiling water bath. At the end of this time the material was neutralized with ammonium hydroxide, centrifuged, and the supernatant submitted to several tests. Both the Molisch test for carbohydrate and the Bial test for arabinose were positive. The presence of purine base was established by positive reactions with ammoniacal silver nitrate and with copper sulfate and sodium bisulfite. Owing to the scarcity of the material, individual purine and pyrimidine bases could not be identified. Analysis of a few batches of nucleoprotein showed the values for nitrogen and phosphorus to be 12 to 15 per cent and 4 to 6 per cent, respectively.

This nucleic acid protein complex—a designation used by Mirsky (1943)—produced anaphylactic shock when injected into sensitized animals, and will be referred to as NPD (nucleoprotein denatured). As was pointed out, our product (NPD), owing to its treatment with strong hydrochloric acid for removal of lipoids followed by prolonged alkali extraction, contains denatured proteins, as indicated by its physical properties. A solution of NPD is very transparent, has very low viscosity, and is not precipitable with 0.14 molar sodium chloride or 0.1 per cent calcium chloride, as is characteristic of some undenatured nucleoproteins; for example, those described by Mirsky and Pollister (1943) for fibrous nucleoprotein from chromatin, and by Hall (1941) for thymus nucleohistone.

By adapting the technique of Hall, we were able to prepare undenaturated nucleoprotein from *H. pertussis* organisms. However, because of its high viscosity and poor solubility this material presented great difficulties for animal experimentation. For this reason, most of our animal tests were conducted with NPD.

We also performed some experiments on the isolation of nucleic acid from NPD. For this purpose NPD was broken down by the following consecutive steps: (1) A solution of protein was diluted at neutrality with 0.85 per cent sodium chloride to 0.5 to 1 mg nitrogen per ml, and immersed in a boiling water bath 5 to 10 minutes. After cooling, the coagulated proteins were removed by centrifugation. (2) The supernatant was then acidified and the resulting precipitate of acid-insoluble proteins (PF) removed by centrifugation. (3) To the second supernatant, without neutralization, was added 20 per cent alcohol; the material was kept for 24 hours at 4 C, and at the end of this period was centrifuged at the same temperature to eliminate a small precipitate. (4) The

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final supernatant containing nucleic acid was adjusted to pH 4 by the addition of sodium hydroxide and mixed with an equal part of alcohol. At approximately 60 per cent alcohol we could precipitate nucleic acid free from protein. The nucleic acid (NA) obtained was redissolved to the original volume. The material gave a negative biuret test but positive Dische and Gurin tests. Quantitative determinations performed with the last two tests indicated that we recovered most of the carbohydrate and nucleic acid in this fraction. However, the fraction might still contain traces of protein in an amount not great enough to be detected by the biuret test. During the procedure described above, most of the proteins were collected in the first two fractions.

EXPERIMENTS WITH MICE

For the experiments described in this section, mice were sensitized by a single intra-abdominal injection of *H. pertussis* vaccine, the degree of sensitivity achieved depending on the amount of vaccine injected. In most cases, each mouse received 50 billion organisms killed by the addition of merthiolate (1:20,000) and 0.25 per cent phenol to *H. pertussis* culture. Such a dose contained 0.15 to 0.2 mg nitrogen. However, the injection of this amount of vaccine killed 20 to 25 per cent of the mice used in some experiments. We found that soaking the organisms for a day or two in chloroform considerably reduced the toxicity of the vaccine without impairing its sensitizing property. Doses of *H. pertussis* smaller than that cited above also induced a noticeable sensitivity in mice.

In contrast to this, the treatment of *H. pertussis* vaccine with a mixture of equal parts of alcohol and ether containing 1 per cent hydrochloric acid greatly reduced its sensitizing properties. In a number of experiments, injection of vaccine treated in this manner failed to produce sensitivity. According to our experiments, the injection of NPD even in massive doses did not produce anaphylactic sensitivity in mice. Table 1 presents data relating to the toxicity of NPD for normal animals as compared with sensitized mice (one experiment).

The lethal dose of NPD for normal mice is equal to 0.6 mg nitrogen per mouse or 30 mg nitrogen per kilogram of body weight. Sensitized mice succumbed from a dose $\frac{1}{20}$, or less, of that required to kill normal mice. In some individual experiments, the difference in the lethal dose for normal and sensitized mice was as great as 100 times. Death in sensitized mice occurred in from 10 to 20 minutes to several hours (12 to 16 hours) after injection, depending on the dose of NPD given. The material caused acceleration of the respiratory movement, which became increasingly difficult with the passage of time. Many mice had convulsions before death occurred. Similarly, we noticed that sensitized mice succumbed more readily than normal mice to intra-abdominal injections of *H. pertussis* vaccine in massive doses.

We also tested the susceptibility of mice sensitized with *H. pertussis* vaccine to different proteins. In the course of this work we prepared NPD from *Brucella abortus*, strain 19, and AF from the filtrate of *Brucella bronchiseptica* culture. Both these materials, together with commercial tuberculin, various sera, broth,

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etc., were used for testing sensitized mice. From these experiments we learned that mice sensitized with *H. pertussis* vaccine could be shocked with the proteins of *B. abortus* and *B. bronchiseptica*, but these mice did not exhibit any increase in susceptibility to injections of proteinic material in general, i.e., horse, rabbit, and guinea pig sera, commercial tuberculin, *H. pertussis* broth, etc.

Some attempts were made to determine which constituent of NPD produced shock in sensitized mice. For this purpose, fractions of NPD, described above, were tested on sensitive mice. The doses were figured in the amount of nitrogen per injection as well as the amount of nucleic acid that the mice received with these injections. As a standard for this test, we accepted the amount of nucleic acid contained in the original NPD, as determined with the Evelyn colorimeter by using the technique of Seibert (1940). The amount of nucleic acid found

TABLE 1

Injection of normal and sensitized mice with H. pertussis NPD

DOSE CALCULATED IN MG N PER MOUSE	RESULTS
Normal mice	
0.6–1.0	4/18*
0.2–0.5	39/50
Sensitized mice	
0.6	0/4
0.12	0/4
0.2	0/5
0.08	2/7
0.04	7/12
0.02	2/2

* In all the tables contained in this paper, the total number of mice is shown in the denominator and the number of survivals in the numerator.

in the different fractions was referred to this standard. The results of these attempts appear in table 2.

According to table 2, the mice received in the preparation containing nucleic acid 5 times more nitrogen and 30 times more nucleic acid than was contained in the original NPD, and still all the mice survived. This indicated that the mice injected with *H. pertussis* antigens became sensitive to the protein portion of nucleoprotein, but not to the nucleic acid.

EXPERIMENTS WITH GUINEA PIGS

Toxicity of H. pertussis protein for guinea pigs. The toxicity of *H. pertussis* NPD was studied with normal and sensitized guinea pigs by intracardial injection of solutions containing different concentrations of the materials. Sensitized guinea pigs were prepared by a single intra-abdominal injection of *H. pertussis* AF or vaccine administered about two months before this test was per-

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formed. Insofar as the results obtained with guinea pigs sensitized with both materials were the same, the data of these tests were combined and tabulated against the results of experiments with normal guinea pigs. The guinea pigs used in one of these tests (table 3) had an average body weight of about 400 grams. The dose injected was calculated in mg of nitrogen of *H. pertussis* NPD per kilogram of body weight of guinea pigs.

Injections of lethal or sublethal doses of NPD into guinea pigs (normal and sensitized) at first greatly accelerates the respiratory movements of the animals. In animals receiving lethal doses of *H. pertussis* protein (NPD) the difficulties in respiration become aggravated with the passage of time. Sensitized guinea

TABLE 2

Sensitivity of mice to different constituents of H. pertussis nucleoprotein

FRACTIONS*	AMOUNT N INJECTED PER MOUSE	MORTALITY OF MICE	AMOUNT OF NUCLEIC ACID GIVEN TO MICE WITH THE DIFFERENT PREPARATIONS (AS COMPARED WITH THE CONTENT OF NUCLEIC ACID IN ORIGINAL NPD)
	mg		
27 C-1 Control original NPD	0.01	1/6	1
27C-1C Coagulable protein obtained in first step	0.005	1/8	1/4
27 C-1D (PF) Acid ppt'd protein obtained in second step	0.005	0/6	1/15
27 C-2F (NA) Fraction freed of protein & containing nucleic acid (step 4)	0.05	4/4	30

* See text for the preparation of these fractions.

pigs injected with the same material develop abundant exudate from the eyes and nose.

Autopsies on normal pigs which died from the injection of NPD and on sensitized pigs in which delayed death occurred after these injections revealed the picture of toxemia: abundant exudate in the peritoneal cavity and inflammation of the peritoneum and of the suprarenal glands, which were dark brown in color and hemorrhagic. These symptoms occurred in guinea pigs that died in three hours (or longer) after injection of *H. pertussis* NPD. Sensitized guinea pigs died shortly after injection with NPD, and on autopsy showed the typical picture of anaphylactic shock, with enlarged lungs and greatly extended heart.

The lethal dose of *H. pertussis* NPD for normal guinea pigs is equal to about 2 mg of nitrogen per kilogram of body weight. Some normal guinea pigs died

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from even smaller doses—0.5 and 0.35 mg of nitrogen. Apparently guinea pigs are much more susceptible to NPD than mice. Sensitized guinea pigs died from 0.15 mg or less of NPD nitrogen.

Another feature is that *H. pertussis* NPD as compared with bacterial toxins (staphylococcus, diphtheria, etc.) kills normal guinea pigs in a rather short period of time, from 3 to 16 hours. It is interesting to notice that, whereas previously described toxic substances isolated from *H. pertussis* filtrates (exotoxin) or from the organism itself (endotoxin) are unstable, the solutions of NPD are very

TABLE 3

Toxicity of H. pertussis NPD for normal and sensitized guinea pigs

H. PERTUSSIS PROTEIN INJECTED: MG N PER KG BODY WEIGHT	NORMAL GUINEA PIGS		SENSITIZED GUINEA PIGS	
	Results	Autopsy	Results	Autopsy
8.5	Died overnight	Toxemia		
5.5	Died overnight	Toxemia		
4	Died overnight	Toxemia	Died in 8 minutes	Anaphylactic shock
3	Died overnight	Toxemia	Died in 5 minutes	Anaphylactic shock
3	Died overnight	Toxemia		
3	Died overnight	Toxemia		
3	Died in 6½ hours	Toxemia		
3	Died overnight	Toxemia		
2	Died overnight	Toxemia	Died in 6 minutes	Anaphylactic shock
0.9	Died in 3½ hours	Toxemia	Died overnight	Toxemia
0.9	Died overnight	Toxemia	Died in 9 minutes	Anaphylactic shock
0.9	Died overnight	Toxemia	Died in 8 minutes	Anaphylactic shock
0.9	Died in 4½ hours	Toxemia	Died in 4 minutes	Anaphylactic shock
0.5	Survived		Died in 4 hours	Toxemia
0.5	Died in 5 hours	Toxemia	Died in 3 hours	Toxemia
0.35	Survived		Died in 3 minutes	Anaphylactic shock
0.35	Died overnight	Toxemia		
0.15			Died in 2 minutes	Anaphylactic shock
0.07			Died in 4 minutes	Anaphylactic shock
Total.....	17 guinea pigs		12 guinea pigs	

stable and can be Berkefeld-filtered and stored at room temperature for many months without losing their toxicity.

Experiments with the Dale [1] *test on isolated guinea pig uterus.* These experiments, adapted from Burn (1928), were performed on isolated uteri of normal and sensitized virgin guinea pigs. Sensitized pigs were prepared by intra-abdominal injection of *H. pertussis* vaccine two or three months before the experiment. The most satisfactory results were obtained when the uterus was kept in Locke-Ringer's solution with a continuous flow of oxygen. This solution was made according to the formula given in the U. S. Pharmacopoeia.

In the course of this work we tested normal and sensitized guinea pigs with different batches of NPD, the concentration of which was expressed in dilution of nitrogen content. At the completion of each test the vitality of the uterus

[1] Dale, 1913, 1929; Dale and Kellaway, 1922.

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was tested with histamine dihydrochloride, 1:10 million. The uteri of normal pigs did not respond to NPD diluted 1:1 million, whereas the uteri of highly sensitized pigs contracted on addition of NPD diluted 1:100 million.

DISCUSSION

The literature contains much information on sensitivity to nucleoproteins and on anaphylactic shock produced by these substances. The earlier observation of Freund (1920*a*, 1920*b*) on the shock produced by the injection of fresh defibrinated blood into animals was later traced by Zipf and Wagenfeld (1930), Zipf (1931), and Barsoum and Gaddum (1935) to the appearance in the blood of adenyl compounds derived from the breaking down of nucleoproteins. Drury (1936) thinks that a combination of histamine and adenyl compounds may be sufficient to account for the whole of the vessel reaction and leucocytosis seen after injury, but he warns that "it would be rash to assume that they are the only substances responsible for the complex response of inflammation."

The significance of nucleoproteins in bacterial allergy has been discussed many times, particularly in connection with sensitivity to tuberculin. The literature on protein in tuberculin cannot be reviewed here. It is necessary, however, to mention the work of Seibert (1940), whose technique we have been using. This author studied the interrelation of nucleoprotein to the other protein of tuberculin in the development of skin sensitivity. Stahl *et al.* (1939) and Huddleson (1943) prepared nucleoprotein from *Brucella* for testing skin allergy in brucellosis. The relation of bacterial allergy to other kinds of hypersensitivity has been reviewed by Rich (1941).

It is interesting to compare the chemical analysis of NPD (nucleoprotein, denatured, prepared from *H. pertussis*) with similar materials prepared from other sources and reported in the literature. For instance, the nitrogen and phosphorus content of several batches of NPD prepared by us were 12 to 15 per cent and 4 to 6 per cent, respectively—the proportion of nitrogen and phosphorus in these preparations being about 3.8:1. Claude and Potter (1943) found 15.58 per cent nitrogen and 3.72 per cent phosphorus in chromatin threads from resting nuclei of leukemic cells. Carter and Hall (1940) found in nucleohistone of calf thymus 16.73 $\pm$ 0.2 per cent nitrogen and 4.6 $\pm$ 0.1 per cent phosphorus, and Mirsky and Pollister (1943) found 15.5 per cent nitrogen and 3.9 per cent phosphorus in fibrous nucleoprotein of chromatin. Tipson (1945) recently discussed in detail the difficulty of establishing the homogeneity of different nucleoprotein preparations in connection with their chemical composition. Sevag *et al.* (1941) and Lackman *et al.* (1941) isolated very pure streptococcal nucleic acid that contained 16.2 per cent total nitrogen and 9.12 per cent total phosphorus. The sample of nucleic acid that Levene and Bass (1931) obtained from tubercle bacilli, which most nearly approaches desoxyribo-*tetra*-nucleotide, contained 14.19 per cent and 9.04 per cent, respectively, of nitrogen and phosphorus as compared with the theoretical values of 16.76 and 9.89.

We were able to break down our product by boiling it at slightly alkaline reaction followed by precipitation of the remaining protein by acidification.

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In this way we could prepare a solution of nucleic acid that reacted negatively to the biuret test and gave a positive reaction for nucleic acid and carbohydrate. This purified nucleic acid did not produce anaphylactic shock in sensitized animals. In this respect our work with *H. pertussis* NPD confirmed the previous findings of other authors insofar as laboratory animals became sensitive to the protein of this material but not to the nucleic acid. Similar findings were reported by Stahl, Pennell, and Huddleson (1939) on nucleoprotein of *Brucella*, and by Seibert (1940) on tubercular proteins.

The allergenic properties of *H. pertussis* organisms apparently represent a complicated mechanism of bacterial allergy. Sabin and Joyner (1938) in very comprehensive experiments demonstrated that injection of a tuberculo-phosphatide accelerated sensitization of guinea pigs to tuberculo-protein. In our case, the removal of lipoids from *H. pertussis* bacilli by extraction with a mixture of alcohol and ether containing 1 per cent hydrochloric acid reduced the sensitizing properties of *H. pertussis* vaccine on laboratory animals. However, our treatment does not destroy entirely the sensitizing properties, and NPD prepared from such extracted organisms can induce some sensitivity in rabbits and guinea pigs, as will be described in the following paper, although the injection of even large doses of denatured *H. pertussis* NPD does not induce anaphylactic sensitivity in mice.

Sensitivity to *H. pertussis* crosses with a few other related gram-negative organisms. However, this condition is not connected with any increase in the susceptibility of mice to injection of proteinic material in general. As compared with normal animals, mice sensitized to *H. pertussis* proteins did not show a noticeable increase in susceptibility to injections of horse serum, rabbit serum, commercial tuberculin, broth used for liquid media, etc.

SUMMARY

Laboratory animals injected with *Hemophilus pertussis* antigens (vaccine or filtrate) develop sensitivity to proteins of these organisms, particularly to nucleoproteins. Fractionation of the nucleoprotein complex revealed that animals sensitized to *Hemophilus pertussis* became highly susceptible to the protein portion of this complex.

The sensitizing properties of *Hemophilus pertussis* antigens were reduced after treatment with a mixture of alcohol and ether containing 1 per cent hydrochloric acid.

Hemophilus pertussis nucleoprotein (NPD) can be used as a shocking fraction for testing sensitivity.

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