practical with the demonstration that concentrated old tuberculin (Lederle) can be substituted as the antigen for sensitization of sheep erythrocytes instead of the polysaccharide fraction previously described(1). The heat stable polysaccharide fraction isolated from mammalian tubercle bacilli is undoubtedly contained in the old tuberculin preparation. Heterologous reactions were noted in approximately 6% of sera from non-tuberculous patients or normal adults in the present series, but these reactions are of lower titer than those obtained from patients with active tuberculosis. Because of the cross reactions, it appears probable that only titers of 1:8 or higher are indicative of tuberculous infection. The potential value of the test is suggested by the fact that 92% of tuberculous patients' sera showed positive reactions in titers of 1:8 or higher, whereas in a precipitin test for carbohydrate antibodies in human tuberculosis recently reported, 50% positive reactions were found(9).

From these observations it seems that this hemagglutination test apparently not only provides a valuable diagnostic tool, but unlike the tuberculin skin test, appears to indicate the presence of tuberculous activity and therein lies its greatest value.

Summary. The sera of 216 normal adults and patients with syphilis or non-tuberculous chest diseases were tested for tuberculous antibodies by the hemagglutination reaction: 203 showed no reactions; 12 had positive reactions in serum dilution of 1:2; and one in dilution of 1:4. The sera of 168 patients with active tuberculosis showed 92% positive reactions in dilutions ranging from 1:8 to 1:512. Sera from 33 apparently cured tuberculous individuals revealed no reactions in 31, and positive reactions in 2 in a serum dilution of 1:2. This hemagglutination reaction is apparently a relatively specific test for active tuberculosis.

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Separation of Pertussal Toxin.* (17814)

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Toxic extracts have been prepared from *Hemophilus pertussis* by a variety of methods(1-9). Pertussal toxin is lethal for mice, guinea pigs and rabbits and is dermonecrotic. It is heat-labile, easily detoxified by formaldehyde, and antigenic. Rabbit pertussal antitoxic sera will neutralize the toxin in multiple proportions and induce passive immunity in guinea pigs(10) and in rabbits and mice(11). The purpose of this investigation is to report methods for the extraction and separation of pertussal toxin from a variant toxin-producing strain of *H. pertussis*. The toxin can be extracted from dried *H. per-*

* Aided by a grant from Lederle Laboratories Division, American Cyanamid Co.

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tussis with 0.05 M $CaCl_2$ and can be separated from this extract by fractionation with methanol-water mixtures under controlled conditions of pH, ionic strength and temperature.

Materials and methods. (a) Preparation of organisms. H. pertussis, strain No. 29†, a variant phase I strain, was grown in peptone broth for 7 days at 35-37°C(8,12,13). The organisms were harvested in a Sharples centrifuge, suspended in distilled water and dried from the frozen state. 0.08% suspensions of these organisms gave turbidities in a Klett-Summerson photoelectric colorimeter equivalent to 10 billion organisms per ml (13).

(b) *Determination of* LD_{50}. The dilutions of toxin were made in 0.9% NaCl containing 1.0% peptone (Bacto). 0.5 ml volumes were injected intraperitoneally into white female mice (Carworth CF No. 1) weighing 12-15 g. Three to 4 dilutions were made per sample; 6 to 8 mice were used per dilution. The LD_{50} value for the toxin was calculated after 96 hours using the method of Reed and Muench(14).

(c) *Chemical and physical methods.* All fractionation procedures were carried out in constant temperature baths. A refrigerated centrifuge (International, PR-I) was used for centrifugation. Nitrogen was determined by the micro-Kjeldahl method of Pregl and phosphorus was determined by the method described by Gomori(15). Hydrogen ion determinations were made on the glass electrode; solutions containing methanol were diluted to less than 5% methanol before pH determination. Ionic strength was determined by calculation. The ultrasonic experiments were carried out with a 9 KC Raytheon Corporation sonic oscillator Type R-22-3 at 0°C. Electrophoretic analyses were carried out in pH 8.6 veronal buffer, ionic strength of 0.10, using the Tiselius apparatus with the Longsworth modification of the optical system(16).

Results. (a) Extraction of organisms with water. A 2.5% suspension of organisms in distilled water (approximately 310 billion organisms per ml) was prepared using a Waring blendor for 15 seconds at 0°C. This suspension was stirred for one-half hour at 0°C and allowed to remain in the 0°C bath for 2 hours. The insoluble residue was removed by centrifugation at 4000 r.p.m. for one hour at 0°C and the supernatant solution recentrifuged under the same conditions. This extract contained 360 LD_{50} per ml and 300 LD_{50} per mg N (average values).

(b) *Ultrasonic treatment of water suspension.* A 2.5% suspension of organisms in distilled water was subjected to ultrasonic treatment in a 9 KC oscillator for one hour at 0°C. The extract, after removal of the bacterial debris by centrifugation at 4000 r.p.m. for one hour at 0°C, contained 1080 LD_{50} per ml and 600 LD_{50} per mg N. The original water extract in this experiment contained 230 LD_{50} per ml and 250 LD_{50} per mg N‡. This represented approximately a 5-fold increase in toxin and only a 2-fold increase in nitrogen. Nevertheless, the ultrasonic method was not used for further extraction of the toxin because of the difficulties involved in processing large volumes of material.

(c) *Extraction of organisms with* $CaCl_2$. In a previous report(9), it was shown that the presence of 0.05 M to 0.1 M $CaCl_2$ in a 2.5% suspension of *H. pertussis* removed large amounts of mucin-like impurities which greatly hindered preparative procedures. The extracts contained all of the toxin originally present. In the present study, the extract obtained from a 2.5% suspension of organisms in 0.05 M $CaCl_2$ at pH 6.5, after removing the bacterial debris by centrifugation at 4000 r.p.m. for one hour at 0°C, contained

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12. Ross, O. A., unpublished data.

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‡This experiment was carried out with a batch of organisms low in toxin.

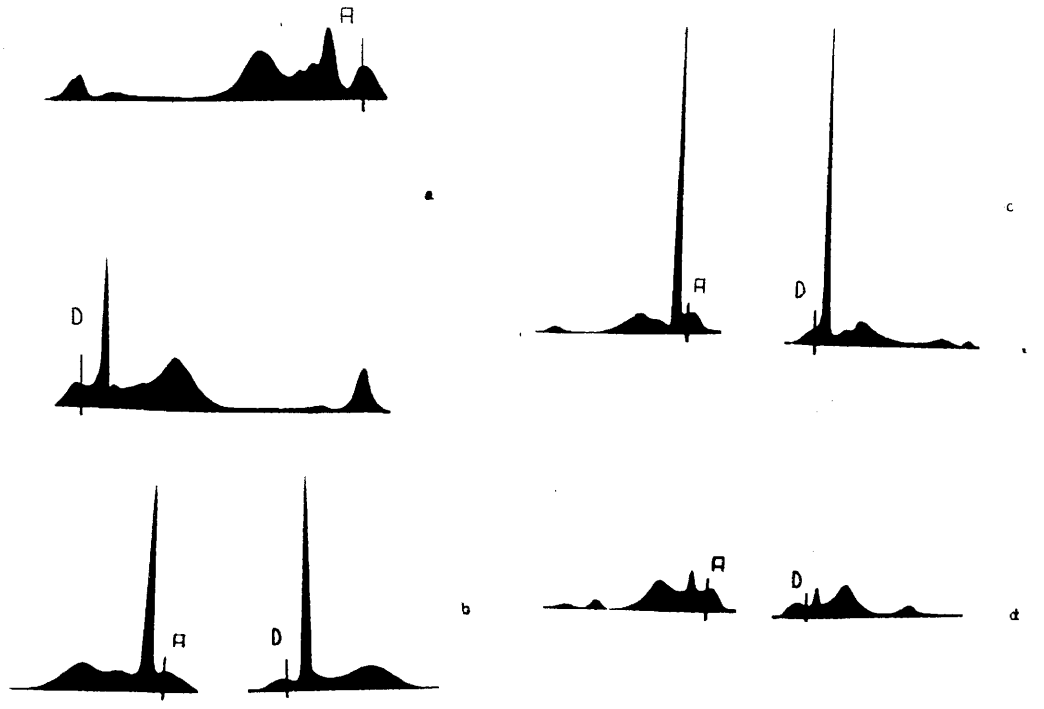

FIG. 1.
Electrophoretic patterns of extracts and fractions of *H. pertussis* in pH 8.6 veronal buffer, ionic strength of 0.10. (a) Water extract. (b) 0.05 M $CaCl_2$ extract. (c) Fraction PI. (d) Fraction PII.

280 LD_{50} per ml and 400 LD_{50} per mg N (average values). This represented an average yield of 78% of the water extract with a purification factor§ of 1.3. The N/P ratio was found to be 19.8. Similar results were obtained with 0.025 and 0.1 M $CaCl_2$ extracts. The optimum pH for extraction of the toxin in 0.05 M $CaCl_2$ was found to be 6.5. Higher hydrogen-ion concentrations gave lower yields. The 0.05 M $CaCl_2$ extract at pH 6.5 was used as the starting material for fractionation.

(d) *Separation of toxin from 0.05 M $CaCl_2$ extract(1). Fraction PI.* Orientation studies revealed that the toxin can be optimally precipitated from the 0.05 M $CaCl_2$ extract at pH 4.4 in 15% methanol, ionic strength of 0.13 at −5°C. The pH was adjusted with hydrochloric acid. The fractionation methods employed have been described in previous publications(13,17,18). The precipitate was dissolved to one-fifth of the original volume in 0.30 M sodium acetate at pH 7.0 and the solution clarified by centrifugation at 4000 r.p.m. at 0°C for one hour. This fraction (PI), which is 5x concentrated, contained 1140 LD_{50} per ml and 1630 LD_{50} per mg N (average values). This represents an average yield of 82% of the 0.05 M $CaCl_2$ extract with a purification factor of 5.4. The N/P ratio was found to be 55.5. In this step, small changes in methanol concentration influenced the solubility of the toxin and the impurities to a greater extent than did small changes in the ionic strength and pH.

(2) *Fraction PII.* Preliminary studies indicated that the toxin could be optimally

§ Purification factor = $\frac{LD_{50} \text{ per mg N in fraction}}{LD_{50} \text{ per mg N in water extr.}}$

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18. Pillemer, L., and Robbins, K. C., *Ann. Rev. Microbiol.*, 1949, v3, 265.

reprecipitated from PI in aqueous solution at pH 5.3, ionic strength of 0.15 at 0°C. The precipitate was removed by centrifugation and was dissolved to the original volume of PI in 0.15 M sodium acetate at pH 7.0 and the solution was clarified by centrifugation. This fraction (PII) contained 920 LD_{50} per ml and 5260 LD_{50} per mg N (average values). This represents an average yield of 81% of PI with a purification factor of 17.5. The overall yield at this step was 51%. The N/P ratio was found to be 43.1.

(e) *Electrophoretic analysis.* Electrophoretic patterns of the water extract, the 0.05 M $CaCl_2$ extract, Fraction PI and Fraction PII from *H. pertussis,* strain No. 29, are shown in Fig. 1. The most active fraction, PII, was found to be heterogeneous.

Discussion. It has been found that the most favorable conditions for the separation of pertussal toxin involve extraction of organisms dried from the frozen state with 0.05 M $CaCl_2$ at pH 6.5 followed by precipitation at pH 4.4 in 15% methanol, ionic strength of 0.13 at −5°C and reprecipitation at pH 5.3, ionic strength of 0.15 at 0°C. The most active fraction, PII, contained 5260 LD_{50} per mg N which represented a 17.5-fold purification over the water extract with an average overall yield of 51%. Fraction PII was found to be heterogeneous by electrophoretic analysis.

The relationship of toxin found in strain No. 29 to that found in true phase I strains has not been definitely established. Strain No. 29 has been shown to satisfy only one of the serological criteria for a phase I organism, namely agglutination by a phase I antiserum(13). This organism contains protective bacterial antigens against the phase I strain used in the mouse protection test(13). The bulk of the available experimental evidence suggests that the toxin found in the variant strain No. 29 is the same as that found in all strains of *H. pertussis.*

Summary. Methods have been described for the separation of pertussal toxin from a 0.05 M $CaCl_2$ extract of *H. pertussis,* strain No. 29, dried from the frozen state. The most active fraction contained 5260 LD_{50} per mg N and was found to be heterogeneous by electrophoretic analysis.

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Influence of Feeding Schedule on Nitrogen Utilization and Excretion. (17815)

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The object of the experiment reported in this paper is to find the answer to a practical question of nutrition: With a given total dietary intake does the feeding schedule have any influence on the efficiency of N utilization, and if so, is it more efficient to have 2, 3 or 4 feedings a day?

According to the current concept of dynamic equilibrium of body constituents, tissue proteins are continually broken down into amino acids and new tissues are continually formed from amino acids. When the rate of protein formation equals the rate of protein breakdown, the body is in protein equilibrium. Besides the reversible reaction between proteins and amino acids, the latter are continually removed irreversibly from the metabolic pool by excretion as such and by conversion into nitrogenous waste products, chiefly urea. The rates of removal of amino acids from the metabolic pool, whether by formation of protein or urea, or by excretion, should vary with the concentration of the assorted amino acids, other things being equal. If the rate of removal of amino acids equals the rate of replenishment from the digestive tract, the concentration of amino acids in the metabolic pool would remain con-