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PURIFICATION AND CHARACTERIZATION OF AN ENDOGENOUS PYROGEN‡

Many microbes and their products are capable of producing fever when injected intravenously in various laboratory animals. Recent studies, involving passive transfer of serum from febrile donor animals to normal recipients, have shown that most, if not all, these agents cause fever indirectly via liberation of a circulating pyrogen of endogenous origin.[1] To date, the source of endogenous pyrogen (EP) has been identified with certainty only in fevers induced by the endotoxins of Gram-negative bacteria. Several lines of evidence indicate that endotoxins liberate pyrogen from polymorphonuclear leukocytes.[2-5] Granulocytic pyrogen has been partially purified and characterized by Wood and his colleagues.[6]

Viruses of the myxovirus group—mumps, influenza, and Newcastle disease virus—are among the most potent fever-inducing microbial agents known. When given intravenously, these viruses evoke large amounts of circulating pyrogen which is clearly distinct from virus and which appears to be the direct cause of this form of fever.[7]

The following studies were undertaken in an attempt to purify and characterize the endogenous pyrogen appearing in the serum of rabbits given NDV and to compare this pyrogen with leukocytic pyrogen obtained from acute inflammatory exudates.[8,9]

A method is described for the assay and purification of NDV-induced serum endogenous pyrogen (hereinafter referred to as NDV-EP) and various features of the febrile response have been correlated with dosage of the pyrogen. The biochemical properties of a partially purified preparation of NDV-EP are compared with those reported by Rafter, Collins, and Wood for granulocytic pyrogen.[6]

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METHODS

General. Male and female albino rabbits of several breeds were used for all experiments. The rabbits were housed in an air-conditioned room and experiments were performed in an adjacent room similarly maintained at 65-70° F.

Temperature recording. Rabbits were placed in wooden or aluminum boxes with openings for the head and rectum. Temperatures were measured with a neoprene-covered resistance coil left in the rectum throughout the experiment and recorded by a Foxboro scanning switch and fever recorder. Measures taken to obtain consistent, stable responses by the rabbits have been described previously.[10] Rabbits showing a variation of over 0.3° C. in the hour preceding injection or an initial temperature higher than 40.5° C. were not used.

Bacterial pyrogen-free technic. All glassware, needles, and reagents that could withstand heating were rendered free of contaminating pyrogen by dry heat at 170° C. for two hours.[11] All solutions were made with sterile doubly distilled water demonstrated to be pyrogen-free in dosages of 20 ml. or more. Reagents that would be destroyed by heat were made up in neutral solutions with physiologic ionic concentration and tested for pyrogenicity in rabbits. Dialysis bags and DEAE cellulose were washed with pyrogen-free saline and the final wash tested for pyrogenicity. In all manipulations of the reagents, serum and its fractions, standard sterile technic was followed.

Tests for pyrogenicity. Four to six rabbits weighing 4 to 5 kg. were injected intravenously with 50 ml. or less of the material to be tested. Temperatures were recorded every 15 minutes for five hours. Serum and its fractions were tested both in normal rabbits (NR) and in rabbits rendered tolerant to bacterial pyrogen (PTR) by daily inoculations of typhoid vaccine.[12] With this bioassay, contamination of any sample by bacterial pyrogen could be detected in the diminished febrile responses of tolerant as compared to normal recipients (especially apparent in the second peak of a biphasic fever). Fractions of serum containing NDV-EP were stated to be pyrogenic only if they produced fever within 15 minutes after injection. A rise of at least 0.5° C. above the baseline was also required as evidence of pyrogenicity. Since a type of tolerance may be induced to endogenous pyrogen by repeated daily injections,[13] recipient rabbits were reused only after a day of rest.

Fever charts. Fever curves were plotted on 1/6-inch graph paper with five lines on the ordinate representing one degree centrigrade and six lines on the abscissa for each hour. The area beneath the five-hour fever curve was measured with a compensating planimeter to derive the fever index.[10] When the temperature failed to return to the baseline by five hours a perpendicular line was drawn from the five-hour reading to the baseline and the area within these lines measured. In cases where a fever was produced but the temperature subsequently dropped below the basal temperature line, only the area above the baseline was measured.

Newcastle Disease Virus (NDV). The Hickman strain of NDV was employed. The methods of cultivation, passage, titration, and storage of NDV have been previously described.[7]

Preparation of serum containing NDV-EP. Five milliliters of pooled allantoic fluid containing NDV with a hemagglutinin titer of 1:1280 were injected intravenously into a fasting donor rabbit weighing 5 to 6 kg. Four hours later the rabbit was

exsanguinated by cardiac puncture. Serum was collected from the clot, cleared by centrifugation, and stored at 4° C. Serum from each rabbit was tested for sterility by inoculation of thioglycollate broth with 1.0 ml. of serum. Sera from several rabbits were pooled prior to testing or fractionation.

Titration of NDV-EP in serum. Seven normal rabbits and seven bacterial pyrogen-tolerant rabbits weighing approximately 5 kg. were reserved for the titration. Only those rabbits which had a stable basal temperature on the day of testing were used. Usually five or six rabbits were available for testing from each group on any given day. To minimize tolerance, smaller doses were injected first and tests were performed on alternate days. In the smaller dose-range ($<$0.1 ml.), serum was diluted with pyrogen-free saline so that the stated dose was contained in a 10 ml. volume. A single, large pool of NDV-EP in serum was used for all doses. A control to determine tolerance occurring during the titration consisted of a comparison of the response to a 3 ml. dose of NDV-EP in serum three days before the titration was started and a 3 ml. dose in the middle of the series of doses.

Best measure of pyrogenicity. Statistical analysis of the data was restricted to the response of normal recipients to undiluted doses of NDV-EP in serum. The maximum fever at the first and second fever peaks as well as the fever index were analyzed for their effectiveness as measures of pyrogenicity. The range of doses that could be represented by a linear relationship with logarithmic conversion of dose or response scales was selected for analysis. For each measure, the regression line was calculated and plotted on a scatter diagram. The variance ratio (F),[14] for the degrees of freedom involved, was used as a test of the departure from linearity of the dose-response relationship. The lambda value described by Bliss[15] was used as the final evaluation of effectiveness of the measure.

Characterization of NDV-EP in serum.

The following methods were used to determine the nature of NDV-EP in serum prior to fractionation:

1. *Saline dialysis.* Fifty milliliters of NDV-EP serum was dialyzed for 24 hours at 4° C. in a cellophane bag against 250 ml. of 0.85 per cent saline. Fifty milliliters of dialysate, equivalent to NDV-EP in 10 ml. of serum was tested for pyrogenicity. The dialyzed serum was also tested for pyrogenicity.

2. *Trichloroacetic acid precipitation.* Ten per cent trichloroacetic acid was added slowly to NDV-EP serum to a final concentration of 2 per cent at 4° C.[16] The supernatant was extracted with ether, neutralized, and tested for pyrogenicity.

Assay of protein. A sample of rabbit serum for which the protein nitrogen concentration was determined by Kjeldahl analysis was used as the protein standard. Protein concentrations of NDV-EP serum or its fractions were determined by the biuret-Folin method of Lowry.[17]

Purification of NDV-EP. Fractionation of serum containing NDV-EP was attempted with various concentrations of ethanol at 0° C. and —15° C.[18] Supernatants were dialyzed against saline to remove the ethanol and precipitates were dissolved in 0.85 per cent saline before their pyrogenicity was tested.

Because a precise separation of pyrogenic activity could not be obtained, the direct ethanol precipitation was abandoned. Since preliminary experiments showed that NDV-EP was stable at low pH and there is diminished coprecipitation of

proteins at low pH,[18] acid precipitation was tried with various concentrations of HCl at 0-4° C. The final purification procedure is summarized in Figure 1. The methods used were as follows:

1. *Acid precipitation.* The entire procedure was carried out at 0-4° C. A solution of 3.0 N HCl was added very slowly with constant mixing of NDV-EP serum to a final concentration of 0.95 N HCl. After two hours the precipitate was separated by centrifugation (3,577 *g* maximum for one hour). The supernatant was neutralized by the slow addition of 3.0 N NaOH with constant mixing. Samples to be tested for pyrogenicity were dialyzed against 20 volumes of 0.85 per cent saline for 24 hours. Three changes of saline were made during this period. Samples of the supernatant to be further fractionated were not dialyzed.

2. *Ethanol precipitation at 0° C.* Absolute ethanol was added slowly with continual mixing to a final concentration of 50 per cent in the neutralized supernatant from the acid precipitation of NDV-EP serum. After one hour the mixture was centrifuged

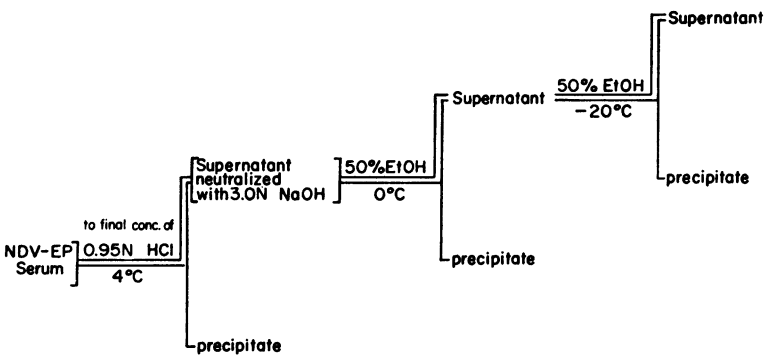

FIG. 1. Procedure for partial purification of NDV-induced serum endogenous pyrogen (NDV-EP).

(3,577 *g* maximum for one hour). Samples of the supernatant to be tested for pyrogenicity were dialyzed against 20 volumes of 0.85 per cent saline for 24 hours. Three changes of saline were made during this period.

3. *Ethanol precipitation at —20° C.* The supernatant from the 50 per cent ethanol precipitation at O° C. was cooled to —20° C. in a refrigerated contrifuge for 12 hours. A further precipitate was obtained which was separated by centrifugation (3,577 *g* maximum for 1½ hours). The precipitate was dissolved in 0.85 per cent saline. The supernatant was dialyzed against 20 volumes of 0.85 per cent saline for 24 hours. Three changes of saline were made during this period. Some of the supernatant preparations were concentrated by flash evaporation at 50° C. for one hour. Under these conditions the pyrogenicity of the preparation was not altered.

4. *Butanol extraction.* Samples of the concentrated supernatant (from the —20° C., 50 per cent ethanol precipitation) were extracted with butanol[19] before column chromatography. Sufficient n-butyl alcohol was added to the concentrated supernatant to reach a concentration of 20 per cent (v/v). The mixture was incubated at 37° C. and shaken intermittently for 15 minutes. The layers were separated by centrifugation

(894 *g* maximum for 20 minutes). The butanol layer and the lipid-containing precipitate were discarded. The aqueous layer was dialyzed against the appropriate saline solution or phosphate buffer prior to column chromatography.

5. *Column chromatography.* The concentrated, dialyzed supernatant after the butanol extraction was made up in samples containing 6 to 8 mg. protein. These

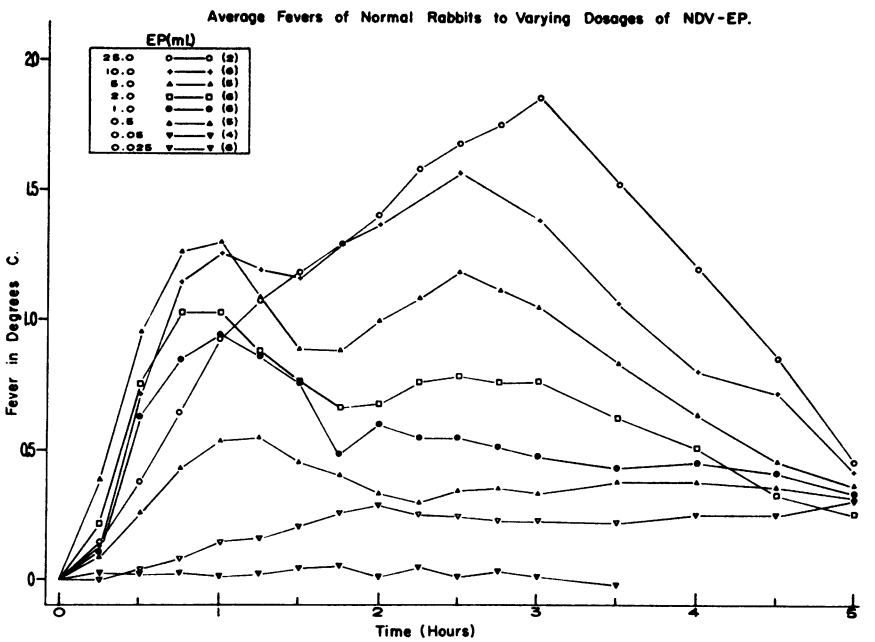

FIG. 2. Mean fever curves of normal recipient rabbits for various doses of NDV-EP. Numbers in brackets on scale represent the number of rabbits used for each dose. Dosages of 0.1 and 0.2 ml. undiluted NDV-EP (not shown) given to same group of rabbits produced prompt, brief elevations of 0.2 and 0.5° C., respectively, which reached a peak at 1-1¼ hours.

samples were added to packed columns containing 0.5 to 3 gm. of DEAE cellulose*[20] or Sephadex G25.†[21] Elution was carried out with gradients of 0.15 to 1.0 M NaCl and 0.02 to 0.4 M phosphate buffer (pH 6 to 7.6). Normal serum was added to washed samples of these adsorbents, eluted and determined to be pyrogen-free before the batch of resin was used.

* DEAE cellulose (diethylamino cellulose), Eastman Kodak Company, Rochester, New York. Before use it was washed with 1.0 N NaOH at 37° C. for one hour, in repeated changes of water and finally with the appropriate buffer for the particular column. Use of NaOH was required to remove contaminating pyrogens evident only when normal serum was eluted from the column. Saline did not acquire pyrogenicity when similarly passed through the column.

† Sephadex G 25, Pharmacia, Uppsala, Sweden. It was heated at 170° C. for two hours to free it of bacterial pyrogen.

Characterization of partially purified NDV-EP.

The dialyzed supernatant obtained from the 50 per cent ethanol precipitation at —20° C. was subjected to chemical and enzymatic tests in order to determine its chemical nature. The methods used were as follows:

1. *Inactivation by heat.* Samples of dialyzed supernatant were heated in a water bath at 50° C. for 3 hours and 80° C. for 2 hours.

2. *Perchloric acid precipitation.* Sixty per cent perchloric acid was added slowly with constant mixing to the dialyzed supernatant to a final concentration of 0.3 M.[22] The precipitate was separated by centrifugation (3,577 *g* maximum for 1 hour), dissolved in 0.85 per cent saline and neutralized slowly with 1 N NaOH. The entire process was performed at 0 to 4° C.

3. *Phenol extraction.* Sufficient phenol was added to 25 ml. of the dialyzed supernatant at room temperature to make a final concentration (w/v) of 90 per cent.[23]

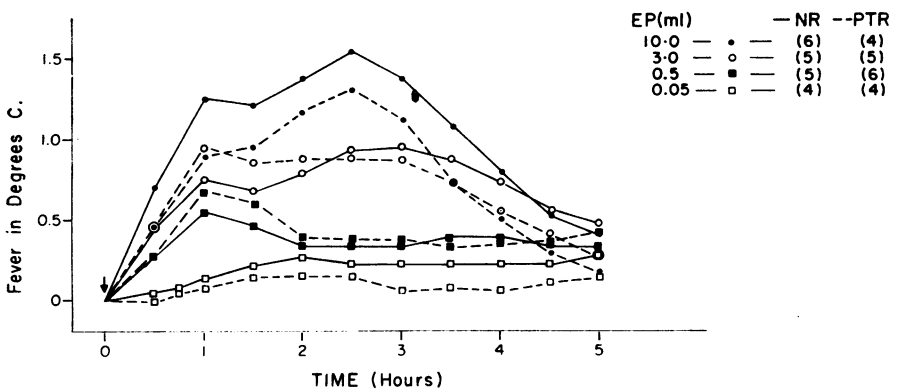

FIG. 3. Mean fevers induced by varying dosages of NDV-EP in groups of normal (NR) and pyrogen-tolerant (PTR) recipients. Numbers of animals given each dose are indicated in brackets.

The mixture was shaken and the supernatant aqueous layer was dialyzed against 0.85 per cent saline prior to testing for pyrogenicity.

4. *Periodate oxidation.* Four ml. of 0.033 M potassium periodate, 37.0 ml. dialyzed supernatant and 4.5 ml. of 0.01 M sodium acetate buffer (pH 5.2) were mixed.[6] The mixture was allowed to stand for five hours in the dark at 0° C. A buffer control lacking the periodate was also prepared.

5. *Action of proteolytic enzymes.*

(a) *Trypsin.* The reaction mixture contained 25 ml. dialyzed supernatant, 3 mg. trypsin,* 4 x 10^{-3} M Ca^{++}[24] and sufficient 0.15 M tris (hydroxymethyl) aminomethane (pH 8) to make a total volume of 30 ml. The mixture was incubated at 37° C. for four hours.[6]

* Trypsin, 3x crystallized, Worthington Chemical Company, Freehold, New Jersey.

(b) *Pepsin.* The reaction mixture contained 25 ml. dialyzed supernatant, 3 mg. pepsin,* and sufficient 0.02 M sodium acetate buffer (pH 4.0) to make a total volume of 30 ml. The mixture was incubated at 37° C. for two hours.[6]

Controls containing (a) no proteolytic enzyme and (b) no dialyzed supernatant were also tested for pyrogenicity. Each sample was neutralized before being tested for pyrogenicity.

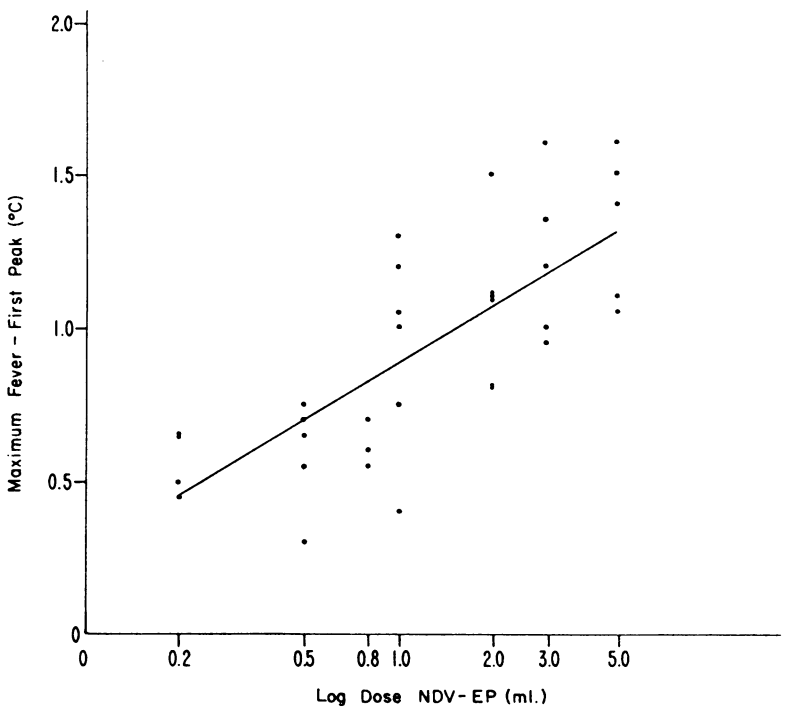

FIG. 4. Scatter diagram and calculated regression line [Y = 0.9230 + 0.606 (x — 0.0595)] for maximum fever of the first fever peak as related to dose of NDV-EP in normal recipient rabbits.

RESULTS

Titration and assay of NDV-EP in serum. The febrile response was measured to various doses of a pooled sample of NDV-EP in groups of normal and pyrogen-tolerant rabbits. The fever curves of the group of normal rabbits are presented in Figure 2. Tolerance following large doses of NDV-EP[13] was minimized by the technics outlined under METHODS. No tolerance was evident in the group of normal rabbits throughout the series of doses.

* Pepsin, 3 x crystallized, Nutritional Biochemicals, Cleveland, Ohio.

With doses of NDV-EP serum greater than 2 ml., a biphasic fever was regularly observed. With progressively lower doses the second fever peak gradually disappeared.

In the range of doses tested, there was little difference in the responses of pyrogen-tolerant rabbits to NDV-EP as compared to those of normal rabbits, indicating that this agent is fully capable of producing biphasic fever without detectable contamination by bacterial pyrogen (Fig. 3). Similar results have been obtained with large doses of leukocyte pyrogen.[25]

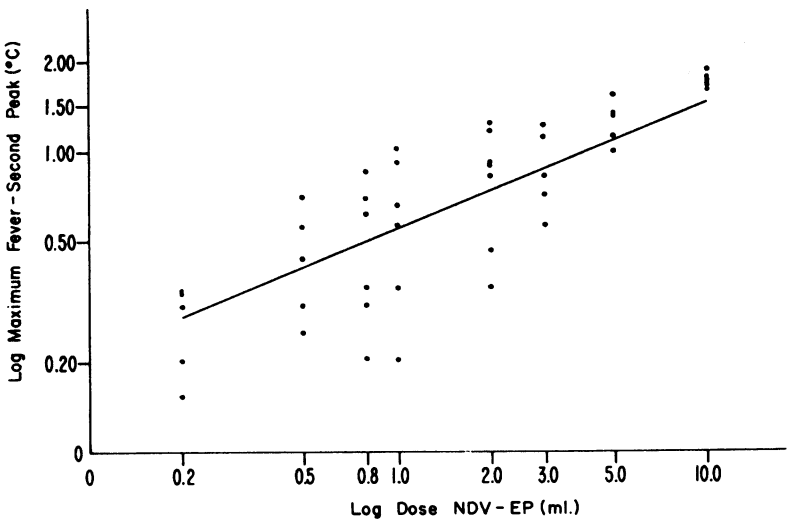

FIG. 5. Scatter diagram and calculated regression line [$Y = -0.1845 + 0.411\ (x - 0.1842)$] for maximum fever of the second fever peak as related to dose of NDV-EP in normal recipient rabbits.

By the statistical analysis described under METHODS, the effectiveness of fever index and maximum fever of the first and second fever peaks were compared as measures of NDV-EP serum pyrogenicity (Figs. 4-6). Fever index (Fig. 6) was determined to be the most effective measure on the basis of the lambda value of Bliss[15] ($\lambda = 0.36$) over the widest dose range (0.2 — 10 ml.). Maximum fever of first peak was nearly as effective ($\lambda = 0.38$) over a narrower dose range (0.2 — 5 ml.). For both these measures the probability of departure from linearity of the dose-response relationship was <0.001.

Characterization of NDV-EP in serum. The pyrogenic component (NDV-EP) was found to be non-dialyzable. There was no loss of pyrogenicity upon dialysis.

When most of the protein in serum containing NDV-EP was precipitated with 2 per cent trichloroacetic acid, the supernatant was pyrogen-free.

Partial purification of NDV-EP. As a result of the preliminary characterization, NDV-EP appeared to be a large molecule, possibly a protein. Methods of protein precipitation were then applied to its purification.

Initial precipitation of serum containing NDV-EP with various concentrations of ethanol at 0 and -15°C. was tried first. At 0°C. most of the

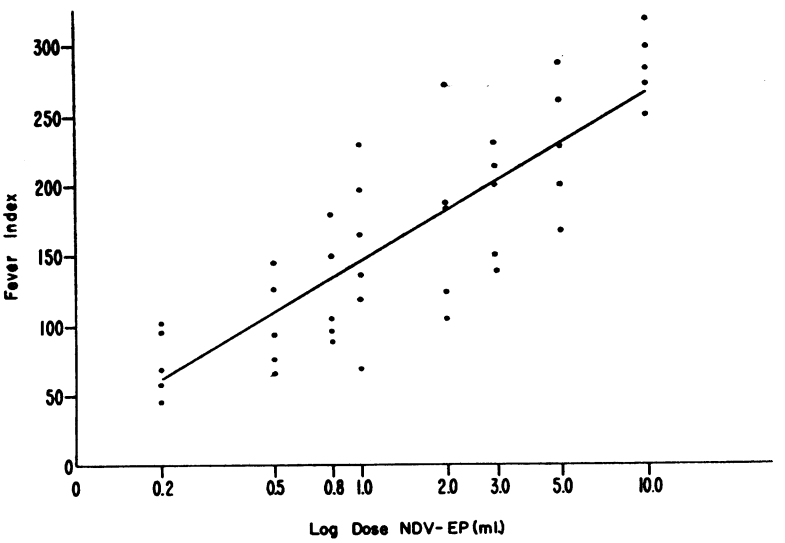

FIG. 6. Scatter diagram and calculated regression line [$Y = 166.58 + 118.89\ (x - 0.1907)$] for fever index as related to dose of NDV-EP in normal recipient rabbits.

pyrogenic activity was found in the supernatant of the 50 per cent ethanol precipitation. At -15°C. the pyrogenicity was found distributed between the precipitates obtained between 40 and 75 per cent ethanol. Because a precise separation could not be obtained by this means, initial ethanol precipitation was abandoned.

The final procedure for partial purification of NDV-EP is summarized in Figure 1. With this procedure, the greatest quantity of the pyrogenic activity was contained in the 50 per cent ethanol supernatant after further precipitation at -20°C. A small quantity of pyrogenic material was contained in this precipitate.

Many attempts were made to obtain further purification of NDV-EP on DEAE cellulose and Sephadex G 25. Gradients of NaCl concentration and phosphate buffer (see METHODS) were tried. The chief problems en-

countered were failure of adsorption to the columns and inactivation upon the column.*

The mean total protein of the sera used for purification of NDV-EP was 6.2 gm. per 100 ml. The relative amounts of total protein remaining after each of the steps of purification are summarized in Table 1.

Upon fractionation of one pool of serum containing NDV-EP by the method outlined, it was found that an equivalent fever index was produced by 1.5 ml. of serum (94 mg. protein) and 9 ml. of -20°C., 50 per cent ethanol supernatant (3.6 mg. protein). Approximately a 25-fold purification was obtained, on the basis of protein content.

TABLE 1. RELATIVE TOTAL PROTEIN REMAINING AFTER STEPS IN PURIFICATION OF NDV-EP FROM SERUM

Fraction	*Per cent original protein*
Serum containing NDV-EP	100
Supernatant, 0.95 N HCl precipitation	35
Supernatant, 0° C., 50% ethanol precipitation	9
Supernatant, —20° C., 50% ethanol precipitation	2

The minimal pyrogenic dose of the partially purified material (capable of eliciting a fever of 0.5° C.) was in the range of 0.5 to 1.0 milligrams of protein. As this response (Text, Fig. 2) was also produced by 0.2 ml. of the original serum (containing 12.5 mg. protein) a 25-fold purification is also evident.

Normal serum fractionated by the same procedures was not pyrogenic.

Characterization of partially purified NDV-EP. The dialyzed supernatant from the -20°C., 50 per cent ethanol precipitation was subjected to various tests in order to learn something about the chemical nature of the pyrogenic component.

When samples of dialyzed supernatant containing 2 mg. of protein were heated at 80°C. for two hours their pyrogenicity was destroyed. When heated at 50°C. for three hours no loss of pyrogenicity was detected. Diluted serum containing endogenous pyrogen showed partial loss of activity when heated at 56°C. for a prolonged period (20 hours) and was almost completely inactivated, as is leukocyte pyrogen,[8] after exposure to 90°C. for 30 minutes (Fig. 7).

*The failure of this material to adsorb to Sephadex G 25 confirms the relatively large size of the pyrogenic molecule, as evidenced by its nondialyzability.

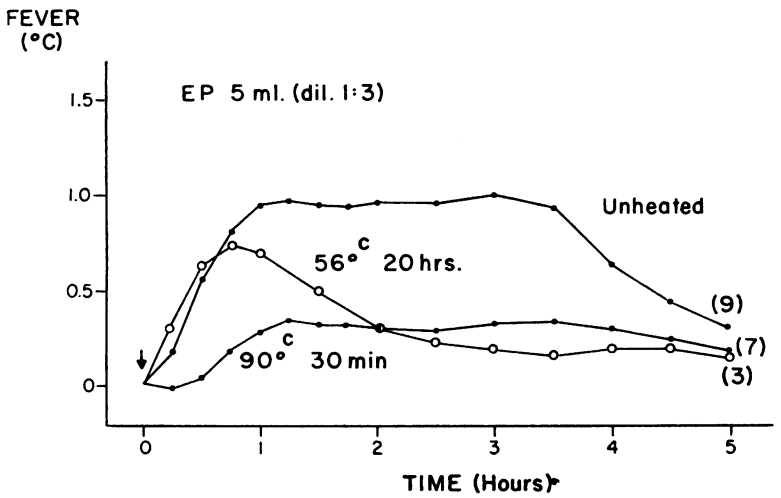

FIG. 7. Effects of heat on pyrogenicity of samples of serum EP diluted 1:3 in pyrogen-free water. Numbers in brackets indicate number of animals.

The pyrogenic activity was completely precipitated by 0.3 M perchloric acid. However, considerable loss of pyrogenicity was noted in the re-dissolved precipitate.

Phenol (90 per cent), a good solvent for most proteins, extracted the pyrogenic activity. The dialyzed aqueous layer was free of pyrogenicity.

Proteolysis of the dialyzed supernatant with either pepsin or trypsin destroyed the pyrogenicity (Fig. 8).

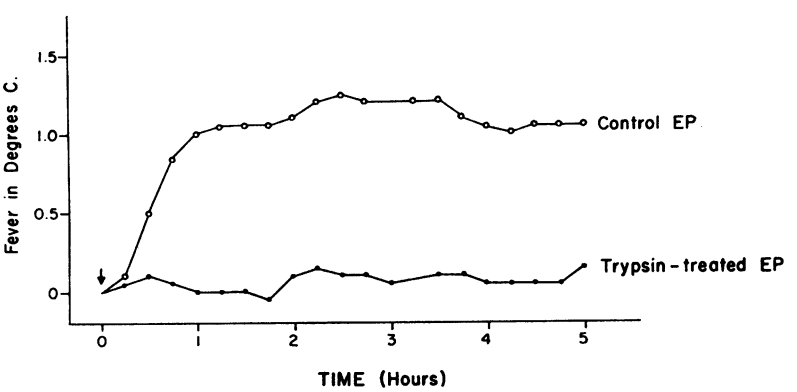

FIG. 8. Inactivation of partially purified NDV-EP (8.5 mg. protein) by trypsin (see text for details). Response to trypsin-treated EP is mean of 3 rabbits; response to control EP is mean of 2 rabbits to same material. Similar results (not shown) were obtained with pepsin. Mean response (2 controls): 0.7° C.; mean response (3 rabbits given pepsin-treated NDV-EP): 0.25° C.

A method of periodate oxidation similar to that used by Rafter, *et al.*[9] on leukocytic pyrogen partially inactivated purified NDV-EP, as indicated by the loss of the second fever peak (Fig. 9).

DISCUSSION

There is now considerable evidence that a circulating endogenous pyrogen (EP) is evoked by a number of microbial products given intravenously as well as by bacterial infection.[1] This agent (or agents) appears to be an essential intermediate in the pathogenesis of many fevers. When rapidly injected in sufficient amounts, serum EP produces a biphasic febrile response with little or no latency in normal recipient rabbits.[13, 26, 27]

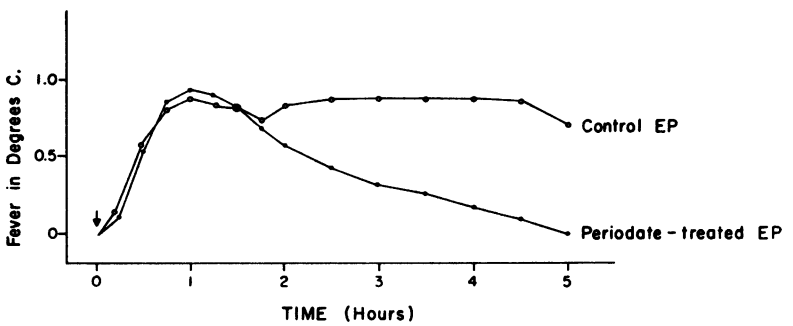

FIG. 9. Mean febrile responses of rabbits given untreated, partially purified NDV-EP and the same preparation oxidized with periodate (see text for details). Each curve represents mean of 3 animals.

Biphasic fever was noted with larger intravenous doses of the virus-induced pyrogen studied here, while smaller doses produced a monophasic fever.* Observations in our laboratory indicate that the biphasic response appears to be related to the mode of injection, since prolonged infusion of similar amounts of the same pyrogen gives rise to a sustained monophasic fever which lasts as long as the injection is continued.

Previous investigators have used fever index as a measure of the pyrogenicity of microbial agents such as endotoxins[28-31] as well as of serum EP[7, 10, 12, 27] and leukocytic pyrogen.[8, 25] The effectiveness of fever index has

* Since serum EP evoked by other unrelated agents such as Gram-negative bacterial endotoxin[18] or pneumococcal infection[9] similarly produces biphasic fever when given in sufficient dosage, as does leukocyte pyrogen obtained from sterile exudates,[25] it seems likely that the biphasic response induced by large doses of NDV-EP is due entirely to EP itself, rather than to the dual effect of EP and a residual component of the initially injected virus.[18]

not previously been determined for biphasic fevers produced by an endogenous pyrogen. With our endogenous pyrogen (NDV-EP), fever index was the best measure of pyrogenicity over the broadest range of doses (0.2 ml. — 10.0 ml.). Maximum fever of the first or second fever peaks appeared to be less reliable indices of dosage.

Partial purification of NDV-EP was aided by its marked stability to acid. Precipitation at 0.95 N HCl, which must be in part a differential denaturation, removed 65 per cent of the serum protein. A better separation of pyrogen by ethanol precipitation was achieved after this initial acid precipitation.

Chemical and enzymatic characterization of the partially purified preparation suggests that its activity is related to a protein. The active component was non-dialyzable through cellophane, precipitable by both trichloroacetic acid and perchloric acid, extracted by phenol and destroyed by proteolysis with either pepsin or trypsin. Extraction of lipid by n-butanol did not alter pyrogenicity.

The characteristics of this virus-induced EP are closely related to those found for leukocytic pyrogen by Rafter, *et al.*[6] The only difference noted was the modification of activity of our pyrogen by periodate oxidation. Leukocytic pyrogen, tested in a dose that produced monophasic fever, was not inactivated. When partially purified NDV-EP was oxidized under similar conditions with periodate, its ability to induce a second fever peak was lost.* This may be due to partial inactivation, since in titration experiments the second fever peak appeared only with larger doses. Alternatively, NDV-EP may consist of two components, one of which was inactivated by periodate oxidation. This possibility seems unlikely since leukocyte pyrogen similarly evokes biphasic fever when given in sufficient dosage.[25] In any event, if a second component exists, it must be partly dependent for its activity on a protein moiety, since the pyrogenicity of large doses of NDV-EP was entirely destroyed by proteolysis with pepsin or trypsin (see Fig. 8).

Partially purified NDV-EP has a similar heat of inactivation to the leukocytic pyrogen described by Bennett and Beeson,[8] but they found no inactivation by trypsin, in contrast to the later work of Wood and his colleagues.[6]

Recent evidence suggests that the NDV-induced serum pyrogen characterized in these experiments may be derived, as is endotoxin-induced

* More recent studies by Wood and his colleagues indicate that purified (as contrasted to crude) leukocyte pyrogen is inactivated by periodate oxidation (personal communication).

serum EP,[4,5] at least in part from circulating leukocytes. When purified parainfluenza virus[32] or NDV† is incubated *in vitro* with blood cells, a rapidly acting pyrogen is released, similar to the serum EP described here. Thus, NDV-EP may be identical to leukocyte pyrogen, as these preliminary biochemical studies suggest.

Evidence has been previously summarized[1] that both serum EP and leukocyte pyrogen differ in their biologic and biochemical characteristics from bacterial endotoxins,[33] Menkin's pyrexin[34] and the pyrogenic "tissue polysaccharides" of Landy and Shear.[35,36] The results reported here lend additional support to this distinction.

Further purification of NDV-induced EP is an obvious necessity before its exact chemical nature can be determined.

SUMMARY

A dose-response relationship has been determined for an endogenous pyrogen (EP) present in the sera of febrile rabbits injected intravenously with Newcastle disease virus (NDV).

By statistical analysis, fever index appeared to be better correlated with dosage than was either the height of the first or second fever peaks.

A 25-fold purification of NDV-induced serum pyrogen was obtained by successive acid and ethanol precipitations.

The pyrogenic activity of this material was associated with a non-dialyzable substance precipitated by trichloroacetic acid.

Further characterization of partially purified NDV-EP revealed that it was a heat labile substance which was precipitable by perchloric acid, destroyed by pepsin or trypsin proteolysis, and removed by extraction with phenol but not with butanol. Oxidation with periodate resulted in partial loss of activity.

In these properties, this substance is clearly distinct from bacterial endotoxin and from other previously described endogenous agents, such as "pyrexin" and "tissue polysaccharides," that are believed or known to have been contaminated by bacterial endotoxins. NDV-induced serum pyrogen appears to be a protein, similar or identical to the pyrogen extracted from granulocytes.

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† Unpublished observations (E.A.).

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