6. Beher, W. T., Anthony, W. L., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 356.

7. Dominguez, O. V., Seely, J. R., Gorski, J., *Anal. Chem.*, 1963, v35, 1243.

8. Boyd, G. S., in *The Control of Lipid Metabolism*, Grant, J. K., Ed., Academic Press, New York, 1963, p90.

9. Beher, W. T., Baker, G. D., *Circulation*, 1963, v28, 648.

10. Kritchevsky, D., Cottrell, M. C., Tepper, S. A., *J. Cell. Comp. Physiol.*, 1962, v60, 105.

11. Strand, O., *J. Lipid Res.*, 1963, v4, 305.

12. Danielsson, H., *Acta Physiol. Scand.*, 1960, v48, 364.

13. Gould, R. G., Cook, R. P., in *Cholesterol* (Cook, R. P., Ed.,) Academic Press, New York, 1958, p290.

14. Wilson, J. D., Siperstein, M. D., *Am. J. Physiol.*, 1959, v196, 595.

15. Wilson, J. D., *ibid.*, 1962, v202, 1073.

16. Hernandez, H. H., Chaikoff, I. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v87, 541.

17. Ivy, A. C., Janecek, H. M., Wojciech, R., *Am. J. Physiol.*, 1961, v201, 194.

18. Bauman, J. W., Hill, R., Nejad, N. S., Chaikoff, I. L., *Endocrinology*, 1959, v65, 73.

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Quantitative Assay of Endogenous Pyrogen in Serum.* (29684)

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Fever has long been used as a bioassay for endotoxin. To quantitate this procedure more precisely, Keene *et al* introduced the concept of the *minimum pyrogenic dose* (mpd) as a point of reference(1). These investigators observed that the amount of endotoxin tested correlated most closely with a fever index determined on the basis of the entire febrile response from time of injection to time of defervescence . Recent studies by Bornstein *et al*(2) demonstrated that when fever was produced with pyrogen derived from leukocytes, both the 1 and 2 hour fever indices, corresponding to the area beneath the curve during the first 1 or 2 hours after injection, correlated well with the number of cells from which the pyrogen was extracted. Kaiser and Wood(3) as well as Bornstein(2) found that the response of rabbits to graded doses of LP was relatively uniform and dose-dependent for individual recipients, allowing the derivation of a dose-response curve which was initially steep and linear, began to fall off with fever approximately 1°C in magnitude, and eventually reached a hyperthermic plateau at somewhat higher levels. Although this graded response was fairly uniform for individual animals, considerable variation was noted between different recipients.

The endogenous pyrogen (EP) found in the serum of febrile animals following a variety of stimuli is thought to be derived from injured leukocytes and to be biologically similar to LP(4). Preliminary experiments from this laboratory showed that the dose response curve for serum EP was similar to that described for leukocytic pyrogen(5). The studies to be described deal with the validity of the quantitative assay of EP in serum.

Materials and methods. All needles, glassware and instruments were sterilized in hot air ovens at 170°C for 3 hours. All serum and other injectables were cultured in thioglycollate broth for 72 hours and discarded unless sterile.

Male white rabbits of mixed breed weighing 1.4 to 4.0 kg were obtained from a single supplier and were housed in air-conditioned rooms. Rabbits used for pyrogen assay were of uniform weight and age, were handled gently and pre-trained in the assay situation for several days prior to use. All injections were made into the marginal ear vein which was shaved and cleansed immediately prior to injection. A minimum of 2 hours was allowed for acclimatization; animals whose temperatures fluctuated more than 0.2°C from the baseline were not used. Recipients

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for individual injections were selected in random fashion from among the animals with stable baseline temperatures.

Rabbits were placed in metal stalls with open backs and tops; they were restrained with loose-fitting metal collars and temperatures were recorded by means of an indwelling rectal thermister (Telethermometer, Yellow Springs Instrument Co.). Temperatures were recorded at 15 minute intervals during the first hour after injection and at 30 minute intervals thereafter for a total of 3 hours.

Groups of 6 donor animals were injected intravenously with heat-killed *Salmonella typhosa* vaccine diluted to a volume of 5.0 ml with saline. The undiluted vaccine contained approximately 3.3×10^9 killed bacterial cells and because of marked toxicity was used in only one experiment. For most experiments, 1:10 (3.3×10^8), 1:50 (6.6×10^7) and 1:100 (3.3×10^7) dilutions of the vaccine were employed. Animals were exsanguinated at the height of the febrile response 120 minutes later. Blood was allowed to clot at room temperature, and serum cleared by centrifugation at 4°C. Sera were cultured, and if sterile were pooled and stored at 4°C until tested.

The assay for EP used in these studies depends upon the determination of dose response curves derived from at least 3-4 different animals at 4 or more graded doses. Fever curves were plotted on standard graph paper with 10 cm on the vertical axis representing 1°C, and 5 cm on the horizontal axis corresponding to 1 hour. The area beneath each curve during the first 2 hours after injection was measured by planimetry and the reading taken directly from the planimeter (Keuffel-Esser 423 M-9788) was defined as the fever index. Characteristically, responses caused by EP in the sensitive range of the dose-response curve were monophasic, peaked within the first hour, and the temperature had returned to near the baseline within 2 hours after injection. Unless these criteria were met, the results of that particular injection were discarded and the assay was repeated in another animal. In the great majority of instances, the fever curves were typical of endogenous pyrogen permitting measurement of 2 hour fever indices and construction of an approximate dose-response curve. To facilitate comparison of the sera from different donors, serum which produced a fever index of 200 was defined as the minimum pyrogenic dose (mpd) of EP. This value was selected because: a) it represented a point approximately midway along the steep, linear part of the dose-response curve; b) the fever curve had a configuration typical of EP but was of minimal magnitude; and c) a fever index of 200 was only slightly greater than the index of a hypothetical fever curve which rises to 0.2°C (range of thermal lability allowed during period of acclimatization) at 15 minutes and remains at that level throughout the 2 hour period of the assay. The ability of each serum sample to produce fever could then be compared on the basis of its mpd. For example, serum having a mpd of 5 ml was said to possess twice the pyrogenic activity of a sample with a mpd of 10.0 ml.

Results. Fig. 1 demonstrates the mean temperature curves of groups of 3 rabbits injected with varying amounts of endogenous pyrogen from a single donor given 3.3×10^9 killed *S. typhosa*. There was an increase

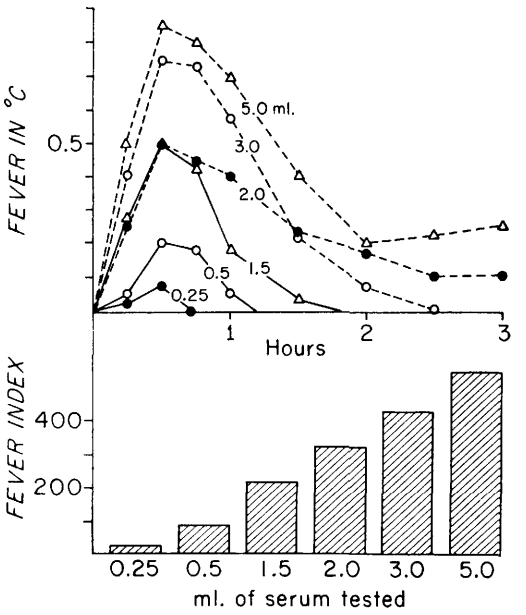
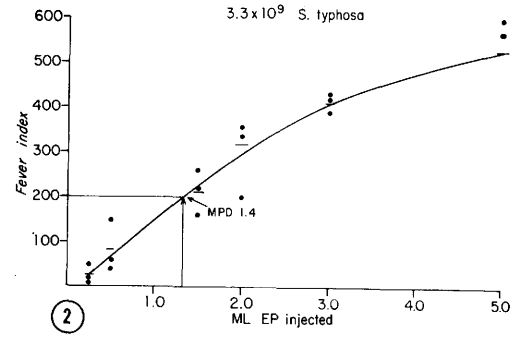


FIG. 1. Magnitude and duration of fever with graded doses of serum EP. Each curve and bar = mean of 3 animals.



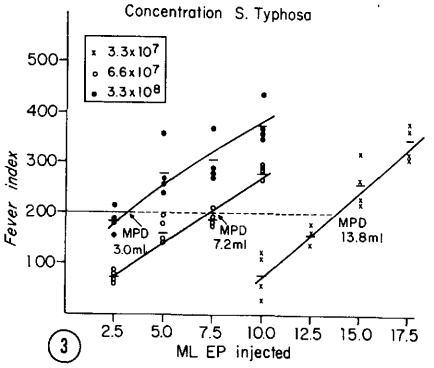


FIG. 2. Method of EP assay. Each point = febrile response of a single rabbit. Point at which curve intersects fever index of 200 = minimal pyrogenic dose.

FIG. 3. Pyrogenicity of serum EP from normal rabbits given 3 graded doses of typhoid vaccine.

in magnitude and duration of fever with each increment in dosage up to a level of 5.0 ml. At this point there was little further increase in fever. The same graded response was obtained when the fever index rather than the absolute increase in temperature was employed. Employing a fever index of 200 as the definition of a minimum pyrogenic dose (mpd), the mpd of this particular sample was approximately 1.4 ml.

It was found further that a more accurate dose-response curve could be constructed by merely presenting the fever index of each animal at different dosage levels on a scatter graph. This is illustrated in Fig. 2, in which the data given in Fig. 1 as the mean responses of 3 rabbits were graphed for individual animals. The curve rose in linear fashion up to a fever index of approximately 500 at which time a plateau occurred. The mpd was again 1.4 ml. This method for presenting the results was followed in all experiments performed subsequently.

There may be considerable variation between the amount of EP elaborated by a *single* donor following a constant pyrogenic stimulus; the example cited in Fig. 1 and 2 represents a brisk release of EP following a large dose of endotoxin; some animals did not produce as much EP following the same dose of endotoxin. In subsequent experiments variability was minimized by comparing the pyrogenicity of pooled serum from at least 6 or more normal donors.

In experiments summarized in Fig. 3, 3 groups of donors were given 3 graded doses of typhoid vaccine and dose response curves were constructed for normal recipients. Amount of EP released varied directly with amount of endotoxin injected. Normal animals given 3.3×10^8 killed *S. typhosa* elaborated approximately twice as much EP as animals given 6.6×10^7 cells. In the first instance the mpd was 3.0 ml; in the second, 7.2 ml (Fig. 3). When the stimulus for release of EP was reduced further by diluting the vaccine to 3.3×10^7 cells (1:100), the mpd rose to 13.8 ml (Fig. 3).

Discussion. These experiments demonstrate that the dose response curve for EP is similar to that for LP. This relationship is valid in different recipients provided at least 3, and preferably 4-6 animals, are used at each dosage level and at least 4 graded doses are tested. The data also emphasize several other factors which must be considered in interpreting experiments in which fever is used as a bioassay. First, it is important that the febrile response fall within the sensitive range of the dose response curve. When the fever index, which encompasses both magnitude and duration of fever, is plotted on the ordinate, the steep linear portion of the curve may be defined as sensitive. Second, when dealing with endogenous pyrogen, it is important to define the amount of EP released in terms of the strength of the stimulus used to release it. In other words, it is quite clear from these experiments that injection of 3.3×10^8 killed *S. typhosa* was capable of producing a great deal more EP than 3.3×10^7 killed cells. This relationship should also

hold true when stimuli other than endotoxin are used to produce EP. Third, the semiquantitative data obtained by testing EP—or any other substance employed in pyrogen assays—improves the accuracy of these assays and yields more meaningful information for comparison of different pyrogenic substances. Fourth, providing it is clearly defined, the concept of the minimum pyrogenic dose is useful in standardizing the pyrogenic response, and to compare data from different laboratories.

It should be realized that, as a form of bioassay, fever is a relatively gross measurement and depends on many variables inherent in the recipient, including the species of animal employed(5). It seems unlikely that differences between individual recipients or even within the same recipient will ever be eliminated. For this reason alone, attempts to control the procedures employed in this assay are indicated.

Summary. A semiquantitative assay for endogenous pyrogen in serum is described. The test depends on using at least 3-4 recipients at at least 4 ranges of dosage, and the use of the fever index rather than height of the fever curve. This permits construction of a dose response curve which delineates the sensitive range of pyrexia. The minimum pyrogenic dose can then be calculated and should serve as a useful standard for comparison of different pyrogenic substances.

1. Keene, W. R., Silberman, H. R., Landy, M., *J. Clin. Invest.*, 1961, v40, 295.

2. Bornstein, D. L., Bredenberg, C., Wood, W. B., Jr., *J. Exp. Med.*, 1963, v117, 349.

3. Kaiser, H. K., Wood, W. B., Jr., *ibid.*, 1962, v115, 37.

4. Atkins, E., *Physiol. Rev.*, 1960, v40, 580.

5. White, L. R., Petersdorf, R. G., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 567.

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Folic Acid Deficiency and Hepatic DNA Synthesis.* (29685)

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Clinical evidence of folate deficiency consisting of a low serum folic acid(1), increased urinary excretion of formiminoglutamic acid (2), and macrocytic anemia(3) are frequently found in cirrhosis. This deficiency appears to result principally from inadequate intake coupled with decreased hepatic storage of this vitamin(4). The demonstration that folic acid is required for cell proliferation(5,6) suggests the regenerative process may contribute to encountered deficits and that folate depletion may interfere with hepatic DNA synthesis and regeneration. The present study was undertaken to assess the influence of liver regeneration on tissue levels of folic acid and folinic acid, and to determine the effect of folic acid depletion on hepatic DNA synthesis.

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Materials and methods. Three hundred weanling Sprague-Dawley rats received a folic acid deficient diet, which was normal in all other basic constituents. At the end of 2 months, by a pre-determined randomized schema, litter mates were each treated with 30 μg folic acid,† 30 μg folinic acid,† 100 μg of vit. B_{12},†, 50 mg uridylic acid,‡ 50 mg thymidilic acid,‡ or 1.0 ml normal saline daily for 10 days. Animals were given 0.2 ml of saline or CCl_4 per 100 g weight orally, and killed by decapitation 12, 24, 48, 72, 96, or 120 hours later. Four hours prior to killing, animals were given 1 μc per g weight of tritiated thymidine (sp. activity 1.9 c per mmole)§(7), intraperitoneally.

† Furnished by Dr. J. M. Ruegsegger, Lederle Laboratories.

‡ Obtained from Calbiochem, New York.

§ Purchased from Schwarz BioResearch, Inc., New York.