

STUDIES ON THE PATHOGENESIS OF FEVER

I. THE PRESENCE OF TRANSFERABLE PYROGEN IN THE BLOOD STREAM FOLLOWING THE INJECTION OF TYPHOID VACCINE*

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Body temperature is controlled by thermoregulatory centers of the brain. Except in conditions involving direct injury to the central nervous system, however, the manner in which these centers are influenced by diseases which give rise to fever is not known (1-3). Numerous studies relating to the problem have been performed with rabbits injected intravenously with pyrogenic substances of bacterial origin (4-14). The fact that the resulting fever occurs only after an appreciable latent period has led to the theory that the thermoregulatory centers are acted upon by a secondary endogenous substance released from cells injured by the originally injected pyrogen (15, 16). The demonstration in leucocytic extracts of a fever-producing substance possessing properties different from those of bacterial pyrogens adds indirect support to this hypothesis (17, 18).

Attempts to relate the leucocytic pyrogen to the pathogenesis of fever, however, have thus far been unsuccessful. First, "pyrexin," the heat-stable fever-producing extract originally obtained from inflammatory exudates by Menkin (19-21), has recently been shown to have the same properties as bacterial pyrogen and appears to have been contaminated with it (18). Secondly, rabbits made leucopenic with nitrogen mustard have been found to be just as responsive to bacterial pyrogen as rabbits with normal leucocyte counts (22). Thirdly, antipyretically effective doses of cortisone have failed to influence the characteristic leucopenia which precedes the onset of pyrogen-induced fever (23, 24). All of these observations have led Bennett and Beeson to conclude that "there is no evidence that the leucocyte changes after pyrogen administration are related to the production of fever (23)."

Contrary to this conclusion, on the other hand, is the fact that the leucopenic reaction which regularly precedes fever induced by typhoid vaccine bears a definite time relationship to the onset of the fever (25). In addition, tolerant rabbits, which respond only minimally to the injected vaccine, fail to exhibit

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a demonstrable fall in white count (25).¹ These observations, together with those of Berthrong and Cluff (28), which indicate that the injected pyrogen affects the rabbits' leucocytes, support the general hypothesis that pyrogen-induced fever is caused by an endogenous factor released from injured cells (including leucocytes). If the hypothesis is valid, it should be possible to demonstrate the endogenous factor in the circulation during fever (16).

The present experiments deal with the speed of clearance of a constant dose of typhoid vaccine given intravenously to unsensitized, "sensitized," and tolerant rabbits respectively. Different rates of clearance are demonstrated in each of the three groups. Of particular significance is the observation that fever-producing activity reappears in the blood of sensitized rabbits after they have apparently cleared the originally injected pyrogen. Reasons for believing that this secondary pyrogenicity may represent the release of the postulated endogenous pyrogen are discussed.

Methods

The pyrogen used was a single lot of typhoid vaccine, monovalent reference standard NRV-LS No. 1, made from *Salmonella typhosa* V-58.² Rabbits were adult healthy males of albino Flemish and New Zealand stock weighing approximately 3 to 4 kg. All studies were carried out at 68 to 70°F. in an air-conditioned room within which the animals were also housed between experiments.

Donor rabbits were divided into three groups: (1) unsensitized donors, (2) sensitized donors, and (3) tolerant donors. The *unsensitized donors* had previously received no injections. The *sensitized donors* had received one or two previous intravenous injections³ of typhoid vaccine from the same source as that used throughout the study. These injections were given at intervals of not less than 3 weeks with an additional 3 to 5 weeks elapsing between the last injection and use of the animal in the experiment. This interval was chosen as a minimum in order to avoid the development of tolerance (26). All *tolerant donors* were given daily intravenous injections of 75 million organisms (1.5 ml. of a 1:10 dilution of the stock vaccine) for a period of 7 to 8 days prior to the experiment. Fever was measured on the 1st and last day of injections to insure that tolerance had been established. The tolerant animals served as donors 24 hours after the last injection.

Recipient rabbits were divided into three equal groups of 5 or 6 animals each and the groups were used in rotation. Experiments were performed on 2 successive days in each week on a single group which was then not retested for 3 weeks. This precaution was taken in order to prevent the acquisition of tolerance by the recipients. None of the recipient rabbits had ever been made tolerant, and all studies dealing with tolerant serum were performed only after those with the two other types of serum had been completed. By the

¹ This finding is not in agreement with statements made by Beeson (26) and by Cluff (27), but since no data on this point are presented in either report their conclusions are difficult to evaluate.

² Kindly supplied by Dr. Geoffrey Edsall of the Army Medical Service Graduate School, Walter Reed Medical Center, Washington, D. C. This vaccine had a bacterial count of approximately 500 million per ml. and a nitrogen content of 0.3 mg./ml. (± 10 per cent).

³ A single animal received three previous injections. In order to make the results strictly comparable, the same individual rabbits were used first as unsensitized donors and later as sensitized donors.

conclusion of the entire experiment all groups had received serum from donors in all three categories.⁴ It should be noted that the recipients were themselves in the sensitized category in that they had received infrequent injections without ever having been made tolerant. Their responses, therefore, to the various sera tested may be considered to have been strictly comparable.

Recording of Temperatures.—Procedures for the handling of the recipient rabbits and the method of recording temperatures have been reported elsewhere (24). Control temperatures were measured for a period of 6 to 7 hours on each of the 2 or 3 days preceding the experiment, and any animal with a fluctuation of more than 1°F., or an initial temperature of more than 103°F. on the day of the experiment, was discarded. A base line for each recipient was established by three or four readings taken during the hour before the experiment was started.

Transfer of Circulating Pyrogen.—All donor animals received a standard inoculation of 1 ml. of undiluted vaccine. The injection was given over a period of approximately 5 seconds into the marginal ear vein. The animals were then bled once by intracardiac puncture at an interval of 5, 30, 60, or 120 minutes following the injection. 50 to 60 ml. of blood were withdrawn and allowed to clot in a flask for 1 hour at 37°C.; the serum was removed and cleared by centrifugation after storage overnight at 4°C. Before use the following day the serum was incubated for a period of 30 to 45 minutes at 37°C. Serum for a single experiment was pooled from 2 donors giving a total of 50 to 60 ml. The volume injected into each of the 5 or 6 recipients within each group was 10 ml., given at a rate of about 1 ml. per second. After inoculation, temperature readings were taken every 5 minutes for the first hour, at 15 minute intervals for the next hour, and thereafter every 30 minutes for a total of 5 to 6 hours.

Calculation of Fever Index.—The resulting fever was charted on $\frac{1}{6}$ inch graph paper with two lines on the vertical axis for each degree F. and six lines on the horizontal axis for each hour. With the temperature at the time of inoculation as a base line, the area beneath the 3 hour fever curve⁵ was calculated by means of a compensating planimeter.⁶ In this way a "fever index" (measured in squared centimeters) was obtained for each animal, an expression of both the height and duration of the fever (26). In cases in which there was no fever, the algebraic sum of the fluctuations above and below the base line was calculated, giving a negative value in some instances.

Exclusion of Extraneous Pyrogens.—Rigid precautions were taken to prevent contamination by pyrogens. Glassware and needles were sterilized by dry heat at 170°C. for 2 hours. All sera were checked for sterility. As a control procedure, normal serum was drawn by these techniques and shown to be nonpyrogenic.

RESULTS

The fever indices of individual recipient animals injected with sera⁷ from the three types of donors are plotted in Fig. 1. From an analysis of the mean curves

⁴ For practical reasons sera obtained at each of the four time intervals from the three donor categories (unsensitized, sensitized, and tolerant) were not tested in all three of the recipient groups. Enough comparable samples, however, were tested in more than one recipient group to demonstrate that the mean responses were practically identical.

⁵ This interval was chosen to obviate late variations in temperature not due to transferable pyrogen. In the few cases in which the fever had not returned to the base line within this time limit, a vertical line was drawn between the 3 hour temperature and the base line to define the area.

⁶ Keuffel and Esser compensating polar planimeter No. 4236M.

⁷ Values similar to those recorded for both unsensitized and sensitized donors have been

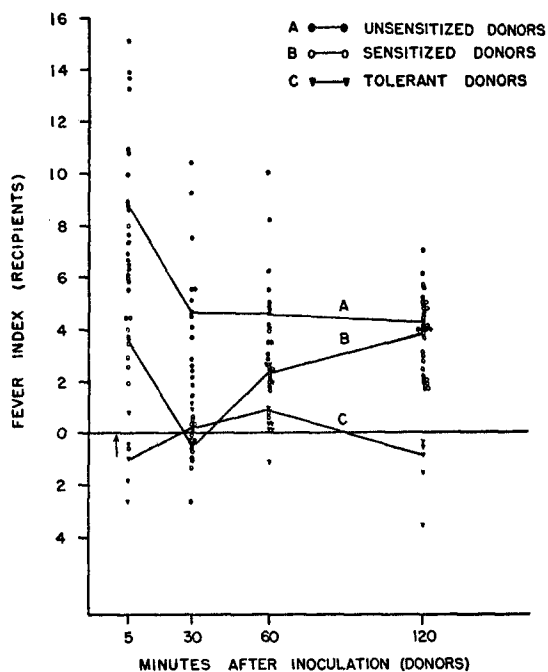


FIG. 1. Individual and mean fevers in recipient rabbits given serum from unsensitized, sensitized, and tolerant donors. Donors were bled at 5, 30, 60, and 120 minutes after inoculation with 1 ml. typhoid vaccine.

TABLE I

*Fever Indices of Recipient Rabbits Injected with Serum from Unsensitized, Sensitized, and Tolerant Donors**

Donor	5 min.	30 min.	60 min.	120 min.
Unsensitized.....	8.9 ± 0.7‡	4.6 ± 0.7‡	4.7 ± 0.7	4.3 ± 0.5
Sensitized.....	3.7 ± 0.8‡	-0.5 ± 0.3‡	2.5 ± 0.4	3.8 ± 0.3‡
Tolerant.....	-1.0 ± 0.7	0.3 ± 0.3	1.0 ± 0.5	-0.8 ± 1.0

* Mean (\pm s.e.) responses of recipients expressed as fever index (square centimeters area beneath fever curve).

‡ The probability is less than 0.01 that the mean responses of recipients are identical for the following:—

- Unsensitized as compared to sensitized donors—5 minute serum.
- Unsensitized as compared to sensitized donors—30 minute serum.
- Sensitized donors—30 minute serum as compared to 120 minute serum.

Thus the augmented clearance of injected pyrogen by the sensitized donors and the reappearance of pyrogenicity in their blood at 2 hours are both statistically significant.

obtained with injections of 20 ml. of heparinized whole blood transfused into recipients immediately after withdrawal.

(see Table I) it is evident that there are three distinct rates of clearance of the injected pyrogen (or its serum combination).

In the unsensitized group the mean value of remaining pyrogenic activity, as reflected in the fever indices of the recipients, falls rapidly to about half of its initial 5 minute value at 30 minutes. Thereafter, in the 60 and 120 minute samples the level of pyrogenicity does not change appreciably.

In marked contrast to the removal of pyrogen in unsensitized animals, the blood of tolerant donors does not contain any significant pyrogenic activity at any of the four intervals tested. The slight rise in the fever index of recipients injected with samples of 60 minute blood from tolerant donors is due to gradual elevations in temperature which occur well after the 1 hour and are not characteristic of the usual pyrogenic response.

In the sensitized group, a rate of clearance intermediate between that of unsensitized and tolerant donors is apparent. The mean value at 5 minutes is slightly less than half of that present in unsensitized animals. Following this there is a rapid removal of pyrogen resulting in complete clearing by the end of 30 minutes. This pyrogen-free interval is then followed by a return of pyrogenic activity in the blood demonstrable at 60 minutes and more marked at the end of 2 hours. It will be noted that the mean level of pyrogenicity finally reached at 2 hours is approximately the same as that shown by 2 hour blood from unsensitized donors.

DISCUSSION

From the results of these experiments it is evident that the rate of removal of the intravenously injected pyrogen was determined by each rabbit's previous exposure to the pyrogenic stimulus. Whereas the blood of unsensitized animals was cleared relatively slowly, that of donors sensitized by one or two injections 3 to 5 weeks previously contained no demonstrable pyrogenicity at the end of 30 minutes. Rabbits made tolerant, on the other hand, by daily injections for at least a week cleared the pyrogen so rapidly that none was detectable in the blood even at 5 minutes.

The exact mechanisms involved in the clearance of pyrogen in each of these three states are not known. Evidence has been advanced that pyrogen tolerance is due to an accelerated removal of the injected substance by cells of the reticulo-endothelial system (29). Humoral factors also have been implicated (30). It is clear, however, that tolerance differs from the usual immune response to injected antigen in that it is relatively non-specific, lasts for only 2 to 3 weeks after cessation of the injections, and is not closely correlated with circulating antibody levels (26, 31). The recent demonstration by Good that tolerance can be produced in patients with agammaglobulinemia adds further support to this conclusion (32).

Previous workers have made no distinction between unsensitized and sensi-

tized rabbits.⁸ This oversight is readily explained by the fact that the fever responses and leucopenic reactions in both are remarkably similar (33). Only by using the passive transfer technique, as employed in the present study, is it possible to demonstrate that sensitized rabbits clear the injected pyrogen more rapidly than do previously unexposed rabbits. The difference is important in itself, but of far greater significance is the fact that pyrogenicity reappears in the blood of sensitized rabbits after the originally injected pyrogen has been completely cleared.

In regard to this latter finding the previous observations of Beeson, LeQuire, and Grant are of particular interest. Using a passive transfer method similar to that of the present study Beeson found that whereas tolerant rabbits had cleared practically all of the injected pyrogen by 4 minutes, the blood of non-tolerant rabbits possessed transferable pyrogenicity for 8 to 10 minutes, but not longer (29). LeQuire independently recorded the same findings (12). Grant, on the other hand, reported the presence of transferable pyrogen in the blood of non-tolerant rabbits for as long as 3 hours after the injection (13). The failure of all three of these investigators to differentiate between unsensitized and sensitized rabbits affords a possible explanation for their apparently conflicting results. Beeson's and LeQuire's findings are in complete agreement with the present observations on sensitized rabbits and can be readily explained if it is assumed that both were using sensitized rather than unsensitized animals. Grant's demonstration of transferable pyrogen at all tested intervals up to 3 hours after inoculation, on the other hand, suggests that he was using unsensitized rather than sensitized donors (compare curves A and B, Fig. 1).

The reappearance of fever-producing properties in the blood of sensitized rabbits after apparent clearing of the originally injected vaccine suggests the entrance into the circulation of a new endogenous pyrogen, which may serve as the effective stimulus to the thermoregulatory centers of the brain. Indeed, it seems not unlikely that the continuous pyrogenicity of the blood from unsensitized rabbits (curve A) may be explained by the resultant effects of both exogenous and endogenous pyrogen. If it is assumed that in unsensitized rabbits the injected pyrogen is not completely removed until after formation of the endogenous factor, the continuous pyrogenicity of the blood may be accounted for. In sensitized rabbits the injected pyrogen is removed more rapidly. Clearance appears to be completed before the entrance of the endogenous factor, and accordingly there is a brief interval during which no transferable pyrogen can be demonstrated (see curve B). But because the endogenous factor does eventually make its appearance in roughly the same concentration as in unsensitized rabbits (compare 2 hour points of curves A and B), the sensitized (donor) rabbits exhibit the same febrile response as the unsensitized donors. In the

⁸ A single exception to this statement is the work of Farr *et al.* which revealed that tolerance develops more rapidly in sensitized than in unsensitized rabbits (30).

presence of pyrogen tolerance, however, the clearance of the typhoid vaccine is so rapid that the injected pyrogen appears not to be present in the circulation long enough to bring about release of a detectable amount of endogenous factor (see 5 minute and 2 hour points of curve C). As a result the febrile response, which is postulated to be due to the endogenous factor, is significantly depressed.

That the secondary pyrogen demonstrated in the present studies is in reality of endogenous origin remains to be established. Its appearance in the blood following the pyrogen-free interval might conceivably be due to a reentrance into the circulation of the originally cleared vaccine. This possibility, however, does not seem likely. As far as the authors are aware, no analogous phenomenon

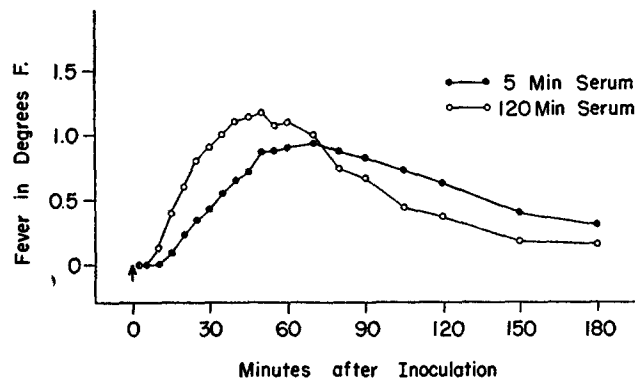


FIG. 2. Mean febrile responses of a single group of recipients to 5 minute and 2 hour sera from sensitized donors (based on 10 and 12 individual curves respectively). The mean rise in temperature caused by the 2 hour sera was significantly faster than that caused by the 5 minute sera ($p = <0.01$ for fever reached at 30 minutes). The mean total febrile responses (fever indices) for the two groups of sera (5 minute and 2 hour) were the same.

has ever been demonstrated involving a reappearance in the circulation of non-viable microbes or any of their constituent products once cleared from the blood stream. Furthermore, careful comparison of the fever responses of the recipient rabbits to the 5 minute and 2 hour sera indicates that the latter produce a significantly faster rise in temperature than do the former (see Fig. 2).⁹ This finding suggests that two different pyrogens are involved and is in keeping with the hypothesis that the fever-producing factor contained in the

⁹ This observation is in accord with Grant's demonstration that blood withdrawn from donors 40 minutes or later after injection produces a more rapid response in recipients than do either injected bacterial pyrogens or blood withdrawn in less than 30 minutes (13). He has attributed the accelerated febrile response to the formation of a new pyrogen-serum complex postulated to be capable of penetrating more rapidly to the thermostatic centers. Contrary to this interpretation is the fact that the combination of pyrogen with serum is completed in as little as 2 minutes *in vitro* (14).

5 minute serum is a product of the injected vaccine whereas that contained in the 2 hour serum is derived from cells of the host. The evidence presented supports the theory that the secondary factor acts upon the thermoregulatory centers of the brain to cause fever.

At least some of the secondary pyrogen would appear to come from injured leucocytes. As already mentioned, the occurrence of prefever leucopenia following the injection of typhoid vaccine has a definite time relationship to the initial rise in temperature (25). Also there is strong evidence that the injected pyrogen injures the leucocytes. Recent observations of Braude with radioactively tagged *E. coli* endotoxin reveal that a large proportion of the injected substance appears promptly in the buffy coat (34). Polymorphonuclear leucocytes thus affected *in vivo* have been shown not only to stick to the endothelium of blood vessels (35, 36) but also to behave abnormally in tissue culture (27). Finally, the fever-producing substance extractable *in vitro* from polymorphonuclear leucocytes produces a febrile response in rabbits different from that evoked by exogenous pyrogen but thus far indistinguishable from that induced by the secondary factor described in the present experiments (18).

The working hypothesis outlined in the foregoing discussion manifestly conflicts with the conclusion of Bennett and Beeson that leucocytic changes are not involved in the production of pyrogen-induced fever (23). Reasons for questioning their conclusion have already been presented elsewhere (16). Further study of the secondary fever-producing factor described in the present experiments will have to be performed before its role as an endogenous pyrogen can be defined. Such studies are already in progress. Whatever their outcome may be, it should be emphasized that cells other than polymorphonuclear leucocytes are probably also involved in the pathogenesis of fever (16).

SUMMARY

The rate of clearance of intravenously injected typhoid vaccine was studied in unsensitized, sensitized, and pyrogen-tolerant rabbits by means of a passive transfer technique.

The blood of unsensitized rabbits which had not been previously exposed to bacterial pyrogen remained pyrogenic for normal recipients throughout a period of 2 hours following the injection.

In contrast, rabbits sensitized by having received either one or two injections of the vaccine at least 3 weeks prior to the experiment cleared their blood of the test vaccine within 30 minutes despite the fact that they exhibit the same febrile response as unsensitized rabbits. After 1 hour, however, a transferable pyrogenic substance was again demonstrable in the sera of this group. Reasons are discussed for believing that this newly appearing substance may be of endogenous origin and may be the factor which directly affects the thermoregulatory centers of the brain.

Rabbits which are made tolerant by repeated daily injections of vaccine have a characteristically depressed febrile response. Not only were the blood streams of such animals cleared of the injected vaccine within less than 5 minutes, but samples of their sera obtained 1 and 2 hours after the injection also failed to contain demonstrable quantities of the secondary pyrogen observed in sensitized animals. The latter observation is in keeping with the suggestion that the secondary pyrogen may play a critical role in the production of fever.

BIBLIOGRAPHY

1. Selle, W. A., *Body Temperature: Its Changes with Environment, Disease and Therapy*, Springfield, Illinois, Charles C. Thomas, 1952, 38.
2. Grant, R., *Ann. Rev. Physiol.*, 1951, **13**, 75.
3. Beeson, P. B., *in Signs and Symptoms*, (C. M. MacBryde, editor), Philadelphia, J. B. Lippincott Company, 2nd edition, 1952, 392.
4. Ranson, S. W., Jr., Clark, G., and Magoun, H. W., *J. Lab. and Clin. Med.*, 1939, **25**, 160.
5. Chambers, W. W., Koenig, H., Koenig, R., and Windle, W. F., *Am. J. Physiol.*, 1949, **159**, 209.
6. Bennett, I. L., Jr., *J. Exp. Med.*, 1948, **88**, 267.
7. Bennett, I. L., Jr., *J. Exp. Med.*, 1948, **88**, 279.
8. Hall, V. E., Field, J., and Grant, R., Memorandum Report (United States Air Force) MCREXD-696-113D, November 4, 1948.
9. Grant, R., *Am. J. Physiol.*, 1949, **159**, 511.
10. Bennett, I. L., Jr., and Beeson, P. B., *Medicine*, 1950, **29**, 365.
11. Farr, R. S., and LeQuire, V. S., *Proc. Soc. Exp. Biol. and Med.*, 1950, **75**, 661.
12. LeQuire, V. S., *J. Infect. Dis.*, 1951, **88**, 194.
13. Grant, R., and Whalen, W. J., *Am. J. Physiol.*, 1953, **173**, 47.
14. Farr, R. S., Clark, S. L., Jr., and Proffitt, J. E., *Naval Med. Research Inst. Rep.*, Bethesda, 1953, Project NM 007 081.12.01.
15. Beeson, P. B., *J. Clin. Inv.*, 1948, **27**, 524, (abstract).
16. Wood, W. B., Jr., *Am. J. Med.*, 1955, **18**, 351.
17. Bennett, I. L., Jr., and Beeson, P. B., *J. Exp. Med.*, 1953, **98**, 477.
18. Bennett, I. L., Jr., and Beeson, P. B., *J. Exp. Med.*, 1953, **98**, 493.
19. Menkin, V., *Arch. Path.*, 1945, **39**, 28.
20. Menkin, V., *Arch. Path.*, 1946, **41**, 376.
21. Menkin, V., *Newer Concepts of Inflammation*, Springfield, Illinois, Charles C. Thomas, 1950, 114.
22. Bennett, I. L., Jr., and Cluff, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1952, **81**, 304.
23. Bennett, I. L., Jr., and Beeson, P. B., *Bull. Johns Hopkins Hosp.*, 1953, **93**, 290.
24. Atkins, E., Allison, F., Jr., Smith, M. R., and Wood, W. B., Jr., *J. Exp. Med.*, 1955, **101**, 353.
25. Atkins, E., and Wood, W. B., Jr., *data to be published*.
26. Beeson, P. B., *J. Exp. Med.*, 1947, **86**, 29.

27. Cluff, L. E., *J. Exp. Med.*, 1953, **98**, 349.
28. Berthrong, M., and Cluff, L. E., *J. Exp. Med.*, 1953, **98**, 331.
29. Beeson, P. B., *J. Exp. Med.*, 1947, **86**, 39.
30. Farr, R. S., Clark, S. L., Jr., Proffitt, J. E., and Campbell, D. H., *Am. J. Physiol.*, 1954, **177**, 269.
31. Morgan, H. R., *J. Immunol.*, 1948, **59**, 129.
32. Good, R. A., *J. Lab. and Clin. Med.*, 1954, **44**, 803, (abstract).
33. Atkins, E., and Wood, W. B., Jr., unpublished observations.
34. Braude, A. I., *J. Clin. Inv.*, in press.
35. Essex, H. E., and Grana, A., *Am. J. Physiol.*, 1949, **158**, 396.
36. Leusen, I. R., and Essex, H. E., *Am. J. Physiol.*, 1953, **172**, 231.