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# MECHANISM OF ENDOTOXIN-INDUCED KININ PRODUCTION IN HUMAN PLASMA*†

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**Abstract**—Formation of kinins in the presence of endotoxin was investigated in cell-free specimens of plasma from normal adult humans, of decomplemented plasma, of plasma deficient in Hageman factor (factor XII), and of plasma from human newborns. Endotoxin activated kinin-forming enzymes and depleted kininogen only in the presence of both complement and antibody to endotoxin. The interaction between plasma and endotoxin was inhibited by soybean trypsin inhibitor but not by ovomucoid trypsin inhibitor. This pattern of inhibition implies that plasma kallikrein or plasmin is the enzyme responsible for conversion of kininogen to kinins in this system. Activity of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) esterase in plasma correlated with formation of kinin during activation of plasma kallikrein by glass, but no such correlation was found when endotoxin was used to activate the kinin-generating system. Salicylates in pharmacological concentrations blocked formation of kinins in the presence of endotoxin but hydrocortisone did not. We conclude that endotoxin reacts with complement and antibody to endotoxin to activate plasma kallikrein or plasmin to form kinin. This reaction can be effectively inhibited by salicylates but not by hydrocortisone, it does not require the presence of Hageman factor, and it does not result in significant increases in the activity of plasma TAME esterase.

THE EARLY phase of endotoxin shock in man and in the unanesthetized primate is characterized by profound hypotension, low total peripheral resistance and nearly normal cardiac output.[1,2] Kinins are potent vasodilators which are formed *in vivo* after endotoxin infusion, and, in the primate, the rise in kinins correlates with the fall in total peripheral resistance in the early phase of shock.[1] The mechanism of production *in vivo* of kinins in the presence of endotoxin is unknown. Four possibilities are: (1) direct activation of plasma kallikrein; (2) interaction with specific immunoglobulins with or without complement to activate kallikreins or other enzymes which can convert kininogens to kinins;[3] (3) activation of the clotting system,[4] which can activate kallikreins or produce kinins by the action of plasmin; or (4) interaction with granulocytes to release or activate kallikrein.[5] This investigation was designed to determine the way in which kinins are formed, in an isolated system, by the interaction of cell-free plasma and endotoxin.

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## METHODS

Heparinized (5 units/ml) blood was obtained from healthy volunteers, from the umbilical vessels of normal full-term infants, and from a patient with Hageman factor (factor XII) deficiency, and the plasma was separated by centrifugation at room temperature. Polypropylene syringes and centrifuge tubes were used for handling the blood, and the plasma was placed in polypropylene or siliconized glass flasks in a 37° water bath.

Endotoxin solution was prepared by adding 1 mg (*Escherichia coli* 0127:B8, Difco Laboratories) per ml of 0·9 per cent saline solution.[1] One ml of the endotoxin solution or 1 ml of saline as a control was added to each 5-ml aliquot of plasma.

In most experiments, 0·2-ml aliquots of plasma were removed at 0 (15 sec), 5 and 10 min after the addition of endotoxin or saline and were assayed for kininogen by the method of Diniz and Carvalho.[6] At 4-min intervals, 0·2-ml aliquots of normal adult and of neonatal plasma were placed directly in a tissue bath containing an estrus rat uterus in order to test for kinin-like activity in the incubation mixture.

To determine whether kinin production correlated with kininogen depletion, a *specific kinin assay* was performed in which 5-ml aliquots of the plasma–endotoxin mixture and the control mixture were placed in 10-ml aliquots of 0·5 M perchloric acid, the supernatant fluid was neutralized and passed over IRC-50 resin, and peptide was eluted from the resin. This method of isolation of kinins has been previously described;[1] it is useful for separation of kinin peptide from other peptides and amines which also contract the estrus rat uterus. In addition, the nature of the eluate was assessed by means of its resistance to digestion with trypsin and its biological inactivation after incubation with chymotrypsin.

Production of kinins by the addition of endotoxin to plasma was compared with that caused by the shaking of plasma with glass beads. Aliquots of each were removed at 0 (15 sec) and 4 min to determine hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAME),[7] concentration of kininogen,[6] and direct kinin-like activity on the estrus rat uterus. The specific kinin assay[1] as outlined above was also done. The TAME esterase activity of plasma was determined by addition of 0·10 ml of 0·2 M TAME to tubes containing 0·5 ml plasma, 0·25 ml of 1 M Tris (pH 8·5) and 0·15 ml of 0·9 per cent saline. After incubation at 37° for 10–15 min, the amount of residual ester was determined colorimetrically.[7] The mixture contained an excess of TAME and the reaction followed zero order kinetics over the time period studied.

Plasma antibody titers to endotoxin were measured by hemagglutination of tanned sheep red cells coated with endotoxin.[5] The hemolytic complement was assayed by the method of Kabat and Mayer.[8] Plasma was decomplemented by absorption at 37° for 40 min with hemolysin-sensitized sheep red cell ghosts and the ghosts were then removed by centrifugation. Antibody to endotoxin was partially purified from hyperimmune rabbit serum by chromatography on a Sephadex G-200 column. Most of the activity of antibodies was observed in the first major peak eluted with phosphate-buffered saline; when analyzed by immunoelectrophoresis, the primary component was 19S immunoglobulin. The 19S immunoglobulin prepared in the same way but without endotoxin antibody was used as a control. Both 19S fractions were added to neonatal plasma as described below.

Effects of certain inhibitors and drugs and of the 19S antibody were investigated in some samples of plasma. Final concentrations in plasma, before addition of endotoxin,

were: soybean trypsin inhibitor, 0·2 mg/ml; ovomucoid trypsin inhibitor, 0·2 mg/ml; acetylsalicylic acid, $10^{-5}$ M; sodium salicylate, $10^{-3}$ and $10^{-5}$ M; hydrocortisone hemisuccinate (Solu-Cortef), $10^{-3}$ M; and 19S antibody, 1:4.

Statistical significance of the data was tested by Student's two-tailed *t*-test.

## RESULTS

### *Plasma from normal adults*

The kininogen levels of normal adult plasma ranged from 3000 to 6000 ng of releasable bradykinin per ml. The results have been normalized by setting the zero time sample as equal to 100 per cent and comparing later samples to this value (Table 1).

TABLE 1. MEAN KININOGEN LEVELS IN HUMAN PLASMA AFTER THE ADDITION OF SALINE SOLUTION OR ENDOTOXIN*

| Type of plasma | No. samples | Complement units | Saline solution | | Endotoxin | |
|---|---|---|---|---|---|---|
| | | | (5 min) | (10 min) | (5 min) | (10 min) |
| Normal plasma | 22 | 103 ± 6 | 94 ± 2 | 93 ± 2 | 73 ± 2† | 58 ± 2‡ |
| Decomplemented plasma | 4 | 11 ± 10 | 92 ± 6 | 95 ± 6 | 94 ± 2 | 94 ± 4 |
| Newborn cord plasma without antibody | 6 | | 100 ± 6 | 102 ± 6 | 94 ± 6 | 92 ± 4 |
| Newborn cord plasma with antibody (Ab titer 1:4) | 1 | | Not tested | 90 | Not tested | 48 |
| Newborn cord plasma plus 19S macroglobulin containing antibody | 2 | | 96 | 95 | 69 | 55 |
| Newborn cord plasma plus 19S macroglobulin without antibody | 6 | | 99 ± 6 | 99 ± 5 | 96 ± 2 | 97 ± 5 |
| Hageman factor-deficient plasma | 5 | | 104 ± 6 | 98 ± 4 | 78 ± 10† | 64 ± 10† |

* Values are expressed as a percentage of the values at zero time (± S.E.M.).
† Significantly ($P < 0·05$) different from saline controls.
‡ Significantly ($P < 0·01$) different from saline controls.

Kininogen was significantly decreased by endotoxin at both 5 and 10 min, and in most cases kinin-like activity was detected directly with the use of the estrus rat uterus assay. That this activity was actually due to kinin peptides was shown in selected instances by the use of the specific kinin assay described above. Mean levels of 5 ng/ml of kinin were found in five samples by this method.

Complement was necessary for production of kinins; endotoxin did not produce significant kininogen depletion or detectable kinin activity in plasma which had been decomplemented prior to the experiment (Table 1). This was true despite the fact that the zero time levels of kininogen in normal and decomplemented plasma

were not significantly different. [The process of complement depletion changed the kininogen levels from a mean of 5880 ± 240 (S.E.M.) ng/ml to a mean of 5700 ± 325 (S.E.M.) ng/ml.]

*Plasma from umbilical artery or vein*

In agreement with previous findings,[9] all samples of normal adult plasma had detectable levels of hemagglutinating antibody to endotoxin. Only one sample of cord plasma had detectable antibody to endotoxin, but all samples had normal levels of hemolytic complement. Endotoxin did not induce kininogen depletion in cord plasma, except for the one sample with detectable antibody (Table 1).

In two instances, we added the macroglobulin fraction of the serum from hyperimmunized rabbits to cord plasma; the final titer of antibody to endotoxin was ≧ 1:4. In these two experiments kininogen depletion was comparable to that which occurred in adult plasma. No significant kininogen depletion occurred after addition of 19S macroglobulin fractions that did not contain antibody to endotoxin (Table 1).

*Hageman factor-deficient plasma*

Plasma from a patient with Hageman factor deficiency, tested on five separate occasions, responded like normal plasma after addition of endotoxin (Table 1).

*Trypsin inhibitors*

Soybean trypsin inhibitor and ovomucoid trypsin inhibitor were added to aliquots of plasma and the response to endotoxin was tested (Table 2). Kininogen depletion was inhibited by soybean trypsin inhibitor but not by ovomucoid trypsin inhibitor. This pattern would be expected if either plasma kallikrein or plasmin were responsible for kinin production.[10]

TABLE 2. EFFECT OF TRYPSIN INHIBITORS AND OF DRUGS ON THE INTERACTION BETWEEN PLASMA AND ENDOTOXIN*

| No. samples | Addition | Plasma plus saline solution | | Plasma plus endotoxin | |
|---|---|---|---|---|---|
| | | (5 min) | (10 min) | (5 min) | (10 min) |
| 6 | None | 97 ± 1 | 94 ± 4 | 81 ± 4† | 66 ± 4† |
| | Soybean trypsin inhibitor (0·2 mg/ml) | 99 ± 4 | 99 ± 2 | 102 ± 5 | 98 ± 3 |
| | Ovomucoid trypsin inhibitor (0·2 mg/ml) | 96 ± 12 | 102 ± 3 | 70 ± 3† | 62 ± 4† |
| 4 | None | 85 ± 4 | 93 ± 3 | 75 ± 4† | 60 ± 6† |
| | Acetylsalicylic acid ($10^{-5}$ M) | 96 ± 4 | 93 ± 4 | 85 ± 10 | 89 ± 12‡ |
| 6 | None | 87 ± 4 | 97 ± 2 | 77 ± 6† | 59 ± 6† |
| | Sodium salicylate ($10^{-3}$ M) | 99 ± 2 | 102 ± 2 | 88 ± 5 | 85 ± 6‡ |
| 6 | None | 98 ± 2 | 94 ± 2 | 72 ± 4† | 57 ± 5† |
| | Hydrocortisone ($10^{-3}$ M) | 94 ± 4 | 99 ± 3 | 78 ± 6 | 68 ± 10† |

* Mean kininogen levels are expressed as a percentage of zero time values (± S.E.M.).
† Significantly ($P < 0.05$) different from saline controls.
‡ Significantly ($P < 0.05$) different from plasma without drug.

*Drugs*

We investigated the effect of three drugs—acetylsalicylic acid, sodium salicylate and hydrocortisone hemisuccinate—on the interaction of endotoxin with normal human plasma (Table 2). In paired experiments, sodium salicylate ($10^{-3}$ M) and acetylsalicylic acid ($10^{-5}$ M) were found to be effective inhibitors of kininogen depletion induced by endotoxin. Hydrocortisone hemisuccinate ($10^{-3}$ M) and sodium salicylate ($10^{-5}$ M) had no effect.

*Comparison with glass activation*

In four paired experiments, incubation of plasma with saline, endotoxin or glass beads was compared by measuring the simultaneous increase of TAME esterase activity, depletion of kininogen and development of direct kinin-like activity on rat uterus, and by the specific assay for kinin production.[1] The kinin-like activity assayed directly was compared with the specific kinin assay in the four paired experiments and in three additional unpaired experiments. The results are shown in Figs. 1, 2 and 3.

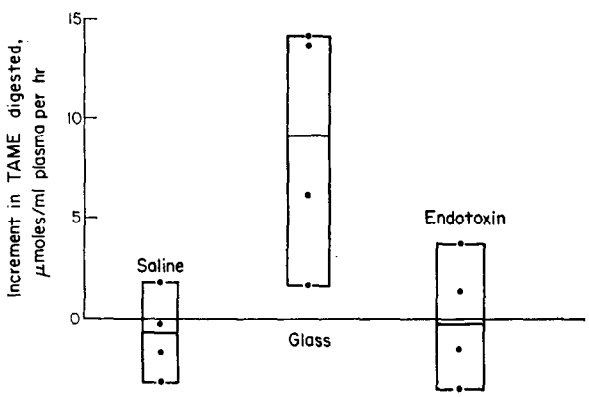


FIG. 1. The mean, range and individual values for the increase in TAME esterase activity of plasma after incubation for 4 min with saline, glass or endotoxin. The mean value for TAME digestion prior to incubation was 6·8 ± 1·06 (S.D.) μmoles/ml of plasma/hr.

Incubation of plasma with glass significantly ($P < 0.05$) increased the TAME esterase activity (mean increase, 9·1 μmoles digested/ml of plasma/hr) as compared with incubation with saline (mean increase, 0·9 μmole digested/ml of plasma/hr) or with endotoxin (mean increase, 0·0 μmole/digested/ml of plasma/hr) (Fig. 1). However, significant ($P < 0.02$) kininogen depletion occurred with both glass and endotoxin incubation, and kinin formation was equivalent and significant ($P < 0.05$) with both endotoxin and glass incubation, as measured by the specific kinin assay. Direct kinin-like activity in the incubation mixture was not always detectable and was not significantly increased from that of saline control samples (probably reflecting the net effect of simultaneous kinin generation and destruction). Correlations of these data are shown in Table 3. They can be summarized by these observations: kininogen depletion occurred in both types of experiments and correlated with kinin production (specific kinin assay) in each experiment; however, TAME esterolytic activity increased only during incubation with glass.

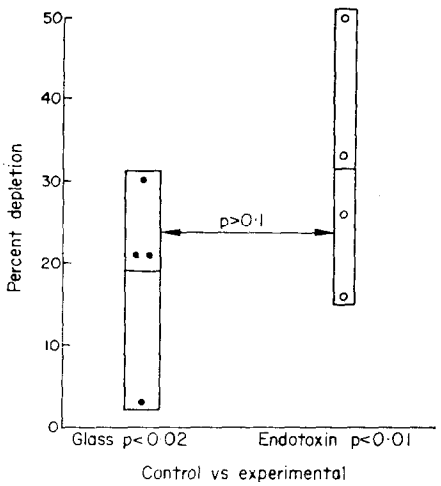


FIG. 2. Depletion of plasma kininogen. Depletion produced by incubation of plasma with glass for 4 min is compared with depletion after incubation with endotoxin for 4 min. Kininogen depletion is significant and equivalent in both systems. Controls were incubations for 4 min of plasma plus saline. The mean, range and individual values are plotted.

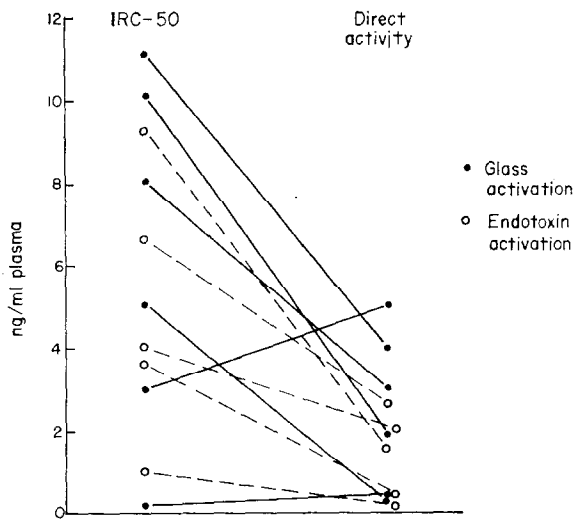


FIG. 3. Results of the specific kinin assay (IRC-50) compared with results of direct application of the incubation mixture to estrus rat uterus after incubation of plasma for 4 min with glass beads or endotoxin. Controls for each method were incubations for 4 min of plasma plus saline. The specific kinin assay demonstrated that, when compared with controls, significant and equivalent amounts of kinin were produced by both glass ($P < 0{\cdot}05$) and endotoxin ($P < 0{\cdot}05$) systems. The direct assay did not detect significant activity in either system (glass, $P < 0{\cdot}5$; endotoxin, $P > 0{\cdot}1$).

## DISCUSSION

Cardiovascular changes which occur promptly after administration of endotoxin to the primate may be mediated by the release of vasoactive substances including kinins.[1] Kinins usually are formed by the action of kallikreins on their substrates, kininogens. The fact that kinins were detected in these experiments proves that at

TABLE 3. CRITICAL CORRELATIONS OF EFFECTS OF BOTH ISOLATED SYSTEMS (GLASS AND ENDOTOXIN) ON THE KININ-GENERATING SYSTEM

| Function | Significance of correlation (P) | |
|---|---|---|
| | Glass | Endotoxin |
| Substrate depletion vs. TAME digestion | < 0·05 | < 0·1 |
| Kinin production vs. TAME digestion | < 0·05 | < 0·5 |
| Substrate depletion vs. kinin production | < 0·05 | < 0·01 |

least some kininogen is converted to kinins and not merely nonspecifically digested Depletion of kininogen is an index of the maximum amount of kinin that could be formed. An active kininase in plasma rapidly destroys any kinin formed, so that at any instant the amount of kinin present is small and a specific assay which halts kinin formation and destruction is required to detect and quantify kinin accurately and consistently. For convenience and with the knowledge that kininogen depletion reflects kinin formation, we routinely have used depletion of kininogen rather than formation of kinin for quantifying the effects of glass or endotoxin on plasma. However, we periodically tested for the presence of kinin.

Kinin can be formed by plasma enzymes other than kallikrein (e.g., plasmin and C'1 esterase).[11] The inhibition of kinin formation by soybean trypsin inhibitor, but not by ovomucoid inhibitor, suggests that the mechanism of kinin formation in the presence of endotoxin in a cell-free system is very likely mediated by a complement-dependent antigen–antibody reaction which activates plasma kallikrein or plasmin. The requirement for complement is shown in the experiments with decomplemented plasma. A role for antibody is suggested by our data with cord plasma. Interpretation of the results on cord plasma is difficult, since cord plasma kallikrein, unlike adult plasma kallikrein, cannot be activated by glass.[12] However, in the newborn, normal amounts of Hageman factor are present, clotting proceeds normally, and plasma kallikrein can be activated by acid.[12] The addition of a plasma 19S fraction containing endotoxin antibody or the presence (in one instance) of endogenous endotoxin antibody allowed the cord plasma to support the endotoxin-mediated depletion of kininogen; this is strong evidence that antibody is necessary in this system. The 19S fraction, which contained no endotoxin antibody, was used to rule out the possibility that nonspecific substances were important in the endotoxin–plasma interaction. In this regard, it is interesting that antibody is not a requisite for the interaction of endotoxin with polymorphonuclear granulocytes *in vitro*, although complement is required.[5]

The role of the Hageman factor in the cell-free system is uncertain. Plasma from one patient with a Hageman factor deficiency showed a normal interaction with endotoxin. This finding suggests that Hageman factor is not a necessary intermediate in this system; however, it is possible that the trace amounts of Hageman factor in the plasma may have been sufficient to activate plasma kallikrein. Although data from Movat's studies[3] with guinea pig plasma suggest that Hageman factor is necessary in

the activation of kallikrein by antigen–antibody–complement complexes, the antigen–antibody system, species, and methods used were so different that valid comparison cannot be made. It is known that antigen–antibody complexes[13] and endotoxin[4] can activate the clotting system and, perhaps in this way, activate plasma kallikrein. Normal Hageman factor was not necessary in our system; this provides some evidence that the clotting system is not involved, and distinguishes endotoxin activation from glass activation of the kinin-forming system.

Other differences in activation of the kinin system by glass and by endotoxin are revealing. It is clear that kinins are produced and kininogens depleted equivalently in both systems (Table 3). However, only glass activation of plasma causes an increase in TAME esterase activity. Many esterases, including kallikrein, plasmin, thrombin E, thrombokinase, permeability factor (PF/dil) and C'1 esterase, are present in human plasma.[14] Glass activates kallikrein, plasmin, PF/dil and C'1 esterase. Perhaps the differences in the response to glass and endotoxin are that each requires plasma kallikrein activation but that endotoxin, in the concentrations used, did not activate as many enzymes capable of esterolytic activity or as much kallikrein as the glass beads did. If TAME activity had been used as the only indicator of kinin generation, the endotoxin results could have been misinterpreted. Our data indicate that TAME esterase activity may be an insensitive index of kallikrein-like activity. This concept is illustrated further by data of Webster and Pierce[14] that show TAME esterase activity of crude plasma kallikrein to be 73 per cent inhibited by a dose of soybean trypsin inhibitor which inhibits only 18 per cent of the kinin-forming activity. For these reasons, we question the significance of data obtained about kallikrein on the basis of TAME esterase activity alone, and suggest that kininogen depletion and kinin formation must also be measured in order to prove activation of the kinin-forming system.

Salicylates can inhibit the increase in vascular permeability caused by kallikreins, but not that caused by kinins.[15,16] Our interpretation of these data is that salicylates, in concentrations of $10^{-3}$ M, block formation of kinin by plasma kallikrein. Others, using different systems, have not confirmed these observations.[17,18] The actions of salicylates on immune systems are complex.[19] Possible modes of action include impairment of antigen–antibody–complement interaction, interference with kallikrein activation or suppression of the action of kallikreins. Apparently, concentrations of salicylate of $10^{-2}$ M or higher are necessary for inhibition of antigen–antibody interaction in an isolated system. Where salicylates act in our system to block the endotoxin-induced kinin formation is not known. It is interesting that, in experimental animals, salicylates have been shown to be effective in decreasing the lethality of endotoxin shock.[20]

The use of hydrocortisone in our system was prompted by the observations that it may block activation of kallikrein by polymorphonuclear granulocytes[21] and by glass in some systems[21] but not in others,[22] and that it has a salutary effect on endotoxin shock in some species.[23] The inability of hydrocortisone to block the effects of endotoxin in cell-free plasma suggests that its effects *in vivo* (if any) in shock must be mediated by some other mechanism.

The relevance of our observations to those observed *in vivo* during endotoxin shock is unclear. Experimental evidence *in vivo* suggests that, in some species, antigen–antibody–complement interactions may account for some effects of endotoxin,[24–27] including the earliest cardiovascular changes, but not the lethal effects.[28–32] We

postulate that endotoxin interacts with antibody and complement to activate plasma kallikrein and form kinins from kininogens. This may have relevance to the early kinin release seen *in vivo* after endotoxin infusion and may account for some of the early cardiovascular changes. Salicylates may be effective in shock partly because of their action on this system.

## REFERENCES


1. A. S. Nies, R. P. Forsyth, H. E. Williams and K. L. Melmon, *Circulat. Res.* **22**, 155 (1968).
2. R. F. Wilson, A. P. Thal, P. H. Kindling, T. Grifka and E. Ackerman, *Archs Surg.* **91**, 121 (1965).
3. H. Z. Movat, in *Int. Symp. Vaso-Active Polypeptides: Bradykinin and Related Kinins* (Eds. M. Rocha e Silva and H. A. Rothschild), p. 177. Pergamon Press, Oxford (1967).
4. P. F. Hjort and S. I. Rapaport, *A. Rev. Med.* **16**, 135 (1965).
5. M. J. Cline, K. L. Melmon, W. C. Davis and H. E. Williams, *Br. J. Haemat.* **15**, 539 (1968).
6. C. R. Diniz and I. F. Carvalho, *Ann. N.Y. Acad. Sci.* **104**, 77 (1963).
7. P. S. Roberts, *J. biol. Chem.* **232**, 285 (1958).
8. E. A. Kabat and M. M. Mayer, *Experimental Immunochemistry*, 3rd edn, p. 135. Thomas, Springfield (1961).
9. J. G. Michael and F. S. Rosen, *J. exp. Med.* **118**, 619 (1963).
10. N. Back and R. Steger, *Fedn Proc.* **27**, 96 (1968).
11. M. R. Klemperer, V. H. Donaldson and F. S. Rosen, *J. clin. Invest.* **47**, 604 (1968).
12. K. L. Melmon, M. J. Cline, T. Hughes and A. S. Nies, *J. clin. Invest.* **47**, 1295 (1968).
13. J. Robbins and C. A. Stetson, Jr., *J. exp. Med.* **109**, 1 (1959).
14. M. E. Webster and J. V. Pierce, *Proc. Soc. exp. Biol. Med.* **107**, 186 (1961).
15. W. G. Spector and D. A. Willoughby, in *Salicylates* (Eds. A. St. J. Dixon, M. J. H. Smith, B. K. Martin and P. H. N. Wood), pp. 141–144. Churchill, London (1963).
16. B. J. Northover and G. Subramanian, *Br. J. Pharmac. Chemother.* **17**, 107 (1961).
17. P. Hebborn and B. Shaw, *Br. J. Pharmac. Chemother.* **20**, 254 (1963).
18. G. P. Lewis, *Nature, Lond.* **192**, 596 (1961).
19. K. F. Austen, in *Salicylates* (Eds. A. St. J. Dixon, M. J. H. Smith, B. K. Martin and P. H. N. Wood), pp. 161–169. Churchill, London (1963).
20. L. B. Hinshaw, L. A. Solomon, E. G. Erdös, D. A. Reins and B. J. Gunter, *J. Pharmac. exp. Ther.* **157**, 665 (1967).
21. M. J. Cline and K. L. Melmon, *Science* **153**, 1135 (1966).
22. V. Eisen, L. Greenbaum and G. P. Lewis, *Br. J. Pharmac. Chemother.* **34**, 169 (1968).
23. W. W. Spink, *Ann. intern. Med.* **57**, 538 (1962).
24. R. W. Schaedler and R. J. Dubos, *J. exp. Med.* **115**, 1149 (1962).
25. G. J. Hildebrand, J. Ng, Y. Seys and S. H. Madin, *Am. J. Physiol.* **210**, 1451 (1966).
26. W. W. Spink, R. B. Davis, R. Potter and S. Chartrand, *J. clin. Invest.* **43**, 696 (1964).
27. V. E. Gilbert and A. I. Braude, *J. exp. Med.* **116**, 477 (1962).
28. R. T. Smith and L. Thomas, *J. exp. Med.* **104**, 217 (1956).
29. J. W. Uhr, *J. exp. Med.* **115**, 685 (1962).
30. L. Lee and C. A. Stetson, Jr., *J. exp. Med.* **111**, 761 (1960).
31. Y. B. Kim and D. W. Watson, *Ann. N.Y. Acad. Sci.* **133**, 727 (1966).
32. P. J. Porter, A. R. Spievack and E. H. Kass, *J. Lab. clin. Med.* **68**, 455 (1966).