Validity and Limitations of Rat Mesoappendix Bioassay Studies

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Bioassay data obtained by the rat mesoappendix method lend themselves poorly to statistical representation. Sufficient reproducibility for quantitative estimation of the titer of vasoactive materials was achieved only with crystalline substances or purified biological extracts. Neither the duration of the displacement of epinephrine reactivity nor the magnitude of the shift in effective constrictor doses of epinephrine provided a satisfactory measure of vasoactivity in blood specimens. A prozone phenomenon, with agents such as Pitressin, histamine and ferritin was encountered with potentiating effects being elicited in high dose ranges and inhibitory effects in low dose ranges.

Bioassay methods in general serve three main purposes: detection, quantitation, and identification. The scope of the rat mesoappendix method within these three categories has not been critically delineated. As a consequence, the relation of the mesoappendix data to experimental conditions has remained controversial and there has been a tendency to assign to the reported findings more precise quantitative implications than warranted. It becomes, therefore, of paramount importance, before advocating the application of the method, to explore the humoral aspects of different syndromes in order to provide a broad framework of basic information. Two specific areas of uncertainty are the reproducibility of the vascular reaction and the extent to which the bioassay data reflect the titer of vasoactive material in biological specimens.

The present appraisal of the bioassay method deals with the problems of quantitation and identification of vasoactive factors in biological samples, such as blood or tissue extracts. Included in the report is a statistical consideration of the dose-response relationship of representative vasoexcitatory and vaso-inhibitory principles, in comparison with crystalline agents with vasoactive properties.

**NEW TERMINOLOGY**

A diversity of biological derivatives and synthetic chemicals have been found to produce equivalent shifts in vascular reactivity. The terms “VDM” and “VEM”, originally introduced to designate inhibitory and potentiating materials, have become associated respectively with the vasoactivity of the protein ferritin and the biological activity of a specific factor of kidney metabolism. It was therefore found preferable to refer to shifts of vascular reactivity in a particular direction by a more general term and to restrict usage of the terms “VEM” and “VDM” to specific substances. In the present report, changes in vascular reactivity were recorded by the symbol “I” to indicate an inhibition of the vasoconstrictor response, and the symbol “P” for a potentiation of the reaction. A subscript before each symbol then served to identify the particular stimulating agent being used to measure the change in vascular reactivity. For example, the change in the threshold response to epinephrine would be recorded as “eI” or “eP”, depending upon the direction of the change. This terminology also circumvented the frequent confusion resulting from use of the term “vaso-depressor” for agents identified by the rat test, with vaso-depressor agents producing a fall in blood pressure.
MATERIAL AND METHODS

Microscopic visualization of the cecal mesentery in the rat was carried out by the method described in detail in a previous report. Bioassays of biological and synthetic materials were made on the basis of the altered vasoconstrictor response to topical epinephrine observed in the mesenteric circulation following i.v. administration of test samples. In all instances, the biological material was prepared just prior to assay, unless otherwise indicated. Known crystalline agents were dissolved in physiological saline, buffered to neutral pH (7.0-7.2) and administered in 0.3-0.4 ml. amounts per 100 grams of body weight. VDM was prepared from fresh rat liver slices incubated at 37.5°C for 90 minutes using 5 parts of Ringer-phosphate medium to 1 part of tissue. VEM was prepared by incubating slices of rat kidney cortex in Ringer-phosphate media for 60 minutes at 37.5°C. Two specimens of crystalline ferritin were available to us, horse spleen ferritin and dog liver ferritin, the activity of which is expressed in terms of the N content per milligram.

A formidable difficulty in evaluating the rat mesoappendix findings arises from the fact that the data lend themselves poorly to statistical presentation. Two aspects of the altered vasoconstrictor response to epinephrine were recorded routinely. The precise amount of epinephrine required to elicit a standard constrictor reaction was determined at selected intervals.

Establishment of Epinephrine Threshold Value (E.T.C). In the terminal vascular bed, although constriction of the metarterioles was visible, the small size of the vessels (10-20 μm) and the uneven narrowing of the vessels precluded accurate measurements of changes in caliber, even under high microscopic magnification (600 X or greater). Changes in the rate or extent of blood flow through the bed were not readily amenable to objective recording and are not in fact indicative of vessel size. This left the possibility of estimating the magnitude of the response in terms of the concentration of a stimulating agent, such as epinephrine, required to reproduce an equivalent constrictor reaction in a selected vessel at intervals throughout the experiment. The equivalence of different constrictor responses was subjectively evaluated by a trained investigator on the basis of a clearly defined effect of the blood flow distal to the vasoconstriction.

A highly reproducible end-point could be established by stoppage of capillary blood flow distal to the metarterioles. Because of its strategic location, narrowing of the precapillary junction sufficed to bring about a disproportionate slowing of blood flow to the sphincters. The epinephrine threshold concentration (E.T.C.) in practice was found to be on the average midway between the smallest effective dose and the concentration required to achieve a maximal constrictor reaction.

In the presence of an inhibitory agent, blunting of the standard response usually resulted in the complete absence of an observable effect on capillary blood flow with the control E.T.C. Potentiating agents induced with this concentration of epinephrine an excessive vasoconstriction which involved other structural components, especially the parent metarterioles and arterioles. Such a distinction allowed the unequivocal establishment of the presence or absence of eP and eF substances.

The displacement of epinephrine reactivity developed progressively and persisted for a variable period of time before returning to the original control level. Thus another parameter, the duration of the change in vascular reactivity, could be measured. It is unfortunate that the representation of the data in the specific numerical terms of a dose-response relationship has given rise to quantitative implications beyond the scope of the assay method.

EXPERIMENTAL DATA

I. Qualitative Aspects: The statistical significance of individual bioassay values was examined by carrying out 10 or more repeat tests of the same sample as unknowns in a representative series of vasoactive compounds. The data were first analyzed on the basis of providing either a negative or positive finding with respect to inhibition or augmentation of the E.T.C. As might be anticipated, varying degrees of reproducibility were demonstrated, dependent both upon the nature of the substance being studied and on its concentration in the test sample. The accompanying table 1 compares the bioassay data for crystalline substances, a number of relatively impure tissue extracts and blood samples with vasoactive properties. L-arternol or angiotonin, produced remarkably consistent potentiating effects (90-92 per cent reproducibility). On the other hand, histamine gave more variable results (60 per cent). A standard preparation of kidney VEM (in vitro anaerobic incubation of rat kidney slices for 60 minutes) showed only a 65 per cent reproducibility (13/20). In the case of samples with inhibitory properties, greater reproducibility was obtained with preparations of liver VDM (18/25) than was possible with crystalline ferritin (21/35).

Saline blanks were also included as unknowns. Out of a total of 50 saline tests, 12 per cent (6 cases) showed activity. Of these, two were falsely labeled as inhibitors and four as...
potentiators. It has been our experience that only infrequently has an incorrect positive diagnosis been established for a particular sample. The more common occurrence has been a false negative finding, due to the failure to detect vasoactivity.

The reproducibility with biological agents was to a considerable extent influenced by the narrow range of concentrations in which many of these materials exhibited activity. The minimum concentration of ferritin detectable in different animals varied by as much as 500 per cent. Hence, repeated tests on unknown samples with titers close to the minimal effective dose showed an exceptionally poor reproducibility (7/20), whereas ferritin samples in the range of concentrations showing maximum vascular effects gave more reproducible results (12/18). Some agents, such as Pitressin, continued to show biological activity over a fairly wide concentration range. Even in these instances, a comparable falling off in reproducibility was encountered with concentrations below levels producing maximal vascular effects. Renin showed a reproducibility of 91 per cent in its most effective concentration range. When diluted to the point of minimum biological activity (0.2 pressor units), only 50 per cent of the tests gave positive assays.

Bioassay data on blood samples from animals subjected to acute stress showed a high degree of reproducibility. For example, eP (epinephrine potentiating) blood samples from rats subjected to hemorrhagic shock, taken during the early compensatory stage (after 30–40 minutes at 55 mm. Hg), or eI (epinephrine inhibitory) samples during the late decompensatory stage (2 hours at 35 mm. Hg), showed a reproducibility of over 85 per cent upon repeated testing. Such samples continued to show vasoactivity even after a 3 to 4 fold dilution with saline. This would indicate that in all probability these bloods contained comparatively high titers of vasoactive material. Assays of blood samples from patients with a variety of chronic disturbances were less definitive, probably because of their mixed content of several vasoactive materials. With this type of material it was occasionally necessary to carry out 4 or 5 repeat assays before a positive qualitative finding could be established. Although individual blood samples from hypertensive patients showed a reproducibility of about 75 per cent, the type of activity encountered in specimens from different individuals differed over the whole range of possibilities; 5/18 had a potentiating effect, 3/18 an inhibitory effect, and the remaining 10/18 a so-called neutral rat test. Results on the neutral samples in this series were the least conclusive. Thus a particular sample produced a transient eP effect in one rat, an inconclusive eI effect in a second rat, and a neutral reaction in a third. This may be the reflection of the differential sensitivity of

<table>
<thead>
<tr>
<th>Biological Materials</th>
<th>Concentration Range (i.v.)</th>
<th>No. Repeat Tests</th>
<th>Type of Activity</th>
<th>Per cent Positive Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arterenol</td>
<td>2.25 µg</td>
<td>25</td>
<td>eP</td>
<td>92</td>
</tr>
<tr>
<td>Angiotonin</td>
<td>1–2 cat units</td>
<td>20</td>
<td>eP</td>
<td>90</td>
</tr>
<tr>
<td>Renin (Hog)</td>
<td>1 pressor unit</td>
<td>12</td>
<td>eP</td>
<td>91</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.5–10 mg</td>
<td>15</td>
<td>eP</td>
<td>60</td>
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<tr>
<td>Ferritin</td>
<td>0.015–0.020 mg</td>
<td>30</td>
<td>eI</td>
<td>67</td>
</tr>
<tr>
<td>Glutathione (-SH)</td>
<td>0.01–0.005 µg</td>
<td>25</td>
<td>eI</td>
<td>92</td>
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<tr>
<td>Kidney VEM</td>
<td>5:1 (orig. supernate)</td>
<td>20</td>
<td>eP</td>
<td>65</td>
</tr>
<tr>
<td>Liver VDM</td>
<td>5:1 (orig. supernate)</td>
<td>18</td>
<td>eP</td>
<td>72</td>
</tr>
<tr>
<td>Muscle VDM</td>
<td>5:1 (orig. supernate)</td>
<td>10</td>
<td>eI</td>
<td>70</td>
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<tr>
<td>Saline</td>
<td>0.9 per cent</td>
<td>50</td>
<td>—</td>
<td>88†</td>
</tr>
<tr>
<td>Normal Control</td>
<td>original</td>
<td>8</td>
<td>neutral</td>
<td>100</td>
</tr>
<tr>
<td>Normotensive (A.S.H.D.)</td>
<td>original</td>
<td>10</td>
<td>neutral</td>
<td>70</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>original</td>
<td>11</td>
<td>eI</td>
<td>73</td>
</tr>
<tr>
<td>Hem. Shock (Rat):</td>
<td>original</td>
<td>12</td>
<td>eP</td>
<td>75</td>
</tr>
<tr>
<td>Early</td>
<td>original</td>
<td>7</td>
<td>eP</td>
<td>85</td>
</tr>
<tr>
<td>Late</td>
<td>original</td>
<td>9</td>
<td>eI</td>
<td>89</td>
</tr>
</tbody>
</table>

eP—epinephrine potentiating
eI—epinephrine inhibiting
A.S.H.D. = Arteriosclerotic Heart Disease
* Number of times same sample was retested in different rats.
† 12 per cent gave false positive bioassays.
individual test rats to separate components of a given sample.

II. Quantitative Implications: In final analysis, the significance of the bioassay information will depend on the extent to which changes in concentration were reflected by a corresponding shift in vascular reactivity. From a practical standpoint, vasoactivity was expressed either as a function of the extent of the shift in E.T.C. value or the duration of the effect. Actually, the two parameters probably represent measurements of separate processes. The magnitude of the shift in E.T.C. would appear to be related more directly to the state of vasomotor tone of the peripheral blood vessels, a variable feature influenced by numerous experimental and biological contingencies. The duration of the effect is most likely a function of the rate of removal of vasoactive materials from the circulation by processes such as inactivation, excretion, or storage. An important consideration was to establish which of the two parameters reflected most accurately the actual titer of vasoactive material in the test sample. This feature was explored initially with known biological agents and with materials containing only one type of vasoactive substance, either e1 or eP.

A. Duration of Effect as Estimate of Vasoactivity. The simplest procedure for bioassay experiments was found to be the application of the control threshold concentration of epinephrine at intervals of 3 to 5 minutes, recording each time the presence either of an increased response, a decreased response, or no change in response. Determinations were continued until the constrictor response had returned to the original base line. For many years this procedure had been used almost exclusively in assays of materials showing vaso inhibitory activity to facilitate routine screening of large numbers of samples. The assumption was made that the duration of the depression of the response to threshold concentrations of epinephrine was quantitatively related to the amount of vasoactive material in the test sample. Although this proved to be a valid estimate in particular animals, the present study indicated it was not sufficiently consistent from one animal to another. This is illustrated in figure 1 which indicates the range of activities recorded with repeated bio-assays of an aliquot of liver VDM. The parameters of duration of effect or magnitude of E.T.C. by themselves are insufficiently discriminating to serve as a measure of vasoactivity. This is clearly indicated by the

![Figure 1](image-url)

**Fig. 1.** Frequency distribution of assay values in 20 rats for single samples of liver VDM. Shift in E.T.C. expressed numerically as $1:\text{X}$ parts of epinephrine.
spread of the bioassay values for liver VDM. For this experiment, rats were selected with a control E.T.C. value of one to two million. Studies with eP substances, such as renin or kidney VEM, indicated that duration measurements alone were equally unreliable for this category of substances as well.

As a corollary of this, a study was made of the extent to which changes in concentration of vasoactive agents were reflected by a corresponding influence on the duration of the eP or el effect. The dose-response relationship, as evidenced by this parameter, was exceptionally poor for 1-arterenol, renin, Pitressin, or kidney VEM, especially in the low dose ranges. In the mid-range, the activity curve showed a comparatively flat plateau, despite a considerable increase in the concentration of the eP agent. With still higher concentrations, two situations were seen: In the case of 1-arterenol and renin, potentiating effects persisted in excess of 60 minutes, the conventional period of observation; with kidney VEM, the duration of vasoactivity occasionally decreased in the high dose range. An unsatisfactory correlation between the duration of the vascular effect and the concentration of the vasoactive agent was found with vasoinhibitory principles, such as ferritin, liver VDM, mecholyl or glutathione.

Summarizing, biological variability, in association with an inconstant dose-duration relationship, ruled out the use of the duration of the epinephrine displacement reaction as an index of the titer of eP or el activity.

**B. Magnitude of E.T.C. Shift in Relation to Vasoactivity.** Studies were then carried out on the validity of estimating the titer of vasoactive materials on the basis of maximal displacement of E.T.C. following the administration of various test substances. As indicated in figure 2, the shift in E.T.C. in the mesentery followed a comparatively uniform course with respect to the concentration of the vasoactive agent. For example, in the case of renin or 1-arterenol as eP agents, and glutathione (GSH) as an el agent, an almost linear relation was shown within the effective range of concentrations. Even with simple tissue washes, such as liver VDM and kidney VEM, a reasonably linear relation existed between the concentration of the vasoactive material and the resulting change in E.T.C. Inasmuch as the exact concentration of VDM in such experiments is not known, a comparative study of the relative E.T.C. displacement produced by particular concentrations of this el agent was made by incubating liver tissues for progressively longer periods and measuring the vasoactivity of incubation aliquots at 30, 60, 90 and 120 minutes respectively. These bioassay values were then compared with the amount of dilution in each case required to abolish vasoactivity. The data presented in table 2 show

**Fig. 2.** Displacement of the E.T.C. value with successive increases in the dose of known potentiators of epinephrine. Slope of the change varies with particular substances in an approximate linear relationship.

**Table 2.—Progressive Change in E.T.C. in Relation to Concentration of Liver VDM**

<table>
<thead>
<tr>
<th>Incubation Period</th>
<th>Concentration*</th>
<th>Magnitude of Shift in E.T.C.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min.</td>
<td>1 unit</td>
<td>-2</td>
</tr>
<tr>
<td>60 min.</td>
<td>3 units</td>
<td>-6</td>
</tr>
<tr>
<td>90 min.</td>
<td>6 units</td>
<td>-10</td>
</tr>
<tr>
<td>120 min.</td>
<td>7 units</td>
<td>-15</td>
</tr>
</tbody>
</table>

* Expressed in arbitrary units on basis of amount of dilution with saline required to abolish el activity of sample.

† E.T.C. on basis of maximal displacement of concentration of epinephrine required to elicit standard vasoconstriction; (ex) indicates experimental threshold concentration, (c) control value.
a close correspondence between the dilution factor and the change in E.T.C.

A comparable trend, but with less consistent agreement, was also found between the relative change in E.T.C. and the concentration of kidney VEM in a given preparation. In these experiments the dose-response curve remained approximately linear to the point where a maximal response was obtained. The slope of such activity curves differed for particular substances.

With blood samples, measurements of the dose-E.T.C. relationship were made by serial dilution experiments. However, it was not possible to demonstrate a consistent dose-response curve with bioassay of blood samples, especially in patients with various chronic disorders. As previously indicated, this may have been a consequence of the presence of several active agents in the blood.

Measurements which included both the factor of time and the magnitude of the shift in E.T.C. were found to provide a more consistent estimate of vasoactivity. It is questionable whether this indicated an interdependence between the two separate parameters of measurement. A plot of E.T.C. values against duration in a random sampling of 100 eP bioassay tests (fig. 3) was suggestive of a relationship between the increase in one parameter relative to the other. However, individual values were not sufficiently reproducible to permit prediction of E.T.C. from duration, and vice versa. The problem is obviously complex and perhaps cannot be resolved on the basis of the assay procedure in its present form.

Several other attempts were made to arrive at a satisfactory quantitative estimate of vasoactivity. The first was a comparison of the vasoactivity of unknown samples with that produced by known concentrations of a standard crystalline substance. Since direct comparison of this sort could justifiably be carried out only in the same test animal, such a procedure proved impractical. A second method involved serial dilutions as a means of establishing the relative order of activity of different samples, unknown specimens being diluted successively with saline to the point where
all evidence of vasoactivity was lost. This was finally adopted as a means of grading different samples on a comparative basis, with, however, no implications concerning the precise titers of vasoactive material in each.

C. Estimation of eI Activity in Biological Material. In earlier studies, duration values by themselves were considered as a valid estimate of the relative order of eI activity for unknown samples. Unqualified acceptance of this premise was not supported by experiments where blood samples with identical durations of vasoactivity were analyzed more critically with reference to their precise quantitative implications. This was done in two ways, by establishing in each case the threshold concentrations of epinephrine required to elicit the control response, and by serial dilution until vasoactivity was lost.

For this experiment five blood samples of patients with hypertension were selected which had given nearly identical assays, in terms of a vasoinhibitory effect (40 to 45 minutes). Subsequent determination of the actual depression of the E.T.C. showed this function to vary in separate instances by factors of from -1 to -20. The E.T.C. value for each of the five blood samples is indicated in Table 3. It is obvious that these five samples, preselected as identical, actually represented a considerable range of vasoactive materials.

Further evidence of the disparity in this regard was obtained by dilution experiments. The same five blood samples were subjected to successive half dilutions with saline and tested at each step for vasoactivity. The number of dilutions necessary to achieve neutrality ranged from three to eight such steps in separate samples. The possibility existed that eI samples could be graded by comparing them with an assay curve of a crystalline standard such as ferritin. Unfortunately, the biological activity of ferritin did not follow a simple linear course with changes in concentration. Several experiments utilizing this procedure were carried out. In practice, attempts to estimate different concentrations of ferritin as unknowns proved only ±400 per cent accurate.

D. Estimation of eP Activity in Biological Material. In previously reported studies, bioassays of eP samples have relied more on the intensity of the change in vascular reactivity than on the duration of the effect. Here again, direct comparison of different samples was not readily achieved. Bioassay values were arrived at in test animals showing control E.T.C. levels ranging from 1:1 million to as high as 1:8 million. As an example, the intravenous injection of one test sample produced a change in reactivity in one animal from 1:1 million to 1:3 million, and a second sample in another animal from 1:6 million to 1:18 million. Both represented a threefold potentiation of vascular reactivity. Could these two samples be therefore considered to have identical titers of vasoactivity?

A comprehensive study along these lines was made with two crystalline agents. For this purpose, l-arterenol was employed as an example of a potentiating agent and ferritin as an inhibitory agent. Over a period of several months, sufficiently large numbers of test rats were encountered with different control threshold concentrations to permit such a comparison. Examination of the data showed that rats with an E.T.C. value of 1:1 million gave the poorest response to both l-arterenol and ferritin. The highest assay values (E.T.C. change) for l-arterenol were obtained in rats with threshold values between 1:6 and 1:8 million. Interestingly enough, ferritin produced a relatively greater depression of the epinephrine response in rats with a threshold value of 1:8 million than in animals with lower values. The data clearly indicated that a direct

<table>
<thead>
<tr>
<th>Blood Samples</th>
<th>Inhibition of Eps. Response</th>
<th>Dilution Factor</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>E.T.C. - E.T.C.</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>44</td>
<td>-1.3</td>
</tr>
<tr>
<td>B</td>
<td>45</td>
<td>-2.0</td>
</tr>
<tr>
<td>C</td>
<td>43</td>
<td>-4.0</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>-6.7</td>
</tr>
<tr>
<td>E</td>
<td>42</td>
<td>-10.0</td>
</tr>
</tbody>
</table>

* Experimental (ex) epinephrine threshold concentration, E.T.C. divided by control value (c).
† Dilution with saline (1:x parts) required to abolish vasoactivity.
VALIDITY OF MESOAPPENDIX FINDINGS

comparison of assay values was possible only between animals having identical E.T.C. values.

Still another method was explored with respect to quantitation of eP activity. This consisted of a comparison of the activity of unknown samples with that of a known agent, such as 1-arterenol or renin on the rat mesoappendix preparation. A direct comparison could be attempted only in instances where the change in vascular reactivity with an unknown sample was attributable to a single agent. Unfortunately, this was the exception rather than the rule in biological material. Even simple serial dilution, as a measure of the relative order of activity, had to be applied cautiously in the case of blood or tissue examples, because of unpredictable dilution effects. The only alternative possibility was biological fractionation, a feature which will be discussed in the subsequent section.

III. Identification of Blood-Borne Agents: Blood or tissue extracts invariably have been found to contain several vasoactive substances, some of which produced changes in vascular reactivity in the same direction, and others, with an opposing effect on the same reaction. Thus, a change in reactivity produced by a blood sample in the direction of an eP effect, for example, may represent the combined influence of several components acting either additively or even synergistically. Superimposed on this, an eI substance may be present to counteract this tendency, with the end result, as measured by the mesoappendix method, representing a complex interaction of multiple factors. The difficulties inherent in quantitation and in the comparison of the relative activity of different blood samples can be readily appreciated.

A. Biological Fractionation. Demonstration of the presence of both potentiating and inhibitory principles in such blood samples has been achieved by in vitro incubation with antiserum to ferritin, or with kidney slices. The latter (pretreatment of plasma specimens by incubation with kidney slices for 60 minutes) neutralized the vasoexcitatory properties of test samples and thereby unmasked any residual eI activity, originally counterbalanced by the presence of oppositely acting materials.

In order to ascertain whether biological fractionation could aid in the more precise measurement of the separate vasoactive components, an extensive examination was made of the blood of five hypertensive patients utilizing every form of fractionation. A total of 40 separate bioassays provided the data indicated in table 4. Although the method presented unequivocal evidence for the simultaneous presence of oppositely acting vasoactive principles in blood, no other implications were warranted. It can be seen that in no case was it possible to match or to cancel out the two opposing vasoactivities, either by simple addition or other mathematical computations. The data were not sufficiently precise to permit separate estimation of eI and eP activities.

IV. Prozone Phenomena: An unusual feature

<table>
<thead>
<tr>
<th>Original Sample</th>
<th>After Treatment With Kidney Tissue*</th>
<th>After Treatment With Liver Tissue†</th>
<th>After Treatment With Antiserum to Ferritin‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.T.C.&lt;sub&gt;ex&lt;/sub&gt;</td>
<td>E.T.C.&lt;sub&gt;n&lt;/sub&gt;</td>
<td>min.</td>
</tr>
<tr>
<td>Neutral</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>eI</td>
<td>34</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>eI</td>
<td>46</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>eP</td>
<td>37</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>eP</td>
<td>38</td>
<td>4</td>
<td>—</td>
</tr>
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</table>

* Incubation with thin slices of kidney tissue from normal rats (as for microrespiration studies) in a 5:1 proportion (5 ml blood sample: 1 gm kidney tissue) under aerobic conditions (95% O₂, 5% CO₂) for one hour at 37.5°C.
† Incubation (as above), using liver slices for 2 hours.
‡ Blood plasma ferritin antiserum (0.2 ml/ml sample) incubated for 30 minutes at 37.5°C.
in mesoappendix bioassay experiments was the appearance of diametrically opposed forms of vasoactivity with changes in concentration of certain test substances. This was a puzzling phenomenon since similar effects were not encountered in assays on blood samples obtained either in acute experimental conditions or in chronic disorders. The prozone reaction was not entirely unexpected since mesoappendix studies, carried out as early as 1940 with a variety of synthetic guanidine derivatives prepared by M. E. Krahl and E. C. Kleiderer, had indicated that some of these compounds produced a depression of epinephrine reactivity at low concentrations, as contrasted with a potentiation of the reaction at higher concentrations. The reverse situation was also encountered with agents which evoked a potentiation at low concentrations and an inhibition of epinephrine reactivity at higher concentration levels.

A. Change in Nature of Vasoactivity with Concentration. Crystalline ferritin (VDM) has been found to exhibit biological activity over an exceptionally narrow range and to give a false "neutral" test at higher concentrations. This is in contrast to the progressive augmentation of vascular reactivity in the mesentery encountered in the present studies as a result of the i.v. injection of successively higher concentrations of substances such as 1-arterenol or renin, or the stepwise el effect with increasing concentrations of mecholyl or glutathione. This led us to investigate the possibility of a comparable prozone phenomenon with other biologically active agents.

In addition to ferritin, two humoral agents—Pitressin and histamine—were found to produce upon intravenous injection in low concentrations an el reaction in the mesentery, and, in contrast, an eP effect with higher doses. The range of vascular effects with changes in concentration are indicated for these two agents in the accompanying figure 4. A possible explanation for this phenomenon was the secondary introduction of indirect systemic effects at higher concentrations. Bioassay experiments were therefore carried out in acute adrenalectomized or nephrectomized test rats, and in chronic hypophysectomized animals (8–10 days). Identical prozone reactions were obtained with all three agents, thereby eliminating the adrenals, kidneys or pituitary as a secondary source of material. A secondary homeostatic effect of higher concentrations of these agents on blood pressure was also ruled out. Ferritin produced no change in blood pressure over an extremely wide range of concentrations in normal rats. In both the instance of Pitressin and histamine, a prozone phenomenon was encountered with doses well below those required to alter the blood pressure or to produce vasoconstriction per se. Blood removed for bioassay during the period of the eP prozone response in the mesentery elicited no potentiating effect in another test rat. Are these vasoactive substances to be characterized as potentiators or inhibitors of vascular reactivity, in terms of their role in physiological processes? The assumption has been made arbitrarily in the case of ferritin that the el changes introduced with minimal effective doses were probably related more closely to the effects produced by these agents operating under physiological conditions. Such a distinction would necessitate classifying Pitressin and histamine as vasoinhibitory sub-

![Fig. 4. Typical dose-response curve with certain vasoactive agents showing el and eP activity in different concentration ranges. Note narrow range over which vaso inhibitory effects were encountered. Pitressin expressed as pressor units, and histamine as mg.](http://circres.ahajournals.org/)

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 VALIDITY OF MESOAPPENDIX FINDINGS

Fig. 5. Shift in direction of vasoactive response with change in concentration of ferritin administered i.v. for bioassay. Ferritin produces characteristic eP effect only with doses between .00015-.005 μg. Beyond this range eP effects were encountered.

stances. The known systemic actions of Pitressin do not support such a classification.

B. Range of Activity with Ferritin. Maximal inhibition of the constrictor response to epinephrine was obtained with concentrations of crystalline ferritin (horse or dog) on the average between .001 to .005 μg/ml. Concentrations in excess of .01 μg. caused even the arterioles and metarterioles to become increasingly hyperreactive to topical epinephrine. Intravenous injection of ferritin in concentrations from 1 to 50 μg. produced no clearly defined effects on the behavior of the mesenteric vessels. Serial dilution below 1.0 μg. revealed the activity curve indicated in figure 5. At successive concentration levels there was a range of hypersensitivity (.05 to .005 μg.), a range of hyporeactivity (.005 to .0005 μg.) and eventually a complete absence of vaso-activity (less than .0005 μg.). The precise concentration levels, at which different zones of activity were obtained, varied with several ferritin preparations, apparently depending upon their purity.

C. Absence of Prozone in Blood Samples. In order to examine the possible prozone phenomenon in blood samples, specimens were drawn during experimental situations which might be anticipated to provide maximal titers of such agents. The dose-response relationship was therefore investigated in i.v. blood specimens withdrawn from rats subjected to profound hemorrhagic shock until irreversibility had set in, and from animals exposed to lethal traumatic shock in the Noble-Collip drum. The bloods were then tested at the original concentration and at successive half-dilutions. In ten experiments of this type, none of the diluted i.v. samples were found to exhibit successively a false prozone of neutral activity followed by increased reactivity upon dilution. Instead, such samples showed a progressive loss of vasoactivity to the point of extinction. Similar dilution experiments were carried out on the blood of rats subjected to bilateral adrenalectomy. Blood samples, 48 to 72 hours after adrenal ablation, produced a profound inhibition of vascular reactivity in test rats.
Although it was possible to dilute such eI samples by as many as four to five half dilutions before vasoactivity was lost, in no case was a reversal of activity encountered.

Further bioassay experiments in this regard were done with venous blood from tourniquet limbs of rats kept totally ischemic for two to four hours before release of the occlusion. It was anticipated that under these conditions maximal amounts of the vasoinhibitory material would accumulate. Here again, no prozone phenomenon was encountered upon dilution of these samples. Evidence for a progressive increase in vasoinhibitory material with longer periods of ischemia was provided by the amount of dilution required to abolish the activity of blood samples taken after varying periods of limb occlusion; a dilution of one to three with saline abolished the vasoactivity of a two hour blood sample; one to five for a three hour sample; one to six for a 3½ hour sample; and one to ten for the four hour sample.

It is clear that the crystalline el agent, ferritin, and blood specimens with el activity, presumed to be due to ferritin, exhibited different behavior patterns with regard to the prozone phenomenon. This discrepancy was all the more puzzling since blood specimens with el activity could be rendered biologically inactive by incubation with anti-ferritin serum, evidence used to establish the identity of the two factors.

DISCUSSION

The rat mesoappendix bioassay serves both as a sensitive detector of vasoactivity and as a guide towards the establishment of the physiological function of these principles by utilizing the in vivo response of a selected segment of the peripheral vascular tree under conditions designed to approximate the normal physiological state. The present studies indicate that the method is beset by a number of uncertainties which stem from the difficulty of controlling the extreme lability of the system. These deficiencies have considerably detracted from the potential usefulness of the preparation as a biologic indicator of vasoactive material. The major contribution of the procedure to our knowledge of vascular homeostasis has been to uncover a hitherto neglected group of biological substances with specific pharmacologic effects on the muscular elements of the minute vessels. Unequivocal evidence has been presented to demonstrate the presence in biological material of substances which potentiate or inhibit the vasoconstrictor response of the mesenteric vessels to epinephrine. Beyond this, further discrimination in terms of identification and quantitation must be made with caution. The evidence would appear to suggest that under normal conditions, local readjustments in blood flow may be established by a shift in the responsiveness of the smooth muscle effector unit, making it more or less responsive to particular constrictor or dilator influences.

The mesoappendix method has been found to be specific only in the sense that it indicates the particular direction in which vascular reactivity has been shifted. The identification of either el or eP activity in a test samples does not by itself constitute evidence that the observed effect is referable either to a single vasoactive component, to several agents with similar biological properties, or to an unbalanced mixture of both principles. This problem was regularly encountered in bioassay of blood samples. Careful examination of the data in the present study indicates that biological fractionation did not provide sufficiently precise information to permit estimation of the separate titers of el and eP activities. Quantitative information of this sort is especially critical to the hepatorenal hypothesis of experimental hypertension, where the assumption has been made that the progressive elevation in blood pressure was accompanied by an initial rise in the titer of VEM, and subsequently by VDM to achieve equilibration.

The quantitative aspects of the mesoappendix method were examined from several points of view. In final analysis, the significance of the bioassay findings depends on the extent to which changes in concentration are represented by a corresponding shift in vascular reactivity. Two major obstacles were encountered. From a practical point of view, it has been found necessary to express vasoactivity as a function of both the extent of the
shift in epinephrine threshold concentration (E.T.C. value) and the relative duration of the effect. Although these indicated the general trend of vasoactivity titers, neither parameter by itself served as a valid measure in this respect. The major deficiency with reference to quantitation was the inability to provide a suitable mathematical representation which took into account the relative importance of the two parameters of measurement. Maximal individual variability was encountered in bioassays of blood samples, which invariably contained mixtures of different vasoactive principles. In the case of crystalline materials containing a single vasoactive component, semi-quantitation was possible, but within prescribed limits. The data emphasize that at the present stage of development, our subjective confidence in the method cannot be supported by an adequate objective expression for it. The more finite implications of the data must be avoided until it is possible to control adequately the influence of biological and experimental factors on the test subject.

CONCLUSIONS

Examination of rat mesoappendix method has emphasized certain inherent limitations, especially with respect to specificity and quantitation. The bioassay represents a relative method for detecting the presence or absence of material with epinephrine potentiating or inhibitory potentialities. The data, however, lend themselves poorly to statistical analysis. The present studies indicate that extrapolations based on the quantitative implications of the data are not possible and that quantitative estimates of vasoactivity in blood samples cannot be made, even after biological fractionation. Until it possible to control adequately the variable influence of a multiplicity of interlocking regulatory features, it is probable that the potential usefulness of the method will not be achieved.

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Validity and Limitations of Rat Mesoappendix Bioassay Studies
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Circ Res. 1955;3:121-132
doi: 10.1161/01.RES.3.2.121

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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