# SCIENTIFIC SECTION

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## THE GUINEA PIG AS A HEMATOPOIETIC TEST ANIMAL.*

(A PRELIMINARY REPORT.)

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Since the introduction of liver extract into clinical medicine, there has been the need of a dependable laboratory assay method to determine the potency of this therapeutic agent. There have been various methods of assay introduced (1, 2, 3) but the practicability and the specificity of these methods may be questioned. In two of the described methods, McGowan (1) and Vaughan, *et al.* (3), used the domestic fowl and the pigeon, respectively. In the other method, McGowan and Sinclair (2) used the domestic pig (*Sus scrofa*).

In a consideration of the first two methods, there are several factors worthy of mention. The erythrocytes of both the domestic fowl and the pigeon belong to the nucleated series. This fact detracts from the practicability of the methods due to the difficulty encountered in the enumeration of the reticulocytes. This difficulty was evidently experienced by Edmunds, *et al.* (4), as they state, in comparing their work to that of Vaughan (3); "This discrepancy may possibly be explained by the method of counting and classification" (page 92, par. 3).

The presence of a nucleus in the erythrocyte causes some difficulty in determining which cell is a reticulocyte and which is not, leaving the final decision to the judgment of the worker. This factor introduces a personal variation which may have considerable bearing upon the final result. When a non-nucleated blood sample is used this difficulty cannot arise. If the cell in question has a small or large amount of reticular material it is a reticulocyte and must be enumerated as such. The worker is not confused by the dual presence of a nucleus and a reticulum and is therefore able to differentiate, without personal influence, those cells which are reticulocytes and those which are not.

Another factor present in the published methods is that of housing the test animal. The average animal room does not readily lend itself to colonies of pigeons and domestic fowls. The confined area of the cages plus a reduced amount of sunshine and exercise must effect the general well-being of the animal. These conditions are rather far removed from the natural habitat of such animals. Peabody and Neale (5) make the following statement concerning this problem ". . . it was noticed that long confinement on the rather restricted diet apparently produced subnormal blood conditions" (page 1231, par. 2).

This difficulty also presents itself in the work of McGowan and Sinclair (2) who used the domestic pig (*Sus scrofa*) of about four months of age weighing about

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43.5 pounds. The difficulties of maintaining an animal colony of such test objects are easily discernible without further elaboration.

Previously suggested methods, in some cases, involve the use of an experimental animal with abnormal blood conditions. Some investigators (1, 2, and 5) have used experimental animals suffering with various degrees of anemia. Such circumstances we feel must certainly create a marked variation in the response of the test animal to the therapeutic agent. As these anemias are present because of pathological conditions and are not produced by controlled artificial methods, the degree of anemia will vary from time to time in the same animal and will certainly differ in a group of animals. The degree of anemia present, accompanied by the changes in the hematopoietic organs, and the efforts of the hematopoietic system to regenerate red blood cells must certainly influence the response produced by liver extract. It is certainly possible and not highly improbable that the same dosage of liver extract would produce a greater reticulocyte response in a hematopoietic system in need of such a stimulant than in a normal one which did not, provided of course, that the blood-producing organs were not partially or completely exhausted.

The present study was undertaken to find a normal, healthy experimental animal meeting the following requirements: (1) having non-nucleated erythrocytes; and (2) thriving under the usual animal room environment. We selected the guinea pig because it satisfied these two requirements and also because of the facility with which we could obtain the blood smears from the marginal veins of the ears of this animal. It was our purpose to determine whether or not a normal, healthy guinea pig would give a reticulocyte response to liver extract and still remain essentially normal.

These studies represent a preliminary endeavor to determine the practicability of using the guinea pig as a hematopoietic test animal and they should be followed by a more elaborate and comprehensive study.

## METHODS.

*General.*—The guinea pigs were selected without reference to particular breed. Normal, healthy, adult pigs, of male sex, were chosen. The weight range was between 500 and 800 Gm. The diet, upon which these pigs had been maintained and which had proved adequate, was not modified during the experiment. Before an animal was used for experimental purposes an erythrocyte count and a blood smear were made to determine whether or not the blood of the animal was normal. Reticulocyte counts were determined on each animal to be studied to establish a normal base line. The animals were weighed before injection, several times during the injection period, and after the injection period. Erythrocyte counts were made before, during and after the period of injection. The incisions on the marginal veins were very small so as not to cause excessive bleeding which might result in a hemorrhagic anemia. During the routine procedure the animal lost only two small drops of blood per day; in some few instances perhaps four or five drops, but at no time was the blood loss sufficient to cause an anemia, as will be demonstrated in the discussion. The first drop of blood obtained was discarded and the second drop used for the reticulocyte enumeration. The subcutaneous injections were given over a period of eight consecutive days. The smears, made every day at approximately the same hour, were immediately followed by the injections.

*Hematological Methods.*—The ear was incised on the marginal vein and a small drop of blood was placed on a chemically clean cover-slip. With a sharpened toothpick a drop of alcoholic brilliant cresyl blue was added to the blood on the cover-slip. The blood and stain were mixed intimately with the toothpick, the second chemically clean cover-slip was placed over the first

and the blood allowed to spread. The smear was then pulled, the two cover-slips were allowed to dry in the air, and counterstained with Wright's stain.

In the enumeration of the reticulocytes five hundred red cells were counted in consecutive fields. The portions of the slides selected were thin and evenly spread in order that the red cells would not overlap. The reticulocyte count was made with oil immersion and the field reduced so that not over twenty-five red cells were counted per field. If the smear was at all difficult to count, one thousand red cells were examined. The reticulocyte counts were made by one person throughout the experiments. Some of the smears (containing the higher percentages of reticulocytes) were sent to a technician, trained by one of the authors, for a reticulocyte count. The technician did not know the reticulocyte count and, in fact, was not aware of the investigations being carried on by us. In all instances our reticulocyte count did not vary over one per cent from that of the technician.

The red corpuscles were counted in a new Newbauer Counting Chamber using the Thomas red cell pipette. Both of these instruments were certified by the Bureau of Standards. The average of two red counts, the difference between the two being not over one hundred thousand, was taken as the red-cell determination.

*Dosage.*—The experimental animals were injected with 1 cc. (per day) of No. 343 Lilly Liver Extract[1] at approximately the same hour each day. As the liver preparations were injected subcutaneously in the abdominal region, great care was exercised not to penetrate the abdominal wall and expel the fluid into the visceral cavity. With one exception, all the animals received the same dosage of liver extract over the same period of time (eight days). (This animal received extract for one day longer than the other animals, and 2 cc. of extract for the last two days.) One cc. of the extract was stated to be equivalent to five Gm. of liver. Each animal, therefore, received a total equivalent of 40 Gm. of fresh liver. We have reason to believe that smaller doses will produce satisfactory responses.

## RESULTS AND DISCUSSION.

*Control Periods.*—As a control measure, each animal was observed for a period of about seven days before it was injected with liver extract. During this time the reticulocytes and the erythrocytes were counted and the animal weighed. The normal averages for reticulocytes ranged from 0.63 per cent to 3.54 per cent (Table I).

*Effect of Iron on Reticulocyte Response.*—It was essential to ascertain if the method used was producing a post-hemorrhagic anemia through blood loss. If the method was producing an anemia, there should be a definite response to iron and ammonium citrate which is used clinically for post-hemorrhagic anemia. On the other hand, if no anemia was produced by the puncture of the marginal veins, but a reticulocyte response occurred as the result of iron present in the extract, this also could be demonstrated.

For this experiment two pigs were used and subjected to the same method of procedure. Normal findings were recorded for five days and the average of these used as a base line. A solution of iron and ammonium citrate (U. S. P.) was made with distilled water of such strength that one cubic centimeter of the solution contained 2.5 mg. of iron and ammonium citrate. The solution was given as described under "DOSAGE." The experimental animals were injected every day for eight days. The reticulocyte counts were made during this period and for nine days following the injection interval to provide for a delayed response. Neither the reticulocyte count, the erythrocyte count nor the weight was influenced by the iron and ammonium citrate.

[1] We are indebted to Dr. H. W. Rhodehamel, of the Eli Lilly Company, for generously supplying us with the liver extract preparations.

Following this procedure the pigs were left in their cages, under the same conditions as those preceding the experiment, for a period of one month. At the end of this interval the reticulocytes were counted on four successive days to obtain a normal range, and the injections of liver extract were begun. The injections were carried on for eight days. The liver extract produced a definite reticulocyte response (Table I, animals number 1 and number 2), but did not effect the erythrocyte count nor the weight.

As there was no response to the injection of iron and ammonium citrate, one may draw two conclusions: (1) that no anemia was produced by the hematological methods; (2) that the experimental animals were not suffering from a nutritional anemia. The hematopoietic system, in cases of post-hemorrhagic anemia, attempts to compensate for the blood loss without therapeutic stimulus which alone would have produced some reticulocyte response. As iron preparations are clinically recognized in the treatment of post-hemorrhagic anemias a response should have occurred if this condition were present. Schultze and Elvehjem (6) have demonstrated that iron (in the presence of copper) brought about a definite reticulocyte increase in conditions of nutritional anemia. Our experimental animals were obtaining sufficient copper from the diet to utilize any iron present, therefore a reticulocyte rise should have occurred if this anemia were present.

TABLE I.

| Experimental. Animal No. | Reticulocytes. Normal Average. | Reticulocytes. Increase. | Reticulocytes. Percentage Increase. | Day of Maximum Increase. |
|---|---|---|---|---|
| 1 | 1.05% | 3.95% | 376 ± 95 | 8 |
| 2 | 0.95% | 3.85% | 405 ± 105 | 9 |
| 3 | 1.70% | 5.10% | 300 ± 58 | 10 |
| 4 | 1.30% | 4.10% | 315 ± 76 | 11 |
| 3 (Reinjection) | 0.63% | 3.77% | 598 ± 158 | 7 |
| 4 (Reinjection) | 1.23% | 4.17% | 339 ± 81 | 10 |
| 5 | 3.30% | 1.70% | 51 ± 33 | 9 |
| 6 | 3.70% | 4.50% | 121 ± 27 | 5 |

*Effect of Liver Extract on Reticulocyte Response.*—The injection of liver extract produced a reticulocyte response in all the experimental animals tested (Table I) without an increase in the erythrocytes or the weight. After the peak of the response had been reached the number of reticulocytes gradually returned to normal. The question immediately arose as to whether the hematopoietic organs had been exhausted by such stimulation or whether they would again respond to liver extract. After a period of one month two experimental animals, previously used, were again injected, the same technique being used. There was a definite response to the liver extract (Table I, reinjection).

These studies are particularly interesting in that they point to the possibility of a standardized laboratory animal. If such is the case, one could not only determine the potency of a preparation, but also could compare directly the action of various preparations upon the same animal.

*Effect of Heat on Liver Extract.*—The liver preparations injected produced a significant rise in the reticulocyte count. With this fact in mind, we were desirous of determining what influence a modified sample of the preparation would produce.

The sample was prepared as follows: 16 cc. of the extract were rapidly boiled to dryness without scorching. To the residue was added enough distilled water to bring the volume to 16 cc.; this sample was again boiled to dryness and the residue dissolved in enough distilled water to make the volume 16 cc.

After the normal base line was obtained the same procedure of injection was carried out as in the previous experiments. The response produced by the injection of the heated sample was definite, but less than that created by the unmodified sample (Table I, animals number 5 and number 6). The heating did not destroy the activity, but may have reduced the potency somewhat. Additional study may clarify this point.

In Table I the reticulocyte responses following the injection of liver extract are given. The erythrocyte counts did not change significantly, and are therefore omitted to conserve space.

From the data thus far obtained, we feel that the method described compares favorably with the other methods presented in the literature and is, therefore, worthy of further study, particularly with reference to its specificity.

In a personal communication from Dr. H. W. Rhodehamel, of Eli Lilly and Co., we have learned that the samples of liver extract used had shown clinical activity.

## CONCLUSIONS.

1. A method suggesting the possibility of using the normal, healthy guinea pig as a hematopoietic test animal is introduced.

2. Experimental test animals that did not respond to injections of iron and ammonium citrate later gave a reticulocyte response when injected with liver extract.

3. The injection of liver extract over a given period caused a definite rise in the reticulocyte count without significantly effecting the erythrocyte count or the weight.

4. A second period of injection produced a response in the reticulocyte count similar to that produced in the first period suggesting the possibility that a given animal may be repeatedly used for test purposes.

5. The active constituent of liver extract which produced a reticulocyte response in the guinea pig is not readily destroyed by heating.

6. This report is of a preliminary nature and is presented merely as a possibility worthy of further study.

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# ALKALOIDAL REAGENTS V. THE ACONITE ALKALOIDS.*

BY JAMES C. MUNCH AND HARRY J. PRATT.

The reactions of the aconitine group of alkaloids with the usual alkaloidal reagents, as well as with certain special reagents reported in the literature, contain

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