SCIENTIFIC SECTION

ON A LABORATORY TEST FOR LIVER EXTRACT.*

BY C. W. EDMUNDS, HAROLD H. BRUECKNER AND AGNES I. FRITZELL.

Among the therapeutic agents which have been introduced into the practice of medicine in the past few years probably none have aroused greater interest than liver and stomach preparations for the treatment of certain anemias. It may also be said that no agents probably ever demonstrated their value to the medical world so rapidly as did these. In a few brief weeks the results described by their discoverers were confirmed by reports emanating from all parts of the medical world; and liver extract and desiccated stomach were placed upon a firm foundation as valuable medicinal agents.

Following the introduction of a liver diet and the recognition of its limitations, efforts were made to isolate the active substance from the liver which was responsible for the clinical improvement and very soon various fractions and extracts were obtained which would produce the same favorable effects that follow the administration of whole liver or stomach. However, in the preparation of these different extracts it was recognized at once that it was necessary to determine their potency—to see whether the individual fraction still possessed the essential action of the liver or whether in the course of preparation it had been lost. It is here that difficulty has arisen as no laboratory method has been discovered which will show whether such extracts will produce the same effects as follow their administration to patients with pernicious anemia. Nor indeed has any other criterion been described in any of the common experimental animals, with possibly one exception, which would seem to serve as an index of potency. The only alternative has been, therefore, to test the activity of these extracts by administering them to patients who are suffering from pernicious anemia. The administration of a potent extract to a suitable patient is followed in due course by an increase in the number of reticulocytes in the blood, by an increase in the number of red blood cells and in hemoglobin, and by clinical improvement. Failure of these signs to appear denotes an extract or fraction without potency.

The difficulties with this method of evaluation are manifest. Not only are patients with this ailment not plentiful but on account of the wide publicity of the value of liver most of the individuals so affected have been taking liver or stomach in some form or other and so are not available for use unless they are in a state of relapse. Then, too, the other side of the question has to be considered; namely, whether it is ethical to administer to a patient with this disease a preparation the potency of which is not known. This latter objection is not a serious one, perhaps, as a delay of a few days is not important in most patients and the benefits to be derived from such tests are so conspicuous and cannot be gained in any other way that the method is entirely justifiable. However, it was in an effort to avoid these difficulties and to try to find a method of laboratory assay that the present study was undertaken.

* From the Laboratory of Pharmacology of the University of Michigan, Ann Arbor.

A survey of the literature shows that many attempts have been made to study the action of liver preparations upon the lower animals and certain effects have been described as following their administration. But in only one instance have these effects been of such a character that they would seem to offer any hope that they could be used for the commercial testing of such products. In 1930, Vaughan, Muller and Zetzel (1) published a paper on the effect of the administration of various liver preparations upon the blood of pigeons. They report in this paper that if preparations which have been shown to be potent by clinical tests are administered to pigeons, their use will be followed by an increased number of reticulocytes in the circulating blood and by an increase in the weight of the birds. These facts, shorn of all details, seemed to offer a hope that the method might be adapted for the testing of preparations in the laboratory and thus to avoid the use of patients for the purpose. Opposed to this idea was the fact that other workers have tried to confirm the findings reported but have failed to do so. However, the importance of the matter seemed to justify further study. Furthermore, it appeared to be an unusually favorable opportunity to carry out such an investigation as we had available for advice and consultation Drs. Sturgis and Isaacs of the Simpson Memorial Institute for Medical Research, and in addition had for study various preparations of liver and stomach which had been used in the Institute and their potency thus determined.

The pigeons we used were derived from two sources. Some were from the Genetics laboratory of the University where these birds have been used for experimental purposes for years so that they had been raised in captivity, as indeed their ancestors had been before them. They were accordingly grain-fed ("Globe pigeon feed"). When this source failed we secured others from a dealer who raised them for the market. Thus they, too, had been raised in captivity and were grain-fed. The blood from the members of the two groups did not show any essential differences.

In regard to the number of reticulocytes in the blood of pigeons Vaughan and her coworkers direct that the pigeons be fed upon grain for some weeks until they show about 10% of reticulocytes in the blood and in any case not over 12%. These figures we have been unable to duplicate. In our pigeons born and bred in captivity we have never found one with a count as low as 10%. Some had 15–17% but most had about 20% and some even as high as 40%. It is probably true that pigeons with only 10% reticulocytes might be preferable for the work but all our birds had higher percentages. This discrepancy may possibly be explained by the method of counting and classification. We used for the purpose of staining the usual alcoholic solution of brilliant cresyl blue which is spread upon cover slips and allowed to dry. A small drop of blood is then spread upon the cover and when the film is dry it is counterstained with Wright's stain. There are in the pigeon's blood what for our purposes may be freely classified as two groups of cells containing reticular material. One group stains very heavily and contains large granules and a marked network giving the cell a dark color. These cells are very noticeable when the film is examined under the microscope. Then there are other cells with smaller amounts of reticular material, some in which it is quite apparent while in others the amount is small, requiring careful focusing. These, too, have been considered reticulocytes—that is, any cells which contain

any granular material were counted as reticulocytes—just as in clinical work. The number of the heavily reticulated cells was usually about 15% and in the fluctuations which occurred from count to count the curve of these cells usually ran parallel to the curve of the total number of reticulocytes in the blood.

Gain in Body Weight.—All the pigeons were weighed during the period they were under observation but the results show that there was no characteristic change in either direction during the time the preparations were being administered. Some pigeons gained weight while many lost. Our results in this respect did not agree with those reported by Vaughan and her coworkers. In their work the administration of liver preparation No. 132E was usually followed by a gain, but with the other preparations gains were exceptional although no losses in weight were reported. We found many which lost weight.

Our general plan of procedure was as follows: The birds were weighed and the blood vessels under one of the wings were exposed by the removal of a few feathers. The vein was then pricked with a surgical needle so as to give a free flow of blood. Small drops were taken and four or five spreads made as described and the liver or stomach preparation to be tested was then administered. In most cases it was injected into the breast muscle where it never seemed to produce any local irritation. In a few cases we injected the material intravenously but this did not seem to offer any advantage to compensate for the extra trouble. Some of the fluid preparations we gave by mouth with a medicine dropper or they were introduced into the crop by means of a small catheter. One desiccated stomach preparation was given in capsules but for the usual fluid preparations the intramuscular route seemed to be the method of choice.

The time when the reticulocyte increase is to be expected is a matter of considerable importance and according to our experience is subject to considerable variation. Vaughan says it is between the third and the eighth day. It is doubtless true that the increase may begin on the fourth or fifth day but after a considerable experience we finally decided on the eighth or tenth day as being early enough to make the count and as being fairly certain to show any increase which might have resulted. Subsequent counts were made at intervals of from five days to a week until about the twentieth day after the injections were begun. In our work all the counts were made by one person (B.) so that they are all strictly comparable.

The preparations studied were from various sources. Some came from material which was being tested in the Simpson Institute while others were kindly furnished by commercial houses from stock which had all been clinically tested. Certain preparations which had been found to be of doubtful value were used as controls.

Dosage.—In an endeavor to secure some uniformity we gave each pigeon the equivalent of about 20 Gm. of liver as indicated on the containers. In only one or two instances did we deviate from this rule and give more as will be noted. The total dosage to be administered was divided into fractions to be distributed over three or four days. A dosage of 4 cc. for instance was given 1 cc. daily, using the two sides of the breast alternately for the injections. In the testing of any single preparation four pigeons were used as a minimum. In a few instances

second or even third groups of four were injected, making a total of from eight to twelve birds for a single study.

Before giving the details of our various assays it might be well to summarize briefly our results and conclusions. In general our work confirms the findings of Vaughan and her coworkers with perhaps certain qualifications. We have discussed, for instance, the discrepancy between their findings and ours in regard to the number of reticulocytes in the pigeon's blood. On the other hand we do find that approximately a week after the beginning of the administration of a potent liver preparation the recticulocytes in the vast majority of pigeons begin to increase in number. The extent of this increase and its duration differs in different birds and is doubtless also dependent upon the amount of the active substance administered. It may possibly be due to smaller dosage but the increases we found in our work were not so great as those reported by Vaughan. In some birds the increase was only 6 or 8%; in most it was between 10 and 20% and in a few it was even higher. In some birds the increase was gradual and sustained, while in others a maximum was reached to be followed by a sharp fall. In an occasional bird no increase occurred, but such findings were, however, infrequent occurrences. In a certain group one bird might fail to react while the others showed a normal increase. It would therefore be advisable when using this method for the testing of such liver or stomach preparations to employ a sufficient number of birds in order to lessen the importance of an erratic reaction.

As to what effect dosage plays we are not prepared to state at the present time. Small doses doubtless produce no appreciable increase but whether the reaction can be utilized for quantitative assays we cannot say without further study. The method apparently will differentiate a potent extract from one without action and probably a strong extract from a weak one, but whether it will go further than that only time and experience will tell. And after all, does even clinical testing of such preparations tell any more?

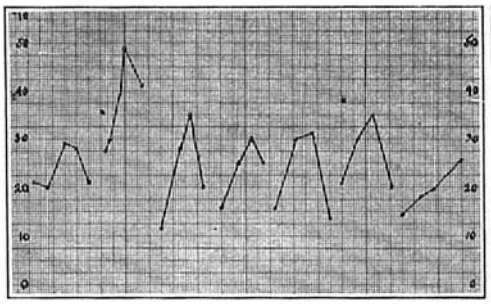
In some preliminary tests we made using this method we got very irregular results so that we seriously considered discarding the method as useless. We were using material from 50-cc. ampuls, withdrawing the small amounts as needed by means of a sterile needle and syringe. The first few injections gave promising results but later these were somewhat erratic so that we contemplated forsaking this line of the research. Just at this time a note appeared in the literature regarding the "stability" of liver extracts and it suggested to us that possibly the explanation of our poor results was to be found in changes in the extract due to frequent exposure to air or possibly to deterioration from other causes during the time over which the injections had extended. In any case we substituted material ampuled in small amounts and with this material our results have been much more encouraging.

In reporting upon our assays we are not giving the names of the firms who kindly furnished us with test material as it must be clearly understood that this is, at the present time, merely a qualitative assay to determine whether a preparation is potent or not. Because an increase in reticulocytes is greater with one preparation than with another need not necessarily mean that the former is the stronger extract. There are so many different factors involved—some of them not understood at present, others, such as the condition of the bone marrow or

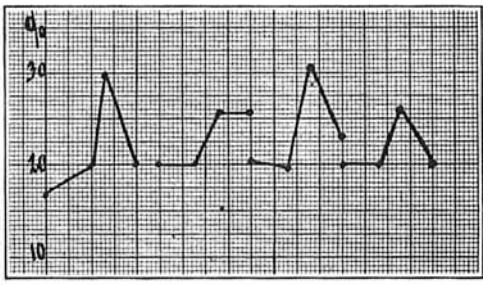
possibly a certain degree of nutritional anemia in a bird—that it would require a much more extensive study and the use of many more birds before any conclusions of a quantitative nature could be drawn. This is manifestly only a preliminary study.

Preparation No. L, Liver Ext.—1 cc. = 5 Gm. Liver. 1 cc. given intramuscularly on each of 4 days. Total equivalent—20 Gm. Liver. See composite Curve I. Average initial reticulocyte count in 7 pigeons, 18%. Average count 11th to 20th day, 34%. Increase, 88%. No increase in weight in any pigeon.

Preparation No. L2, Experiment I. Solution Liver Extract.—1 cc. = 33½ Gm. Liver. Two injections of 0.3 cc. each given = 22 Gm. Liver. See Curve II. Average initial count in 4 pigeons, 19%. Average count 13th day, 29%. Increase, 53%. Slight decrease in weight in every pigeon.



Curve I.—Percentage increase in reticulocyte counts of seven pigeons following intramuscular injection of 4 cc. liver extract.



Curve II.—Percentage increase in reticulocytes of four pigeons following two injections of 0.3 cc. each of liver extract—total 0.6 cc. = 22 Gm. liver.

Preparation No. L2, Experiment II.—Same preparation as in Experiment I but given in double the dose, total equivalent of 44 Gm. Liver in four daily injections. See Curve III. Average initial count in 4 pigeons, 25%. Average count 10th to 14th day, 30% Increase. 20%. Preparation No. L2 was preserved with 0.5% phenol so that the birds in Experiment II were given about 7 mg. phenol and it is possible that this amount of phenol might account for the small rise in reticulocytes; considerably smaller with the equivalent of 44 Gm. of liver than with 22 Gm. On the other hand, two of the pigeons showed an increase in weight—the largest increases we had in any of our series, *viz.*, from 307 Gm. to 369 Gm. in 13 days and in the other bird from 306 Gm. to 350 Gm. in the same time. The other two birds lost slightly.

Preparation D1.—"Intramuscular." 1 cc. = 5 Gm. Liver. 4 cc. given in four daily injections. Average initial count in 8 pigeons, 22%. Average count 10th day, 26%. Increase, 18%. This preparation was interesting as with it four of the eight pigeons reacted poorly while the other four gave good percentage increases and it illustrates the necessity of using a sufficient number of birds.

Preparation D2.—"Experimental Intravenous." Total dose given 4 cc. = 20 Gm. Liver. Average initial count, 18%. Average count 9th to 16th day, 31%. Increase, 72%. No change in weight of pigeons.

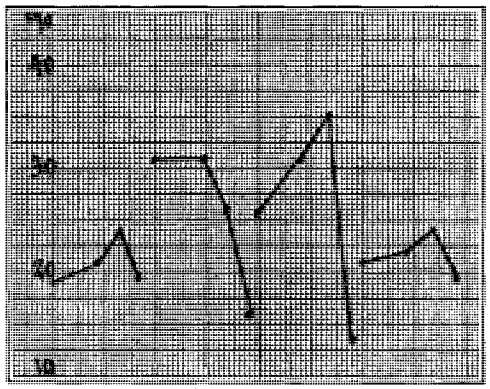
Preparation V.—"Subcutaneous liver extract made from equine livers." Total dose given 2.5 cc. = 25 Gm. Liver. Average initial reticulocyte count, 23%. Average count on 14th day, 27%. Increase, 17%.

As additional material for study and as a control Dr. Guy W. Clark, pharmacologist of the Lederle Laboratories, very kindly sent us ampuls of three lots of liver extract, as follows:

No. 56 G. being their commercial product containing 0.5% phenol and which had been tested in a clinic making a special study of pernicious anemia and reported as being fully potent. One cc. of this material was the equivalent of 33.3 Gm. liver and we gave two injections of 0.5 cc. each. Average initial count in 4 pigeons, 19%. Average count 10th to 14th day, 31%. Increase, 63%.

Preparation 57 C. was the same as 56 G. except that it contained 1% sodium chloride. The equivalent of 33.3 Gm. liver was administered as in the previous experiment. Average initial count in 4 pigeons, 19%. Average count later, 31%. Increase, 63%.

It doubtless is a coincidence but it is at least interesting that these two preparations which were identical except for the sodium chloride content give exactly the same percentage increase in reticulocytes in two groups of four pigeons each. The results obtained with these two preparations, however, are remarkably close to those obtained with another preparation received weeks earlier. In the former preparation with the equivalent of a smaller amount of liver the increase was from an initial count of 19% to 29%, while with the present preparations, 56 G. and 57 C., the increase was from 19% to 31%. Certainly it was a close agreement but one that we would dislike to have to promise to duplicate, at least at this stage of our study. It might be well to remark that these three preparations sent by Dr. Clark were examined by us (B.) as unknowns and only when the curves were completed was the key consulted.



Curve III.—Percentage increase in reticulocytes of four pigeons from same liver preparation as Curve II but given in double the dose; 1.2 cc. = 44 Gm. liver.

The third preparation sent by Dr. Clark—57 D.—was the regular product except that the filled ampuls had been heated at 75° for 15 minutes. It contained no phenol. Average initial count on 4 pigeons, 23%. Average count on the 11th day, 20%. Decrease, 13%. (Curve IV.)

The conclusion to be drawn, therefore, is that this preparation was inactivated by the heating. The reticulocytes graphs of all four pigeons were almost straight lines—no increase being found outside the limits of error and the results constitute an excellent control to the other two preparations and to the possibilities of this method of assay.

Other control tests carried out were as follows:

An extract was autoclaved at the Simpson Institute for two hours. Administered to one bird it gave on the eleventh day an increase of 2.4% and in the other a loss of 0.3%. Later, on the 14th day, both showed a slight increase, 4% in one case and 7% in the other. The results with this autoclaved preparation may be said to be practically negative. (Curve V.)

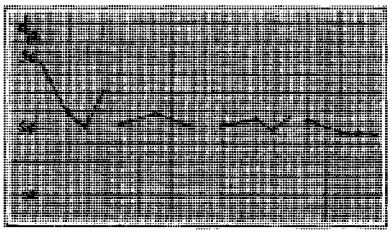
A further control was made using a dried extract which had been heated at the Simpson Institute. The equivalent of 20 Gm. of liver were given and the results were as follows:

Initial counts for the two pigeons were 22% and 19%. Ten days later the counts were 21% and 19%. (Curve VI.)

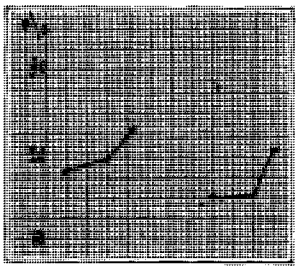
No change having taken place, this extract, too, was considered to be without potency.

Ventriculin.—This substance, the dried and powdered defatted hog's stomach, as is well known is being used extensively in the treatment of pernicious anemia.

In man, it produces the typical increase in reticulocytes in cases of such anemia and it was therefore a matter of considerable interest to see whether it would have such an action in pigeons. In addition it would serve also as a control on the method. Being an insoluble powder it was administered in capsules which the birds were allowed to swallow. Fifty mg. were placed in each capsule and each

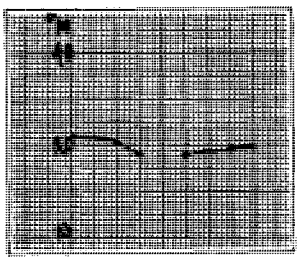


Curve IV.—Changes in reticulocyte count of four pigeons receiving a Lederle liver extract which had been heated and apparently rendered inactive.

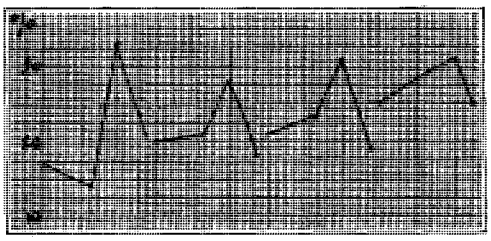


Curve V.—Percentage changes in reticulocyte count of two pigeons given a liver preparation which had been autoclaved for two hours. Very doubtful activity.

bird was given eight of these capsules on each of four days, making a total dosage of 1600 mg. In two of the pigeons the amount was doubled but the response was not greater than with the smaller dose. The result was a typical increase in num-



Curve VI.—Reticulocyte count from pigeons given a dried liver extract which had been heated and thus apparently inactivated.



Curve VII.—Increase in reticulocytes following 1.6 Gm. ventriculin given in four daily doses by mouth to each of four pigeons.

ber of reticulocytes as is shown in the composite Curve No. VII. The percentage increase was as follows:

Average initial count in 4 pigeons, 22%. Count on 10th day, 21%. Count on 16th day, 31%.

It will be seen that the increase in reticulocytes is between 40% and 50% but that it occurs considerably later than in the case of the liver preparations which could be given by intramuscular injection.

Inasmuch as the stomach wall preparation itself had given a satisfactory reticular response it was thought that possibly an extract of the stomach wall might be effective. We therefore administered to another group of pigeons a commercial aqueous-glycerin extract of the gastric mucosa of the hog presumably containing besides enzymes such organic and inorganic constituents as would result from such extraction. Being fluid it could be administered by mouth to the pigeons by means of a fine catheter introduced into the crop. The early dosage was 3 cc. daily but this having little effect the dosage was increased until a total of 18 cc. was given over a period of 4 days.

The results were as follows:

Average initial count, 17%. Average count of 13th day, 26%. Increase, 53%.

It will thus be seen that this gastric mucosa extract gave a very satisfactory response—in fact, practically as good a response as had been given by the liver extract. This reaction of the pigeons to the gastric extract is interesting and entirely logical inasmuch as they had reacted well to the administration of the desiccated material itself. It must be noted, however, that the dosage used was large but in considering the dosage of this extract it must not be overlooked that the preparation was given by mouth while the liver preparations were given by intramuscular injection, and it will be remembered that liver preparations given by mouth require a much larger dosage than when they are given by injection. The response to the extract, moreover, constitutes a corollary to the findings of Morris and his coworkers (2). They report that the intramuscular injection of concentrated normal gastric juice also produces a rise in the number of reticulocytes in patients and an increase in red blood cells and in hemoglobin. In the reports of these experiments, too, it is to be noted that enormous doses of unconcentrated gastric juice were given by mouth. In one case 10,000 cc. of hog gastric juice were administered without any effect on the reticulocytes. Apparently in the case of all these products a large proportion of the active substance must be destroyed in the alimentary canal inasmuch as oral administration requires a dosage much higher than is effective when the product is injected.

Comments.—The early efforts to determine the potency of antianemic agents by means of an increase in the number of reticulocytes in pigeons were unsatisfactory, apparently due to deterioration of the extracts tested. Later assays employing only small ampuls of material so that repeated exposure to the air was avoided gave very promising results. A number of extracts and other antianemic preparations which had been proved clinically to be potent were found to react in a positive manner when tested by this method. Other preparations which had been rendered clinically inactive by heat failed also to react here. Pigeons also responded to desiccated gastric tissue in a positive manner and also to a glycerin-aqueous extract of the gastric mucosa.

Our experience during the past year with this method of testing antianemic preparations leads us to feel that it is worthy of further study. The present situation of clinical testing is unsatisfactory not only for the commercial production of such preparations but also from the standpoint of scientific progress. On the other hand, experience on a much wider scale with the method herein described

is very desirable. It is not offered as a final answer to the problem but as a procedure worthy of trial.

In its use careful technic is essential—not only in regard to the staining of the blood but especially with respect to the counting of the reticulocytes. In the testing of a preparation it would seem important that the responsibility for the counts should rest upon one worker. Inasmuch as an occasional pigeon apparently fails to "react" it is important to utilize a sufficient number of birds. Experience only will tell how many, but we would suggest ten or twelve. In some birds the increase may begin later than the ninth or tenth day resembling the so-called delayed type of reaction.

BIBLIOGRAPHY.

(1) Vaughan, Muller and Zetzel, *Brit. J. Exptl. Path.*, 11 (1930), 456.
(2) Morris, Schiff, Burgen and Sherman, *J. Am. Med. Assoc.*, 98 (1932), 1080.

THE STANDARDIZATION OF ERGOT—A MODIFICATION OF SMITH'S QUANTITATIVE COLORIMETRIC ASSAY.*[1]

BY ASA N. STEVENS.

In view of the chemical nature of ergot and its preparations physiological methods of standardization have long been considered more satisfactory. None of the usual alkaloidal assay procedures have been found adequate. More recent investigations into the chemistry of ergot and the application of certain color reactions have given considerable promise that advantage might be taken of a more rapid and a more accurate colorimetric chemical method of standardization.

It is the object of this paper, therefore, to present some data accumulated over a period of about two years, using a modified colorimetric chemical assay method together with the Cock's Comb (Official U. S. P. X) and the Reversal Uteri (Broom and Clark (2)) methods All the comparative figures that are presented in tabulated form have been obtained from samples that were assayed by the indicated method within a period of one week.

DEVELOPMENT.

The observation that certain color reactions can be obtained with the constituents of ergot is not new. Tanret (6) observed color changes when concentrated sulphuric acid was poured on ergotinine. Later, Keller (3) improved the reaction by dissolving the alkaloid in glacial acetic acid, adding a trace of ferric chloride and pouring the sulphuric acid underneath. He obtained an immediate intensely blue color.

Van Urk (8) using a modification of the general reaction for indol derivatives, mixed 1 cc. of a one per cent solution of para-dimethyl-amino-benzaldehyde with an equal volume of an alcoholic solution of ergot alkaloid and poured sulphuric acid down the side of the tube. Smith (5) further modified van Urk's method by mixing 2 cc. of a one per cent tartaric acid solution of the alkaloid with 1 cc. of

* Scientific Section, A. PH. A., Toronto meeting, 1932.
[1] A contribution from the Analytical Laboratories, Eli Lilly & Company.