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THE RESPONSE OF THE GUINEA PIG'S RETICULOCYTES TO SUBSTANCES EFFECTIVE IN PERNICIOUS ANEMIA.

A BIOLOGIC ASSAY OF THE THERAPEUTIC POTENCY OF LIVER EXTRACTS.^{1, 2}

By BERNARD M. JACOBSON

(From the Medical Clinic, Massachusetts General Hospital, and the Department of Medicine and Biochemical Laboratory of the Harvard Medical School, Boston)

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If a suitable quantity of mammalian liver, or an extract thereof, be given to unselected guinea pigs, a significant increase in the number of circulating reticulocytes will ensue in the majority of the animals. This phenomenon was first observed seven years ago, and since that time considerable data have been obtained which indicate that the reticulocytosis may be utilized as a valid indicator of the therapeutic potency of materials

because of the presence of evidence of hemolysis, have been said to simulate human pernicious anemia. But to most investigators has been lost the fact that the therapeutic efficacy of liver, in human disease, is confined to pernicious anemia and related macrocytic anemias, and the fact that none of the toxic materials have induced in animals a condition which even remotely resembles Addison's anemia.

The extensive literature dealing with biological tests is summarized in Table I. Space does not permit a

TABLE I

Summary of literature concerning biological tests for therapeutic potency of liver extracts

	Positive results	Negative results
Normal animals.....	(11) (35) (7) (20)	(1) (34) (38) (13) (37) (14)
Animals rendered anemic by hemorrhage.....	(18)	(1) (9) (27) (36) (38)
Animals rendered anemic by infection.....	(2) (6)	(26) (29) (36)
Animals rendered anemic by dietary deficiency.....	(23) (29) (39)	(36)
Animals rendered anemic by toxic materials.....	(17) (30) (33) (27)	(1) (5) (8) (12) (13) (27)
Miscellaneous anemias.....	(19) (22)	(32) (36) (38) (17)

which are effective in pernicious anemia. A preliminary note concerning this work has already been published (40).

Many attempts have been made in the past to devise a method of biologic assay of liver extracts. As far as the author is aware, only one of the many heretofore proposed has been demonstrated fully valid.

HISTORICAL

Most of the studies previously reported have taken the form of investigations of the effects of liver extracts upon the blood of either normal animals, or of animals rendered anemic in various ways. Several toxins have been known to produce in laboratory animals anemias, which,

critical discussion of the many proposed tests, but, in the author's opinion, none give promise of serving as a valid indicator of the therapeutic activity of substances which are effective in pernicious anemia. To this generalization there are three exceptions, which are discussed below.

Of interest is the recent communication of Landsberg and Thompson (20). These authors, independently of the present writer, found that normal guinea pigs, maintained on a normal diet, reacted to the subcutaneous administration of commercial liver extract with a reticulocytosis. Six guinea pigs, after the injection of liver extract, exhibited reticulocytoses ranging from 4 to 8 per cent. One month later, two of these animals again reacted with a reticulocytosis to the administration of liver extract. Two animals did not react to the injection of an iron salt. The authors conclude that the phenomenon is worthy of further study.

Recently Miller and Rhoads (24) have proposed a different type of test. These authors noted that five adult guinea pigs, when fed a Goldberger diet which was productive of canine black tongue, lost weight, and died in two to three weeks. Five other guinea pigs, when fed the same diet together with commercial liver extract, gained weight, and survived. A group of four animals which received the same diet together with 1 gram of

¹ This study was made possible by assistance from the William W. Wellington Research Fund and the DeLamar Mobile Research Fund of Harvard Medical School and by Therapeutic Research Grants Numbers 206 and 244 of the Council on Pharmacy and Chemistry of the American Medical Association.

² Presented in abstract before the American Society for Clinical Investigation, April 30, 1934.

vegex (autolyzed yeast) lost weight and died. Similar results were obtained with a group of three guinea pigs fed the diet plus 2 grams of ventriculin. But a group of four guinea pigs given the diet together with 1 gram of ventriculin and 1 gram of vegex gained weight and survived. The conclusion was reached that the death or survival of the guinea pig may serve as a useful test for evaluating the potency of various substances used in pernicious anemia.

It is the opinion of the present writer that the validity of the above phenomenon, as a test for all types of substances effective in pernicious anemia, is not yet demonstrated. In the author's laboratory the administration to guinea pigs, fed the basic diet of Miller and Rhoads, of large amounts of a more highly purified but therapeutically active commercial liver extract did not prevent the weight loss exhibited by the control animals on the basic diet alone.

The most recent attempt to produce an anemia by means of a dietary deficiency has yielded very significant results in the hands of Miller and Rhoads (39). By feeding a modified canine-black-tongue-producing diet to swine, a symptom-complex marked by oral mucous membrane lesions, achlorhydria, and macrocytic anemia, was produced. This anemia was relieved by the administration of an extract of the livers of normal swine, but not by the extract of the livers of the anemic swine. Further similarity of this pathological condition with pernicious anemia of man was evidenced by the facts that in the anemic swine, the femoral bone marrow became hyperplastic, and the gastric juice and the liver were devoid of hematopoietic activity in pernicious anemia. These findings of Miller and Rhoads suggest the use of such anemic swine as test animals for the assay of liver extracts. The quantitative relation between the amount of liver administered to the swine and the resultant hematopoietic effects remains to be investigated.

METHODS

The care of the guinea pigs

The animals used in the present study were adult male guinea pigs, weighing, at the time of acquisition, between 300 and 800 grams. They were obtained from various sources. No reason, other than the desirability of avoidance of pregnancy, accounts for the use of only male animals. The animals are maintained in small cages, each holding from six to eight guinea pigs, and each fitted with a wire bottom, the holes of which are large enough to eliminate coprophagy. The diet of the animals consists solely of oats, carrots, and lettuce. The quantity of food is not measured. No hay, sawdust, or wood shavings are present in the cages.

The method of estimation of reticulocytes

For the purposes of this study the following method has been found most satisfactory, affording a high degree of accuracy with the expenditure of a minimum amount of labor. A clean glass slide is warmed over a flame and is then coated with a thin film of saturated alcoholic solution of brilliant cresyl blue. The ear of the guinea pig is wiped off with alcohol, and a very small drop of capillary blood is taken up on a clean cover slip, which is then inverted upon the stained slide. The blood film is spread out by gentle pressure on the cover slip, and then the sides of the cover slip are rimmed with melted paraffin. This preparation is suitable for the estimation of reticulocytes for not longer than forty-five minutes, for after the lapse of a longer interval excessive hemolysis takes place. The eyepiece of the microscope is blocked off, with holes cut in paper, so that each oil immersion field contains from twenty-five to thirty erythrocytes. The reticulocytes in this wet preparation are readily identified; all red blood cells containing either punctate or thread-like particles of purple staining reticulum are classified as reticulocytes. The reticulum can readily be distinguished from platelets or from particles of the dye by the position of the latter outside the erythrocyte. The counting is carried out in areas in which the erythrocytes are distributed in a fairly uniform and unicellular layer. The reticulocytes among two hundred and fifty red blood cells in one corner of the preparation are counted, and a like number in an opposite corner; the number of reticulocytes observed multiplied by 0.2 yields the per cent of reticulocytes.

The above method of estimation of reticulocytes has been described in detail, for it is the opinion of the author that repetition of the studies reported in this paper requires the exact duplication of the author's technique.

Comparative reticulocyte counts by the wet method and by the conventional method, on a dry smear counterstained with Wright's stain, performed on samples of blood from nine guinea pigs, yielded no significant differences in the percentage of reticulocytes. The accuracy of the wet method, in the hands of trained observers, was demonstrated by the following experiment. Two observers, independently of one another, counted

the reticulocytes in six different areas, each area comprising 500 erythrocytes, of samples of blood from five guinea pigs. A highly uniform distribution of reticulocytes in different parts of the wet preparation was observed. The greatest differences in distribution were of the same order of magnitude as the greatest differences between the counts of the two observers. In neither case was the divergence large enough to render invalid the differentiation between a negative and a positive reticulocyte response (*vide infra*).

Further evidence for the reliability of the reticulocyte method is furnished by the relative constancy, in the individual animal, of the negative responses presented in Tables III and V.

The method of administration of liver extracts

Unless otherwise stated, the experiments to be described below concern the effects of substances introduced parenterally. All solutions, containing water as the diluent, are made up to a volume of approximately 5 cc., and are injected intraperitoneally, without any aseptic or antiseptic precautions. No peritoneal inflammatory reaction following such an injection has ever been noted.

Substances which are to be administered orally are best given in a volume not exceeding 2 cc.

The selection of reactive guinea pigs

The reticulocyte levels of unselected guinea pigs. The reticulocyte counts of thirty unselected guinea pigs were studied over a period of eight successive days after the installation of the animals in the environmental conditions described above. It was observed that the initial reticulocyte counts of many animals were above 1.2 per cent, that during the first four days many counts remained above this level, but that, with the exception of three animals, during the last four days the reticulocyte counts remained at 1.2 per cent or lower. It has been the practice of the writer to discard guinea pigs which exhibit fluctuant reticulocyte levels during the first week of observation.

The animals which exhibit stable reticulocyte counts of not over 1.2 per cent, which comprise the majority of all newly acquired guinea pigs, are then tested for their capacity to react with a reticulocytosis to the administration of therapeutic

tically active liver extract. For this purpose commercial liver extract,³ in an amount derived from 4.3 mgm. of fresh liver per kilogram weight of guinea pig, is injected intraperitoneally, on one occasion only. During the subsequent six days a large proportion of the injected animals will exhibit a positive reticulocyte response, in a manner to be described fully below. The proportion of animals which are found to be reactive, on the initial test, varies amongst different batches of guinea pigs; from 30 to 70 per cent is the proportion most often encountered. Only those guinea pigs which react to the initial test with a positive response are retained for further experimental purposes.

The uninjected guinea pigs offer no clue that might serve to differentiate reactive from non-reactive animals. Neither the weights of the animals, the height of the erythrocyte counts, the initial reticulocyte levels, or the appearance of the stained blood smear, show any significant differences between the two classes.

The variations in the proportions of reactive animals do not seem to bear any relation to the season of the year.

It has been the practice of the author, in the past, to discard those animals which have not reacted to the initial test with a reticulocytosis. It is not to be inferred, however, that there necessarily exists a fundamental qualitative difference between the two classes of guinea pigs. For, further tests for reactivity of the initially non-reactive animals may remove a large proportion from this class. In Table II are presented the results of the retesting, at approximately monthly intervals, of a group of twenty-five unselected

TABLE II
Responses of 25 unselected guinea pigs to successive retesting

Number of animals	7	6	7	3	2
Mgm. of fresh liver injected per kilo of animal					
43.0.....	+	—	—	—	—
43.0.....	+	+	—	—	+
4.3.....	+	+	+	—	+
4.3.....	+	+	+	—	+
4.3.....	+	+	+	+	—

³ The author has used Solution Liver Extract-Lederle, N. N. R.

animals. It is evident that the seven guinea pigs which were initially reactive remained reactive through the subsequent tests; while of the initially non-reactive animals, thirteen became reactive and exhibited positive responses on three or more successive occasions. Of the remaining five initially non-reactive animals, three became responsive on the fifth test, while only two did not remain consistently reactive after the first positive response. In no case, therefore, did any animal constantly exhibit a non-reactive state.

The hematopoietic response to the administration of liver extracts

The reticulocyte response. In order to study the reticulocyte level during a control period, counts on twenty-seven reactive guinea pigs (i.e., those animals which have reacted to the initial test with a positive response) were performed on six successive days, during which nothing was administered. Only one animal exhibited values as high as 1.6 per cent reticulocytes. A statistical treatment of these data yields the following results:

Arithmetical mean 0.85 per cent reticulocytes
Standard deviation 0.33 per cent reticulocytes
Probable error 0.22 per cent reticulocytes
Probable error $\times 3.2$ 0.70 per cent reticulocytes

It is fair to conclude, therefore, that practically all resting, reactive guinea pigs, over a period of six successive days, will not exhibit reticulocyte counts greater than 1.55 per cent.

On the other hand, following the administration of therapeutically active liver extract, the reticulocytes almost invariably, during the subsequent six days, rise to at least 2.0 per cent. Numerous examples of these responses are to be seen in Table III. A *positive response* is defined by the author as a rise of the reticulocyte count to at least 2.0 per cent on two successive days, within six days after the administration of the substance to be tested. A *weakly positive* or *doubtful* response is defined as a rise of the reticulocyte count to at least 2.0 per cent on only one day, or on two non-successive days, of the six day period. All other reactions, within the six day period, are termed *negative responses*.

Many examples of weakly positive and of negative responses are contained in the tables cited above.

TABLE III
Responses of erythrocytes and of absolute number of reticulocytes

Guinea pig number	Negative responses				Guinea pig number	Positive responses			
	Days after injection	Reticulocytes	Red blood cells	Number of reticulocytes		Days after injection	Reticulocytes	Red blood cells	Number of reticulocytes
		per cent	mil-lions				per cent	mil-lions	
297	1	1.0	6.10	61,000	42	1	1.2	4.99	60,000
	2	0.2	6.27	12,500		2	1.8	6.24	112,400
	3	0.8	6.21	49,700		3	1.8	6.64	119,600
	4	1.2	6.05	72,600		4	2.2	4.95	109,000
	5	0.6	6.39	51,200		5	2.6	4.94	128,500
	6	0.4	6.42	38,500		6	1.6	6.30	101,000
31		0.8			296		0.8	4.97	39,700
	1	1.2	5.48	65,800		1	1.2	5.03	60,800
	2	1.0	5.76	57,600		2	0.2	4.94	9,900
	3	0.8	5.91	47,250		3	2.0	4.60	92,000
	4	0.8	5.79	46,300		4	1.8	4.75	85,050
48	5	0.8	5.73	45,800	298	5	2.6	4.84	116,000
		0.6				6	2.4	5.38	140,000
	1	0.6	6.54	39,200			1.0	5.15	51,500
	2	0.6	6.52	39,100		1	1.4	5.70	80,000
	3	0.4	6.58	26,300		2	2.0	6.54	113,000
40	4	0.2	6.31	12,600	39	3	2.8	5.56	156,000
	5	0.6				4	2.4	6.18	148,000
	1	1.4	5.80	34,800			1.0	5.72	57,200
	2	1.4	5.87	82,200		1	1.6	5.43	86,800
	3	1.6	4.96	78,400	114X	2	1.4		
114X	4	1.6	5.70	91,200		3	1.0	4.75	47,500
	5	1.6	5.66	90,600		4	2.0	5.35	107,000
	6	1.8	5.40	97,200		5	2.0	6.24	124,800
						6	2.2	5.70	125,000
114X	1	1.0	6.83	68,300	63		0.8	6.58	52,600
	2	1.0	6.19	61,900		1	0.6		
	3	1.4	6.56	91,800		2	2.6	6.72	175,000
	4	1.4	6.77	94,800		3	2.2	6.40	141,000
	5	1.4	6.50	91,000		4	2.4	7.21	173,000
	6	0.8	6.47	51,800		5	2.4	6.80	163,000
	7	0.8	6.59	52,700	131X	6	2.0	6.60	132,000
131X		0.8	5.83	46,700		7	1.8	7.05	127,000
							0.4	6.08	24,300
	1					1	0.2	5.21	10,400
	2					2	1.0	5.60	56,000
	3					4	2.4	5.44	131,000
	4					5	2.0		
	5					6	2.4	6.56	157,000
131X	6					7	1.0	7.74	77,400
	7					8	1.4	7.68	107,000

That the reticulocytosis is both relative and absolute is demonstrated by the protocols of Table III.

The peak of the reticulocytosis is always attained within six days after the administration of the liver extract, but the reticulocytosis of at least 2.0 per cent persists for several days, and the values recede, in an irregular fashion, to the control level of 1.2 per cent, or less, within ten to twenty-one days, in most instances.

It is an invariable practice in this laboratory to await the return of the reticulocyte count to 1.2 per cent or less, before another experiment is undertaken. Values of 1.6 or 1.4 per cent may be followed by a slight transient rise. On the

other hand, the return of the reticulocyte count to at least 1.2 per cent insures a low level during a subsequent six day period.

The above definition of a positive response is an arbitrary one, and, to be sure, necessarily implies the distinction between a reticulocyte count of 2.0 per cent and one of 1.8 per cent. It is not the contention of the writer that such a distinction is possible. Fortunately, the necessity of making such a fine distinction is rarely encountered. In Table IV are presented the frequency distribution

of the experiment on another animal; only *positive* or *negative* responses, as defined above, are to be accepted. Doubtful responses are not repeatedly observed in any one animal.

To the infrequency of reticulocyte peaks which fall on the border line between a positive and a negative response, and to the necessity of repetition of every doubtful reaction, is to be added another safeguard of the validity of the observation, namely, the procedure used in the quantitative assay, whereby a series of graded amounts

TABLE IV
Frequency distribution of reticulocyte peaks

Per cent reticulocytes	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	5.4	5.8	6.2	7.6
Negative responses (131 experiments)	4.4	16.6	17.4	24.2	19.1	12.2	6.1																
Positive responses (193 experiments)								2.7	8.9	10.4	6.8	10.3	17.6	19.7	11.4	5.7	1.6	1.6	0.5	0.5	0.5	0.5	1.0
Doubtful responses (30 experiments)								37.0	23.1	10.0	0.0	6.5	6.7	6.7	6.7								

of the reticulocyte peaks of a large number of experiments. These data are derived from 354 experiments on 115 reactive guinea pigs, following the administration of a large variety of materials. The reticulocyte responses are classified as follows: Positive, 55 per cent, negative, 37 per cent, and weakly positive or doubtful, 8 per cent. It is evident that among the negative responses, the reticulocyte peak was *under* 1.8 per cent in 93.9 per cent of all cases, and in 81.7 per cent of all instances was *under* 1.6 per cent. Similarly, of the positive responses, 88.4 per cent of all peaks were *over* 2.2 per cent, and 68.0 per cent were *over* 2.4 per cent. Thus, in approximately 82 per cent of all responses, following the administration of a variety of materials, the borderline values of 1.8 to 2.2 per cent reticulocytes may not be expected to present themselves. The distribution of the peaks of the weakly positive or doubtful responses are illustrative of this interpretation. Thus, inasmuch as the summit of the distribution curve is found at a reticulocyte count of 2.0 per cent, with a steep downward slope, it seems fair to conclude that these values of 2.0 and 2.2 per cent reticulocytes, observed on only one day of the six day period, are an expression of the unavoidable error inherent in the method of estimation of the reticulocytes. For this reason, every doubtful result necessitates the repeti-

tion of the experiment on another animal. This procedure will be described below.

The reticulocyte response of the reactive guinea pig appears to be an all-or-none reaction. There is no relation between the amount of liver administered, above a minimal effective dose, and the height of the subsequent reticulocyte peak (Fig. 1). Regardless of the dosage, 97 per cent of all reticulocyte peaks of positive responses are distributed between 2.0 per cent and 4.0 per cent reticulocytes, inclusive (Table IV). On the other hand, the data of Figure 2 suggest a slight tendency of the reticulocyte peak to appear earlier in the six day period, with increasing amounts of liver material administered.

There is no correlation between the height of the reticulocyte peak and the initial erythrocyte count of the animal.

The reticulocytosis that the *oral* administration of liver extract induces differs qualitatively in no way from that following the intraperitoneal administration.

As far as the present data indicate, the reactive state is maintained indefinitely. The animal which has been in this laboratory the longest period has exhibited a total of 18 positive responses during the past 21 months. Another animal, during the 16 months preceding death, reacted positively 14

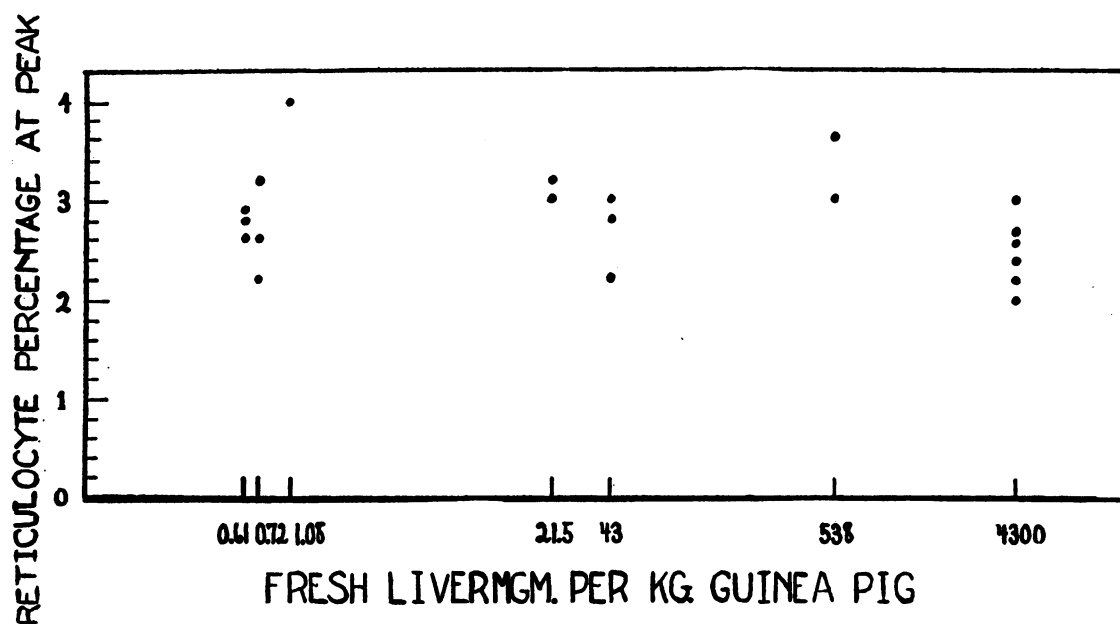


FIG. 1. THE RELATION BETWEEN THE DOSAGE OF LIVER EXTRACT AND THE MAGNITUDE OF THE RETICULOCYTE RESPONSE

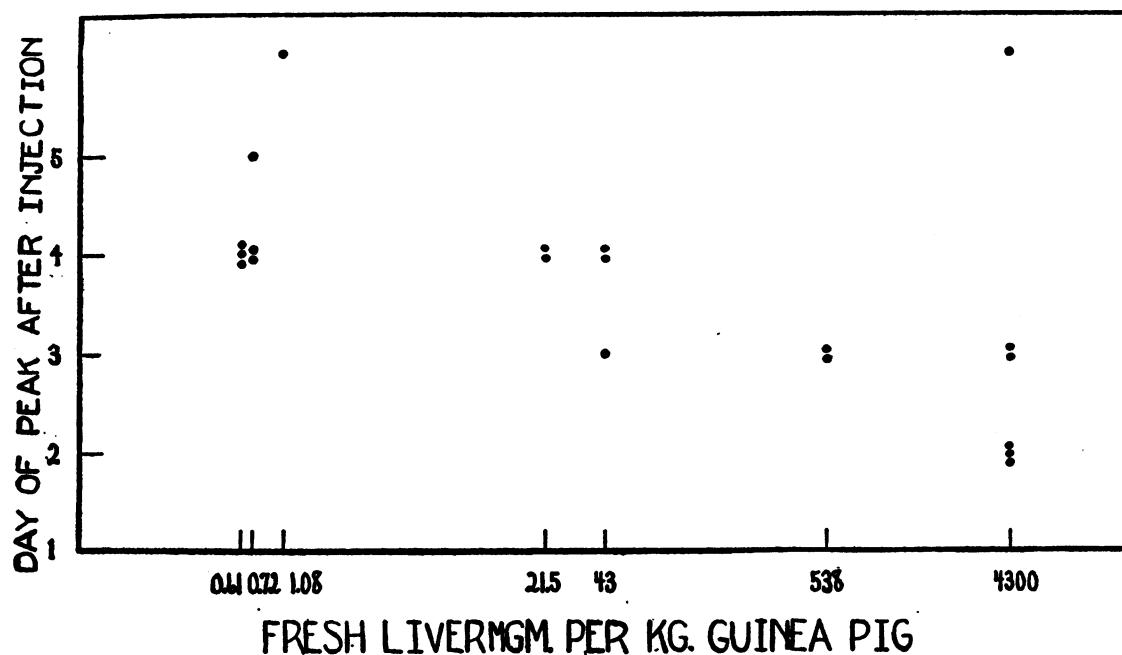


FIG. 2. THE RELATION BETWEEN THE DOSAGE OF LIVER EXTRACT AND THE TIME OF THE RETICULOCYTE RESPONSE

times. On retesting 15 reactive guinea pigs, all of which had exhibited reactivity on at least 4 occasions over a period of 4 months or longer, 14 positive responses were observed. One guinea

pig, which reacted negatively, showed 6 subsequent positive responses during the following 5 months.

The erythrocyte response. The erythrocyte responses to the administration of several inert ma-

terials, to five reactive guinea pigs, and of several active materials, in various amounts to nine animals, are depicted in Table III. The averages of all counts in each group are as follows:

	Erythrocytes	
	Negative response million per cu. mm.	Positive response million per cu. mm.
Day of injection	6.24	5.64
Day after injection		
1	6.07	5.52
2	6.26	5.77
3	6.05	5.53
4	6.14	5.56
5	6.07	5.87
6	5.73	6.10
7		6.81

The only inference from these scanty data that can be drawn is that the erythrocytes of the positively reacting animals tend to rise slightly, after the fourth day following the administration of liver material. A more complete investigation of these changes is under way.

The quantitative assay of the hematopoietic activity of liver extracts

Minimal effective dose or standardization curve.

The quantitative application of the induced reticulocytosis involves the definition of the unit of hematopoietic activity. This unit might be arrived at by one of two different procedures. If the assumption is made that all unselected guinea pigs are of an approximately equal degree of sensitivity, and that each individual animal may exhibit, in successive tests marked variation in degree of sensitivity, it should be necessary to follow the procedure described by Gaddum (10) and applied to the assay of oestrin by several workers (4, 21). This method consists of the construction of a curve which depicts the percentage of positive reactions among a large number of animals as a function of several different doses of oestrin; the amount of oestrin which induces oestrus in 50 per cent of the animals is termed the unit. In a similar fashion, a material of unknown potency is administered to a large number of animals, and by reference to the standardization curve the number of units in the material is ascertained.

Table II contains data bearing on this question. Of the twenty-five unselected, newly acquired guinea pigs initially injected with material derived from 43 mgm. of liver, 36 per cent exhibited a

positive response; while after the second administration of the same amount, this proportion rose to 68 per cent; and a third test, with *one-tenth* of the previous dosage, yielded 92 per cent of positives. But if the concept of a standardization curve be applicable, one would expect that the percentage of positive responses in the second test would be approximately equal to that in the first test; and that the proportion of positive responses in the third test would drop considerably. On the other hand, a similar increase in the percentage of positive responses during the second testing occurred in one group of unselected animals injected on each occasion with liver extract from 4.3 mgm. of liver. These observations demonstrate that all unselected guinea pigs, at the time of the initial test, are not equally sensitive; while a large amount of data have demonstrated that guinea pigs which have reacted positively to the initial test maintain, indefinitely, an approximately equal degree of sensitivity.

The hypothesis that a majority of reactive guinea pigs respond to the administration of a *minimal effective dose* of liver material in a consistent fashion has been rendered valid by the results of over one thousand experiments on several hundred animals, with a large variety of liver extracts. This method of assay will be described below.

The definition of the unit of hematopoietic activity. In Table V are presented the protocols of the assay on guinea pigs of a therapeutically potent, commercial liver extract. The potency of this lot of extract was ascertained on two patients suffering from pernicious anemia. In each case the resultant hematopoietic response was indicative of a high degree of therapeutic effectivity (Table VIII, second periods of Patients 5 and 6). In Table V it is seen that decreasing doses of this extract were administered, on one occasion only, to groups of two or three reactive guinea pigs. All the animals injected with amounts exceeding 0.54 mgm. (of fresh liver, per kilogram of guinea pig) reacted positively; one of three animals was positive to 0.54 mgm.; while smaller doses were inert. These data illustrate the method used in this laboratory for the assay of a liver extract. All weakly positive or doubtful responses, as described above, call for the repetition of the same dose in another animal; and a majority of three definitive

TABLE V
Assay of commercial liver extract (per cent reticulocytes)

Amount of fresh liver from which extract derived, mgm. per kilo	4,300		538			43			22		1.08	0.72			0.61			0.54			0.48			0.43	
Day of injection	1.0	0.4	0.8	1.0	0.8	0.8	0.6	0.8	0.8	1.0	0.8	1.0	1.0	0.6	0.8	1.0	0.8	1.0	0.8	1.2	0.8	1.0	1.0	1.0	1.0
Days after injection																									
1.....	1.6	1.2	0.6	1.4	1.6	1.4	0.4	0.4	1.2	1.2	0.8	0.6	0.8	0.6	0.8	1.0	0.8	1.2	0.8	1.4	1.0	0.8	0.8	1.0	1.0
2.....	1.4	2.0	2.6	2.0	3.0	2.0	0.8	1.4	0.8	0.8	0.8	1.0	0.8	0.4	0.8	1.0	1.0	1.2	0.8	2.0	0.8	0.8	1.0	1.0	0.8
3.....	1.0	2.4	2.2	3.0	3.6	3.0	2.4	0.8	1.6	1.6	1.4	1.0	2.2	1.8	2.0	2.0	2.2	1.0	0.8	2.8	0.8	0.8	1.0	0.8	0.8
4.....	2.0						2.8	2.2	3.2	3.0	2.0	2.0	3.2	2.2	2.8	2.6	2.8	1.2	0.8		1.0	1.0	0.8	1.0	0.8
5.....	2.0							2.2	2.0	2.4	3.6	2.6		2.0				1.2	0.8		0.8	1.0	1.4	1.0	0.8

Assay: 100,000 mgm./0.61 mgm. = 164,000 G. P. U.

responses identify the minimal effective dose. Thus, the minimal amount of material per kilogram guinea pig which, after a single intraperitoneal administration, induces a positive reticulocyte response in at least two of three reactive guinea pigs, is termed the Guinea Pig Unit (G.P.U.). The amount of fresh liver commonly dealt with in considerations of liver therapy is 100 grams; hence the above assay indicates a potency of 100,000 mgm./0.61 mgm. = 164,000 G.P.U. per 100 grams of fresh liver.

It is to be remembered that the above value is that of the extract *in terms of* fresh liver, and is not an expression of the potency of the fresh liver from which the extract was derived.

The preceding assay was carried out by means of a single intraperitoneal injection in each animal. One commercial liver extract was assayed in the above fashion, and also by means of a single oral administration. The minimal effective dose by the parenteral route was 0.34 mgm., while by the oral route, the minimal effective amount was 7.3 mgm. This difference, of 22 times, is of an order of magnitude not far removed from the difference in the therapeutic effects in pernicious anemia of orally and of parenterally administered liver.

The significance of the induced reticulocytosis

Control observations. That the intraperitoneal injection of water, used in the dilution of all materials administered, does not induce a reticulocytosis, is evident from the many *negative* re-

sponses that are presented in Tables III and V. Space does not permit the presentation of the protocols of all the negative responses that have been encountered. These negative reactions are summarized in Table VI.

TABLE VI
Summary of negative responses

	Number of experiments	Number of guinea pigs
A. Pure substances		
Sodium nucleinate.....	1	1
Spermine phosphate.....	1	1
Methionine.....	4	4
Tryptophane.....	3	3
Ferric chloride.....	2	2
B. Mixture of crude substances		
Human urine.....	3	3
Mixtures of inactivated, intrinsic and extrinsic factors.....	10	10
Orange juice.....	3	3
Extracts of human sera.....	19	17
Extracts of porcine stomach....	33	32
Commercial liver extracts.....	96	91
Experimental liver extracts.....	155	153

The assay of the human liver, normal and pernicious anemia

The writer has long been convinced that the relation of the induced reticulocytosis in the guinea pig to the substances in liver which are effective in pernicious anemia cannot be elucidated by the above mentioned control observations. For, regardless of the number of different therapeutically inert materials that might be tested in the guinea pig, the possibility remains, as prominently as ever, that there may have been over-

looked one or more substances exhibiting no therapeutic potency, but capable of inducing a positive response in the guinea pig. A different type of crucial experiment must be resorted to. Such an experiment is described in detail in another place (16). It consists of the assay on guinea pigs of crude extracts of four human livers. That of a control subject yielded a value of 127,000 G.P.U. per 100 grams of fresh liver; a case of pernicious anemia in partial remission a value of 47,000 G.P.U.; while two cases of pernicious anemia in relapse values of 650 and 348 G.P.U., respectively. It is evident that the liver of the patient dying of pernicious anemia contains only a negligible amount of the material which induces the reticulocytosis in the guinea pig; that the liver of the patient in remission contains considerably more of the reticulocytogenic material; and that the liver of the non-anemic individual contains almost as much as is present in commercial liver extract. These results accord with the assays, on patients suffering from pernicious anemia, of similar livers (16). The above data demonstrate that the vast number of inorganic and organic substances in liver, in the absence of the pernicious anemia principle, exert no effect on the reticulocytes of the guinea pig. The inference may be drawn that the material in liver which is reticulocytogenic in the guinea pig is closely related to the material in liver which is effective in pernicious anemia.

The response of the guinea pig to the administration of Castle's intrinsic and extrinsic factors

That the reactive guinea pig responds with a reticulocytosis to the materials in liver which are effective in pernicious anemia has been demonstrated above. But liver is not the only source of materials which are effective in this disease. There remain to be considered the effects on the guinea pig of other therapeutically potent substances.

The writer has attempted to carry out on guinea pigs the classical experiments of Castle and his co-workers (3). To this end, the fasting gastric juice of a non-anemic patient was mixed with minced beef, the mixture brought to a pH of about 4, incubated for two hours, and was then fed to guinea pig number 17 daily for five suc-

TABLE VII

Responses to the oral administration of Castle's intrinsic and extrinsic factors.

(Per cent reticulocytes)

Guinea pig number	17	42	256	61	14
	Gastric juice, 6 cc. per kgm., and beef muscle, 4.3 grams per kgm., after incubation	Same as Guinea Pig 17, except gastric juice boiled 5 minutes before incubation	First period: gastric juice, 5 cc. per kgm., heated 90° 30 minutes; beef muscle, 5 grams per kgm., pepsin, 0.2 gram per kgm. Second period: same as above, except gastric juice not heated	Rice polishings extract, 1 cc. per kgm., to Guinea Pig 61; same with 4.3 cc. gastric juice per kgm. to Guinea Pig 14	
Days					
1	→ 0.4	→ 0.8	→ 0.8	→ 1.0	→ 1.0
2	→ 0.8	→ 0.6	→ 0.6	→ 0.8	→ 1.8
3	→ 1.2	→ 1.2	→ 0.4	→ 1.4	→ 2.6
4	→ 1.0	→ 0.8	→ 0.2	→ 1.6	→ 3.2
5	→ 2.2	→ 1.0	0.2	→ 1.2	→ 3.4
6	2.8	0.8	0.4		
7	3.0	0.8	→ 0.4	1.2	3.0
8	2.8	1.0	→ 0.6		
9			→ 0.8		
10			→ 1.0		
11			1.0		
12			3.6		
13			3.2		
14			2.0		

cessive days (Table VII). A positive response was evoked. On the other hand, when the same gastric juice was boiled five minutes before incubation of the mixture, and the mixture administered as before to guinea pig number 42, a negative response followed. The same materials, with the addition of pepsin U. S. P., were administered in two successive periods to guinea pig number 256. Again, the results were identical with those of the preceding experiment. A third experiment was carried out with an extract of rice polishings, a material which Castle has demonstrated to be a source of extrinsic factor. As guinea pigs number 61 and 14 demonstrate, the extrinsic factor alone was inert, while the mixture of normal human gastric juice and extrinsic factor was reticulocytogenic.

These experimental results exhibit a striking similarity with those obtained on patients suffering from pernicious anemia (3). In both patients and guinea pigs the administration of a mixture of intrinsic and extrinsic factors is followed by an hematopoietic response; in both, the adminis-

tration of extrinsic factor alone is devoid of effect; and, in both, the administration of intrinsic factor that has been inactivated by heat is also without effect.

It seems to the writer that the results of the foregoing experiments furnish more evidence for the view that the reactive guinea pig responds with a reticulocytosis to the same materials that are effective in pernicious anemia.

A comparison of the guinea pig assay, and of the therapeutic potency, of liver extracts

In Table VIII are presented the data of the assays, on guinea pig and on patient, of seven different, experimentally produced, partially purified liver extracts. These materials were administered by intramuscular injection to previously untreated patients suffering from classical pernicious anemia. In most cases the material from not more than 100 grams of fresh liver was administered, but in an amount sufficiently large to possibly induce a maximal hematopoietic effect.

It has already been demonstrated (Table V) that one particular commercial liver extract, which was therapeutically potent, exhibited on guinea pig assay an activity of 164,000 G.P.U. per 100 grams of fresh liver. Assays of nine different lots of commercial liver extracts yielded values ranging from 140,000 to 305,000 G.P.U., per 100 grams of fresh liver. It is not the writer's intention to discuss, at this time, the possible significance of the variations in the degree of activity which these different extracts exhibit, but only to point out that 7 of these 9 extracts assayed between 164,000 and 210,000 G.P.U.

The foregoing data bear on the interpretation of the assays of extracts number 114 and number 118 (Table VIII).⁴ Both extracts yielded the same guinea pig value of 47,000 G.P.U., that is, the amount of activity that might be derived from 25 to 35 grams of fresh liver. The very similar results yielded by both patients, to whom this extract was administered, clearly indicate that a markedly sub-maximal effect was induced. As far as the author is aware, the only observations of the hematopoietic effects in pernicious

⁴ The author is indebted to Dr. Randolph West for the material and the clinical data of extracts number 232, 233, 234, and 252; and to Dr. William B. Castle for the material and clinical data of extract B.

TABLE VIII
Comparative assays on guinea pigs and on patients of liver extracts

Liver extract number	B										233
Guinea pig assay, in terms of G.P.U. per 100 grams fresh liver	Less than 77										170
Patient number	13	14	15	2.49	1.96	2.58	1.33	5	6	375	232
Red blood cells at beginning of experimental period	2.50	2.05	2.40	1.4	1.2	1.4	2.2	3.2	8	11	233
Red blood cells at termination of experimental period	1.4	1.2	1.4	8	10	8	8	8	8	8	233
Reticulocyte peak, per cent	8	10	8	—	—	—	100	100	170	200	233
Length of experimental period, days	—	—	—	—	—	—	100	100	170	200	233
Total amount of fresh liver, from which extract derived, grams	—	—	—	—	—	—	100	100	170	200	233
Response to subsequent administration of commercial liver extract	—	—	—	—	—	—	100	100	170	200	233
Red blood cells at beginning of period	—	—	—	—	—	—	100	100	170	200	233
Red blood cells at end of period	—	—	—	—	—	—	100	100	170	200	233
Reticulocyte peak	—	—	—	—	—	—	100	100	170	200	233
Length of period, days	—	—	—	—	—	—	100	100	170	200	233
Amount of fresh liver, from which extract derived, grams	—	—	—	—	—	—	100	100	170	200	233

anemia of the parenteral administration of small amounts of liver are those of Strauss and Castle (31). The erythrocyte and reticulocyte responses which these authors observed subsequent to the administration of liver extract derived from 20 to 30 grams of fresh liver exhibit variations in a range into which fall the responses induced by extracts number 114 and number 118.

It is the author's conviction, based on an adequate experience in the attempt to assay liver extracts on patients, that a closer correlation of the guinea pig and of the human potency of extracts number 114 and number 118 cannot be expected.

The assays of the remaining extracts yield corresponding activities on the guinea pig and on the patient.

DISCUSSION

The foregoing data describe the phenomenon of the induced reticulocytosis exhibited by the reactive guinea pig, and demonstrate the application of this phenomenon to the quantitative determination of the therapeutic activity of liver preparations. The factors which condition the procedure include (1) a particular and fixed diet and environment of the guinea pigs; (2) the attainment by the observer of a high degree of accuracy in the estimation of the reticulocytes; (3) the rejection of guinea pigs the reticulocyte counts of which fluctuate, during a preliminary control period; and (4) the use, in tests of guinea pigs for reactivity, of liver extract of a high degree of therapeutic potency.

As far as the author is aware, the guinea pigs which are found to be reactive do not suffer from any disease acquired prior to the initial test, or from a pathological state induced by the dietary and environmental conditions under which the animals are maintained in this laboratory. The erythrocytes of the reactive guinea pigs do not show any resemblance to those observed in pernicious anemia, nor are the animals anemic. On the other hand, there are two facts which bear on this question. In the first place, both reactive and initially non-reactive guinea pigs possess a femoral bone marrow which is exceedingly rich in primitive elements of both the white and red blood cell series. The large number of megaloblasts, in proportion to the number of normoblasts, simulate

the classical picture of the bone marrow findings in pernicious anemia.

In the second place, it has been demonstrated that the reactive guinea pig forms reticulocytogenic material from a source of extrinsic factor only in the presence of active intrinsic factor, a phenomenon which, as far as the writer is aware, has been observed only in pernicious anemia.

The facts known at present concerning the guinea pig phenomenon do not permit any further speculation on a possible deficiency state simulating that considered to exist in pernicious anemia.

Regardless, however, of the obscurity of the basis of the phenomenon, the data presented in this study furnish evidence for the conclusion that the guinea pig test is a valid indicator of the therapeutic efficacy of materials which are effective in pernicious anemia.

SUMMARY

From thirty to seventy per cent of guinea pigs, maintained on a diet of oats, carrots, and lettuce, exhibit a significant rise in the number of reticulocytes following the administration to them of therapeutically active liver material. For every active material there exists a minimal effective dose, which is termed the Guinea Pig Unit of hematopoietic activity, and which is a quantitative expression of a degree of activity. Evidence of an indirect nature is presented that the capacity to induce the reticulocytosis is confined to materials which are effective in pernicious anemia, and that the guinea pig test is a valid indicator of the therapeutic potency of liver preparations.

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