THE PIGEON AS A HEMATOPOIETIC TEST ANIMAL.*

BY WM. A. PEABODY[1] AND R. C. NEALE.

The need of a dependable laboratory assay method for the antipernicious anemia principle has been obvious for some time. Vaughan, Muller and Zetzel (1) in 1930 described the use of grain-fed pigeons as test subjects. Orally or parenterally, effective liver extracts gave marked increase in pigeon reticulocyte percentages without significant rise in red blood cells or hemoglobin. Normal saline, histamine, vitamins B_1 and B_2, human gastric juice and casein gave essentially negative reticulocyte responses. Beef steak gave results similar to those from liver, while leucine produced suggestive effects although known to be negative clinically. Recently Edmunds, Brueckner and Fritzell (2, 3) reported encouraging results with this method.

The present study was undertaken to learn more about the practicability of the test and its specificity for the principle in question. The effects of injecting normal saline, leucine, histidine, tryptophane, ash of liver extract and several intramuscular liver preparations were chosen for investigation. Histidine and tryptophane were included because of the claim of Fontes and Thivolle (4) that these amino acids together are the essential factors lacking in primary anemia. Determinations of erythrocyte and hemoglobin levels were not contemplated, but they were followed in a number of experiments after it was noticed that long confinement on the rather restricted diet apparently produced subnormal blood conditions.

METHODS.

General.—Birds were taken without reference to sex or breed, and caged singly or in pairs. Diet was restricted throughout to the mixed grain, and water. In the earlier experiments, weights and reticulocyte percentages were determined at intervals during the fore-periods as well as the experimental periods. Later, weighings were made only for the purpose of comparing the dosages per unit of body weight. Most injections were made after the initially high (around 20%) reticulocyte levels had subsided to 11–15% and were fairly constant or only slowly changing. The counts were made at one- to two-day intervals just preceding and throughout the injection periods, but at longer intervals after recession of the reticulocyte curves from their peaks. When red cell and hemoglobin were determined, samples were taken shortly before injection, at or near the peak of the reticulocyte rise, and again about a week later.

Blood Collection and Staining.—Blood samples were taken at about the same hour in the morning, always before injections. One or two drops of blood from a wing vein were mixed in small glass cups with about twice that volume of a solution of 0.85% NaCl containing about 1% potassium oxalate. Smears, two for each sample, were spread by the slide method, with a spreading-slide having smoothly ground, beveled edges. The present procedure was developed when it was found that the use of brilliant cresyl blue, before or after drying of the smear, with or without alcohol fixation or the use of Wright's stain, variously caused the pigeon erythrocytes to hemolyze, fragment or stain too lightly. The presence of a nucleus in these cells obviated the necessity of using a counter-stain when it was found that methylene blue (Löffler's, in 0.85% NaCl) satisfactorily stains both nucleus and reticulum. The air-dried smears simply were suspended in a jar of this stain for three minutes or so, removed, washed briefly in running water, and again air-dried. With this relatively simple procedure, a very high proportion of excellent preparations resulted. These stains tended to fade when covered with oil for several hours. For the earlier experiments, the reported values for reticulocyte percentage are based upon

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average counts made by two individuals; later, by one and the same individual. In either case, at least 1000 cells were counted for each sample, and 2000–4000 cells when the distribution was irregular. Except early in the first few experiments, all but the most doubtful reticulated cells were counted as reticulocytes.

Blood for red counts and hemoglobin were collected from the same veins in the usual pipettes. The instruments (improved Neubauer, Sahli) were not specially standardized, but as the same set of each apparatus was used throughout, and by the same individuals, the figures reported for either determination are strictly comparable.

Preparations, Dosages, etc.—The lots of intramuscular liver extract used (excepting sample "X," 1 cc. = 33.3 Gm. liver) were of approximately the same liver equivalence (1 cc. = 5 Gm. of liver extracted). Phenol or tricresol 0.05% was used as preservative. Dosages were chosen to provide about 156 mg. of total solids, as determined before neutralization and sterilization. This required 0.70–0.75 cc. of extract in all. This amount, whether given in 1, 3 or 6 injections hereinafter is referred to as a "single dose," half the amount as a "half dose," etc. Organic solids, approximated by deducting ash from total solids, amounted to 147 mg. per cc. in one representative lot.

Saline 0.85% for injection contained phenol 0.05%.

The amino acid dosages were about equivalent to the organic solids contained in the single dose of liver extract. Histidine-HCl and tryptophane were about equal by weight in Experiment 9*b*, and 2:1 molecularly, respectively, in Experiments 5*a*, 10*b* and 11*c*.

Ash was dissolved in HCl, filtered, neutralized and made to double the volume of the extract ashed; ash obtained from the single dose of extract was given in each of the two tests.

Only the liver extracts were Berkfeld filtered; other solutions were heat treated excepting those of histidine-tryptophane designated later. Injections were intramuscular or subcutaneous into the breast excepting the 3 saline tests made on leg muscles.

All birds which gave negative responses after administration of inert substances were shown to be responsive to liver extract at some time or other.

RESULTS AND DISCUSSION.

Control Periods.—The reticulocyte percentages (hereinafter abbreviated to R. P.) for six of the first seven birds fell from 15–22 per cent down to 8–10 per cent (in one case, to 5 per cent), after about 25 days of confinement. Instead of leveling off at the minimum as expected, many of the curves climbed again before coming to a fairly constant level in the range 11–13 per cent. For brevity these parts of the curves, as well as most of the negative curves for other substances, are omitted from the figures and tables.

The first saline control experiment unfortunately was begun during the unexpected rise following the first minimum, and resulted in an apparent boost from 13% to 19%. The same bird later responded to a single dose of liver extract, one injection, by a rise from 13% to over 21%. Three other initial saline controls were entirely negative.

Effect of Liver Extract.—Liver extract injections produced significant reticulocyte responses in all instances. While the double dose (Experiment 1*a*, Table I) gave only slightly greater absolute rise in R. P. than did the average single dose (2*a*–11*a*), the latter averaged about twice as effective as the half doses (7*d*, 10*d*). The relative rises in R. P. vary much more than the absolute increases, since in general the greater responses occurred with the lower starting levels of R. P. Evaluation of the absolute rise in R. P. per unit of dosage per kilo of body weight should give better comparative figures ("Response Index"). The greatest variation of this index occurs between Experiments 6*b* and 4*c*—about 61 per cent, based on the lower value (2.3). It is felt that the variations between some of the other

experiments would have been less if only those birds weighing 300–400 Gm. had been used, since the smaller birds, as compared with the larger, usually did not seem to respond (to the same amount of extract) quite in inverse proportion to the body weights. Perhaps the single dose was above the optimum dose for these smaller pigeons. In Experiments 2*a*, 4*a* and 4*c*, delayed secondary peaks in the R. P. curves occurred. The higher points appear to be nearer the correct values, as compared with the average results. Apparently, the double rises are more likely to occur after multiple injections. In 4*c* (1 injection) the difference between the two peaks, both in time and in R. P., was much less than after 6 injections in 2*a* and 4*a*. Obviously, a considerable number of tests would have to be made to obtain a quantitative assay.

In a number of the earlier experiments, small hematomas formed around the wing veins, but probably caused no stimulation of hematopoiesis, since some of them accompanied the level or descending curves after salt or other inert sub-

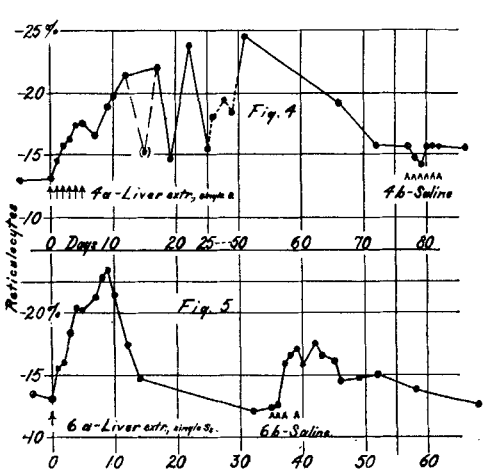

Fig. 4 and Fig 5.—Effect of intramuscular liver extract, followed by saline, on pigeon reticulocyte percentages.

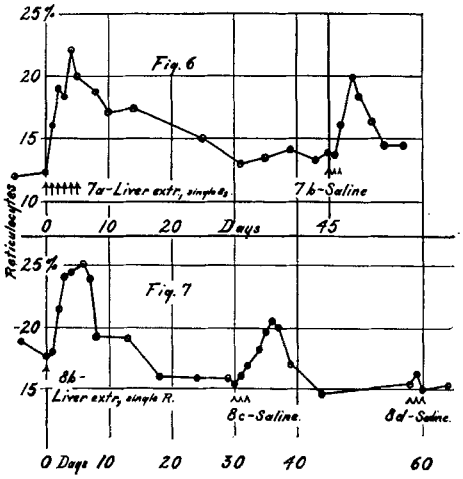

Fig. 6 and Fig. 7.—Effect of intramuscular liver extract, followed by saline, on pigeon reticulocyte percentages.

stances. Significant hemorrhage occurred in only one instance, a salt experiment to be discussed later. All birds treated with liver extracts appeared to be in good general health throughout the experiments.

Red cell and hemoglobin determinations were undertaken after the diet and the prolonged confinement appeared to produce blood paler in color, with increased erythrocyte size, while liver extract treatment seemed to deepen the color. The results (Table II and Fig. 8) indicate probable increases in hemoglobin and notably higher red cell counts after the single doses. It was necessary to dilute the third sample 1:1 in Experiment 4*c* in order to obtain a hemoglobin reading on the (Newcomer) instrument scale, but the values have been omitted because of probable error in readings for the first two samples. In Experiment 11*a* the r. b. c. increased one million even with a high starting level.

Except, possibly, for earlier response, the results agree qualitatively with those of Edmunds, Brueckner and Fritzell, but close comparisons are difficult

because of the differences in dosage and starting levels. As compared with the results of Vaughan *et al.*, for equivalent doses of similar extracts, the reticulocyte responses are of about the same order. Their findings that red cell counts were not appreciably influenced, very likely may be explained on the basis that their last samples were taken the day after the last injection, *i. e.*, the seventh day after the first injection. In Table II it appears that the maximum values for red cells are reached somewhat later, and that the increase at 7 days (Experiment 4*c*) may be slight. The fact that our doses were given in one injection, or in three at the most, that unknown hereditary or environmental factors may have operated, or that there may have been actual differences in the extracts used, must also be considered. That the extracts employed in the present work produced no weight increase beyond the normal fluctuation possibly indicates a difference in composition. Over *long* periods, the diet and inactivity maintained or increased the weight of all birds used.

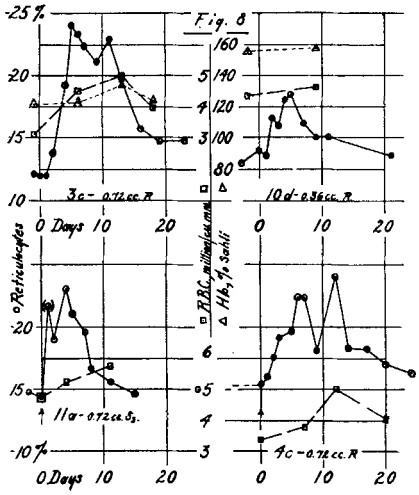

Fig. 8.—Effect of liver extract intramuscularly on reticulocytes, red blood cells and hemoglobin in pigeons.

Salt Injections after Liver.—When the R. P. of the first pigeon injected with liver extract had fallen to a level, at 15–16%, saline injections into the breast were begun. The day after the second injection, the R. P. had risen to 22.3%, when the bird was discarded. It was then decided to follow this effect in other birds. (Fig. 4 to 7; Fig. 1 to 3 have been omitted to save space.) Thus it was possible to show that, for the dosages used, the magnitude of the effect gradually and rather evenly decreased until it became negligible at 70–77 days after the last injection of liver. The only satisfactory explanation available was that the saline "flushed out" from the site of the injections a quantity of the liver substance which otherwise but gradually would become absorbed. In 2 later tests, brief massage of the injected areas on several different days produced no response, although distilled water gave a 2.5% rise in one. Injection of salt was then made in the leg muscles after liver in the breast, with a tremendous rise resulting. This bird, however, had bled heavily after blood sampling on the day before the first salt injection. A second such experiment, soon after a half dose of extract, brought a 3.8% increase, practically to the peak after liver, though from a higher starting level. Salt was then injected into the leg muscle of a pigeon which had received no previous liver treatment. No R. P. rise resulted. Previous liver administration apparently is necessary if the salt effect is to be elicited by injection either into the leg or the breast. These results render the explanation of the salt-after-liver effect still more obscure, since it scarcely is conceivable that the injection into the leg of as little as 0.72 cc. of physiological saline, divided into 2 or 3 doses, could directly cause liberation of any active principle stored at some remote site.

Ash of Liver Extract; Leucine.—Two experiments with each of these preparations showed no reticulocyte response.

Histidine and Tryptophane.—The first trial with the combined amino acids (9*b*), in which about 114 mg. were given in 4 injections, brought a prompt but transient R. P. rise of 6.8%. The peak was reached before the third injection and the curve had descended below the starting level two days after the last injection. This bird undoubtedly was sick, as shown by ruffled feathers and marked diarrhea during and for at least a month after the injection period, but whether the condition preceded the first injection was not noticed. In the next test (5*a*), 100 mg. produced

a 3.5% rise, lasting somewhat longer, and without pathological symptoms. This experiment was the only one of this series in which red cell and hemoglobin determinations were made (Table II). Although there was an apparent slight increase in hemoglobin, the red cell count continued to decrease. Each of these solutions had been made definitely alkaline to litmus by addition of NaOH before sterilization in the boiling water-bath, upon which, particularly in the first, a yellow discoloration resulted. Because of this, and in view of the known lability of tryptophane, it seemed possible that decomposition products might have been responsible for the observed effects. For the next experiment (10*b*), with 100 mg. of the combined acids, Na_2CO_3 was used for neutralization and the solution was not heated. The results were definitely negative, as also in a fourth trial (11*c*) in which were used 112 mg. from a new source of supply, neutralized with NaOH and heat treated. Two more experiments, respectively using histidine-HCl (78 mg.), and tryptophane (75 mg.) separately, made slightly alkaline with $NaHCO_3$ and heated, also gave no increase in R. P. although the tryptophane solution was fairly yellow in color.

The positive results in Experiment 9*b* may as reasonably be attributed to sickness of the bird as to injections of the amino acids, since in two instances subsequently, R. P. increases were found to accompany diarrhea when nothing had been injected. Whether to attribute the moderate R. P. response in 5*a* to histidine-tryptophane, to decomposition products thereof, or to unknown causes remains debatable. The existence of difference in degree of decomposition as compared with the subsequent negative experiments is a possibility. Whatever the rôle of tryptophane and histidine in pernicious anemia it seems justifiable to conclude that they, as free amino acids, are not the substances in liver extract effective in the pigeon. Although histidine was found present by qualitative test, only combined tryptophane was demonstrated, and it is extremely unlikely that more than a small fraction of the organic solids of the extracts is composed of these amino acids. The pigeon-active crystalline material isolated from liver extract, and used in Experiment 5*b* (Table II) gave negative tests for histidine, tryptophane and tyrosine. Further details are omitted since the crystals later were found to be impure and too little was available for a complete study.

Copper.—Although ash was ineffective, the fact that liver extract produced much more increase in erythrocyte count than in hemoglobin indicated that the possible effect of copper should be checked separately. Each of 3 birds was given 0.01 mg. Cu (0.1 cc. of $CuSO_4$) in single injections. This amount of copper was practically twice that found by analysis in the single dose of preparation T. Two of the birds showed no response; the third, an R. P. rise of 3.5 per cent, about equal to the result after a half dose of liver extract. Thus the presence of traces of copper appears to account for little more than a quarter, if any, of the response noted after liver extract injection.

Clinical Comparison.—Unfortunately, circumstances have prevented extensive clinical testing of the extracts used in the present work. Preparation "R" in four cases of tropical sprue exhibited low potency as compared with a well-known commercial product. Another sample failed to provide maintenance in a case of pernicious anemia, although equal volume dosages of commercial extract "X" proved adequate.[1] Since equal volumes of "R" and "X" produced about the same response in the pigeon, the assumption that the pigeon test is specific for the anti-pernicious anemia principle may be unjustified Also, Edmunds, *et al.* observed destruction of pigeon potency upon heating an extract at 75° for 15 minutes, yet clinically potent preparations often have been sterilized by heat treatment.

It should be noted that the dose necessary to give significant reticulocyte increase in the "normal" pigeon is tremendously large compared (on the basis of body weight) with the clinically therapeutic dose. Thus, 0.36 cc. of "X" in the

[1] We are indebted to Dr. Rafael Molina, University of Porto Rico, San Juan, P. R., and Dr. Wm. B. Porter, Medical College of Virginia, Richmond, for the reports on sprue and pernicious anemia, respectively.

400-Gm. pigeon (8*f*) is equivalent to 54 cc. in a 60-kilo patient. Yet the pigeon reticulocyte increase was only 3.6 per cent, 27 per cent over the starting level, and only about twice as great as a possible normal fluctuation.

General Recommendations for Performance of the Pigeon Test.—To attain more uniform results, certain recommendations seem warranted. Birds weighing 300–400 Gm. are preferable. Those which exhibit diarrhea should be discarded. To guard against spontaneous reticulocyte rises which occasionally occur, two or three blood samples had best be examined at close intervals before, and including the day of injection. The flow of blood should be stopped promptly after sampling (this usually offers no difficulty). The dosage for active extracts should be chosen to give an absolute increase in reticulocyte percentage of at least 8–12 per cent when the optimum initial level of 11–13 per cent is used. Single injections are preferred if the resultant volume is

TABLE I.—RETICULOCYTE RESPONSES IN GRAIN-FED PIGEONS AFTER LIVER EXTRACT INTRAMUSCULARLY.

Pigeon.	Experiment.	Total Dose, Cc.	No. Injs.	Ret. Span.	Ret. Abs.	Rise % Rel.	Wt. at Inj., Gm.	Response Index.[1]
1	1*a*	1.80Q	6	10.8–23.1	12.3	114	253	1.6–
2[3]	2*a*	0.72R	6	11.9–20.3	8.4	71	211	1.8–
				–25.1	13.2	111		or 2.8–
3	3*a*	0.75S_3	6	11.0–22.3	11.3	103	264	3.0
	3*c*	0.72R	3	12.0–24.0	12.0	100	(280)	(3.4–)
4	4*a*	0.90Q	6	13.2–22.0	8.8	67	229	2.0
				–24.5	11.3	86		or 2.6
	4*c*	0.72R	1	15.4–22.4	7.0	45	260	(1.8)
				–24.1	8.7	56	est.	(2.3–)
	4*d*	0.72T	2	14.0–25.2	11.2	80	245	2.7
6	6*b*	0.75S_3	1	13.1–23.4	10.3	79	360	3.7
7	7*a*	0.78S_3	6	12.3–22.0	9.7	79	323	3.1
8	8*b*	0.72R	1	17.7–25.0	7.3	41	(400)	(2.9)
11	11*a*	0.72S_3	1	14.4–23.1	8.7	60	(316)	(2.7)
7[4]	7*d*	0.36R′	1	12.3–17.2	4.9	40	336	3.3
10	10*d*	0.36R′	1	14.0–18.5	4.5	32	379	3.3
6	6*e*	0.36X[2]	1	12.3–15.9	3.6	29	396	2.9
8	8*f*	0.36X[2]	1	12.7–16.1	3.4	27	400	2.7

[1] Response index = $\frac{\text{Absolute rise in R. P.}}{\text{No. of "Single dosages" per Kg. of body weight}}$

[2] X = A commercial preparation.

[3] Experiments 2*a*–11*a*, "single doses." Bracketed figures indicate a second, delayed peak in reticulocyte curve. Parentheses indicate figures are subject to some error because of failure to weigh birds at time of injection.

[4] Experiments 7*d*–8*f*, "Half doses."

not too large, as this saves time and labor and probably gives smoother curves. Reticulocytes should be determined at one- to two-day intervals until after the maximum appears to have been passed. The same pigeon can be used for repeated tests if disappearance of the active substance is demonstrated by salt injection before each new experiment, or if the time between tests is known to be ample from previous experience.

CONCLUSIONS.

Inorganic constituents (liver extract ash, copper), leucine and likely histidine and tryptophane have been eliminated as constituents of liver extract effective in increasing the reticulocyte percentage in the blood of grain-fed pigeons. Liver extract injection also significantly increases the concentration of red blood cells

and probably the hemoglobin in such birds. While these results strengthen the assumption that the pigeon response is a measure of the substance or substances effective in pernicious anemia, limited clinical comparisons indicate that the pigeon

TABLE II.—RED CELL AND HEMOGLOBIN RESPONSES.

Experiment.	Dose.	Days after 1st Inj	Retic. %.	Red Cells, Million Per Cu. Mm.	Hemoglobin, Sahli %
3*c*	Single R, in 3 injs.	0	12.1	3.12	122
		7	23.3[1]	4.54	123
		14	19.7	5.00	135
4*c*	Single R, 1 inj.	0	15.4	3.40	
		7	22.4	3.81	Unchanged (?)
		12	24.1[2]	5.02	Increased[3]
		20	17.0	4.08	
5*a*	Histidine, tryptophane, 100 mg. in 2 injs.	−3	15.8	3.84	83
		−1	15.6	3.74	
		1	16.0		
		3	16.8	3.14	
		7	19.3[4]	3.39	90
		13	15.0	3.36 (10/31/32)	
5*b*	Crystals (L. E.) 44 mg. 3 injs.	0	14.8		
		9	19.2[5]	4.91 (1/4/33)	
10*d*	Half R, 1 inj.	−2		4.37	156
		0	14.0[6]		
		9	15.1	4.63	158
11*a*	Single S_8, 1 inj.	0	14.4	4.77	
		4	23.1[7]	5.23	
		11	15.6	5.77	

Remarks: [1] Max., 24.0%, 6th day. [2] Max. [3] Figures (Newcomer) omitted because of error in reading. [4] Max.; R. P. rise not typical (?). See discussion. [5] Max., 22.5%, 5th day. [6] Max., 18.5%, 5th day. [7] Max.

effectiveness may not parallel the clinical response. Further clinical comparison is necessary to settle the question of specificity or practical utility.

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A METHOD FOR THE DETERMINATION OF MINUTE AMOUNTS OF ALDEHYDES IN ETHER.*

BY M. W. CAREY, L. W. GREEN AND R. E. SCHOETZOW.

Several investigators (1, 2) have shown that the U. S. P. X test, for aldehydes in ether, is not sensitive to small amounts. The use of solid potassium hydroxide instead of the solution which is directed by the U. S. P. X, will increase the sensitivity so that between 50 and 100 parts per million of acetaldehyde may be detected

* Scientific Section, A. PH. A., Toronto meeting, 1932.