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THE RESPONSE OF RETICULOCYTES TO POTENT DIETS IN SEVERE EXPERIMENTAL ANEMIA DUE TO HEMORRHAGE.

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The appearance of reticulated red blood cells in the peripheral circulation has for several years been a matter of considerable clinical interest. Investigation has indicated that definite increase in reticulocytes may be obtained following liver and iron therapy in posthemorrhagic anemias.[6,3]

This paper reports the results of a study of the reticulocyte response to potent diets in carefully controlled experimental anemias. The animals used are highly standardized dogs, suffering from a severe anemia existing for a period of years due to blood removal.

Methods. The present experiments were carried out on dogs which are being used for the study of hemoglobin and red cell regeneration. The general experimental procedures to which these animals are subjected have been described before.[5]

The experimental animals were born and raised in our kennels and are kept under practically constant conditions with regard to exercise, ventilation and uniform temperature in the dog rooms. The dogs are rendered anemic by four or more bleedings spaced at 1- or 2-day intervals. The desired anemia level of 40 to 50 per cent hemoglobin is then maintained as near a constant as possible by further hemorrhages graded as to frequency and amounts. Some of the hemoglobin values recorded on the tables may appear as high ones. These high figures, however, represent the levels just prior to further removal of blood in order again to reach the desired anemia level. Blood plasma volume determinations are essential since fluctuations of plasma volume must be taken into consideration in estimating the required bleeding. Hemoglobin pigment in grams is determined in every aspirated bleeding. Hemoglobin is measured in the form of acid hematin colorimetrically against a standard which at 100 per cent is equivalent to 13.8 gm. of hemoglobin per 100 cc. The animals are kept on a standard basal ration consisting of a bread mixture prepared in the laboratory. This basal ration bread is adequate to maintain the anemic dog in health for years and yet permits of but minimal hemoglobin and red cell regeneration over and above the maintenance factor. This bread ration is used in the control periods alternating with or combined with other food factors or drugs to be tested. The animals used in this experiment belong to this carefully standardized anemic colony and their response to various diets is well known. The degree of regeneration is measured by the amount of hemoglobin produced each week over and above the maintenance factor. This maintenance factor indicates the hemoglobin and red cells used up by the general wear and tear of the circulation in the body. We also speak of a "carry over." By this is meant that a dog on a favorable diet will store somewhere in its tissues or organs the ingredients for hemoglobin and red cell construction which later during unfavorable diet periods will be drawn upon for the manufacture of hemoglobin and red cells.

It must be emphasized that the bone marrow is under sustained maximal stimulation because of the long existing anemia and repeated bleedings. The diet is adequate for general maintenance requirements. There were no complicating factors of an infectious or toxic nature. After the control period on the standard bread diet lasting 2 or more weeks, diets known to be very potent for hemoglobin regeneration were fed during a 2-week period which was again followed by the control basal bread ration. In one case, iron was administered intravenously. Daily reticulocyte and erythrocyte counts were made in addition to the usual weekly sampling and blood volume determinations, all blood being obtained by jugular puncture. For reticulocyte counts, the wet method was used. As the blood flowed freely from the needle in the jugular vein, a small drop was rapidly transferred on the tip of a glass rod to a cover-slip. The cover-slip was then gently inverted on a slide on which a film of 0.5 per cent alcoholic solution of brilliant cresyl blue had been dried. The preparation was rimmed with vaseline and the number of reticulocytes in 1000 red cells were counted under oil immersion. Care was taken to count all of the cells in several different areas of the slides in order to avoid errors due to clumping of reticulocytes. The number of reticulocytes per cubic millimeter of blood can be readily calculated from the total red blood cell count and the reticulocyte per cent.

Dry Method. Permanently stained smears, using the method of Hawes[1] and Cunningham,[2] were made in one series of experiments. It was found that the cells failed to stain well with the brilliant cresyl blue. A satisfactory explanation of this has not as yet been found. It is possible that the cells did not remain sufficiently long in contact with the dye, before the smear was pulled. Increased speed of coagulation necessitated immediate pulling of smears. Citrated blood was not used. There is a possibility that there is some change in the permeability of the red cells of the anemic dog. They are known to be much less fragile than the normal. Because of the discrepancies in the results obtained by the dry method, they are not included in this paper.

Experimental Data. The blood picture, in general, presented by these animals is that of an extreme, secondary anemia, with aniso- and poikilocytosis and erythrocytes of very small diameter. Microcytes and what appear to be cell fragments are also present in large numbers. The reticulocytes vary from large pale cells to cells of very small size. The reticulum is often very dense, as noted by Seyfarth in anemias with rapid regeneration.[4] Normoblasts appear very rarely.

The blood platelets present an extremely interesting picture. They attain an enormous size, approximating in many cases the diameter of a normal erythrocyte. They appear singly or grouped in clusters. In certain instances these platelets are present in extraordinarily large numbers, particularly in Dog 27-241, Table 3.

TABLE 1.—LIVER AND IRON (DOG 25–97, BULL, FEMALE, ADULT).

Diet, Grams per day.	Date.	Reticulocytes.		R. B. C.	Hb. removed by bleeding.	Hb. level.	R. B. C. vol. hematokrit.	Plasma vol.
		Thous- per c.mm.	Per cent.	Millions per c.mm.	Gm.	Per cent.	Per cent.	Cc.
Bread (S) 450*	July 11	255	4.9	5.2	1.4	49	21.5	1177
	12	210	4.5	4.7				
{Liver 300	13							
{Fe. 40 mg.	14	323	6.6	4.9				
{Bread (S) 400	15	...	4.0					
	16	140	3.1	4.5				
	17	143	2.8	5.1				
	18	222	3.9	5.7	....	69	30.6	1058
	19	260	4.0	6.5	16.5			
	20							
	21	204	3.0	6.8	17.5			
	22	180	3.1	5.8	12.3			
	23	125	3.3	3.8				
	24	243	4.2	5.8	....	67	30.6	
	25	213	3.8	5.6	....	71	30.7	1034
	26	297	5.4	5.5	17.0			
Bread (S) 450*	27							
	28	155	3.1	5.0	16.6			
	29	69	1.5	4.6	11.2			
	30	100	2.0	5.0				
	31	143	2.7	5.3	....	67	28.3	
	Aug. 1	141	3.0	4.7	....	62	27.3	1075
	2	105	2.3	4.6	16.7			
	3							
	4	103	2.4	4.3	11.4			
	5	70	2.0	3.5				
	6	121	3.2	3.8	....	52	22.4	
	7	144	3.9	3.7				
	8	138	3.3	4.2	....	45	19.5	1124
	9	140	3.6	3.9				

* Salmon 75, Klim 25.

The detailed observations are recorded in Tables 1 to 6 and show certain points which are worthy of emphasis. In each dog the total red blood cell curve does not parallel the hemoglobin curve, the rise in red blood cell not being proportional to the amount of hemoglobin.

Dog 25-97. This animal has been raised on a liver diet since weaning and has always responded much more actively with hemoglobin regeneration to any diet than the usual anemic dogs. This experiment demonstrates a high reticulocyte level during the control period when hemoglobin regeneration is low. The pre-control period was preceded by a potent dietary régime with much hemoglobin production. When the animal is put on liver and iron which constitutes one of the most potent hemoglobin-building diets the reticulocyte response is only a questionable one. The hemoglobin production during this two-week test diet period amounts to 63 gm. over and above the maintenance level. The total hemoglobin output due to the liver and iron feeding is figured as approximately 120 gm. in this experiment.

TABLE 2.—LIVER AND IRON (DOG 24–45, BULL, FEMALE, ADULT).

Diet, Grams per day.	Date.	Reticulocytes.		R. B. C.	Hb. removed by bleeding.	Hb. level	R. B. C. vol. hematokrit.	Plasma vol.
		Thous. per c.mm.	Per cent.	Millions per c.mm.	Gm.	Per cent.	Per cent.	Cc.
Bread (S) 500*	July 7	...	...	4.7	18.8	45	19.5	1228
	14	68	1.4	4.9	....	47	20.3	1256
	15	43	1.1	3.9				
Liver 300	16	33	1.0	3.3				
Fe 40 mg.	17	37	1.1	3.4				
Bread (S) 300*	18	31	0.9	3.4				
	19	59	1.5	3.9				
	20							
	21	325	5.8	5.6	....	66	27.9	1155
	22	211	4.4	4.8	15.1			
	23	129	3.0	4.3	15.1			
	24	215	3.9	5.5				
	25	75	1.7	4.4	....	58	25.5	
	26	79	1.8	4.4				
	27							
	28	264	4.0	6.6	....	90	38.9	1068
	29	70	1.3	5.4	20.5			
Bread (S) 500*	30							
	31	92	1.7	5.4	18.9			
	Aug. 1	39	0.9	4.3	17.0			
	2	17	0.4	4.2	....	56	24.3	
	3							
	4	17	0.4	4.2	....	59	24.9	1180
	5	25	0.6	4.1	13.9			
	6	65	1.5	4.3	11.5			
	7	29	0.9	3.2				
	8	26	0.8	3.3	....	47	21.7	

* Salmon 75, Klim 30.

The actual reticulocyte per cent increase is but 20 per cent whereas the reticulocyte concentration (red cells x per cent reticulocytes) shows a rise of 48 per cent. No acute response of reticulocytes follows the various bleedings. When the dog is again placed on the control diet, one notices a definite drop in reticulocytes which reaches a lower level than that during the previous control period.

This occurs in spite of a carry over of hemoglobin production of 56 gm.

Table 2 depicts another experiment of liver and iron feeding. The reticulocyte level during the control bread period is considerably below that presented by other dogs of this experimental series.

Dog 24–45 demonstrates a definite reticulocyte response on the 6th day of the potent diet feeding, increasing to 5.8 per cent or a reticulocyte concentration of from the beginning average of 55,000

TABLE 3.—LIVER (DOG 27–241, COACH, FEMALE, ADULT).

Diet, Grams per day.	Date.	Reticulocytes.		R. B. C.	Hb. removed by bleeding.	Hb. level.	R. B. C. vol. hematokrit.	Plasma vol.
		Thous. per c.mm.	Per cent.	Millions per c.mm.	Gm.	Per cent.	Per cent.	Cc.
Bread (S) 325*	June 27	...	....	4.9	....	42	19.5	820
	July 2	258	4.7	5.5				
	3	285	5.6	5.1	....	47	22.2	860
	4	312	5.9	5.3				
	5	296	5.6	5.3				
{Liver A-924-230	6	557	8.7	6.4				
{Bread (S) 325*	7	550	11.0	5.0				
	8	676	10.9	6.2				
	9	324	5.6	5.8				
	10	383	6.5	5.9				
	11	233	3.5	6.7	....	70	31.5	787
	12	288	4.0	7.2	18.3			
Bread (S) 325*	13							
	14	240	4.0	6.0	19.3			
	15	212	5.6	3.8				
	16	190	3.8	5.0	....	61	27.0	
	17	138	3.3	4.2				
	18	131	2.8	4.7	....	53	23.1	805
	19	211	4.6	4.6	13.6			
	20							
	21	181	4.2	4.3	....	50	21.6	
	22	202	4.8	4.2				
	23	184	4.1	4.5				
	24	280	5.1	5.5				
	25	165	3.6	4.6	....	57	23.1	797

* Salmon 100, Klim 30.

to 325,000 on the liver and iron diet. This constitutes about a 500 per cent increase in reticulocyte concentration. This result is in marked contrast to the previous experiment, Table 1. Six days later there is another rise to 4 per cent or 264,000 reticulocytes per c.mm. with a subsequent drop to the control level.

Dog 27–241 shows the result of the feeding of liver. The reticulocytes again are high in the control bread period which in this case has not been preceded by a potent-hemoglobin regenerating diet. They average 280,000 per c.mm. and 5.45 per cent in spite of low hemoglobin production. On the liver diet there is a marked response lasting 3 days, rising to 676,000 and 11 per cent. This constitutes a reticulocyte concentration increase of 130 per cent. During the second control period there is a drop in reticulocytes to below the initial level, although there is a carry over of hemoglobin production.

TABLE 4.—SECONDARY ANEMIA LIVER FRACTION (DOG 24-2, BULL FEMALE, ADULT)

Diet, Grams per day.	Date.	Reticulocytes.		R. B. C.	Hb. removed by bleeding.	Hb. level	R. B. C. vol. hematokrit.	Plasma vol.
		Thous. per c.mm.	Per cent.	Millions per c.mm.	Grams.	Per cent.	Per cent.	Cc.
Bread (S) 325*	July 2	126	2.8	4.5	11.9	51	23.5	945
	3	175	3.8	4.6				
	4	...	3.5					
	5	126	3.6	3.5	....	46	20.1	
	6	229	5.6	4.1				
	7	118	3.2	3.7				
	8	102	3.0	3.4				
	9	176	4.0	4.4	....	49	22.4	942
	10	253	6.5	3.9				
Sec. anemia fraction† Bread (S) 325*	11	...	3.5					
	12	110	2.9	3.8				
	13							
	14	253	6.5	3.9				
	15	198	4.6	4.3				
	16	196	3.7	5.2	....	59	24.3	939
	17	176	4.0	4.4	12.2			
	18	172	4.3	4.0	12.0			
	19	189	5.4	3.5				
	20							
	21	209	5.5	3.8	....	58	24.2	
	22	176	4.3	4.1				
	23	271	6.6	4.1	....	54	23.5	961
	24	225	4.9	4.6	17.3			
Bread (S) 325*	25	179	4.7	3.8				
	26	129	3.0	4.3	....	58	23.6	
	27							
	28	106	2.8	3.8				
	29	125	3.2	3.9				
	30	...	...	4.7	....	64	26.3	926
	31	167	3.8	4.4	14.9			
	Aug. 1	117	3.9	3.0	10.9			
	2	133	3.8	3.5				
	3							
	4	80	2.5	3.2	....	52	22.5	
	5	131	4.1	3.2				
	6	122	3.6	3.4	....	47	20.2	1016

* Salmon 75, Klim 25.

† 500 gm. fresh liver equivalent.

We again observed a high reticulocyte count in the first control period and there is considerable variation from day to day, the average for the period being 163,200 per c.mm. and 4 per cent.

With addition of the secondary anemia liver fraction W-635, potent for hemoglobin regeneration, to the diet there is only a questionable reticulocyte response beginning on the 4th day and continuing throughout the period with considerable daily variation. This response is more significant in relation to the drop in the second control period, than it is with relation to a rise after the first.

TABLE 5.—LIVER AND SECONDARY ANEMIA LIVER FRACTION PLUS IRON (DOG 23-1, COACH, FEMALE, ADULT).

Diet, Grams per day.	Date.	Reticulocytes.		R. B. C.	Hb. removed by bleeding.	Hb. level.	R. B. C. vol. hematokrit.	Plasma vol.
		Thous. per c.mm.	Per cent.	Millions per c.mm.	Grams.	Per cent.	Per cent.	Cc.
Bread (S) 500*	July 9	...	...	4.7	....	45	20.1	1042
	10							
	11	160	3.9	4.1				
	12	155	3.6	4.3				
Liver 300	13							
Sec. an. fraction‡	14	317	6.6	4.8				
Plus Fe. 220 mg.†	15	...	3.3					
	16	161	2.6	6.2	....	60	24.3	995
	17	192	3.7	5.2	13.2			
	18	171	3.8	4.5	12.3			
	19	352	8.0	4.4				
	20							
	21	428	6.8	6.3	....	74	33.0	
	22	221	4.8	4.6				
	23	336	7.0	4.8	....	71	30.2	992
	24	205	3.4	5.9	13.9			
Bread (S) 500*	25	160	3.2	5.0	14.7			
	26	181	4.1	4.4	10.7			
	27							
	28	236	5.9	4.0	....	56	23.2	
	29	108	2.7	4.0				
	30	...	...	...	....	52	22.2	1050
	31	168	3.9	4.3	9.9			
	Aug. 1	119	3.4	3.5				
	2	154	3.5	4.4	....	53	21.7	
	3							
	4	108	2.7	4.0				
	5	53	1.4	3.8				
	6	152	4.0	3.8	....	45	19.2	1095
	7	81	2.3	3.5				

* Salmon 75, Klim 25.
† Bread (S) 350.
‡ 300 gm. fresh liver equivalent.

Dog 23-1. The reticulocyte level is again seen to be high in the first control period. On the addition of liver, plus a secondary anemia liver fraction plus additional iron to the diet, there is a reticulocyte response which seems definite on the 7th day. The reticulocyte response occurs several days after the active regeneration of hemoglobin had begun. There is again a drop in the reticulocyte level during the second control period. This dog differs from the animals described above in that the drop during the first week of the after control period is less than that during the second week, 3.98 per cent and 2.88 per cent averages, respectively.

TABLE 6.—IRON, INTRAVENOUS (DOG 27–239, BULL MONGREL, FEMALE, ADULT).

Diet, Grams per day.	Date.	Reticulocytes.		R. B. C.	Hb. removed by bleeding.	Hb. level.	R. B. C. vol. hematokrit.	Plasma vol.
		Thous. per c.mm.	Per cent.	Millions per c.mm.	Grams.	Per cent.	Per cent.	Cc.
Bread (S) 400*	July 28	...	...	4.2	....	39	18.1	800
	29	116	3.3	3.5				
	30	170	3.7	4.6				
	31	204	4.0	5.1				
Fe, i.v. 20 mg.† Bread (S) 400*	Aug. 1	249	5.3	4.7				
	2	258	6.3	4.1				
	3	490	8.3	5.9				
	4	486	9.0	5.4	....	46	22.1	737
	5	276	5.3	5.2				
	6	209	4.1	5.1				
	7	350	6.6	5.3				
	8	211	3.9	5.4				
	9	255	4.4	5.8				
	10							
	11	234	4.1	5.7	....	68	31.5	725
	12	420	5.6	7.5	14.3			
	13	346	6.3	5.5	14.1			
	14	285	6.2	4.6				
Bread (S) 400*	15	320	6.8	4.7	....	57	26.8	
	16	355	7.1	5.0				
	17	460	9.2	5.0				
	18	...	...	5.6	....	66	30.9	747
	19	...	...	...	16.3			
	20	...	...	...	12.3			
	21							
	22	...	...	...	....	56	24.8	
	23							
	24							
	25	...	...	5.1	....	57	25.3	723
	26	...	...	...	13.6			

* Salmon 75, Klim 25.
† Iron administered as colloidal—Loeser.

Dog 27–239. The reticulocyte level in the first control period for 3 days averages 167,000 per c.mm. and 3.67 per cent, which is similar to Dogs 24–2, Table 4 and 23–1, Table 5.

During the administration of iron intravenously there is a definite reticulocyte response which starts on the first day, reaches its maximum on the 4th day amounting to a reticulocyte concentration increase of 167 per cent, continues throughout the test diet period and carries over into the second control period. The daily variations are of considerable magnitude.

Following a gradual drop we again observe a rise to 420,000. Another rise occurs on the 3d day of the control period. Unfortunately the reticulocyte counts had to be discontinued before the lower bread control level was reached.

Discussion. Diets potent for hemoglobin regeneration produce but a slight or only moderate reticulocyte response under the experimental conditions reported. Most of these animals show a high reticulocyte level during the control bread period preceding the test diet régime. One also observes marked individual variations in the reticulocyte response elicited. For example Dog 25–97, Table 1, with a high initial level of reticulocytes was fed an optimum diet for hemoglobin regeneration with the usual result of a very high hemoglobin output. As has been mentioned previously this animal has been raised on a diet very rich in liver since weaning and reacts always with much greater hemoglobin manufacture than the other anemic animals of the series. Further stimulation by the liver and iron diet elicits only a very questionable response in reticulocyte output.

Dog 24–45, Table 2, on the other hand, with a much lower reticulocyte level during the control period shows a much more definite reaction to the liver and iron diet as concerns reticulocytes.

If one considers the reticulocyte concentration rather than the percentage rise this increase amounts to 500 per cent in Table 2 as compared to but 48 per cent in Table 1 on the same diet. If one uses this figure for comparison the reticulocyte increases appear to better advantage.

These experiments of course are only a few in number as we were mainly interested in ascertaining whether diets very potent in hemoglobin building properties would also demonstrate a marked increase in the reticulocytes. We observe that in the cases cited feeding the secondary anemia extract gives negative results, adding liver to this diet gives a value just slightly above that of the liver alone. Liver plus iron gave better results than either liver or iron alone. One must remember however that a large series of such experiments may give different results.

One may argue that the red blood cells are at a much higher beginning level than reported in human cases of secondary anemia and that therefore the response would be but a slight one. In considering the bone marrow response of these dogs, however, it must be emphasized that their normal hemoglobin level is approximately 130 to 160 per cent with a red cell count of 8 to 10 millions and that a red cell count of 5 millions in these animals is comparable to one of 3 million in a human being. As the majority of these dogs show red cell figures of 5 million or below at the beginning of the test diet period the anemia level is more severe than one would realize when considering the figure indicated on the tables. These dogs have been kept at an anemia level of one-third of normal for several years. The bone marrow therefore has been under maximal stimulus due to the anemia *per se*, for a long period of time. This is evidenced by the high level of reticulocytes. The added stimulus of a diet, potent in hemoglobin regeneration, does not therefore bring a marked response since stimulus is already nearly maximal. Red cell production might be considered as approaching a normal mechanism in these animals, since there is no toxic factor at work, nor is there any nutritional deficiency. The frequent bleeding masks the level to which the total red blood cell count would rise.

That there is at least some disturbance in the maturation of the erythrocytes, is evidenced, however, by the high control level of reticulocytes. The normal level for dogs is given by Seyfarth[4] as 0.2 to 0.5 per cent. Under the stress of the demand for erythrocytes, the bone marrow turns out some immature cells with the great mass of mature cells. There does not appear to be a correlation between reticulocyte formation and hemoglobin output. The reticulocyte level might be as high with a 10 gm. hemoglobin output as with one amounting to 100 gm. of hemoglobin.

Summary. The response of the reticulocytes on feeding potent diets for hemoglobin regeneration to dogs suffering from a long continued severe anemia produced by blood removal has been compared with the reaction due to the feeding of the basal bread ration. The reticulocyte level shows considerable individual variation. The results obtained demonstrate only a slight or moderate reticulocyte response to diets very potent for hemoglobin regeneration. There seem to be two factors in the potent diets fed. The most important one is the factor influencing hemoglobin regeneration. The second assists in stroma formation.

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