BLOOD PICTURE IN GUINEA PIGS DEFICIENT IN THE ANTI-STIFFNESS FACTOR

by

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Introduction

A deficiency disease of guinea pigs characterized by muscular stiffness was described by Wulzen and Bahrs in 1936 (40). The animals were raised on grain diets lacking in green feeds but adequate in minerals and necessary vitamins. This deficiency was also found by them to occur when guinea pigs were fed on pasteurized milk, supplemented by skim milk powder, copper and iron, carotene and orange juice (41).

The first overt sign of the deficiency was a muscular stiffness in the wrist region which frequently progressed until the wrist could no longer be bent (41, 38). The disease increased in severity until the animals with rare exceptions died in periods of from one month to a year or two. Autopsies showed the muscles to be extremely atrophied and in most cases finely streaked with calcium deposits running parallel to the muscle fibers. Large deposits of tricalcium phosphate were often found in and under the skin, in the joint regions, between the ribs and in many body organs including the heart and aorta (41). Raw cream fed by mouth was capable of curing the original wrist stiffness. Also molasses (37) and many green

vegetables[1] were good sources of the "anti-stiffness" factor.

Metabolic studies of the effect on guinea pigs of lack of this dietary factor have been carried on by van Wagtendonk et al (30, 31, 32, 33, 34, 35, 36, 39).

Differential counts and leucocyte enumerations on the blood of supposedly normal guinea pigs have shown wide variations both from investigator to investigator and from animal to animal (12, 13, 16, pp.590-591; 26). Lucia and Lucia (15) summarized the findings of eight investigators and reported their own findings on 303 differential counts made over a period of one year on carefully selected animals. Their findings were as uniform as those found on human blood.

Joyner (12), in 338 counts, encountered so much variation that he summarized:

> "The variations in the counts demonstrate that a fairly large range must be considered for practically all types of cells. Extreme variations suggest that the guinea pig is frequently subject to disturbances of its differential blood picture without alteration of its external appearance of health."

Scarborough (26) attempted to find correct values by averaging the differential counts of thirty-four investigators. Leucocyte values reported by many of the same investigators also show wide variations (12, 13).

[1]Unpublished material.

"Normal" figures for differential counts, red and white cell enumerations, platelets, hemoglobin estimation, and erythrocyte diameters for guinea pigs are also to be found in hematological texts and handbooks (6, pp.810-11; 16, pp.590-91).

The Kurloff inclusion has been observed in guinea pig blood cells of the lymphocyte or monocyte series (6, pp.810-811). According to Lucia and Lucia (15) it usually occurs as a homogeneous azurophilic mass varying in size from that of lymphocyte granules to twice the diameter of a red cell. They have also observed it as a spireme-like thread contained in a vacuole. Semenskaja (6, pp.810-11) found that Kurloff inclusions are not present in new-born guinea pigs but appear at the end of the second week after birth and slightly earlier in females than in males. He observed them to be slightly more numerous in pregnant than in non-pregnant females.

After the castration of male and female guinea pigs, Kurloff inclusion cells diminished but did not disappear; even after seven weeks Severi (27) found them in the blood stream, spleen, and bone marrow.

Loewenthal (14) regards the Kurloff masses not as inclusions but as "probably an intracellular reaction produced by a parasite." Lucia and Lucia (15) found the inclusion to stain supravitally as a homogeneous mass and

considered it to be dead phagocytosed material. Jarczyk (6, pp.810-11) concludes that Kurloff bodies "consist of an albuminoid substance elaborated *in situ* in the protoplasm of lymphocytes." Ferrata thought the Kurloff body to be giant azurophilic granules (6, pp.810-11).

Hintererreger (6, pp.810-11) reports that Kurloff cell inclusions stain reddish-violet with Giemsa and Wright. They are negative to the Prussian blue test for iron; they are insoluble in alcohol. They are negative to osmic acid tests for fat and to tests for glycogen and phosphorus. He also found that administration of adrenalin and pilocarpin stimulated a sharp increase of Kurloff cells. He observed that azure granules of lymphocytes responded by numerical increase to the same stimuli as produced an increase in the number of Kurloff bodies and he regarded them as genetically and functionally similar though not related. He believed that Kurloff body cells were probably related to metabolism and that their number signified increased metabolic rate.

Experimental

Guinea pigs used to establish the normal blood picture were segregated as to sex and raised on a diet of rolled barley, liberal amounts of kale or grass, iodized salt, and straw ad libitum. None showed wrist stiffness. Gravid animals were not tested.

Experimental animals were segregated as to sex and fed the following diet deficient in the anti-stiffness factor:

	a. m.	
Skim milk powder	20.0	g.
Water	80.0	g.
Copper sulfate	0.078	mg.
Ferric chloride	0.480	mg.
Ascorbic acid	10.0	mg.
	p. m.	
Skim milk powder	20.0	g.
Water	80.0	g.

Fat- and water-soluble vitamins were added alternately to the evening diet to make the average daily consumption per animal for each vitamin as follows:

Thiamin hydrochloride	0.20 mg.
Pyridoxine hydrochloride	0.10 mg.
Riboflavin	0.50 mg.
Nicotinic acid	1.00 mg.
Pantothentic acid	0.10 mg.
Inositol	10.00 mg.
P-aminobenzoic acid	2.00 mg.
Choline	50.00 mg.
Biotin (SMA conc. S200)	0.01 mg.
Beta carotene	150 I. U.
Viosterol	40 I. U.
Alpha-tocopherol	0.10 mg.
2-methyl-1, 4-naphthoquinone	0.10 mg.

All received straw and iodized salt ad lib.

For all tests animals were chosen at random from this laboratory's colonies of stock and deficient guinea pigs. All blood samples were taken from the ear which was gently stroked or warmed to cause an hyperemia (17, p.20). Incisions were made with sharp Bard-Parker "11" blades. Animals were loosely wrapped in a dish towel, sampled, and returned to pens within approximately five to eight minutes. Frightened or struggling animals were returned to pens and tested after a lapse of several hours since physiological leucocytosis is known to result from exercise and fright (2, p.182; 3, p.123). An eight-key "Lab-Count

Denominator" was used for all cell counts.

The "Student's" t-test (8, Table III) was used in testing the difference between the means of the stock animals and the deficient animals.[1] The formula used for determining this is as follows:

Let $\bar{x}$ be the mean of the observations for stock animals.

Let $\bar{y}$ be the mean of the observations for deficient animals.

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{\left(\frac{\Sigma(x - \bar{x}) + \Sigma(y - \bar{y})^2}{N_1 \quad N_2 - 2}\right)\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}} = \frac{\bar{x} - \bar{y}}{\sigma_{\bar{x} - \bar{y}}} .$$

I. The Erythrocyte

A. Enumeration.

B. Hemoglobin Content.

C. Color Index.

D. Diameter.

E. Erythropoietic Function.

F. Sedimentation.

A. Enumeration. Normal values were determined for forty stock animals including both males and females.

[1] Computations made under the supervision of Dr. J. C. R. Li, Department of Mathematics, Oregon State College.

Twenty-six determinations were made on animals subjected to the deficient diet for periods ranging from 24 to 120 weeks, average 60 weeks. See Table I.

Both normal and deficient estimations were made in June, July and August. Human erythrocyte values are said to be 500,000 higher in the winter (2, p.92; 20, p.184).

Dilutions were made 1:100 with Toisson's solution. Five groups of sixteen squares each were counted with no variation of more than ten cells from group to group permitted[1] (2, p.90; 20, p.173 and p.473). A home-made shaker which gave approximately a figure-eight rotation was used to minimize variations from sample to sample.

Table I. Erythrocyte Enumeration.
Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 24 to 120 Weeks Compared with Stock Animals.

Diet	Number of Weeks on Diet	Number of Animals	Mean	Compared with Stock	
				t	Degrees of Freedom
Stock	--	40	4,964,000		
Deficient	24-120	26	3,935,000	9.39	64

These figures indicate a significant difference in numbers of erythrocytes.

B. Hemoglobin Content. Normal values were determined for sixteen stock animals. Eighteen determinations

[1]Usual variation permitted is 20 cells from group to group.

were made of animals on the deficient diet for intervals ranging from 26 to 110 weeks, average 57 weeks. See Table II.

Estimations were made with the Sahli-Haskins technique (20, p.463) which uses a color standard unchanged over a period of sixteen years. The color standard, at 20°C, has the same color as blood containing 13.8 g. of hemoglobin per 100 cc. when it is diluted exactly to the 100 mark in the Sahli tube. Heating at 55-60°C for seven minutes completes the reaction between the hydrochloric acid and hemoglobin, a change which requires 24 hours at room temperature.

The hemoglobin coefficient[1] for this laboratory was determined to be 15.7 g. for normal female guinea pigs. In general hemoglobin is lower for women in grams per 100 cc. than in men but is approximately the same per 5,000,000 red cells (2, p.110).

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[1] "Hemoglobin coefficient" is a term suggested by Osgood (20, p.179) which replaces the expression, "the number of grams of hemoglobin per 100 cc. calculated to a count of 5,000,000 red cells" (2, p.110).

Table II. Hemoglobin Content.
Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 26-110 Weeks Compared with Stock Guinea Pigs.

Diet	Number of Weeks on Diet	Number of Animals	Mean	Compared with Stock	
				t	Degrees of Freedom
Stock	--	16	98.12%		
Deficient	26-110	18	75.80%	14.49	32

These figures indicate a significant difference in per cent of hemoglobin.

C. Color Index. The color index is the ratio between the amount of hemoglobin per unit number of red cells in the blood and the amount of hemoglobin per unit of cells in the blood of the average normal individual of the patient's sex and age group (2, p.111). Human normal values average 1.0 (range 0.85 - 1.15). Results above 1.20 and below 0.80 are considered to be definitely pathological (2, p.112). Values below normal are called hypochromic, above - hyperchromic, and normal - normochromic. Iron anemias, for example, are typically hypochromic (2, p.314; 16, p.212; 20, p.203).

Normal values were determined for sixteen stock animals. Eighteen determinations were made for animals on the deficient diet for periods ranging from 26 to 110 weeks, average 57 weeks. See Table III.

Table III. Color Index.
Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 26-110 Weeks Compared with Stock Guinea Pigs.

Diet	Number of Weeks on Diet	Number of Animals	Mean	Compared with Stock	
				t	Degrees of Freedom
Stock	--	16	0.98		
Deficient	26-110	18	0.94	1.41	33

These figures indicate a statistically insignificant difference in the color index.

D. Red Cell Diameter. Red cell diameters were measured with the Haden-Hauser Erythrocytometer (9, 10) which utilizes the principle of light diffraction for the measurement of the average diameters of large numbers of minute objects, a method originated by Thomas Young (42) and A. Pijper (24). A home-made diffractometer which gave almost exactly the same results as the Haden-Hauser Instrument was also used. The Haden-Hauser erythrocytometer is reported to be the most accurate instrument of its kind (9) and gives results which compare favorably with direct measurements by means of an eyepiece micrometer. It is probably even more accurate than the eyepiece micrometer since it measures thousands of cells at one time. Readings can be taken in one minute.

Accuracy of the instrument is said to be about one-half micron (9; 16, p.626) but is dependent upon the

quality of the slide being examined. No variation of more than one-fifth micron from slide to slide for any one animal was found in the following experiment. The elimination of any slide not giving an absolutely bright clear halo is probably the reason for this lack of variation.

Normal values were determined for thirty-nine stock animals. Forty-six determinations were made for animals on the deficient diet for periods ranging from 30 to 110 weeks, average 51 weeks. See Table IV.

Table IV. Red Cell Diameter.
Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 30-110 Weeks Compared with Stock Guinea Pigs.

Diet	Number of Weeks on Diet	Number of Animals	Mean	Compared with Stock	
				t	Degrees of Freedom
Stock	--	39	7.35μ		
Deficient	30-110	46	7.69μ	-8.56	83

These figures indicate a significant difference in red cell diameter.

E. Erythropoietic Function. This was determined by the measurement of basophilic aggregations in erythrocytes.

Pearlman and Limarzi (22) have found a close correlation between basophilic aggregation and reticulocyte levels in several diseases. They found basophilic aggregations and reticulocytes to give the same response to liver therapy in pernicious anemia. McCord, Holden and Johnston

(18), who studied over six thousand cases in the lead-poisoning epidemic of 1934-35, found the test to be accurate and concluded as did Hawes (11) that no distinction should be made, as had been customary, between polychromatophilia and stippling.

The basophilic aggregation test was used in this study in preference to reticulocyte determination because of its greater simplicity.

Technique as carried out in this laboratory - modified from Kracke (16, p.672):

Stain (Manson's methylene blue):

Borax	1 g.
Methylene blue	2 g.
Distilled water (boiling)	100 cc.

Add the borax to the boiling water. The stain is stable for two weeks and must be filtered before each using. Make smears as usual; stain not before one hour and not later than three hours after making smears. Before staining fix one half of smear (longitudinally) with a strip of filter paper carefully wetted with methyl alcohol. Allow to dry until filter paper is loose. The other half is left unfixed so that laking may take place during staining. Stain for ten minutes. Rinse carefully with distilled water. Air dry.

Seek with low power or high dry power the point on

the unfixed part of the slide where the maximum number of basophilic aggregations is to be found. This, for guinea pigs, was at the point near the termination of the smear where the erythrocytes were still quite evenly distributed but were just beginning to be arranged in circles.

Count with oil immersion only. Many aggregations cannot be seen at all with high dry power. Count five or more sets of ten fields each from the center of the slide towards the edge in this region of maximum concentration. Select the four highest sets - e.g., typical figures are 2, 2, 4, 7, 5, 3, 2. Just opposite this area, on the fixed side, count several fields of red cells. Basophilic aggregation is expressed as a percentage of the red blood cells. In this laboratory twenty fields of fixed cells were counted to determine the average number of erythrocytes. Counting of erythrocytes and basophilic aggregations was facilitated through restricting the field that could be observed at one time by inserting in the eyepiece a cardboard with a hole cut to limit the field to approximately sixty red cells at 1080 X. This was further subdivided with hairs to four squares.

The technique of counting the maximum number of basophilic aggregations made this test accurate whereas counting in scattered areas gave wide variations from slide to slide or even on the same slide if recounted. Slides can

be counted in five to seven minutes.

Pearlman and Limarzi, in comparing reticulocytes with basophilic aggregations, criticized the basophilic aggregate technique on the following points:

Pearlman and Limarzi (22)	This laboratory and others.
1. The technique is poor because cells stained 12 hours to 5 days after prepared gave lower results than smears stained within 12 hours after preparation.	1. Time limits for staining were specifically stated to be 1 to 3 hours after preparation of the slide (18: 16, p.672). The criticism on this point is weak since reticulocyte stains are "vital" stains.
2. Stain precipitates; stain unreliable.	2. Always filter stain before using. No Difficulty with unreliability if stain is made up fresh (in this lab. the stain is made up fresh each week). Inaccurate results are obtained if slides are stained in Coplin jars without filtering stain immediately prior to use; scum settles over the smear and prevents the proper reaction between erythrocytes and stain, giving areas on the slide where the reaction has occurred and others almost totally negative.

Pearlman and Limarzi	This laboratory and others.
3. Difficulty in microscopic identification of basophilic aggregations (experienced technician could not differentiate some small lymphocytes from basophilic aggregations).	3. Basophilic aggregations must be examined under oil immersion only. With oil immersion no difficulty was experienced in over 300-400 slides in distinguishing small lymphocytes and basophilic aggregations. Inability to make such a distinction is comparable to saying that nucleated red cells cannot be distinguished from small lymphocytes.
4. Too much variability from slide to slide and even on reexamination of same slide by same technician.	4. The same difficulty was experienced in this laboratory. Discrepancies disappeared when maximum number of basophilic aggregation cells were counted as described under technique. The only sets of slides made on one animal in constant condition which showed any discrepancy are listed below: (1) 0.53% (2) 1.33% 0.50% 1.31% 1.31% (3) 1.17% (4) 0.92% 1.15% 0.94% (5) 1.61% 1.60%

Normal values for basophilic aggregations were determined for twenty-five stock guinea pigs. Nineteen estimations were made on guinea pigs fed the deficient diet 3 to 10 weeks and fifty-one on guinea pigs fed the deficient diet 21 to 74 weeks, average 30 weeks. See Table V.

Table V. Basophilic Aggregations.
Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 3-10 Weeks and for Periods of 21-74 Weeks Compared with Stock Guinea Pigs.

Diet	Number of Weeks on Diet	Number of Animals	Mean	Compared with Stock	
				t	Degrees of Freedom
Stock	--	25	2.06%		
Deficient	3-10	19	0.98%	7.69	42
Deficient	21-74	51	0.68%	17.23	74

These figures indicate a significant difference in per cent of basophilic aggregations for both groups of deficient animals.

F. Sedimentation Rate. Sedimentation rates were determined with the Landau-Adams Microsedimentation apparatus. The reliability of the apparatus was demonstrated by J. L. Rogatz, M. D., in a series of one hundred hospital cases chosen at random, with the proved Smith-Cutler method used as a control (25).

Normal values were taken on twenty-one stock male and female guinea pigs. Experimental values were taken on

fifteen guinea pigs ranging from 2 to 6 weeks on the deficient diet and on thirty-seven males, chosen at random, fed the skim milk diet for 16 to 81 weeks, average 42 weeks. See Table VI.

Table VI. Sedimentation Rate.
Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 2-6 Weeks and for Periods of 16-81 Weeks Compared with Stock Guinea Pigs.

Diet	Number of Weeks on Diet	Number of Animals	Mean	Compared with Stock	
				t	Degrees of Freedom
Stock	--	21	2.24 mm.		
Deficient	2-6	15	3.87 mm.	-4.05	37
Deficient	16-81	37	5.12 mm.	-4.31	59

These figures indicate a significant difference in sedimentation rate for both groups of deficient animals.

Table VII.
Summary of the Effects of Deficiency of the Anti-Stiffness Factor on Erythrocytes of Guinea Pigs.

Test	Diet	No. of Weeks on Diet	No. of Animals	Mean	Compared with Stock	
					t	Degrees of Freedom
Red Cell Count	Stock	--	40	4,964,000		
	Deficient	24-120	26	3,935,000	9.39	64
Hemo-globin	Stock	--	16	98.12%		
	Deficient	26-110	18	75.80%	14.49	32
Color Index	Stock	--	16	0.98		
	Deficient	26-110	18	0.94	1.41	33
Red Cell Diam.	Stock	--	39	7.35μ		
	Deficient	30-110	46	7.69μ	-8.56	83
Baso-philic	Stock	--	25	2.06%		
	Deficient	3-10	19	0.98%	7.69	42
Aggreg.	Deficient	21-74	51	0.68%	17.23	74
Sed. Rate	Stock	--	21	2.24mm.		
	Deficient	2-6	15	3.87mm.	-4.05	37
	Deficient	16-81	37	5.12mm.	-4.31	59

These figures indicate a significant difference for all tests except color index.

II. The Leucocyte

A. Enumeration.

B. Differential Count.

C. Non-filamented Neutrophils.

A. Enumeration. Blood was collected in a Thoma white cell pipette, diluted 1:10 with a solution of 0.5 cc. glacial acetic acid, 99.5 cc. of distilled water, and enough gentian violet to give a slight violet color. Tubes were shaken with the shaker described under "Erythrocyte Enumeration." Cells were counted in four areas one square millimeter each in size. No variation greater than five cells from square to square was permitted (2, p.68; 20, pp.172-3).

Normal values were determined for fifty-five stock male and female guinea pigs. Twenty-six determinations were made on guinea pigs ranging from 15 to 98 weeks on the skim milk diet. See Table VIII.

Table VIII. Leucocyte Enumeration.
Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 15-98 Weeks Compared with Stock Guinea Pigs.

Diet	Number of Weeks on Diet	Number of Animals	Mean	Compared with Stock	
				t	Degrees of Freedom
Stock	--	55	7,459		
Deficient	15-98	26	17,668	-14.33	79

These figures indicate a significant difference in numbers of white cells.

B. Differential Count. Lucia and Lucia (15), in a study of 303 normal guinea pig differential counts, found a considerable variation in the distribution of cells even in each smear. In this laboratory guinea pig blood smears made with the usual technique were found to give counts varying as much as twenty per cent in lymphocytes and neutrophils from slide to slide on slides made from consecutive drops of blood. Many slides gave a majority count of lymphocytes or neutrophils depending on the way the slide was counted.

It was decided that the most representative count of any section of a slide was one which ran from edge to edge, the width being dependent upon the size of the microscopic field. Smears were made with heavy parchment paper roughly about 7 mm. in width on standard microscope slides. This gave a narrow smear (about 1/4" in width). The

counting area was limited to a space approximately 3/8" to 3/4" long near the termination of the smear. However, several hundred cells can be counted in this area. The greatest variation from slide to slide from consecutive drops of blood with this method was about two to three per cent. The cells appeared to be much better distributed than in the ordinary technique and cell breakage was decreased.

Method of counting: Count across the slide from edge to edge, each crossing limited to one microscopic field in width, until enough cells have been differentiated. Do not stop the count in the center of the slide.

Differential counts were made for twenty-five normal male and female guinea pigs. Values obtained were very close to those of Lucia and Lucia (15) so no further normal counts were made. The small difference is probably accounted for by the inclusion of percentages of disintegrated cells by this laboratory.

Differential counts were made on nineteen guinea pigs fed the milk diet 4 to 8 weeks, twelve guinea pigs fed the milk diet 10 to 14 weeks, and eighty-four guinea pigs fed the milk diet 24 to 110 weeks. See Tables IX and X.

Guinea pigs fed the basal skim milk diet 24 to 110 weeks were classified into four groups: (1) relatively

limber females, (2) stiff females, (3) relatively limber males, and (4) stiff males. See Tables X and XI.

Table IX, Part I. Differential Count.
Guinea Pigs Deficient in the Anti-Stiffness Factor
for Periods of 4-8 Weeks and for Periods of 10-14 Weeks
Compared with Stock Guinea Pigs

Cell Type	25 Stock Guinea Pigs Mean %	19 Guinea Pigs 4-8 weeks on deficient diet Mean %	Compared with Stock t	Degrees of Freedom	12 Guinea Pigs (Male) 10-14 weeks on deficient diet Mean %	Compared with Stock t	Degrees of Freedom
Granulocytes	20.42	26.58	1.92	132	<u>47.83</u>	7.17	132
Agranulocytes	73.99	67.76	-1.83	133	<u>52.30</u>	-5.52	133
Disintegrated cells	5.59	5.38	-0.24	133	3.76	-1.87	133
Neutrophils	17.76	23.77	1.79	133	<u>41.55</u>	6.13	133
Lymphocytes	70.09	64.94	-1.49	132	<u>51.43</u>	-4.70	132
Eosinophils	2.06	2.30	0.20	133	2.01	-0.03	133
Kurloff	1.95	1.29	-1.80	133	<u>0.14</u>	-4.29	133
Basophils	0.52	0.52	-0.03	133	0.27	-0.91	133
Monocytes	1.63	1.41	-0.61	133	0.94	-1.64	133

All values underscored are significantly different from the stock animals.

Table IX, Part II. Differential Count.
Guinea Pigs Deficient in the Anti-Stiffness Factor
for Periods of 24-110 Weeks Compared with Stock Guinea Pigs

Cell Type	25 Stock Guinea Pigs Mean %	11 Relatively Limber Females Mean %	Compared with Stock t	d.f.*	10 Relatively Stiff Females Mean %	Compared with Stock t	d.f.*	Mean for all F %	9 Relatively Limber Males Mean %	Compared with Stock t	d.f.*	55 Relatively Stiff Males Mean %	Compared with Stock t	d.f.*	Mean for all M %
Granulocytes	20.42	24.65	1.11	132	<u>38.88</u>	4.67	132	31.42	<u>55.36</u>	8.51	132	<u>50.21</u>	11.65	132	<u>51.14</u>
Agranulocytes	73.99	72.29	-0.42	133	58.41	-3.72	133	65.68	<u>41.59</u>	-7.45	133	<u>46.40</u>	-10.20	133	<u>45.64</u>
Disintegrated	5.59	<u>3.00</u>	-2.57	133	<u>2.58</u>	-2.89	133	<u>2.79</u>	<u>2.95</u>	-2.44	133	<u>2.91</u>	-3.98	133	<u>2.90</u>
Neutrophils	17.76	22.71	1.24	133	22.42	1.13	133	22.56	<u>54.74</u>	8.61	133	<u>43.23</u>	9.53	133	<u>44.49</u>
Lymphocytes	70.09	70.41	0.08	132	<u>55.18</u>	-3.53	132	63.16	<u>39.34</u>	-7.01	132	<u>44.98</u>	-9.11	132	<u>43.68</u>
Eosinophils	2.06	1.54	-0.37	133	<u>16.53</u>	9.38	133	<u>8.22</u>	<u>0.30</u>		133	<u>6.09</u>	4.21	133	<u>5.23</u>
Kurloff	1.95	<u>0.89</u>	-2.44	133	1.97	0.04	133	1.35	<u>0.03</u>	—	133	<u>0.00</u>	—	133	<u>0.00</u>
Basophils	0.52	0.41	-0.40	133	0.88	1.19	133	0.63	0.32	-0.68	133	0.79	1.38	133	0.60
Monocytes	1.63	0.69	-2.18	133	1.10	-1.19	133	0.88	1.68	0.11	133	1.32	-1.08	133	1.33

*d.f. = degrees of freedom. All values underscored are significantly different from the stock.

Table X, Part I. Differential Count.
Guinea Pigs Deficient in the Anti-Stiffness Factor for 24 - 110 Weeks
Relatively Stiff Males Compared with Relatively Limber Males

Cell Type	9 Relatively Limber Males Mean (%)	55 Relatively Stiff Males Mean (%)	t	Degrees of Freedom
Granulocytes	55.36	50.21	1.35	132
Agranulocytes	41.59	46.40	-1.19	133
Disintegrated	2.95	2.91	0.04	133
Neutrophils	54.74	43.23	2.89	133
Lymphocytes	39.34	44.98	-1.39	132
Eosinophils	0.30	6.09	-4.02	133
Kurloff	0.03	0.00	—	133
Basophils	0.32	0.79	-1.65	133
Monocytes	1.68	1.32	0.85	133

Values underscored are significantly different.

Table X, Part II. Differential Count.
Guinea Pigs Deficient in the Anti-Stiffness Factor for 24 - 110 Weeks
Relatively Stiff Females Compared with Relatively Limber Females

Cell Type	11 Relatively Limber Females Mean (%)	10 Relatively Stiff Females Mean (%)	t	Degrees of Freedom
Granulocytes	24.65	38.88	-3.08	132
Agranulocytes	72.29	58.41	2.84	133
Disintegrated	3.00	2.58	0.35	133
Neutrophils	22.71	22.42	0.06	133
Lymphocytes	70.41	55.18	3.10	132
Eosinophils	1.54	16.53	-8.36	133
Kurloff	0.89	1.97	-2.05	133
Basophils	0.41	0.88	-1.35	133
Monocytes	0.69	1.10	-0.78	133

Values underscored are significantly different.

Table XI.

The Mean Value of Each Blood Cell Type per Cubic Millimeter Male and Female Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 24 - 110 Weeks Compared with Stock Guinea Pigs.

Cell Type	25 Stock Guinea Pigs	64[1] Male Guinea Pigs Deficient Diet 24-110 Weeks	21[2] Female Guinea Pigs Deficient Diet 24-110 Weeks
Granulocytes	1575	9027	3906
Agranulocytes	5550	7965	8278
Disintegrated	375	513	351
Neutrophils	1275	7788	2847
Lymphocytes	5250	7611	7938
Eosinophils	150	885	1008
Kurloff	150	0.5	170
Basophils	41	106	79
Monocytes	127	226	110

[1]Mean value of each cell type for both stiff and relatively limber male guinea pigs X 17,700 (the mean for the white blood count for deficient guinea pigs).

[2]Mean value of each cell type for both stiff and relatively limber female guinea pigs X 12,600 (the mean for all white blood counts on deficient females to date.)

C. Non-filamented Neutrophils. Percentages of non-filamented neutrophils were determined for twenty-five stock guinea pigs and fifteen guinea pigs fed on a diet deficient in the anti-stiffness factor 10 (one case) to 100 weeks, average 63 weeks. All counts were made at 1080 X according to Osgood's interpretation of a filamented cell (19, pp.48-49). See Table XII.

Table XII. Non-filamented Neutrophils.
Guinea Pigs Deficient in the Anti-Stiffness Factor for Periods of 10-100 Weeks Compared with Stock Guinea Pigs.

Diet	Number of Weeks on Diet	Number of Animals	Mean %	Compared with Stock	
				t	Degrees of Freedom
Stock	--	25	4.43		
Deficient	10-100	15	14.53	6.29	39

These figures indicate a significant difference in numbers of non-filamented neutrophils.

Discussion

The Normal Guinea Pig. One of the difficulties encountered in studying blood changes in abnormalities experimentally produced in laboratory animals is the great variation in "normal" values as found in the literature. Much time is lost by each experimenter since he must attempt to determine these values for his own laboratory. Frequently after he has taken his normal values he finds so much variation in them that they are almost useless to him (6, pp.810-11; 12; 13; 16, pp.590-91).

The following data taken on "normal" guinea pigs as they arrived at this laboratory shows at least one important cause of this variation in guinea pigs. It also shows the universal susceptibility of guinea pigs to deficiency of the anti-stiffness factor. These guinea pigs appeared externally to be in good health. See Table XIII.

Table XIII. The Normal Guinea Pig.
A Tabulation of Amounts and Severity of Stiffness upon Arrival of Stock Guinea Pigs Supplied to this Laboratory.

Number of Animals	Source of Animals	Basal Diet	Green Feed	Amount of Stiffness		
				Per cent not stiff	Per cent moderately stiff	Per cent severely stiff
86	Supply House	?	Lettuce	3%	39%	58%
20	Stock Colony another lab.	Rabbit pellets	Lettuce	0%	25%	75%
11	Farmer	?	Liberal amounts fresh hay	82%	18%	0%
20	Stock Colony another lab.	Rolled barley	Carrots	30%	50%	20%

Stock animals in this laboratory fed rolled barley with large amounts of kale or mixed grasses daily showed no wrist stiffness. If lettuce was substituted as the source of green feed, stiffness soon appeared. The following table shows a group of eight stock animals from this laboratory fed on barley and kale or grass and the same group two months later fed on rabbit pellets and grass. See Table XIV.

Table XIV. The Normal Guinea Pig.
Stock Guinea Pigs on a Diet Adequate in the Anti-Stiffness Factor Compared with the Same Guinea Pigs Two Months Later on a Diet Poor in the Anti-Stiffness Factor.

Number of Animals	Source of Animals	Basal Diet	Green Feed	Amount of Stiffness		
				Per cent not stiff	Per cent moderately stiff	Per cent severely stiff
8	Stock Colony this lab.	Rolled barley	Kale or grass	100%	0%	0%
		Rabbit pellets	Grass	12%	62%	26%

Blood values taken in this laboratory from stock animals (both young and old) which showed no wrist stiffness had no greater variation, and sometimes less, than the corresponding human values while in almost every type of test the deficient animals showed much wider variations. See Table XV.

Table XV. The Normal Guinea Pig.
A Comparison of the Range of Values
in Clinical Tests of Stock Guinea Pigs
and Guinea Pigs Deficient in the Anti-Stiffness Factor.

Test	Range of Stock Animals	Range of Deficient Animals
Hemoglobin	88% - 108%	59% - 93%
Red count	4,260,000 - 5,625,000	2,880,000 - 4,960,000
Color index	0.87 - 1.06	0.75 - 1.12
Red cell diameter	7.1μ - 7.6μ	7.2μ - 8.2μ
Sedimentation rate	1.0 mm. - 3.5 mm.	2.0 mm. - 19.0 mm.
White count	3,900 - 11,900	11,050 - 30,000
Per cent neutrophils	6% - 31%	5% - 88%
Per cent lymphocytes	60% - 79%	8% - 84%
Per cent eosinophils	0% - 6%	0% - 26%

This research indicates that the anti-stiffness factor plays an important role in erythropoiesis in guinea pigs. One of the most important effects on the blood of animals deficient in the anti-stiffness factor is the decrease in basophilic aggregations in the erythrocytes, which may be considered another way of measuring reticulocytes (22). The anemia produced is hypocythemic, normochromic, and normocytic or macrocytic. One-third of the

cases observed were definitely macrocytic. In general the more severe the hypocythemia, the greater was the macrocytosis. There were no cases of microcytic anemia. This eliminates a possible inadequacy of iron in the milk diet as a cause of the anemia since iron anemias are typically hypochromic and microcytic.

In most cases deficiency of the anti-stiffness factor did not produce a severe anemia; however, nineteen per cent had red counts of 3,500,000 - 2,880,000 as compared with the normal count of 4,260,000 to 5,625,000.

In rheumatoid conditions there is usually a moderate reduction in erythrocytes and hemoglobin (6, 7, 21, 23, 29). Severe cases of rheumatism may produce severe anemia (6). The nature of the anemia is undetermined. Moderate simple hypochromic anemia is reported by Comroe (5) to occur.

The sedimentation rate showed an early (2 to 6 weeks on the deficient diet) and statistically significant increase over the normal values even though fifty per cent of the cases were still normal. In the young guinea pigs the first to show elevated sedimentation rates were also the first to show wrist stiffness. Nearly all the guinea pigs 17 to 39 weeks on the skim milk diet had abnormally high sedimentation rates, only 18.75 per cent remaining within the normal range. However, animals deficient in the anti-stiffness factor 45 to 81 weeks again showed a

high percentage of normal values although the mean is still significantly higher than normal; at this age 42.10 per cent had normal sedimentation rates.

Steinbrocker (29), Short, Dienes, and Bauer (28) report the sedimentation rate to be positive in rheumatoid arthritis in ninety per cent of the cases. Especially is this true in the acute stages of rheumatoid arthritis (21, 29). In osteoarthritis Short, Dienes, and Bauer (28) reported a positive sedimentation rate in fifty per cent of seven patients, but it is usually considered to be only slightly increased in this condition (2, p.249). The Review of American and British literature on rheumatism and arthritis for 1939 (1) reported the sedimentation rate to be normal in eighty per cent of the cases of rheumatoid arthritis.

The differential count for guinea pigs deficient in the anti-stiffness factor shows numerous deviations from those of stock guinea pigs. It is possible to have changes in the percentage of a cell type without having any deviation from the total number of that type normally present per cubic millimeter. This is termed a "relative" change (2, p.181). Absolute changes occur when there are actual decreases or increases of any cell type per cubic millimeter.

Tables IX and X show percentages of cells for stock guinea pigs and guinea pigs deficient in the anti-stiffness factor. Table XI shows the means for each cell type per cubic millimeter of blood in stock animals and the mean for guinea pigs deficient in the anti-stiffness factor 24 to 110 weeks.

> The mean number of leucocytes for stock animals X the mean number of each cell type for stock animals = the mean normal value for each cell type per cubic millimeter of blood.

The mean number of each cell type per cubic millimeter for the deficient guinea pigs is determined by the same method, using, of course, the mean values of leucocytes and cell types corresponding to the sex and length of time on the deficient diet.

The differential count for deficient guinea pigs will be discussed under the following headings:

A. Male and female guinea pigs 4 to 8 weeks on the deficient diet.

B. Male guinea pigs 10 to 14 weeks on the deficient diet.

C. Male guinea pigs 24 to 110 weeks on the deficient diet.

(1) Relatively limber males.

(2) Relatively stiff males.

D. Female guinea pigs 24 to 110 weeks on the deficient diet.

(1) Relatively limber females.

(2) Relatively stiff females.

A. Male and female guinea pigs deficient in the anti-stiffness factor for 4 to 8 weeks show no real variation from stock guinea pigs. See Table IX. There is a very slight increase in percentage of neutrophils.

B. Male guinea pigs fed the deficient diet for periods ranging from 10 to 14 weeks: See Table IX.

1. Granulocytes - increased percentage from normal (statistically significant).

2. Agranulocytes - decreased percentage from normal (statistically significant).

3. Disintegrated cells - slightly decreased percentage from normal (not significant).

4. Neutrophils - increased percentage from normal (statistically significant).

5. Lymphocytes - decreased percentage from normal (statistically significant).

6. Eosinophils - normal.

7. Kurloff - decreased percentage from normal (statistically significant).

8. Basophils - normal.

9. Monocytes - normal.

C. Male guinea pigs fed the deficient diet for periods ranging from 24 to 110 weeks: See Table IX, Part II, Table X, Part I, and Table XI.

1. Granulocytes - increased percentage; absolute increase in number of cells per cubic millimeter.

2. Agranulocytes - decreased percentage; normal number of cells per cubic millimeter.

3. Disintegrated cells - decreased percentage; normal number of cells per cubic millimeter.

4. Neutrophils - increased percentage; absolute increase in number of cells per cubic millimeter.

5. Lymphocytes - decreased percentage; normal number of cells per cubic millimeter.

6. Eosinophils - increased percentage; absolute increase in number of cells per cubic millimeter.

7. Kurloff - none seen.

8. Basophils - normal percentage; absolute increase in number of cells per cubic millimeter.

9. Monocytes - normal.

Both relatively limber and relatively stiff male guinea pigs fed the deficient diet for periods of 24 to 110 weeks have significantly abnormal percentages of exactly the same cell types. There are, however, two important differences between the two kinds of animals. Relatively stiff guinea pigs have a significantly higher

number of eosinophils than do relatively limber male guinea pigs. The more limber animals actually have a significantly lower number of eosinophils than do the stock animals. Frequently they have no eosinophils at all.

On the other hand, the more limber male guinea pigs of this age group have significantly higher percentages of neutrophils than do the stiffer males.

D. Female guinea pigs fed the deficient diet for periods ranging from 24 to 110 weeks: See Table IX, Part II; Table X, Part II, and Table XI.

1. Granulocytes - normal percentage; slightly increased number of cells per cubic millimeter.

2. Agranulocytes - normal.

3. Disintegrated cells - decreased percentage; normal number of cells per cubic millimeter.

4. Neutrophils - normal.

5. Lymphocytes - normal.

6. Eosinophils - increased percentage; increased number of cells per cubic millimeter.

7. Kurloff - normal.

8. Basophils - normal percentage; perhaps increased number of cells per cubic millimeter.

9. Monocytes - normal.

(1) Relatively limber female guinea pigs 2 4 to 110 weeks on the deficient diet: See Table IX, Part II, and Table X, Part II.

There are normal percentages of all cells except disintegrated cells which are significantly decreased from normal in percentage as are Kurloff inclusions.

(2) Relatively stiff female guinea pigs 24 to 110 weeks on the deficient diet: See Table IX, Part II, and Table X, Part II.

1. Granulocytes - significantly increased in percentage from normal.
2. Agranulocytes - significantly decreased in percentage from normal.
3. Disintegrated cells - significantly decreased in percentage from normal.
4. Neutrophils - normal.
5. Lymphocytes - significantly decreased in percentage from normal.
6. Eosinophils - significantly increased in percentage from normal.
7. Kurloff - normal.
8. Basophils - normal.
9. Monocytes - normal.

Relatively stiff female guinea pigs show more deviations from normal in the differential count than do the

more limber female guinea pigs. The neutrophil count is normal for both groups but the stiffer females have an eosinophilia of fifteen per cent.

Both relatively stiff male and female guinea pigs fed the deficient diet for periods ranging from 24 to 110 weeks showed a persistent eosinophilia. The amount of eosinophilia generally rose with increasing stiffness. In every case eosinophilia was associated with moderate or severe stiffness. In only two cases out of eighty-five was severe stiffness not accompanied by eosinophilia, and one of those cases showed six per cent basophilia.

The deficient males alone had a neutrophilia and an absolute decrease in Kurloff cell inclusions.

The loss of the Kurloff cell inclusion by the deficient males is especially interesting since normal female guinea pigs tend to have a higher number of Kurloff cell inclusions than do males and since the deficient females showed no decrease from normal of their Kurloff inclusions.

White cell counts were increased in all but one male who had 11,050 white blood cells. Usually the white cell count did not become elevated in the deficient guinea pig until about three to five months of deficiency in the anti-stiffness factor.

Very few (six) white blood counts were made on deficient females but half of these were lower than the white blood count of any deficient male.

In rheumatoid conditions the leucocyte count is reported to range from normal in quiescent stages to 20,000 in acute stages (1, 5, 6). In chronic arthritis there may be a leukopenia (5, 7). Steinbrocker (29), Comroe (5) and Eaton (7) found the non-filament count to be increased. Neutrophils were reported in Downey (6) to be increased somewhat during acute stages. The Review of American and British literature on rheumatism and arthritis for 1939 (1) reported ninety-six per cent of the cases of rheumatoid arthritis had neutrophilia. Pemberton (23) and Comroe (5) found a persistent eosinophilia in severe cases.

Summary

1. Deficiency of the anti-stiffness factor in guinea pigs produced a hypocythemic, normochromic and normocytic or macrocytic anemia.

2. Poor erythropoietic function was indicated by a significant drop in per cent of basophilic aggregations in erythrocytes even in the first weeks of the deficiency.

3. The sedimentation rate of red cells was elevated within two weeks after the beginning of the deficiency, the high values paralleling the development of wrist stiffness. In only fifty-eight per cent of the cases was there a high sedimentation rate in animals fed on the deficient diet approximately one year.

4. There was a leucocytosis averaging 17,700 cells with granulocytes and non-filamented neutrophils showing statistically significant increases.

5. Stiff males and females showed persistent eosinophilia whereas relatively limber males showed a decrease in eosinophils from normal.

6. Male guinea pigs deficient in the anti-stiffness factor showed an absolute increase in all types of granulocytes, a relative decrease in lymphocytes and disintegrated cells, and an absolute decrease in Kurloff cell inclusions.

Female guinea pigs deficient in the anti-stiffness factor showed a slight absolute increase in all types of granulocytes and a relative decrease in disintegrated cells.

Bibliography

1. American rheumatism association. Rheumatism and arthritis. Review of American and English literature, 1939.

2. Beck, Regena Cook. Laboratory manual of hematologic technic, including interpretations. Philadelphia and London, W. B. Saunders company, 1938. 389p.

3. Best, C. H. and Taylor, N. B. The physiological basis of medical practice. Third edition. Baltimore, The Williams and Wilkins company, 1943. 1942p.

4. Bloom, W. Zur frage der Kurloffkorper. Jahrb. morph. u. mikrosk. anat. forsch. 13:1/2, pp. 320-342, 1928.

5. Comroe, B. I. Arthritis and allied conditions. Third edition. Philadelphia, Lea and Febiger, 1941. 1359p.

6. Downey, Hal. Handbook of hematology. New York, P. B. Hoeber, Inc., 1938. 4v.

7. Eaton, E. R. Chronic arthritis report based on study of cell count in 250 cases. J. American institute of homeopathy 25, pp. 125-136, March 1932.

8. Fisher, R. A. and Yates, F. Distribution of t statistical tables for biological, agricultural and medical research. London, Oliver and Boyd, 1938. 90p.

9. Haden, R. L. Diffraction methods for measuring the diameter of the red blood cell. The journal of laboratory and clinical medicine 20:6, p. 567, March 1935.

10. Haden, R. L. A new instrument for the diffractometric measurement of the diameter of red blood cells. The journal of laboratory and clinical medicine 25:4, p. 399, January 1940.

11. Hawes, J. B. Basophilic aggregations. Boston M. & S. J. 161, p. 493, September 1909.

12. Joyner, A. L. A study of the white blood cells of the normal guinea pig. The American journal of anatomy 62: 4, pp. 497-506, May 1938.

13. Le Goyon, R. et Houot, A. Variations des leucocytes dans le sang d'animaux sains an cours de l'abces de fixation. Compt. Rend. Soc. Biol. 109:3, pp. 222-225, 1935.

14. Loewenthal, N. De l'origine parasitaire des corps de Kurloff. Bull. Hist. Appl. Phys. et Path. 6, pp. 338-351, 1929.

15. Lucia, S. P. and Lucia, E. L. The differential blood count of the normal guinea pig. Archives of pathology 5: pp. 616-625, April 1928.

16. Kracke, R. R. Diseases of the blood and atlas of hematology. Second edition. Philadelphia, London, Montreal, J. B. Lippincott company, 1941. 712p.

17. Krogh, August. The anatomy and physiology of capillaries. Revised. New Haven, Yale university press, 1930. 422p.

18. McCord, C. P., Holden, F. R. and Johnston, Jan. Basophilic aggregation test in the lead poisoning epidemic of 1934-35. The American journal of public health 25, pp. 1089-1098, October 1935.

19. Osgood, E. E. and Ashworth, C. M. Atlas of hematology. San Francisco, J. W. Stacey, Inc., 1937. 255p.

20. Osgood, E. E. A textbook of laboratory diagnosis. Third edition. Philadelphia, the Blakiston company, 1944. 676p.

21. Oxford University Press. A survey of chronic rheumatic diseases, 1938.

22. Pearlman, M. D. and Limarzi, L. R. Correlation studies of basophilic aggregations in various clinical conditions. The American journal of clinical pathology 8, p. 608, 1938.

23. Pemberton, Ralph. Arthritis and rheumatoid conditions, their nature and treatment. Second edition. Philadelphia, Lea and Febiger, 1935. 455p.

24. Pijper, A. Diffraction phenomena in cultures of micro-organisms. Journal of the medical association of South Africa 14, p. 211, 1918.

25. Rogatz, J. L. A simple micrometric apparatus for determining the sedimentation rate of the erythrocytes. The journal of laboratory and clinical medicine 28, pp. 1842-1849, December 1943.

26. Scarborough, R. A. The blood picture of normal laboratory animals. Yale journal of biology and medicine 3, pp. 63-80, October 1930.

27. Severi, R. I. Corpi di Kurloff nella castrazione. Pathologica 23:472, pp. 79-83, 1931.

28. Short, C. L., Dienes, Louis and Bauer, Walter. Rheumatoid arthritis: a comparative evaluation of the commonly employed diagnostic tests. The journal of the American medical association 108:25, p. 2087, June 1937.

29. Steinbrocker, Otto. Arthritis in modern practice. Philadelphia and London, W. B. Saunders company, 1941. 606p.

30. van Wagtendonk, W. J. A dietary factor essential for guinea pigs: III. Changes in the distribution of acid-soluble phosphorus in the liver and kidneys during deficiency. The journal of biological chemistry 155:1, pp. 337-343, September 1944.

31. van Wagtendonk, W. J. A dietary factor essential for guinea pigs: X. Purine metabolism during deficiency. The journal of biological chemistry 167:1, pp. 219-224, January 1947.

32. van Wagtendonk, W. J., Freed, A. M. and Ballou, C. E. A dietary factor, essential for guinea pigs: V. Phosphorus and calcium content of the blood and muscle during deficiency. Archives of biochemistry 5:3, pp. 329-335, December 1944.

33. van Wagtendonk, W. J. and Lamfrom, H. A dietary factor essential for guinea pigs: VI. Changes in the distribution of acid-soluble phosphorus in the muscle during a deficiency of the anti-stiffness factor. The journal of biological chemistry 158:2, pp. 421-424, April 1945.

34. van Wagtendonk, W. J., Rathkey, A. A., Ballou, C. E. and Wulzen, R. A dietary factor, essential for guinea pigs: IV. Serum phosphatase during deficiency. Archives of biochemistry 5:2, pp. 273-278, October 1944.

35. van Wagtendonk, W. J., Schocken, V. and Wulzen, R. A dietary factor, essential for guinea pigs: II. A comparative study of the creatine excretion of animals on a diet deficient in this factor and in vitamin E. Archives of biochemistry 3:3, pp. 305-310, February 1944.

36. van Wagtendonk, W. J. and Simonsen, D. H. A dietary factor essential for guinea pigs: IX. Tissue metabolism during the deficiency. The journal of biological chemistry 165:2, pp. 449-461, October 1946.

37. van Wagtendonk, W. J. and Wulzen, R. A dietary factor essential for guinea pigs: VIII. The isolation of the anti-stiffness factor from cane juice. The journal of biological chemistry 164:2, pp. 597-602, August 1946.

38. van Wagtendonk, W. J. and Wulzen, R. A dietary factor, essential for guinea pigs: I. Isolation from raw cream. Archives of biochemistry 1:3, pp. 373-377, February 1943.

39. van Wagtendonk, W. J. and Zill, L. P. A dietary factor essential for guinea pigs: VII. Changes in the distribution of the plasma protein during the deficiency. The journal of biological chemistry 159:2, pp. 247-253, July 1945.

40. Wulzen, R. and Bahrs, A. M. A common factor in planarian and mammalian nutrition. Physiological zoology 9:4, pp. 508-529, October 1936.

41. Wulzen, R. and Bahrs, A. M. Effects of milk diets on guinea pigs. The American journal of physiology 133:2, p. 500, June 1941.

42. Young, Thomas. Introduction to medical literature. London, Underwood and Black, 1913.