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Sulfated Mucopolysaccharides and Basophilic Leucocytes in Rabbits on a High Cholesterol/Oil Diet[1] (38237)

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Although the basophilic leukocyte was discovered in 1879 by Paul Ehrlich (1), its physiological function is still not fully understood. At present, most investigations on basophils concern its involvement in allergic situations, due to its histamine content. Besides histamine, basophilic leukocytes contain metachromatic material that provides a means for examination, but the nature of this material is still not clear. It has been suggested that this is a heparin-like substance (2, 3). The development of a microelectrophoresis procedure for examination of heparin and of mucopolysaccharides by Jaques *et al.* (4, 5) has made possible a further study of this metachromatic material in basophils. Since an increase is observed in the number of basophils in diseases associated with an increase in circulating lipids (myxedema, nephrosis, diabetes) and a decrease in the number of basophil in patients with low levels of circulating lipids (hyperthyroidism), Braunsteiner (6) postulated that the basophils have a regulating function in fat metabolism. The rabbit is distinctive among mammals for its high blood basophil count and susceptibility to atherosclerosis produced by a diet high in cholesterol and fat. This study was designed to investigate the relationship between blood basophil, its metachromatic substance, serum lipids, and the development of atherosclerosis in rabbits on a high cholesterol–oil diet.

Methods and Materials. Animals. New Zealand white male rabbits were used. The animals were housed in individual cages and cared for in accord with the principles of Care of Experimental Animals—A Guide for Canada. They were fed pellets of rabbit chow (Early Seed & Feed Ltd.). For the experimental animals, there was incorporated in the chow before pelleting, cholesterol (1 kg/100 kg) and peanut oil (3 liters/100 kg). Food and drinking water were supplied *ad libitum*.

In the first experiment, in which determinations were made of blood basophil counts, serum cholesterol, and serum triglycerides at fortnightly intervals, the experimental group consisted initially of 28 rabbits. Four rabbits were killed for tissue studies each 2 weeks, starting with the introduction of the diet. Three rabbits on a normal diet were used as controls throughout the study.

In the study of mucopolysaccharides in white blood cells, 20 rabbits were used. Six were fed on normal diet to serve as control. However, one died during operation, before enough blood was collected. Hence, data were obtained for only 5 of the control group. Fourteen were fed on experimental diet. One died during the period of experiment before being sacrificed. Six were sacrificed after receiving the experimental diet for 8 weeks. Seven were sacrificed at the 13th week on the experimental diet.

Determination of blood basophils. The method of Cooper and Cruickshank (7) was used. Rabbits were fasted overnight and bled from the lateral ear vein bi-weekly. The ear was cut with a sharp knife. After the

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first drop of blood was removed, 0.025 ml blood was sucked into a micro-pipette and delivered into a tube containing 0.1 ml EDTA solution (0.1% EDTA in saline) and mixed gently. 0.1 ml of a mixture of 0.5% cetylpyridinium chloride, distilled water and 0.8% toluidine blue in 5% aluminum sulfate (5:5:4) was added to the tube, and mixed well. Four improved Neubauer counting chambers were filled with the mixture. Basophils which were seen as purple/red metachromatically stained cells were counted in the whole ruled area. When the counts of a sample in different counting chambers varied widely, more chambers were filled and counted. When a blood sample gave values considerably different to that of the average of the group, more blood samples were taken for further counting.

Determination of serum triglyceride and cholesterol. After taking blood samples for basophil counts, the rabbits were fasted overnight. Blood was collected from the lateral ear vein and serum was prepared. 0.3 ml serum was pipetted into a tube containing 5.0 ml of isopropyl alcohol and an extraction mixture which consists of activated alumina, copper sulphate and calcium hydroxide. After being shaken thoroughly, the tube was centrifuged to obtain a clear supernatant which was then processed for determination of triglyceride and cholesterol. The triglyceride determination was a colorimetric procedure based on the Hantzsch reaction (8). Triglycerides were saponified to yield glycerol which was oxidized by sodium metaperiodate to formaldehyde, which in turn combined with acetylacetone to produce a color which was directly related to the amount of triglycerides present. The optical absorbances of samples were measured at 405 nm. The cholesterol determination procedure was based on the principle of Kiliani-Zak (9). Cholesterol reacted with ferric ion in sulfuric acid and acetic acid to produce a brown color. The optical densities of the samples were measured at 560 nm.

Examination of mucopolysaccharides in white blood cells. Animals were anesthetized by sodium pentobarbital. Blood was then collected by cardiac puncture with a syringe containing 3.8% sodium citrate solution (in a ratio of 9:1). The blood samples were placed in plastic tubes (2.6 × 10 cm), and centrifuged at 600 rpm for 5 min at 5°. The supernatant was pipetted out carefully. The boundary layer was then removed into a smaller plastic centrifuge tube (1.5 × 12 cm). A few milliliter of saline were added to the red cell layer which was stirred up gently. Then the boundary layer and the red cell fraction were centrifuged at 600 rpm again. The above procedures were repeated three times. The supernatants were then centrifuged at 2600 rpm for 10 min. The white cell layer was collected and extracted for mucopolysaccharides as follows. The cells were suspended in 5 vol of acetone. The resulting precipitate was collected by centrifuging, washed with the mixture of isopropyl alcohol and petroleum ether. After drying from the organic solvents, the cell precipitate was digested with pronase at 37° and pH 8.0, maintained by simultaneous dialysis against 0.1 *M* Tris–HCl buffer. The digestion mixture in a small bag of dialysis tubing was immersed in the buffer inside an incubator. After proteolysis, the digestion mixture was heated in a boiling water bath for 15 min. It was then centrifuged at 3000 rpm for 15 min. Sodium chloride was added to the supernatant in the amount of 1%. Mucopolysaccharides were precipitated from the supernatant by 5 vol of acetone. The crude mucopolysaccharides were collected by centrifuging at 3000 rpm for 15 min, then dissolved in 1% sodium chloride solution, and reprecipitated by 5 vol of methanol. After the removal of methanol by centrifuging, the mucopolysaccharide extract was lyophilized. The products were then examined and evaluated by the improved microelectrophoresis method of Sue & Jaques (5) in which two μl of each test solution and of reference heparin (from Leo Pharmaceutische Production N. V. Emmen, The Netherlands) were applied to an agarose gel slide side by side, then subjected to electrophoresis, 6.3 v/cm for 20 min. For each sample one set of the electrophoresis slides was fixed with 0.1% cetylpyridinium chloride (CPC), another set was fixed with 0.1% CPC + 1.0 *M* NaCl followed by washing with 0.1% CPC thoroughly. The slides were then stained by

toluidine blue and decolorized in 1% acetic acid. The color of the spot produced by the test solution in the slide was recorded, the migration distance relative to the Leo reference heparin measured, and the optical density measured with a Chromoscan densitometer (Joyce, Loebl & Co. Ltd., England) using #545 filter. Optical densities were converted to equivalent metachromatic units from a graph prepared using slides to which International Standard Heparin had been applied.

For the extraction of mucopolysaccharides from whole blood, blood samples were put into acetone directly without differential centrifuging, and proceeded as above.

Enzyme studies. Chondroitinase ABC (Lot 7104) extracted and purified from *Proteus vulgaris,* was obtained from Seikagaku Kogyo Co., Tokyo, Japan; chondroitinase AC (Lot 7004P) extracted and purified from *Flavobacterium heparinum,* was purchased from the same company. The enzymes were contained in ampules with five units in each. Each ampule of enzyme was assayed with authentic substrate before using for experiments. The assay method described by Yamagata *et al.* (10) was followed for the incubation of the enzymes with the mucopolysaccharide extracts from blood basophils. The incubation mixture contained 10 μl of mucopolysaccharide substrate, 10 μl Tris–HCl buffer (pH 8.0 for chondroitinase ABC and pH 7.3 for chondroitinase AC), 10 μl sodium acetate solution, and 5 μl albumin solution. Enzyme was added in excess. Incubation was at 37° for 16 hr to ensure complete degradation of the substrates. After the incubation, the samples were subjected to microelectrophoresis examination. Ribonuclease was used for the elimination of ribonucleic acids in the mucopolysaccharide extracts. Ribonuclease-A, from bovine pancreas, 5X crystallized, was purchased from Sigma Chemical Co. (Lot 79B-0750), St. Louis, U.S.A. The enzyme preparation was assayed with authentic ribonucleic acid before use for experiment. Excessive enzyme was incubated with mucopolysaccharide extracts at pH 7.7, 40° for 16 hr.

Hyaluronidase, from ovine testes, (690 N.F. units/mg), was purchased from the Sigma Chemical Company (Lot 53C-2170), St. Louis, U.S.A. Mucopolysaccharide extracts were incubated with excessive enzyme in 0.1 *M* sodium phosphate buffer (pH = 5.3) in the presence of 0.15 *M* sodium chloride.

Mucopolysaccharides. For identification of the mucopolysaccharides in the rabbit blood basophil, pure preparations of mucopolysaccharides were used as reference. Heparin, Lot #4439, of 5000 units/ml, was obtained from Leo Pharmaceutische Production N. V. Emmen, The Netherlands. Chondroitin sulfate A, sodium salt, Code 97-D53, Batch WP 4307, was obtained from Miles-Seravac (Pty.) Ltd., Berkshire, England. Chondroitin sulphate C, sodium salt, super special grade, Lot No. 4702, was obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Hyaluronic acid, Lot 12C-2730, grade 1, prepared from human umbilical cord, was obtained from Sigma Chemical Company, St. Louis, U.S.A. Dermatan sulphate, sodium salt, super special grade, Lot No. 4701, was obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan.

Results. Gross appearance of tissues on autopsy. No macroscopic changes were observed in the rabbits receiving a normal diet. The aortas of rabbits on the high cholesterol–oil diet showed discrete elevated spots on the intimal surface as early as 2 weeks on the diet. These areas increased markedly by eight weeks and at 13 weeks they covered up to 90% of the surface of the aortic arch and became yellow. The area covered by lesion decreased with distance down the aorta. They were located particularly at the areas of origin of vessel branches. Fatty livers were observed. Spleens were swollen and grey necrotic spots were observed on the surface.

Serum cholesterol and triglyceride levels. Changes of serum cholesterol and triglyceride levels during the experiment are shown in Fig. 1. Total serum cholesterol value increased very rapidly during the first 2 weeks on the high cholesterol–oil diet. Thereafter, a high level of serum cholesterol was maintained which slightly increased during the rest of the periods. Serum triglyceride level increased in the first 6 weeks.

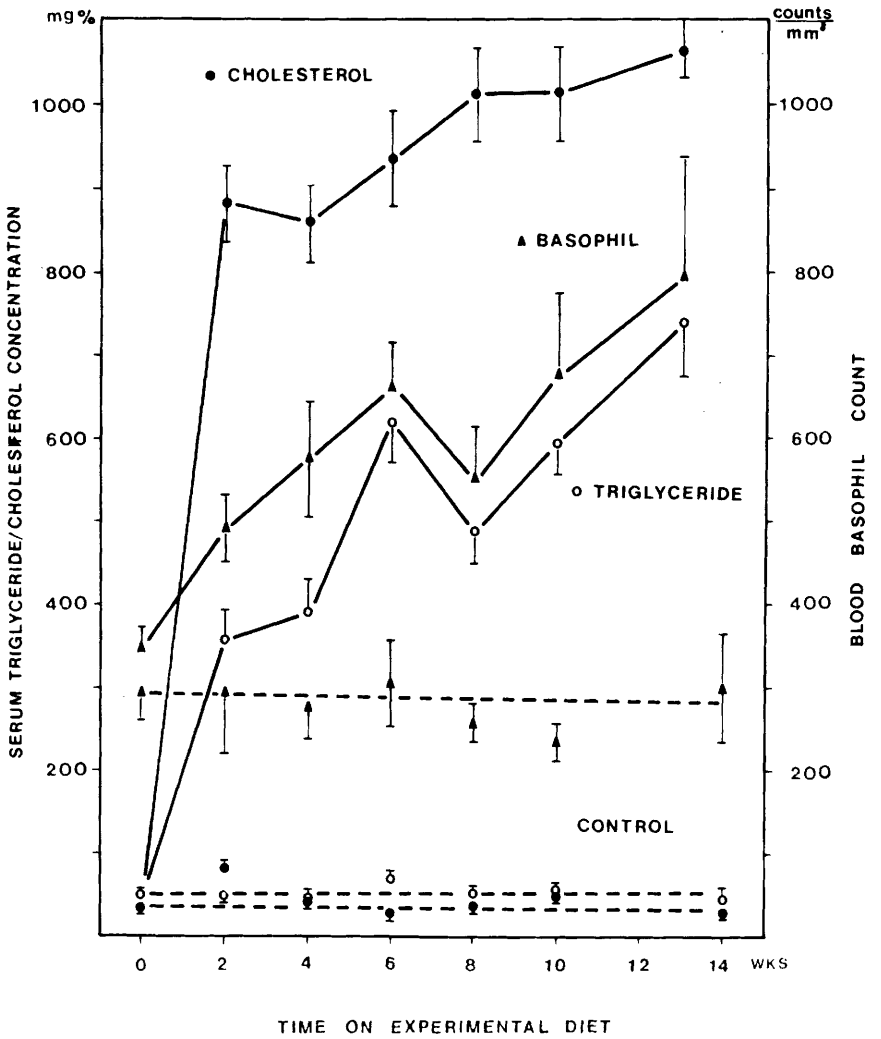

FIG. 1. Changes of serum cholesterol, triglyceride, and blood basophil level of rabbits on high cholesterol–oil diet. Values given for mean and standard error. $n = 28$ initially and reduced by 4 for each group.

There was a slight decrease at the 8th week and then increased again.

Blood basophil count. The changes of the blood basophil counts are shown in Fig. 1. The basophil counts increased during the time course on cholesterol–oil diet. At the end of the experiment, it was more than twice of the value at the beginning and the changes of blood basophil counts followed the same pattern as serum triglyceride with a slight decrease at the 8th week. Statistical test showed a high correlation (correlation coefficient, $r = 0.96$) between mean basophil counts and mean triglyceride concentrations. The blood basophil values do not correlate as well with serum cholesterol levels (correlation coefficient, $r = 0.80$).

Mucopolysaccharides in rabbit white blood cells. In the first group of rabbits, 4 were sacrificed biweekly to obtain blood and tissues. Whole blood samples were processed to extract mucopolysaccharides. No mucopolysaccharides can be detected in the samples containing less than 300 basophil counts/mm^3 of blood. In many samples there was a brown syrup instead of solid precipitate. The interfering substance(s) was thought to come from red blood cells.

The difficulties in extraction of mucopolysaccharides from whole blood were probably due to basophils being only a minute fraction in the blood. In order to obtain satisfactory determinations, white blood cells had to be isolated from other blood components.

In the second experiment mucopolysaccharides were extracted from the white blood cells of rabbits on the experimental diet for 0, 8 and 13 weeks. The extracts of the animals in the control group (0 wk) produced bluish–purple spots similar to that produced by chondroitin sulphates in the agarose electrophoretic slides stained by toluidine blue with one exception. The extracts from the white blood cells of the rabbits on the high cholesterol–oil diet for 8 and 13 weeks produced blue spots which were slightly purple in the front parts of the spots. After the treatment with ribonuclease, the substances producing the blue spots were removed and bluish purple spots were obtained in the agarose gel electrophoretic slides. This indicates the presence of ribonucleic acids in the extracts.

After the removal of ribonucleic acids, the major mucopolysaccharides in the rabbit white blood cells extracts were identified as chondroitin sulphates. This is concluded from the following evidence:

(1) Specific colour reaction with toluidine blue. The mucopolysaccharide extracts of rabbit white blood cells react with the dye in the same way as authentic chondroitin sulphates, changing the blue dye to red in the wet agarose gel media, and to bluish-purple when the gel is dry. Meanwhile, heparin-toluidine blue complex remains red in wet and dry agarose gel.

(2) Electrophoretic mobility. The mucopolysaccharide extracts migrate slower than heparin, only 0.85 of the distance migrated by the reference Leo heparin, during electrophoresis. Authentic chondroitin sulphates have similar electrophoretic mobility as that of the mucopolysaccharide extracts.

(3) Differentiation by electrolytes. The cetylpyridinium chloride (CPC) complex of chondroitin sulphates in agarose gel is soluble in 1.0 *M* sodium chloride and consequently is removed from the gel. On the other hand, the complex of CPC with heparin is not soluble in this electrolyte concentration (5). When the agarose gel slides containing the mucopolysaccharides extracts from rabbit white blood cells were treated with the solution of 0.1% CPC + 1.0 *M* NaCl, the materials were removed from the gel and subsequently no metachromatic spots resulted. This indicates the material in the extracts is not heparin but chondroitin sulphates.

(4) Specific enzyme reaction. The chondroitinase ABC from *Proteus vulgaris,* and chondroitinase AC from *Flavobacterium heparinum* have rather specific substrate spectra for sulphated mucopolysaccharides. They do not attack keratin sulphate, heparin or heparitin sulphates, but chondroitin sulphate A, B, and C for chondroitinase ABC and only chondroitin sulphate A and C for chondroitinase AC. The mucopolysaccharides in the rabbit white blood cells were digested and removed by both chondroitinase ABC and chondroitinase AC.

The results of determination of sulphated mucopolysaccharide content of white blood cells of rabbits on the high cholesterol–oil diet by microelectrophoresis are summarized in Table I. In each group the amounts of sulphated mucopolysaccharide extracted from white blood cells were related to the basophil counts, with correlation coefficients (r) of 0.775, 0.782, 0.723 for the animals sacrificed at 0, 8 and 13 weeks on the experimental diet respectively. However, this positive correlation did not exist between groups. In spite of the increase of blood basophil counts in the course of high cholesterol–oil diet, there was no increase of sulphated mucopolysaccharide (SMPS) contents in the leucocytes.

Besides the major metachromatic spot produced by chondroitin sulphates, there is another spot in the electrophoretic slides containing the mucopolysaccharide extracts from rabbit basophils. This component migrates much slower than heparin and chondroitin sulphates, with a relative migration of 0.44 to the reference heparin. With toluidine blue staining, it produces a red spot in the agarose gel but becomes blue when the gel dries. This spot is removed from the gel during the decolorizing procedure by 1% acetic acid. The complex of this component

TABLE I. Sulphated Mucopolysaccharide (SMPS) Contents of Blood Basophils of Rabbits on High Cholesterol–Oil Diet.

Weeks on diet								
0			8			13		
SMPS[a]	Basophil[b]	SMPS[c] / basophil	SMPS[a]	Basophil[b]	SMPS[c] / basophil	SMPS[a]	Basophil[b]	SMPS[c] / basophil
0.337	312	1.080	0.254	655	0.388	0.348	1093	0.318
0.123	255	0.482	0.118	303	0.389	0.112	456	0.246
0.327	375	0.872	0.412	1363	0.302	0.154	727	0.212
0.152	283	0.537	0.200	452	0.442	0.315	933	0.338
0.233	264	0.883	0.311	525	0.592	0.193	872	0.221
			0.231	405	0.570	0.335	703	0.477
						0.174	480	0.363
0.234 ±0.044[d]	298 ±22[d]	0.771 ±0.113[d]	0.254 ±0.041[d]	617 ±157[d]	0.447 ±0.046[d]	0.233 ±0.037[d]	752 ±88[d]	0.311 ±0.035[d]

[a] SMPS content is expressed in equivalent metachromatic units of International Standard Heparin per ml of blood.
[b] Number of cells/ml of blood.
[c] $\times 10^{-3}$ units/cell.
[d] Values represent mean and standard error.

with CPC is soluble in 1.0 *M* sodium chloride. At first, it was thought to be hyaluronic acid, since hyaluronic acid has similar properties, and the presence of hyaluronic acid in basophils has been suggested by Astaldi *et al.* (11). However, in the present study, this material showed resistance to testicular hyaluronidase. It is also not susceptible to chondroitinase and ribonuclease. The nature of this material is not clear. It is only a minor component in the mucopolysaccharide extract from rabbit basophils. In the present study, the quantity of this material obtained was insufficient for further analysis.

Discussion. The metachromatic stainability of blood basophils by basic dyes, indicates the presence of acidic mucopolysaccharide substance. From the similarity to the tissue mast cell, many authors believe that blood basophils, or "blood mast cells" as Ehrlich called them, contain heparin. However, the evidence for blood basophils is not as complete as it is for the cousin cell line, the tissue mast cell. Many of the suggestions for heparin are only based on histochemical observations (2, 12–14). In the present study, mucopolysaccharides were isolated from the leucocytes. Since the basophil is the only type of leucocyte containing metachromatic substances, it can be concluded that the mucopolysaccharides which were isolated mainly came from the basophils. This is supported by the positive correlations found between the sulphated mucopolysaccharide content and the blood basophil counts of each group of animals. Based on several criteria (specific color reaction with toluidine blue, electrophoretic mobility, differential effect of inorganic electrolyte, and destruction by specific enzymes), the sulphated mucopolysaccharide substance in rabbit blood basophils has now been identified as chondroitin sulphate A/C.

Astaldi *et al.* (11) reported the diminution of metachromasia of the basophils after exposure to testicular hyaluronidase and suggested that hyaluronic acid may be a component of their granules. Hyaluronic acid was not found in the mucopolysaccharide extracts of rabbit basophils in this study. Since testicular hyaluronidase hydrolyzes chondroitin sulphate A/C in addition to hyaluronic acid (16), the observation of Astaldi *et al.* with the use of testicular hyaluronidase probably indicates the presence of chondroitin sulphates instead of hyaluronic acid in the basophils.

An involvement of the basophil in fat metabolism has been suggested by a few investigators (6, 17, 18). From the data obtained from thirty persons, Braunsteiner and his co-workers (17) found a positive correlation between the level of triglycerides in plasma and the number of basophils, but less correlation between the number of basophils and cholesterol, free fatty acid, or phospholipids. The results of this study demonstrate that changes of basophil count parallel the change in serum triglyceride rather than in cholesterol during the development of experimental atherosclerosis. This supports the hypothesis that blood basophils are involved in lipid metabolism. The significance of a relation between blood levels of basophils and triglycerides may involve lipoprotein lipase. A positive correlation between the level of triglycerides in plasma and endogenous lipoprotein lipase has been demonstrated (19). Furthermore, Shafrir and Biale (20) reported that serum lipoprotein lipase appeared after intravenous infusion of very low density lipoproteins. Endogenous lipoprotein lipase is probably activated by the endogenous liberator/activator. The basophils contain sulphated mucopolysaccharides which activate lipoprotein lipase. Based on the above we suggest that elevation of triglyceride level in the blood triggers the release of sulphated mucopolysaccharides from blood basophils which in turn activate endogenous lipoprotein lipase and results in the removal of lipids from blood. The prolonged hyperlipemia will subsequently cause (i) an elevation in the number of basophils in the blood as a result of the mechanism of homeostasis, (ii) exhaustion of the sulphated mucopolysaccharides content of basophils as a result of the prolonged overdemand. The observation of degranulation of basophils after a fatty meal by Shelley and Juhlin (18) and the results of the present study (increase in blood basophil number but decrease in sulphated mucopolysaccharide content per basophil

during a long term hyperlipemia) are in good agreement with the above predictions.

Summary. Rabbits were fed a diet containing 1% cholesterol–3% peanut oil for 13 weeks. It was found that blood basophil count, serum triglyceride and cholesterol increased during the development of atherosclerosis. The changes of blood basophil number showed a significant correlation with the changes of serum triglyceride level but less correlation with serum cholesterol. The major component in the mucopolysaccharide extracts of the rabbit blood basophils was identified as chondroitin sulphate A/C. The sulphated mucopolysaccharide content per basophil decreased in the course of hyperlipemia and hypercholesterolemia. These findings give evidence of the physiological role of basophils in metabolism of triglycerides.

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1. Ehrlich, P., Arch. Physiol. 3, 166 (1879).

2. Behrens, M., and Taubert, M., Klin. Wochenschr. 30, 76 (1952).

3. Turchini, J., and Van Kien, L., Rev. Belge. Pathol. 24, 520 (1955).

4. Jaques, L. B., Ballieux, R. E., Dietrich, C. P., and Kavanagh, L. W., Can. J. Physiol. Pharmacol. 46, 351 (1968).

5. Sue, T. K., and Jaques, L. B., Can. J. Physiol. Pharmacol. 51, 994 (1973).

6. Braunsteiner, H., "The Physiology and Pathology of Leukocytes," p. 57. Grune & Stratton, New York (1962).

7. Cooper, J. R., and Cruickshank, C. N. D., J. Clin. Pathol. 19, 402 (1966).

8. Fletcher, M. J., Clin. Chim. Acta 22, 393 (1968).

9. Zak, B., Am. J. Clin. Pathol. 27, 583 (1957).

10. Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S., J. Biol. Chem. 243, 1523 (1968).

11. Astaldi, G., Rondanelli, E. G., and Bernardelli, E., Rev. Hematol. 8, 105 (1953).

12. Sylven, B., Acta Radiol. Suppl. 59 (1945).

13. Lavas, W., and Thoma, K., Klin. Wochenschr. 28, 95 (1950).

14. Minni, M., and Belloni, F., Haematologica, Paris 37, 1455 (1953).

15. Piette, C., and Piette, M., C. R. Acad. Sci. (Paris) 242, 2775 (1956).

16. Ludowieg, J., Vennesland, B., and Dorfman, A., J. Biol. Chem. 236, 333 (1961).

17. Braunsteiner, H., Sailer, S., and Sandhofer, F., Metabolism 14, 1071 (1965).

18. Shelly, W. B., and Juhlin, L., Amer. J. Med. Sci. 242, 115 (1961).

19. Sailer, S., Sandhofer, F., and Braunsteiner, H., Dtsch. Med. Wschr. 90, 865 (1965).

20. Shafir, E., and Biale, Y., Eur. J. Clin. Invest. 1, 19 (1970).

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