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## EXPERIMENTAL ANEMIA PRODUCED BY PHENYLHYDRAZINE DERIVATIVES

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Soon after the discovery of acetyl phenylhydrazine (pyrocin) by Liebreich it was observed that this chemical possessed marked antipyretic effects, and it therefore came into widespread use in diseases accompanied by fever. Further experience indicated that in addition to reducing the temperature, the drug also produced an anemia that varied in degree according to the dosage. Experiments performed by Dreschfeld (1) on the effects of pyrocin in rabbits showed that an anemia accompanied by hemoglobinuria was produced, and that many phagocytic cells containing intact erythrocytes and hemoglobiniferous particles appeared in the spleen. He also noted that spectroscopic examination of the blood showed the presence of methemoglobin.

There have been many observations upon the type of anemia produced by phenylhydrazine since Dreschfeld's early report. Ziegler (2), Lafleur (3), Wertheimer (4), Fraenkel (5), Albertoni (6), Gatti (7), Ballistini (8), Morawitz and Pratt (9), Gilberti (10) and Foti (11) have carefully described the peripheral blood changes produced by phenylhydrazine. Lafleur (3), Albertoni (6), Gatti (7), Morawitz and Pratt (9) examined the various organs affected by this drug both macroscopically and by histological methods. Suzuki (12) and Taschenberg (13) reported increased resistance on the part of the red blood cells to solutions of phenylhydrazine. In 1915 Eppinger and Kloss (14) recommended the use of phenylhydrazine hydrochloride in the treatment of polycythemia vera. They found that under its influence they could reduce the number of red blood cells and the percentage of hemoglobin to normal, and with this reduction in cell count an amelioration of symptoms took place. These observations stimulated interest in the use of phenylhydrazine deriva-

tives, and in view of the fact that no complete morphological and physiological study has been made, it seemed that a careful investigation of the effect of these substances upon the blood and blood forming organs in animals might be of value.

#### METHODS

Adult rabbits weighing about 2 kg. were used, and daily studies of the blood were made upon each animal. These studies consisted of total white blood cell counts, total red blood cell counts, hemoglobin determinations (Newcomer) (15), hematocrit determinations, reticulated red blood cell counts, and differential counts after the vital technique of Sabin (16). The fragility of the red blood cells of certain animals was tested against solutions of sodium chloride and phenylhydrazine hydrochloride. Rosenthal's (17) phenoltetrachlorphthalein test was used in determining the liver function. The presence of methemoglobin was determined spectroscopically. The bile pigments in the blood plasma were studied by Van den Bergh's (18) bilirubin method. At death studies of the fresh bone marrow, spleen and lymph nodes were made with the supravital dyes, neutral red and Janus green, according to the technique devised by Doan, Cunningham and Sabin (19). Histological studies of sections of the bone marrow, spleen, liver, lungs and kidney were made on autopsy material from each animal.

#### EXPERIMENTAL

In the first series 9 rabbits were used and after a control period of three days each animal received an injection of 30 mg. of phenylhydrazine hydrochloride into the peritoneal cavity. One rabbit was then killed on each successive day.

Within twenty-four hours after the drug had been administered an anemia had begun to develop, and this anemia ran essentially the same course in all the animals. It increased in severity until the fourth day, at which time the destruction of red blood cells was balanced by red blood cell production. Following this the red blood cell production increased over destruction and the red blood cell count and hemoglobin returned towards normal. The details of the peripheral blood studies are shown in figure 1, which illustrates the

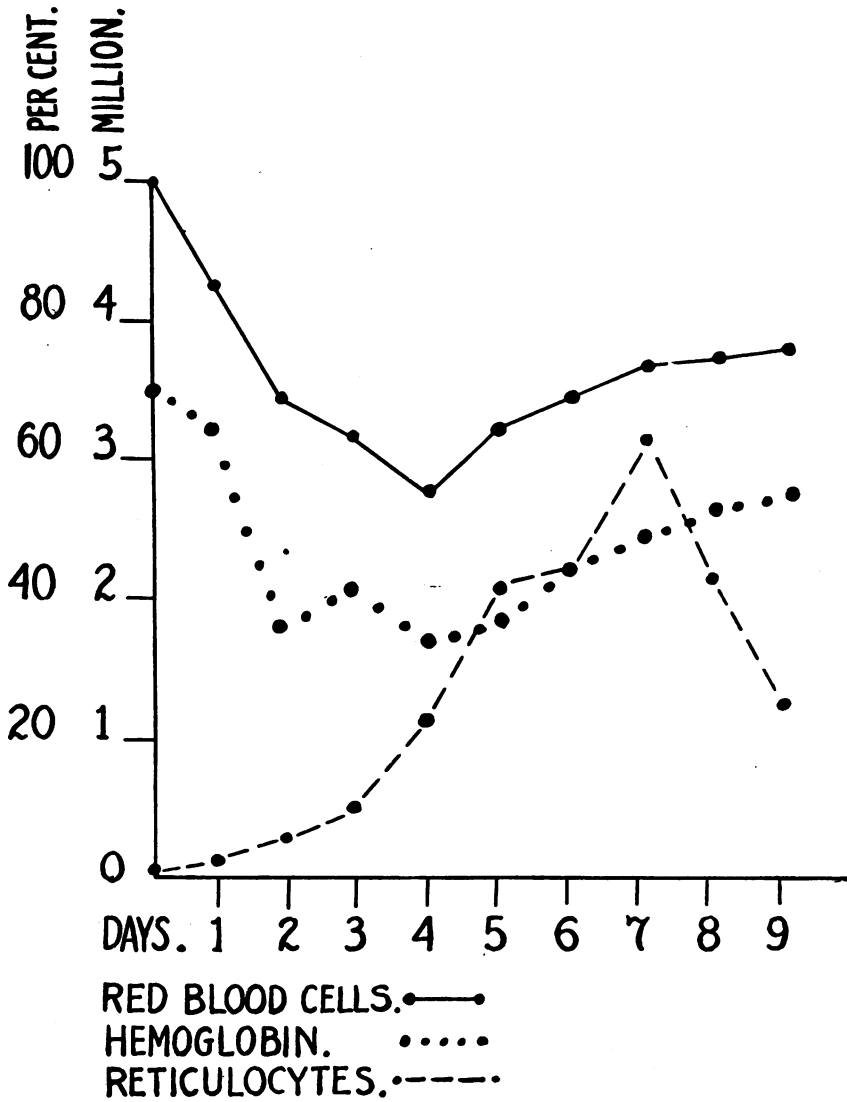


FIG. 1

course of the anemia in a representative animal. At the period of most severe anemia the peripheral blood contained large reticulated red blood cells which numbered as high as sixty per cent of the

erythrocytes. With the return of the hemoglobin and red cells to normal these reticulated cells decreased. Very few microcytes or poikilocytes were seen in the peripheral blood and the color index remained constantly at about the normal level for rabbits. Occasionally during the anemic stage monocytes containing hemoglobiniferous particles were observed in the peripheral blood.

It has been suggested that the clinical administration of phenylhydrazine hydrochloride may be controlled by daily white blood cell counts and that when a slight leukocytosis appears the drug should be stopped. In experimental animals no definite leukocytosis was observed that could in any way be correlated with the injection of the drug.

The resistance of the red blood cells to hypotonic solutions of sodium chloride and to phenylhydrazine hydrochloride was determined in 6 rabbits on the control days and on the third, sixth and ninth days of the anemia. The resistance to hypotonic solutions of sodium chloride was determined according to standard technique. In determining the resistance of the red blood cells to phenylhydrazine hydrochloride, 20 cu. mm. of blood taken from the marginal ear vein of the rabbit were placed in tubes containing 1 cc. of freshly prepared phenylhydrazine hydrochloride solutions varying in strength from 0.20 to 0.68 per cent, and the tubes were immediately shaken to make a suspension. The tubes were then allowed to stand for two hours at room temperature. At the end of this time the tube showing a complete lack of corpuscular residue was recorded as the point of complete hemolysis. It was found, as is shown in figure 2, that the resistance of the red blood cells to sodium chloride solutions remained the same throughout the anemia, but there was a marked decrease in the resistance of the red blood cells to phenylhydrazine on the third day of the anemia, followed by an increase in resistance on the ninth day—a marked increase over that of the control period.

The liver function of each rabbit was determined by the phenol-tetrachlorophthalein test of Rosenthal (17) just before the animal was killed. In all the experiments the retention of the dye was approximately the same regardless of whether the animal had been injected with the drug one day or nine days before the test. At the end of fifteen minutes about 3.3 per cent to 4.4 per cent of the dye remained

in the blood in contrast to 0.5 per cent in normal animals, but at the end of forty-five minutes the retention was only slightly above normal. Figure 3 shows curves of the average figures for each group of animals.

Methemoglobin was shown to be present in the blood by spectroscopic examination in 5 of the animals—on the third day of the

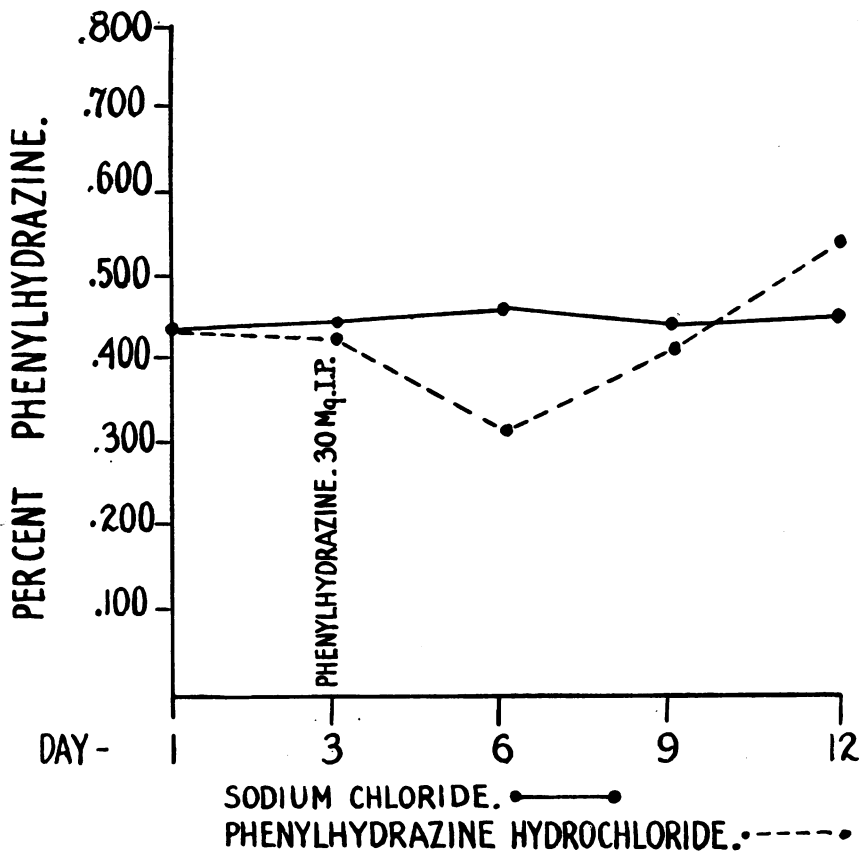


FIG. 2

anemia. When a solution containing 440 mg. of phenylhydrazine in 10 cc. of Ringer's solution was injected into the ear vein of a rabbit, it was found that the animal died after the first 2 or 3 cc. had been introduced. On examination the blood was found to be chocolate brown in color and gave a strong methemoglobin band in the spectrum.

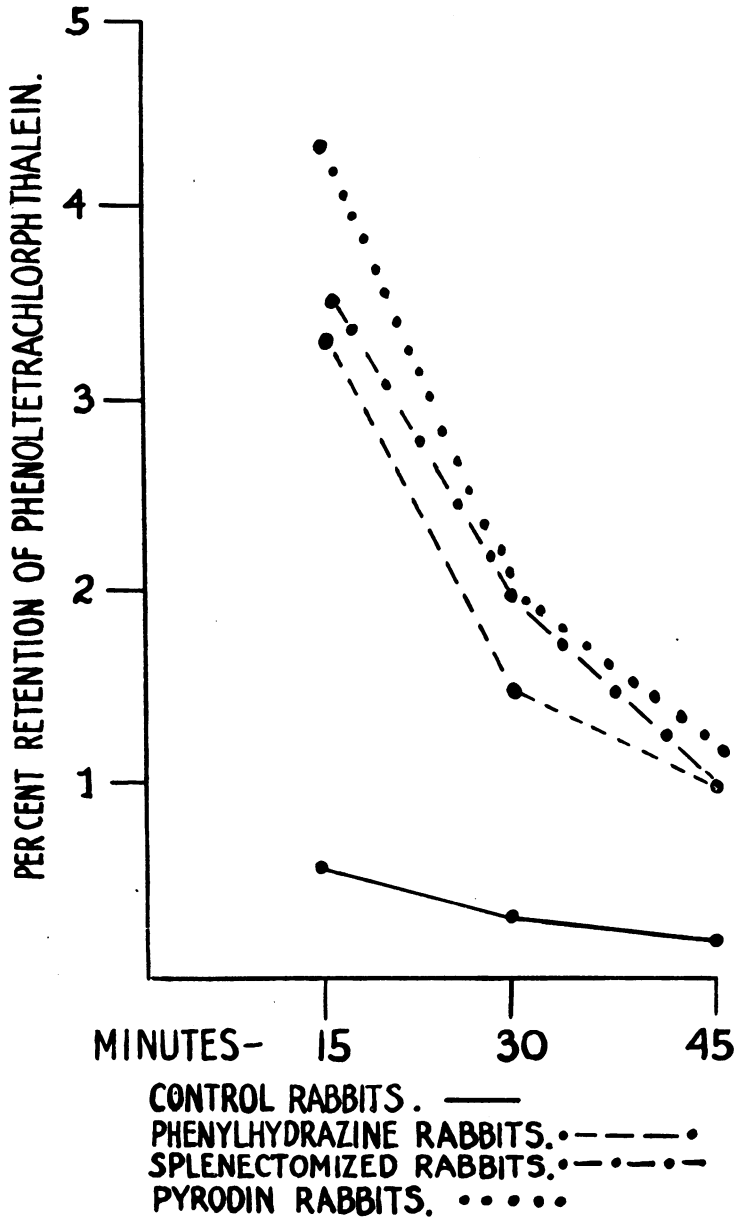


FIG. 3

Blood bilirubin determinations were made on the blood plasma of several rabbits during the period of anemia, but no bilirubin was detected in the plasma. The experience of others, however, has shown that it is very difficult to demonstrate the presence of this pigment in the blood of rabbits. It is generally supposed that the threshold for bilirubin is low in these animals. A short time after the appearance of the anemia it was possible to demonstrate the presence of urobilinogen in the urine of the rabbits. The amount of urobilinogen increased until the eighth or ninth day of the anemia, after which there was a gradual decrease and it was not until three weeks after the injection of the phenylhydrazine hydrochloride that the urine was free from urobilinogen.

The animals were placed under ether anesthesia and just before death studies after the vital technique of Doan et al. (19), were made upon fresh tissues from the bone marrow, spleen and mesenteric lymph nodes. The femoral bone marrow showed a hyperplasia of the red cell elements as the anemia progressed. There was a definite increase in the number of erythroblasts and normoblasts which on the seventh or eighth day of the anemia numbered as high as 80 per cent of the total cells. With the progression of the anemia the number of the earliest form of red blood cells—the megaloblast—increased until, in the very hyperplastic marrows, on the six and seventh day, the megaloblasts formed 3 or 4 per cent of the total cells. In contrast to these observations, it was found in the femoral marrows of normal rabbit that no megaloblasts were seen, and that only 15 to 30 per cent of the total cells were erythroblasts and normoblasts. No hyperplasia of the myelocytic series was present in these animals. The clasmotocytes of the bone marrow increased in numbers, reaching a total of 5 or 6 per cent, in contrast to 1 per cent in the normal controls. In the early stages of the anemia these cells were loaded with phagocytized red blood cells, but as the anemia progressed they contained mainly debris. The megakaryocytes were increased. It was interesting to note that the polymorphonuclear cells would stream up to the periphery of the megakaryocytes, and slowly invade the outer rim of the cytoplasm of the giant cells. Once this rim was passed they would stream faster, often going around and around the nucleus of the megakaryocyte. The polymorphonuclear leuko-



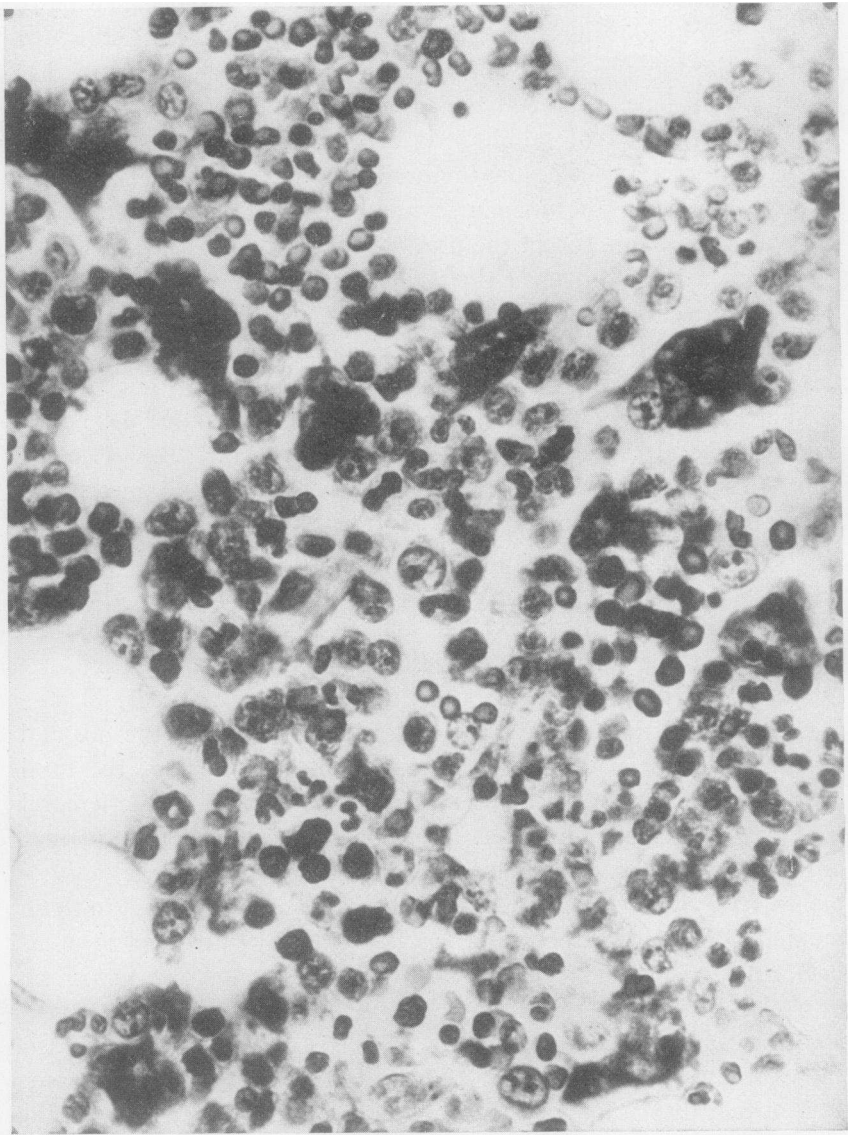


FIG. 4. RABBIT VI. POTASSIUM FERROCYANIDE AND FUCHSIN. SECTION OF HYPERPLASTIC FEMORAL BONE MARROW SHOWING LARGE PIGMENT-BEARING CLASMATOCYTES WHICH GIVE THE IRON REACTION.  $\times 1000$

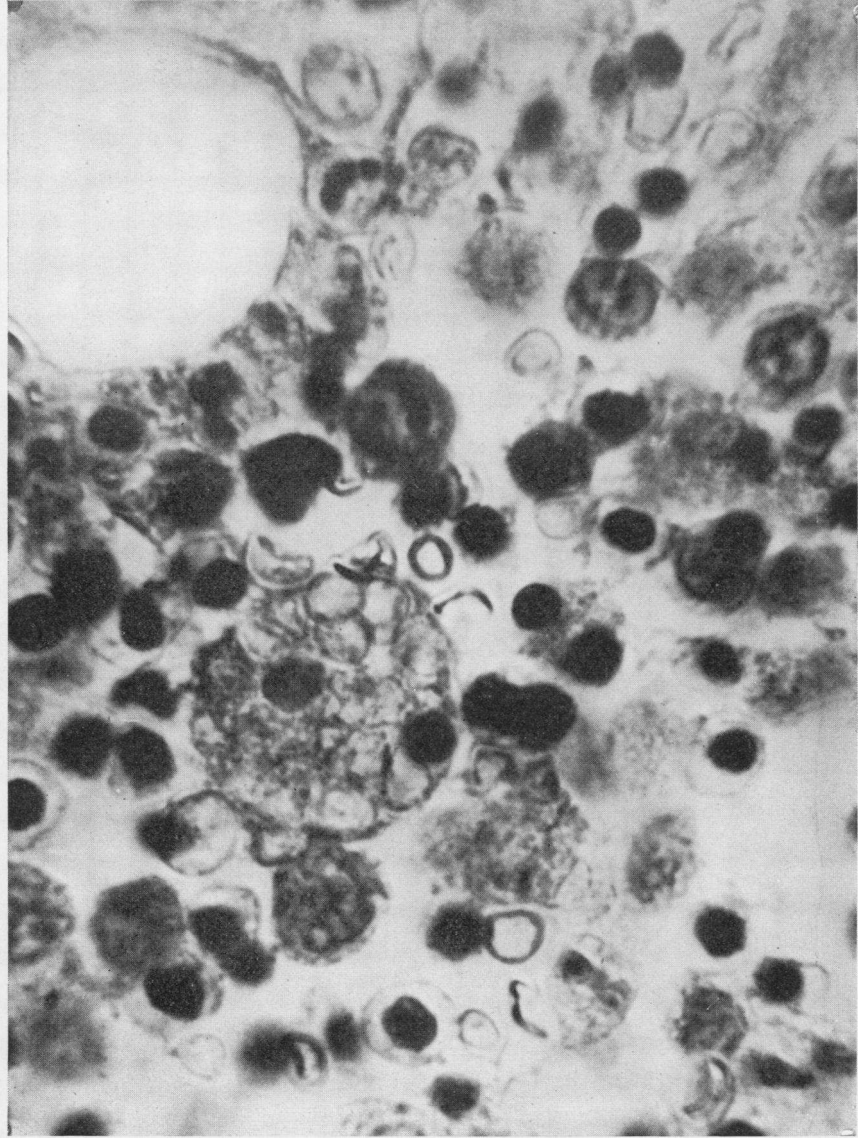


FIG. 5. RABBIT VI. EOSIN AND METHYLENE BLUE. SECTION OF HYPERPLASTIC FEMORAL BONE MARROW SHOWING A LARGE CLASMATOCYTE PACKED WITH RED BLOOD CELLS.  $\times 2000$

cytes, then, either became inactive in the cytoplasm of the giant cells or streamed off through the cytoplasm and passed out of the cell.

The histological sections of the bone marrow also showed an increase in the erythrocytic series that became more marked as the anemia progressed. With the hyperplasia of the red blood cell elements there was a diminution of the fat cells, and on the eighth and ninth days of the anemia the marrow was practically a solid erythropoietic tissue. At all times, however, the blood sinuses of the bone marrow were wide open and packed with red blood cells. There was a marked increase in the clasmatocytes of the marrow, many containing red blood cells; all of the phagocytic cells also contained pigment giving an iron reaction with potassium ferrocyanide. The megakaryocytes increased in number with the hyperplasia of the erythrocytic tissue and often contained polymorphonuclear leukocytes. There were no constant changes in the leukocytic series.

The spleens were slightly enlarged but appeared otherwise normal. The studies of the living tissue showed a marked increase in the number of clasmatocytes. These increased rapidly as the anemia progressed, and on the third or fourth day from 8 to 13 per cent of the cells from the spleen were clasmatocytes in contrast to the normal splenic count of 3 or 4 per cent. These cells were all packed with red blood cells, and some of them had as many as ninety red blood cells included in their cytoplasm. As the anemia progressed the red blood cells were broken down and on the eighth or ninth day the clasmatocytes contained debris. In the histological sections there were no constant changes in the lymphoid or fibrous elements of the spleen. There was, however, a marked increase in the number of clasmatocytes containing intact red blood cells, and a pigment which took the stain for iron.

The mesenteric lymph nodes gradually increased in size during the anemia until on the ninth day they reached three or four times their normal size. Supra-vital studies showed an increase in the reticular cells and in the lymphoblasts. In these nodes were many clasmatocytes which contained debris, but occasionally one would find phagocytic cells packed with erythrocytes. In the histological

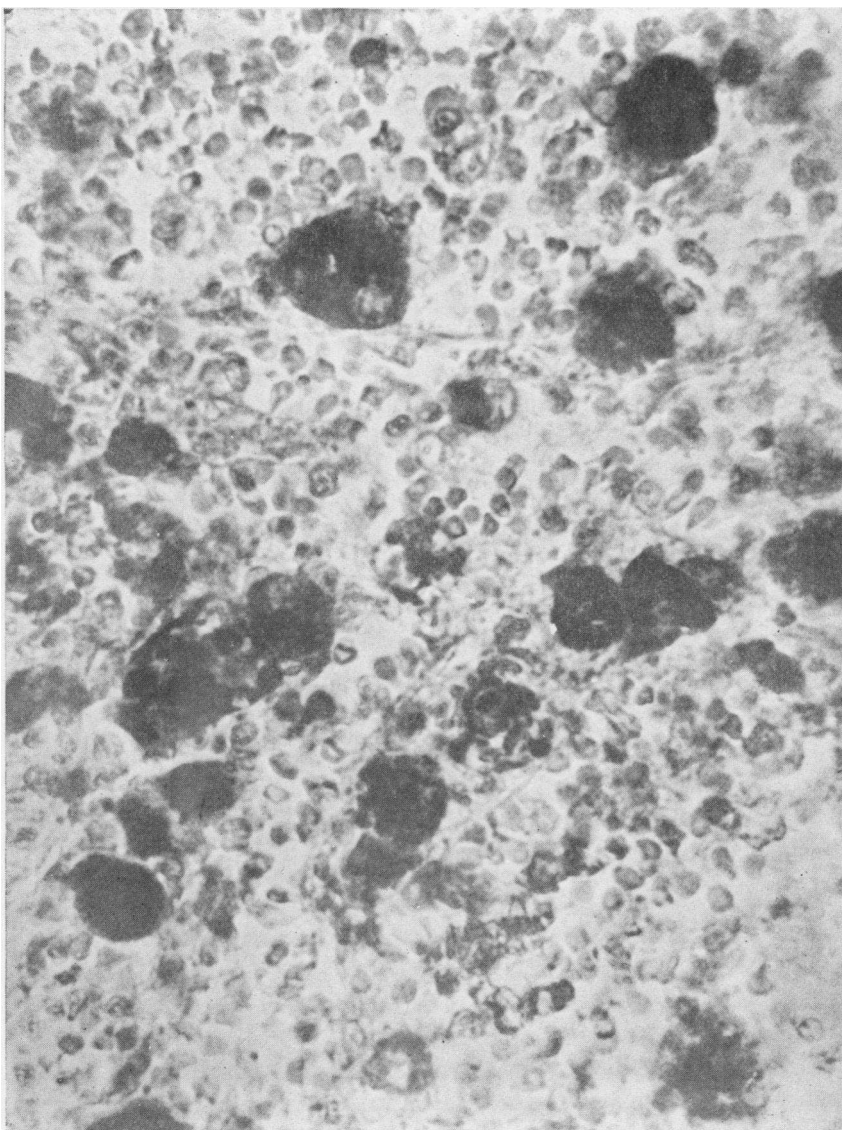


FIG. 6. RABBIT XVII. POTASSIUM FERROCYANIDE AND FUCHSIN. SECTION OF SPLEEN SHOWING NUMEROUS PIGMENT-CONTAINING CLASMATOCYTES WHICH GIVE THE IRON REACTION.  $\times 1000$

sections there was a marked hyperplasia of the lymphoid tissue with numerous phagocytic cells, a few of which contained red blood corpuscles. The majority of the phagocytic cells contained a yellow pigment which did not give the iron reaction.

The histological findings in the livers were inconstant, but the liver parenchyma was apparently not damaged in any instance. There was no evidence of fatty change or necrosis. As the anemia progressed there was a gradual increase in the Kupfer cells, these cells containing particles which gave the iron reaction. The glycogen content of the liver as evidenced by Best's carmine stain was variable. There were no changes of any note in the lungs and kidneys.

In the second series of nine rabbits, acetylphenylhydrazine (pyrodin) was injected and the same technique as in the first series was followed. The changes in the peripheral blood were identical with those produced by phenylhydrazine hydrochloride. The liver function was determined and gave a slight retention of the dye at the end of fifteen minutes which fell to normal after forty-five minutes. Studies of living tissue and histological studies gave the same results as in the animals receiving phenylhydrazine hydrochloride.

The third series of experiments was done on animals after splenectomy. Phenylhydrazine hydrochloride was injected in the same manner as in the first series. The anemia produced was in no way different from that produced in normal rabbits. There was no change in the liver function curve. Studies of the living tissue and histological studies showed that with the extirpation of the spleen, the clasmatocytes of the bone marrow and the Kupfer cells of the liver were increased in number and activity, thereby assuming the phagocytic function of the spleen.

#### SUMMARY

An anemia, pursuing a similar course in all the experiments, was produced by the intraperitoneal injection of phenylhydrazine derivatives in rabbits. When the anemia was established it was possible to demonstrate the presence of methemoglobin in the blood serum. There was a marked increase in the percentage and total number of reticulated red blood cells in the peripheral blood during the period

of the anemia, and the hematopoietic system underwent a marked hyperplasia which progressed until red blood cell production over-balanced red blood cell destruction. A marked increase in the number of phagocytic cells containing intact red blood cells and pigment was found in the spleen, bone marrow and lymph nodes during the anemia. It was not possible to demonstrate the presence of bilirubin in the blood plasma of the rabbits during the anemic stage. Urobilinogen appeared in the urine on the first day after the injection of phenylhydrazine and remained in demonstrable amounts for about three weeks.

After the administration of phenylhydrazine there was a decreased resistance of the red blood cells to solutions of phenylhydrazine which lasted until there was an outpouring of new red blood cells from the bone marrow. These young red blood cells showed an increased resistance to phenylhydrazine.

There was a disturbance in liver function as evidenced by the early retention of phenoltetrachlorphthalein but it did not seem to be marked. No histological changes denoting liver injury could be found in any of the rabbits.

There was no essential difference between the type of anemia produced by phenylhydrazine hydrochloride and acetylphenylhydrazine. The anemia produced in splenectomized animals did not vary from that produced in normal animals except that in animals with the spleens removed there was more phagocytosis in the bone marrow and liver.

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