STUDIES OF THE PROCESS OF INTESTINAL IRON ABSORPTION IN RATS. Elmer B. Brown and Mary Rother, Department of Internal Medicine, Washington University, St. Louis, Mo.

Radioactive iron was given orally to rats which were then killed at various times in order to study the distribution of labeled iron within intestinal mucosal cells during the process of iron absorption. Rats, fasted for 16 hours, were given 100 μg. of FeSO₄ labeled with 5 μc. of Fe⁵⁹. Periods of absorption varied from one minute to 48 hours. Segments of proximal intestine were washed and the mucosal cells were harvested, homogenized, and fractionated by differential centrifugation into subcellular particles and a nonparticulate solution. Radioiron was detected in plasma samples within one minute after instillation into the duodenum. Mucosal radioactivity increased to a maximum at 60 minutes and then declined; most absorption from a single bolus of radioiron occurred during the first three hours. Subcellular fractionation of mucosal homogenates one minute to 48 hours after radioiron administration showed: increasing percentages of Fe⁵⁹ in the “nuclear” fraction; constant low values in “mitochondria”; rising “microsomal” radioiron for approximately three hours with a subsequent decline; and a progressive decrease in nonparticulate radioactivity. Further fractionation of the nonparticulate material with perchloric acid produced a protein-free solution containing most of the radioactivity during the first 15 minutes of the absorptive process; later the radioiron was predominantly protein bound. Characterization of these two fractions suggests that the iron-binding protein is distinct from ferritin; the nonprotein fraction migrates with the amino acids glycine and serine. In iron-deficient rats the amino acid-associated radioiron predominates for longer periods after administration with relatively little accumulation of protein-bound iron. The addition of ascorbic and succinic acids enhances radioiron absorption, especially when these two acids are combined. These observations suggest that pathways of iron absorption in the rat are different from those proposed in the classic “mucosal block” theory.

STUDIES ON EFFECTIVE ERYTHROPOIESIS IN MAN: I. “LOW-OUTPUT” PATTERNS OF ERYTHROPOETIC FAILURE. A. J. Samuels, Department of Medicine, University of California Medical Center, and the Cedars of Lebanon/Mt. Sinai Hospitals, Los Angeles, Calif.

Employing a new method described recently by the author, effective erythropoiesis (EE), the rate of entry of newly formed red blood cells into the circulating blood, was studied in 50 anemic patients. Among a group of patients characterized by low output erythropoietic failure (LOEF), in which evidence of accelerated erythropoietic response is lacking, three different kinds of curves representative of impaired EE were observed. The purpose of this paper is to describe these curves and to indicate their hematopoietic significance.

EE was determined by measuring the rate of erythrocyte cholinesterase (RBC-ChE) regeneration. In eight normal human subjects the curve representing RBC-ChE regenera-
tion was a straight line, the slope of the curve was positive throughout, and the average rate of regeneration (R) was 1.0 per cent per day (0.8–1.3). In four patients with aplastic anaemia and total aregenerative anaemia the slope of the curve was negative and its shape was exponential. In a patient with pancytopenia in relapse, the slope of the curve was negative but the shape of the curve was linear. On the sixth day of vitamin B12 therapy, 48 hours after the appearance of the reticulocyte response, the slope of the curve abruptly became positive and R was calculated to be +34.1 per cent per day. Thereafter the VPRC rose progressively in linear fashion. A similar response was observed in two patients after the administration of iron for iron deficiency anaemia. Similar curves were observed in two patients with hypoplastic anaemia without therapy. In a patient with Gaucher's disease and in two patients with myelofibrosis the curve of RBC-ChE regeneration was essentially linear, the slope was positive, and R was 0.3–0.5 per cent per day.

With this method it appears that various types of "low output" erythropoietic failure can be documented and the response to therapy titrated.

ERYTHROBLAST MATURATION IN BONE MARROW CULTURES: EFFECT OF ERYTHROPOIETIN.
Edward R. Powsner and Lawrence Berman, Medical Research Department and Radioisotope Service, Veterans' Administration Hospital, Dearborn, and Department of Pathology, Wayne State University College of Medicine, Detroit, Mich.

Development of an in vitro model for the study of erythroblast maturation has been reported previously from these laboratories. Maturation is measured both as the incorporation of C14 glycine or Fe59 (ferrous) into heme and morphologically as the accumulation of orthochromic normoblasts.

This model has now been used to study the effect on maturation of preparations of erythropoietin known to be active in vivo. They include extracts of the urine of anemic patients (A. Gordon; F. Stohlman), extracts of the plasma of anemic sheep (W. F. White; L. O. Jacobson, E. Goldwasser, et al.), and extracts of plasma of anemic rabbits (G. Keighley and H. Borsook; W. Rambach).

Preliminary results strongly suggest that the addition to the cultures of some, but not all, of the preparations of erythropoietin results in an increase in C14 heme synthesis.

Consistent but statistically significant small increases (up to 17 per cent) in heme synthesis have been obtained with extracts of plasma of both anemic rabbits and sheep as compared to controls. One urinary extract decreased heme synthesis. The decrease was less marked when a more purified preparation was used.

In addition to these cultures of human bone marrow, preliminary cultures of normal rabbit marrow cells have been tested with an active erythropoietin preparation. Results indicate somewhat larger increases in heme synthesis (30 to 50 per cent). A small but significant increase is present when the concentration of erythropoietin is decreased as low as 0.0001 mg (protein) per ml of bone-marrow suspension.

These experiments demonstrate a direct effect of erythropoietin on the erythroblast in vitro measured by an increase in C14 heme synthesis and by quantitative morphologic data. They do not indicate whether erythropoietin acts solely or directly on the intracellular synthetic pathway for hemoglobin synthesis or indirectly on other aspects of cell growth and proliferation.

AN EVALUATION OF THE MEASUREMENT OF RED CELL SURVIVAL WITH DISOPROPYLFLUOROPHOSPHATE-P32 (DFP32). Martin I. Cline and Nathaniel I. Berlin, National Cancer Institute, National Institutes of Health, Bethesda, Md.

DFP32 has not been widely used to measure red cell survival in man or in experimental animals although it was first suggested as an erythrocyte label by Grob, Lilienthal, Harvey and Jones in 1947 (Bull. Johns Hopkins Hosp. 81:217, 1947).

We undertook to evaluate DFP32 as a red cell marker using a simplified technique of isotope measurement and using C14-glycine and chromium-51 as independent measures of red cell life span.
**Dogs:** The uptake of intravenously administered DFP32 by the erythrocytes of adult dogs was found to be dose-dependent with a gradual increment in uptake in the range between 0.03 and 0.26 mg/Kg. Random labeling of erythrocytes with DFP32 gave survival times of 103 to 139 days with a mean of 121 days in five dogs. Effective blocking of erythrocyte uptake of DFP32 could be achieved by prior administration of unlabeled DFP. If there was a sufficient timelag between administration of unlabeled DFP and DFP32 a cohort of cells of similar age could be labeled. Using this technique and the C14-glycine method as cohort labels the erythrocytes formed in response to acute hemorrhage were found to have a shortened survival with a pattern of random destruction.

**Man:** Erythrocyte survival in seven patients with hematologic disorders was determined simultaneously by random labeling with DFP32 and with chromium-51 and cohort labeling with C14-glycine. Red cell survivals of 59 to 124 days were found by the DFP32 method. These data were in good agreement with the erythrocyte life spans obtained with C14-glycine. The chromium-51 technique failed to reflect moderate shortening of red cell life span.

It is concluded that DFP32 can provide a technically simple and useful method of measurement of red cell survival by either random or cohort labeling.

**A Quantitative Radioactive Antiglobulin Test.** Nicholas Costea, Robert Schwartz, Matthew Constantoulakis, and William Dameshek, Blood Research Laboratory, Pratt Diagnostic Clinic—New England Center Hospital, and Department of Medicine, Tufts University School of Medicine, Boston, Mass.

Previous experience with antiglobulin sera and elution techniques indicated the impracticability of obtaining a quantitative estimate of the degree of erythrocyte coating in autoimmune hemolytic anemia. For this reason the present studies with 1131-labeled rabbit antihuman globulin serum were undertaken. A method was developed in which the non-specific uptake of radioactive antiglobulin serum (RAG) was reduced to a minimum, while sensitization of cells could be easily detected. The uptake of RAG was found to be dependent upon pH, temperature, incubation time and the amount of sensitizing antibody used. Within certain limits a linear relationship between RAG uptake and titer of sensitizing antibody was observed. Eighty samples of normal erythrocytes had an uptake of 1.43 ± 0.4 μg. N/ml. RBC; the values in 12 cases of autoimmune hemolytic anemia were 2.8-29.0 μg. N/ml. Quantitation of erythrocytes sensitized by anti-A, B, D, CD, Fy*, JK* and Lu* was possible. The test was reproducible on a day-to-day basis and was independent of the particular sample of RAG used, provided it was present in excess. Weakly sensitized, Coombs-negative red cells could be detected by this method. The “4-plus” serologic reaction was found to have a wide range of antibody coating. The correlation between RAG uptake, severity of autoimmune hemolytic anemia, and therapy will be discussed in the light of these findings.

**Current Status of the Hereditary Human Gamma-Globulin Groups.** Hugh Fudenberg, Department of Medicine, University of California School of Medicine, San Francisco, California.

Since Grubb’s demonstration of a genetically determined variation in human γ-globulin in 1956, six additional genetic factors in human γ-globulin have been defined by family and population studies. In Caucasians, two loci, segregating independently, control the production of six well defined factors. Genes at the first locus determine the production of four factors, tentatively termed Gm 1(a), (b), (x), and (r). Gm 1(a) and (b) are inherited as simple Mendelian codominant, non-sex-linked factors. Gm 1(x) and (r) occur only in Gm 1(a+) whites, in a manner somewhat analogous to the subgroups of the A antigen in human erythrocytes. Genes at a second locus, tentatively termed Gm 2, determine the production of the allelic factors Gm 2(a) and Gm 2(b). A seventh factor, “Gm-like,” occurs only in Negroes, and is independent of the Gm 2 locus. Its relation to the Gm 1 locus has not as yet been clarified (Steinberg).
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The data to be presented demonstrate that the Gm factors are confined to the 7S component of normal and abnormal γ-globulin, and are absent in the 19S and γ1S, components. 7S monomers obtained from purified 19S and γ1S, materials by treatment with sulfhydryl reagents also lack Gm properties. The data further indicate that in heterozygous subjects the allelic factors are present in different molecules of the 7S γ-globulin.

CHROMOSOME STUDIES IN HEMATOLOGY. John F. Jackson (Sponsored by Charles C. Sprague), Tulane University School of Medicine, New Orleans, La.

Chromosome counts were performed in acute leukemia, chronic lymphocytic leukemia, chronic granulocytic leukemia, multiple myeloma, polycythemia vera, leukemoid reaction and hypoproteinemia. Metaphase squash preparations from bone marrow cultured in TC199 for 24 hours, or peripheral blood leukocytes cultured in TC199 for 72 hours in the presence of phytohemagglutinin, was the basic material for study. Leukemic peripheral blood leukocytes cultured 48 hours without phytohemagglutinin were also suitable. Treatment of cultures one to four hours with colcemid to arrest mitoses in metaphase, followed by hypotonic treatment in one per cent sodium citrate, fixation in 50 per cent glacial acetic acid and squashing in acetoorcein produced satisfactory spreads for chromosome analysis. Aneuploid cells were of much greater occurrence in the hematologic malignancies in comparison to normals and such diseases as cretinism, muscular dystrophy, hepatolenticular degeneration, and intersex problems. Hyperdiploid cell populations with 47 chromosomes in addition to the normal 46 chromosome mode were frequently found in the hematologic malignancies, but were not present in leukemoid reaction or dysproteinemia. Tetraploid cells were frequently noted, especially in multiple myeloma, where octoploid cells were also noted. Simplified techniques for chromosome analysis provide an additional tool for the further characterization of many diseases. Specificity of chromosomal abnormality may be associated with certain hematologic diseases.

A SIMPLE METHOD FOR DETECTION OF ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PD) DEFICIENCY. Virgil F. Fairbanks and Ernest Bent Butler, City of Hope Medical Center, Duarte, Calif.

There is a continued need for a simple and rapid screening test for erythrocyte G-6-PD deficiency. We have investigated the possibility of linking tetrazolium dyes to TPN reduction to develop such a test. A method has been developed which can be performed with a minimum of equipment, using stable, premeasured mailable reagents, which permits visual interpretation of a strongly colored and relatively stable dye.

Whatman DE-20 paper is treated with 0.02 cc. of a 0.26 M KH2PO4 solution per square cm., and dried. Approximately 0.004 cc. of packed erythrocytes are applied to a 6 mm.-diameter circle on the paper, and are dried. The paper is placed in distilled water at room temperature for 20 minutes, blotted, and placed in distilled water for an additional 20 minutes and blotted again. This removes hemoglobin and some other proteins, leaving a very light yellow spot. The paper is now dried. A "spot reagent" is applied. A strong purple color develops in two minutes with normal erythrocytes; little or no color develops with G-6-PD-deficient erythrocytes. A ml. of the "spot reagent" contains the following: glucose-6-phosphate, 12.0 mg.; MTT [3(4,5 dimethylthiazolyl 1-2) 2.5 diphenyl tetrazolium bromide] 2.0 mg.; TPN, 1.0 mg.; phenazine methosulfate, 0.02 mg.; MgCl2, 6H2O, 3.0 mg.; and 0.02M phosphate buffer, pH 7.0, 0.5 cc. These reagents can be prepared together in a test tube in the dry state and are stable for at least eight weeks.

We have compared this test with the glutathione stability test and the spectrophotometric assay of G-6-PD in normal subjects, and in Negro and Caucasian subjects with G-6-PD deficiency. Good correlation has been established. Since the reagent for this test can be prepared in advance in mailable, stable form, any laboratory should be able to perform this test with only a pipette, beaker, and distilled water.
The Reduction of Methemoglobin in Normal, Glucose-6-Phosphate Dehydrogenase Deficient, and Congenital Methemoglobinemia Erythrocytes. Ernst R. Jaffe, Department of Medicine, Albert Einstein College of Medicine, New York, N. Y.

Although the precise mechanism by which methemoglobin (MetHgb) is reduced to hemoglobin (Hgb) in human erythrocytes is still not completely understood, evidence has accumulated to indicate that the process is associated with metabolic activity. Requires regeneration of reduced pyridine nucleotides, and involves an electron transport system. In the present study, MetHgb was produced in erythrocytes from normal adults, Negro males whose erythrocytes were deficient in glucose-6-phosphate dehydrogenase activity (G-6-PD-D), and from a woman with congenital methemoglobinemia without abnormal hemoglobin (Cong. MetHgb), by incubation with sodium nitrite. Washed erythrocytes were further incubated without substrate or with glucose or inosine. Reduction of MetHgb to Hgb occurred at equivalent rates in normal and G-6-PD-D erythrocytes, but failed to occur in the Cong. MetHgb erythrocytes. The reduced triphosphopyridine nucleotide (TPNH) methemoglobin reductase system was assayed by incubating hemolysates of MetHgb-containing erythrocytes with TPNH and methylene blue. No difference was observed in the rate of reduction of MetHgb in normal, G-6-PD-D, and Cong. MetHgb hemolysates. The reduced diphenolphosphoryl nucleotide (DPNH) methemoglobin reductase system was assayed by incubating similar hemolysates of MetHgb-containing erythrocytes with DPNH and 2,6-dichlorobenzenone indophenol. No difference was observed in the reduction of the dye by normal and G-6-PD-D hemolysates, but reduction by the Cong. MetHgb hemolysate was markedly deficient. These observations confirm other reports that there is no abnormality in the reduction of MetHgb in intact G-6-PD-D erythrocytes in the absence of methylene blue and demonstrate that the TPNH- and DPNH-utilizing methemoglobin reductases are normal in these cells. The findings also support the theory that normal reduction of MetHgb to Hgb is dependent upon a DPNH-utilizing system that is deficient in erythrocytes of certain patients with congenital methemoglobinemia of the enzymatic type.

Pyruvate Kinase (PK)-Deficiency Hereditary Nonspherocytic Hemolytic Anemia. Kouichi R. Tanaka, William N. Valentine, and Shiro Miwa, Department of Medicine, University of California Medical Center and Wadsworth Hospital, Los Angeles, Calif.

The congenital nonspherocytic hemolytic anemias represent a heterogeneous group. The seven cases (three males and four females, aged 2 to 38 years) here reported correspond in all major respects to those classified as Type II by Selwyn and Dacie. They are nonspherocytic, hemolytic, Coombs-negative anemias, and exhibit increased autohemolysis, not correctible (or only partially correctible) by glucose, but corrected by adenosine triphosphate (ATP) or adenosine diphosphate. Splenectomy in four patients has not resulted in significant improvement. The clinical severity and transfusion requirements vary widely.

The erythrocytes were assayed for both glycolytic and nonglycolytic enzyme activity. The significant finding was the markedly reduced activity of red cell pyruvate kinase (PK) in all seven patients. Interestingly, the leukocytes have normal PK activity. Glucose-6-phosphate dehydrogenase and enzymes in the Embden-Meyerhof pathway other than PK, as well as a group of nonglycolytic erythrocytic enzymes, were normal or increased in activity. Low pyruvate kinase values similar to those of these patients have not been found in a survey of a variety of other hematologic and nonhematologic disorders. Family data (including 43 members of one kindred) are consistent with an autosomal recessive mode of transmission of the defect. The heterozygotes demonstrate about one-half the normal level of red cell PK activity, but are clinically well and have normal hemograms.

Red cells are dependent primarily upon glycolysis for their energy-producing mechanism. Since the pyruvate kinase step is essential for regeneration of ATP, the demonstrated defect would be expected to impose a serious metabolic handicap. This presumably is related to the hemolytic process. It is suggested that this disorder be called "Pyruvate kinase (PK)
deficiency hereditary nonspherocytic hemolytic anemia," which appears to represent another instance in which clinical disease is definable at the molecular level.

**AN ABNORMAL FETAL HEMOGLOBIN PROBABLY BASED UPON AN ALPHA CHAIN ABNORMALITY.**

Helen M. Ranney and Cathleen O'Brien, Albert Einstein College of Medicine, New York, N. Y., and University of Vermont, Burlington, Vt.

Biochemical studies of hemoglobin have led to the theory that inherited abnormalities in the \( \alpha \) polypeptide chain of hemoglobin will result in abnormal fetal hemoglobins, whereas abnormalities in the \( \beta \) chain will be unapparent or nearly so at birth. There are, however, few clinical observations to corroborate the theory. The family to be described was detected because an abnormal fetal hemoglobin was found in a survey of cord bloods in Burlington, Vermont. This unusual fetal hemoglobin had anodal electrophoretic mobility at pH 8.6 greater than the mobility of hemoglobin A. The rapid hemoglobin component exhibited increased alkali resistance and was initially thought to represent Bart's hemoglobin \( (4\gamma^a) \). The mother of the child was of Italian and French-Canadian extraction, while the father was of Northern European ancestry.

Hematologic examination including hemoglobin electrophoresis of the blood of the mother and of her parents disclosed no abnormalities. However, an abnormal hemoglobin comprised some 20 per cent of the father's total hemoglobin. The father's rapid abnormal hemoglobin component had greater electrophoretic mobility than the abnormal component of his newborn son; the father's hemoglobin exhibited the same alkali resistance as normal hemoglobin A. The father's abnormal hemoglobin has been tentatively designated as hemoglobin I; the abnormal fetal hemoglobin as hemoglobin Burlington \( (2\alpha^a 2\gamma^a) \).

Results of recombination experiments and of amino acid analyses will be discussed.

Three points of clinical interest are illustrated by this family study: (1) It lends further support to the idea that abnormal fetal hemoglobins will result from \( \alpha \) chain abnormalities, whereas \( \beta \) chain abnormalities will appear only after birth. (2) It indicates that fetal hemoglobins other than Bart's may have both rapid electrophoretic mobility and increased alkali resistance. (3) It illustrates again that abnormal hemoglobins in the United States are found in persons who do not have known Mediterranean or Negro ancestry.

**FORMATION OF DIFFERENT HEMOGLOBINS IN TISSUE CULTURE OF HUMAN BONE MARROW TREATED WITH HUMAN DEOXYRIBONUCLEIC ACID.**

Lorraine M. Kraus, Division of Chemistry, University of Tennessee Medical Units, Memphis, Tenn.

Tritiated thymidine-labeled deoxyribonucleic acid (DNA) extracted from human bone marrow cells from a person having only hemoglobin A was incubated for 10 days with bone marrow obtained from a patient with sickle cell anemia with hemoglobin S and eight per cent hemoglobin F. Autoradiograms demonstrated the labeling of the DNA from Hb-A bone marrow and the presence of the HDNA in nucleated erythrocytes and other marrow cells of the Hb-S bone marrow. After 10 days' incubation with HDNA the bone marrow was incubated with Fe\(^{59}\) and iron\(^{59}\)-labeled hemoglobin resulted, thereby showing the synthetic ability of the 10-day bone marrow tissue culture. Cell hemolysates were prepared from the tissue culture and from peripheral blood of Hb A (source of DNA) and Hb S (original tissue culture). The absorption spectra of all these in the range 220 to 700 m\(\mu\) were superimposable and typical for oxyhemoglobin with a small amount of methemoglobin. Hemoglobins electrophoretically different from Hb A and Hb S were demonstrated on paper and agar electrophoresis of the cell hemolysate from the DNA-treated tissue culture. Electrophoresis of the polypeptide chains of the hemoglobins from DNA-treated tissue culture showed the presence of the original \( \alpha^a, \beta^a, \gamma \) polypeptide chains, and in addition \( \beta^s \) and possibly an altered \( \alpha \)-polypeptide chain. The results of these experiments will be discussed in reference to hemoglobin as an indicator of DNA-induced transformation of mammalian cells.
ABNORMALITIES OF THE IMMUNE RESPONSE IN CHILDREN WITH HEMOGLOBINOPATHIES.
G. William G. Thurman (Sponsored by Charles C. Sprague), Emory University School of Medicine, Atlanta, Ga.

In a study reported to the Annual Meeting of the American Society of Hematology in 1959, there was some indication that children with abnormal hemoglobin patterns did not respond as well to some antigenic stimuli as did children with normal patterns. The following study was planned to demonstrate this difference:

Five groups of children were studied:
Group 1: Twenty children with homozygous A hemoglobin patterns by paper electrophoresis; no elevation of $A_2$ or $F$ demonstrable.
Group 2: Twenty-two children with homozygous $S$ hemoglobin patterns by paper electrophoresis.
Group 3: Twenty children with both A and $S$ patterns by paper electrophoresis; also no elevation of $A_2$ or $F$.
Group 5: Four children with thalassemia minor.

After complete history and physical examination, each child had baseline agglutinations for typhoid, tularemia, and brucella antibodies. Study of all those with detectable levels of antibody was discontinued; all of the other children were immunized first with typhoid vaccine; 21 days after the last dose of vaccine, circulating antibody levels again were determined. One month later, they were immunized with acetone-extracted tularemia vaccine; after ten days, antibody levels were drawn for comparison with the prevaccination levels. Following a further interval of one month, the children were immunized with brucella antigen; in a similar fashion, circulating antibody levels were determined for comparative purposes. Serum electrophoretic patterns were obtained on all patients in an attempt to identify specific abnormalities of gamma globulin or other fractions.

The results to be presented indicate a significant difference between group 2 and group 1; an even more striking finding was that group 3 also differed from group 1; minor differences were noted in group 4 and 5 when compared with controls. The relationship to various electrophoretic patterns will be discussed.

FOLIC-ACID DEFICIENCY AS A LIMITING FACTOR IN THE ANEMIAS OF THALASSEMIA MAJOR.
A. Leonard Lashly, Jack M. Cooperman, Robert Feldman, Joseph Cerado, Julia Herrero, and James F. Marley, New York Medical College, New York, N. Y.

A recent report from our laboratory (Lancet 2:490, 1961) indicated that folic acid deficiency is a heretofore unsuspected limiting factor in the acute anemic crises as well as in the recurrent anemia of patients with thalassemia major. In such individuals, administration of folic acid produced hematologic, biochemical, and clinical improvement.

The acute anemic crisis in patients with thalassemia major who do not require frequent transfusions, although similar to that observed in other chronic hemolytic anemias such as sickle cell and congenital spherocytic anemia, is less widely recognized than in the latter disorders. We have found that folic acid deficiency is frequently associated with these acute anemic episodes. This was most clearly revealed by increased urinary formiminoglutamic-acid (FIGlu) excretion following histidine metabolic loading (Proc. Soc. Exp. Biol. & Med. 101:350, 1959). It is of theoretic and diagnostic interest that, although megaloblastosis was present in some instances, erythroid and myeloid morphology was "normoblastic" in many cases where increased formiminoglutamic-aciduria after histidine loading indicated folic acid deficiency existing biochemically. Serum vitamin-$B_{12}$ levels were normal in all cases; serum folic acid activity was low to normal.

The etiology of folic acid deficiency in these patients is not completely understood. Jandl and Greenberg (New England J. Med. 260:461, 1959) suggested that there is an increased local tissue requirement for folic acid due to increased red cell turnover. We found, however, that many patients have a decreased ability to absorb folic acid from the gastrointestinal tract. This may account, in part, if not entirely, for the folic acid deficiency.
In patients with thalassemia major who require frequent transfusions, we found that, when folic acid deficiency was demonstrable, administration of folic acid increased or maintained hemoglobin levels so that the interval between transfusions was prolonged.

Results of experiments in progress on the folic acid metabolism in these patients will be presented.

By reducing transfusion needs, these findings give promise of a new therapeutic approach to the acute and recurrent anemia in this chronic disease.

**Correlation Between Serum Folic-Acid Activity and Response to Antifolate Therapy.** Barth Hoogstraten, Herman Baker, and Peter Reizenstein, Departments of Hematology and Chemistry, The Mount Sinai Hospital, New York, and The Brookhaven National Laboratory, Upton, N. Y.

The sensitivity or resistance of neoplastic diseases to antifolate therapy may be determined by assay of the serum folic acid activity (SFAA); the SFAA levels appear to correlate with the state of the diseases studied.

Microbiologic assay of SFAA of 150 patients with hematologic neoplasias revealed low values in patients with active disease, three of whom had associated megaloblastic anemia, and normal levels in patients in complete remission. No such correlation existed with serum vitamin-B12 levels. With serial SFAA determinations, significant changes were noted during the varying stages of the underlying diseases.

Acute lymphoblastic leukemia (ALL) was the only disease thus far studied in which patients with active disease had normal or near-normal SFAA levels. Of three patients with ALL treated with antifolates, two had a normal SFAA and both responded to therapy, while the third patient with a low SFAA value failed to respond. In addition, during relapse, low SFAA levels were noted and repeat courses of antifols were ineffective.

Three patients with Hodgkin’s disease and low SFAA levels did not respond to antifolate therapy, while one patient with Hodgkin’s disease and one with lymphosarcoma, both of whom had normal SFAA values, had complete remissions after therapy.

The possible mechanism of resistance and sensitivity to antifolate therapy will be discussed.


There is considerable and unpredictable individual variation in prothrombinopenic response to coumarin anticoagulant drugs, which has not been resolved by elegant clotting factor tests. We propose to approach the problem by relating the pharmacologic behavior of these drugs to their biologic activity. No previous studies of this type have been done with Warfarin. To this end we have developed a Warfarin assay in which the biologic material is extracted with acidified ethylene dichloride, washed with mild alkali, recovered with strong alkali, and measured spectrophotometrically.

Extinction coefficients of Warfarin and Dicumarol UV spectra were obtained with pure drug in 2.5N NaOH. Correlation graphs of optical density and apparent Warfarin concentration were prepared from appropriate standards. Beer’s Law was obeyed over the concentrations tested. The pH effect on aqueous-organic phase drug distribution was studied to predict drug behavior and loss during the assay.

Assay specificity was evaluated by comparing the UV spectra of pure drug, assayed standard, and test plasma—virtually identical. These drug sources were carried through an 8-transfer countercurrent distribution scheme. The distribution spectra have single peaks in the same tube; the experimental and predicted values agree and yield a calculated purity of 98.5 per cent for the assay.

The following data were obtained for Warfarin and Dicumarol after single doses in the same individuals:
Stool elimination (per cent of dose)  
Urine excretion (per cent of dose)  
Maximum blood level (hours)  
Average biological half-life (hours)  
Plasma nonprotein-bound drug (per cent)  

These preliminary data indicate that gastrointestinal absorption of Warfarin is rapid and complete, and of Dicumarol is often slow and incomplete. Plasma nonprotein-bound drug may relate to drug urinary excretion.

A direct correlation was found for Warfarin biologic half-life and prothrombinopenic response in the same individual on varying doses, and in different individuals on a standard dose.

ROLE OF AC-GLOBULIN IN PROTHROMBIN ACTIVATION. Walter H. Seegers and Ricardo H. Landaburu, Department of Physiology and Pharmacology, Wayne State University, College of Medicine, Detroit, Mich.

Methods have been developed for obtaining concentrates of Ac-globulin from bovine plasma. First the prothrombin is removed by adsorption on barium sulfate. The Ac-G is precipitated at pH 5, dissolved in saline, and stabilized by adding S-mercaptosuccinic anhydride. The solution is fractionated with the use of cold alcohol followed by chromatography on DEAE-cellulose or Amberlite IRC-50. Finally it is fractionated by iso-electric precipitation. The main Ac-G fraction precipitates at pH 5.4.

Thrombin becomes more active in the presence of Ac-globulin. The activity measured as Ac-globulin is actually thrombin activity accelerated by Ac-globulin in the activation of prothrombin. In other words, thrombin is an enzyme that activates prothrombin, and it is more active in the presence of Ac-G. Our best experimental demonstration is with the use of p-toluenesulphonyl-L-arginine methyl ester (TAME) as a substrate. Under certain conditions, thrombin hydrolyzes this compound more than 24 times as fast in the presence of Ac-globulin than when none is there.

A total of 4.4 units of thrombin was placed into each of a series of test tubes. This thrombin was prepared by chromatography on phosphate cellulose by methods already described (W. H. Seegers and R. H. Landaburu: Can. J. Biochem. Physiol. 38:613, 1960) and the preparation was homogeneous by several criteria. From tube to tube the Ac-globulin concentration was increased from nothing in the first to 100 Ware-and-Seegers units in the last. The TAME concentration was 0.04 M and thus was there as substrate in excess. After 30 minutes, the reactions were stopped with the addition of an equal volume of neutral 40 per cent formaldehyde. Then the extent of TAME hydrolysis was determined by titration with 0.025 M NaOH. Each ml. of this reagent was equivalent to 20 units of thrombin on the standard curve. The substrate hydrolysis was accelerated as the Ac-globulin concentration increased.

The acceleration of thrombin activity was applied in the quantitative determination of Ac-globulin. Analyses of several plasma samples have been completed and the concentration in human plasma was relatively low; then, in order of increasing concentration, were plasma from dog, cow, and rabbit.
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FIRST SYMPOSIUM

Monday, November 27, 1961, 2:00–5:30 p.m.

LEUKOCYTIC ENERGIES

Moderator: John W. Rebuck

A BIOCHEMICAL VIEW OF THE PHAGOCYTIC PROCESS. Manfred L. Karnovsky. Biophysical Laboratory and Department of Biological Chemistry, Harvard Medical School, Boston, Mass.

The ingestion of particulate matter by phagocytic cells is a phenomenon which has been studied for many years but which has received very limited attention from the biochemical viewpoint.

The phagocytic phenomenon could, however, serve as an excellent model for studies of the metabolic basis of physiological action. Leukocytes could be permitted to ingest particles in a defined medium, and changes in the utilization of substrates and appearance of metabolic products that accompany the phagocytic process could be measured. The use of radioactive substrates would provide additional information.

Such studies have been performed in the last few years. In our laboratory, for example, an investigation of the metabolic basis of phagocytosis in polymorphonuclear leukocytes, monocytes and alveolar macrophages of the guinea pig has been conducted. In general, it was found that for the first two cell types glycolysis provided all the metabolic energy needed for the ingestion of particles. In the third type of cell, aerobic metabolism was necessary. Polymorphonuclear leukocytes have been more intensively studied than the other cells mentioned and exhibit two striking metabolic changes during phagocytosis: (1) Oxidation of glucose via the hexosemonophosphate pathway and oxygen uptake are greatly stimulated, even though this apparently provides no utilizable energy; (2) incorporation of labeled precursors, particularly inorganic phosphate-$^{32}P$, into lipid substances is enhanced. Only certain phosphatides, i.e., those with a net negative charge, are affected. These phenomena in polymorphonuclear leukocytes have probable relevance to cytoplasmic changes involving, in the first case, the neutrophilic granules, and in the second case cellular membranes.

PROFIBRINOLYSIN LOCALIZATION IN THE EOSINOPHILIC SERIES. Marion I. Burnhart and Jeanne M. Riddle, Department of Physiology and Pharmacology, Wayne State University College of Medicine, Detroit, Mich.

The fluorescent antibody technic provided a direct method to study the cellular site of profibrinolysin synthesis; also, attention was focused on the function of the eosinophil. Antisera to human profibrinolysin, produced in rabbits, was coupled to a fluorescent dye and used to mark those cells containing a sufficient quantity of profibrinolysin. Specific staining occurred in the eosinophilic series of bone marrow from normal as well as pathologic material. Liver and spleen exhibited occasional cellular staining which was correlated with eosinophils present in the tissue. Normal peripheral blood and that from a few asthmatics revealed only weakly fluorescing mature eosinophils. The specificity of the cellular fluorescence was established by adsorption with various purified plasma proteins. Only adsorption with profibrinolysin completely abolished the activity of the fluorescent antibody. Partial blocking was achieved when the antigenic sites were occupied by unlabeled antiprofibrinolysin. Fluorescent normal rabbit gamma globulin did not stain eosinophils specifically. A nonspecific attraction of acid dye to eosinophils was ruled out since fluorescent antiprofibrinolysin stained when the pH varied from 6–8.3. All members of the eosinophil series exhibited specific staining, with the intensity of fluorescence increasing with maturity. The number and stage of granule differentiation correlated well
with the degree of fluorescence. Smears from a case of acute granulocytic leukemia, in which many of the developing eosinophils were ruptured, provided a unique opportunity to study the free granules. These fluoresced brilliantly when treated with rhodamine antiprofibrinolysin, as did eosinophil granules isolated from a leukocyte concentrate obtained from the blood of a patient with a 40 per cent eosinophilia.

Thus, eosinophils appear to synthesize and transport profibrinolysin to function in maintaining the fluidity of the blood.


Distinction is made between blood basophilic granulocytes and tissue mast cells: although separate in origin and individual in structure they possess similar functions. Basophilic leukocytes were detected in exudates in experimental animals by Ringoen (1923) and Plimpton (1940). Basophilic granulocytic migration in inflammatory exudates in man was studied in human skin windows.

Two conditions—ulcerative colitis (Priest, Rebuck and Havey, 1959, 1960) and interstitial cystitis—were found to predispose to increased extravasation of blood basophilic leukocytes. In ulcerative colitis, basophilic granulocytes appeared in increased numbers three hours after application of diphtheria toxoid, reaching their peak in 14–27 hours. Such basophils showed pericellular metachromatic diffusion, hypersegmentation of polymorphous nuclei, cytoplasmic edema, and specific granular hypertrophy and vacuolation. In interstitial cystitis, a still greater migration of basophils occurred, reaching its maximum at 26 hours. Such basophils showed metachromatic halos, and shedding of granules and cytoplasm with some ingestion by mononuclears. Lakes of metachromatic material were ringed by basophils. Biopsies of pyoderma, colon, or bladder, in the respective conditions, revealed increases in both basophilic leukocytes and tissue mast cells. The significance of overflooding the exudate with histamine, heparin, and serotonin is underscored. Increased eosinophilic migrations were noted in skin lesions and organ lesions. Archer (1960) noted that eosinophils are mobilized by histamine. Windows in a patient with basophilic granulocytic leukemia yielded the same sparse number of basophils present in control lesions. The increased basophil-mast-cell complex in ulcerative colitis has been confirmed by McAuley and Sommers (1961). Recently, Juhlin and Shelley (1961) have implicated a similar basophil-mast-cell complex as the basis of cold urticaria, stressing specific degranulation (histamine release) of both cell types in the cold, in addition to the antihistaminic blood eosinophilia noted by Duke (1960).

Role of the Lymphocyte in Delayed Hypersensitivity and Transplantation Immunity. Joseph D. Feldman and John S. Najarian, Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif.

Lymphoid-cell suspensions were derived from lymph nodes and spleen of guinea pigs sensitized to B.C.G. bacilli and which also had received tritiated thymidine to label dividing cells. In homologous guinea pigs the concomitant intravenous injection of cells prepared in this fashion, and the intradermal injection of PPD, elicited a classical delayed inflammatory reaction at the skin site. Tritiated cells were present in significant numbers among the infiltrating cells. The majority of the labeled cells were mature and immature lymphocytes. When a mixture of nonlabeled sensitized cells and tritiated nonsensitized lymphoid elements was used, few or no labeled cells appeared at the PPD test site; nor were labeled sensitized cells found at the skin-test site when coccidioidin was employed as a provoking agent in the skin of animals given B.C.G.-sensitized cells.

Lymphoid-cell suspensions were prepared from lymph nodes and spleen of mice sensitized to a homologous strain both by skin grafts and intraperitoneal spleen cell injections and which also had received tritiated thymidine to label dividing cells. Isologous mice received these cell suspensions from three days before to three days after homografting. In all instances, grafts were rejected in an accelerated fashion at from one to six days after
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transplantation. Few or no labeled cells were observed in the homograft rejection bed. Similar accelerated rejection of homografts was achieved when the sensitized cells were enclosed in millipore chambers which were then placed around the periphery of the graft or within the peritoneal cavity.

In the two experimental models described, sensitized lymphocytes played two roles. In the passive transfer of tuberculin sensitivity they were physically present in the test site and were unable to elicit a reaction from a distance. In contrast, in homograft rejection the sensitized cells were absent from the graft bed and were able to destroy grafts from a distance.

OBSERVATIONS IN DIFFUSION CHAMBERS ON THE RELATION OF MONONUCLEAR LEUKOCYTES OF BLOOD TO CONNECTIVE AND HEMATOPOIETIC TISSUES. Nicholas L. Petrakis, Departments of Preventive Medicine and Medicine, and the Cancer Research Institute, University of California School of Medicine, San Francisco, Calif.

Experiments are in progress dealing with the in vivo growth and differentiation of normal and leukemic leukocytes of man, employing millipore diffusion chambers. The chambers function as in vivo tissue culture vessels and permit the enclosed cells (autologous and homologous) to be exposed to various humoral host environments. It has been shown that cellular homografts in diffusion chambers survive in nonimmunized hosts if host lymphocytes do not enter the chamber.

The blood of normal adult humans and animals has been found to contain mononuclear leukocytes (lymphocytes and/or monocytes) capable of differentiating into connective tissue histiocytes and fibroblasts when cultivated in diffusion chambers in vivo. Studies are in progress to evaluate the capacity of these cells to differentiate into hematopoietic tissues under the simulation of certain nutritional, endocrine, and local tissue influences. These have included ascorbic acid deficiency, testosterone, bacterial polysaccharides, and exposure to the heteroplastic bone- and marrow-inducing action of transitional epithelium of the urinary bladder. Significant findings include:

1. Ascorbic acid plays a direct cellular role in the differentiation of guinea-pig mononuclear leukocytes into fibroblasts.
2. Testosterone and the "inducing" action of urinary epithelium can activate the "mesenchymal" capacity for hematopoietic differentiation of certain mononuclear leukocytes found in blood of adult vertebrates.

These findings are of interest in respect to the pathogenesis of connective tissue diseases, and the abnormal differentiations characterizing the leukemias and myeloproliferative syndrome.

PARTICIPATION OF BASOPHILES IN INFLAMMATION. Earl P. Benditt, Seattle, Wash.

The basophiles of tissue and blood presently appear to be the principal repositories of heparin and histamine. In rats and mice serotonin is present in the tissue mast cells. An enzyme with properties like chymotrypsin is present in tissue basophiles and possibly in circulating basophiles. Evidence on the mode of activation and release of these several substances and their participation in injury reactions will be discussed.

The question of the relationship of mast cells and circulating basophiles is still open. Present evidence indicates that the several characteristic substances are present both in the free and fixed cells. The two cell types appear to have a different origin, one from pre-existing mast cells in tissues and the other from leukocyte precursors in the bone marrow. Relationship of these cells in terms of their operation in disease will be discussed.
MECHANISMS REGULATING LEUKOCYTE RELEASE FROM PERFUSED FEMURS. Albert S. Gordon, Burton S. Dornfest, Joseph LoBue, Eugene S. Handler and Henry Quastler, New York University, New York, and Brookhaven National Laboratory, Upton, N. Y.

We have developed a method for the precise estimation of leukocyte release from isolated perfused femurs of rats. Femurs are dissected free of all surrounding tissue and capped at both the proximal and distal ends with rubber tubing. Blood or plasma is perfused through these preparations with a peristaltic pump. The foramina at the epiphyseal extremities, through which nutrient arteries supply the marrow, allow direct passage of the perfusate through the marrow vasculature. Such perfused marrows are kept in good physiologic condition for four to six hours as indicated by (1) the normality of time marrow cytology, (2) the absence of extravasation of blood, (3) the response to vasoactive agents, (4) the synthesis of DNA, and (5) the maturity of the leukocytes released.

The data indicate that perfusate flow rate and the numbers of circulating white blood cells influence marrow leukocyte release. Using leukocyte-free blood, a positive correlation was established between flow rate and the numbers of marrow neutrophils and lymphocytes discharged. Perfusion with blood containing normal numbers of leukocytes resulted in suppression of marrow white-cell release and in the sequestration of lymphocytes within the marrow. Autoradiographic studies with H3 thymidine-labeled femurs indicated that the decrease in perfusate lymphocytes could not be wholly accounted for in the marrow, suggesting disintegration of the sequestered cells.

Whole blood containing the leukocytosis-inducing factor (LIF) from repeatedly leukocytapheresed rats evoked either a marked increase in blood flow rate, or prevented the reduction in flow due to a progressive increase in vascular resistance encountered in control experiments. These alterations in flow rate were accompanied by increased rates of discharge of leukocytes into the perfusate. Addition of known vasoactive agents (acetylcholine, histamine, endotoxin) did not reproduce the flow rate or leukocyte release patterns obtained with the LIF.


Thirteen patients with various types of leukemia (7 chronic granulocytic, 4 acute granulocytic, 1 chronic lymphocytic, 1 monocytic) underwent continuous leukocyte withdrawal (leukapheresis) on eighteen occasions; 78 to 1614 billion leukocytes were removed in 94 to 275 minutes, representing 14 to 233 per cent of the initial circulating leukocyte number.

In marked contrast to the leukocytosis observed during leukapheresis in 23 hematologically normal subjects, all leukemic patients exhibited a profound and protracted fall in total circulating leukocyte number, usually during and always following leukapheresis. The decline in the peripheral leukocyte count continued for 2–7 days thereafter. The return of the leukocyte count toward the initial level was generally slow (1 to 69 days in 11 of 18 studies). There was no significant return in 7 studies. A significant decrease in platelet concentration was observed in all 18 studies, lasting from 30 minutes to 8 days, which was similar to that observed in normal subjects. The hematocrit was not significantly altered.

These data indicate that:

1. Despite excessive numbers of leukocytes in the peripheral blood, leukemic patients cannot mobilize tissue reservoirs of leukocytes as readily as the normal subject.

2. Contrary to expectations, the readily available leukocyte reservoir (RAR) in the leukemic patient is less than in the normal subject, with a ratio to the circulating leukocyte reservoir of 2–20 to 1, when compared with at least 60 to 1 measured in normal subjects.
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(3) The not readily available reservoir (NRAR) was hypercellular in 16 of 18 studies, suggesting an encroachment upon the BAR.
(4) One major defect in these leukemic patients was an impairment of release of leukocytes from the tissues into the blood.
(5) The rate of proliferation of leukocytes in these leukemic patients is far less than has been generally anticipated in the past.

THE EFFECT OF PHYTOHEMAGGLUTININ ON LEUKOCYTE CULTURES. C. Ross McIntyre and Franklin G. Ebaugh, Jr., Dartmouth Medical School and Hitchcock Clinic, Hanover, N. H.

The addition of phytohemagglutinin (PHA), a mucoprotein derived from the common bean (Phaseolus vulgaris), has been demonstrated by Nowell (Cancer Research 20:462, 1960) to be a factor necessary for cell division in leukocyte cultures. Using a recently developed membrane fractionation procedure for the determination of P32 in the DNA, RNA, and acid soluble fractions of tissue cultures (Kahan, F. M.: Analyt. Biochem. 1:107, 1960) we have investigated the metabolic events occurring in leukocyte cultures. Cultures not containing PHA show no increase in DNA-P32 activity whereas those containing PHA display rising DNA activity at 24 to 48 hours, which reaches a peak of 10-20 times that of the controls after seven days. The activity of the DNA fractions has been used as a means of assaying growth. The plant growth factors indoleacetic acid (2.0 y/ml.), kinetin (0.2 y/ml.), and traumatic acid (0.2 mg./ml.), do not duplicate the effect of PHA. Also ineffective are conjugated estrogens (0.1 to 1.0 y/ml.), fetal calf serum 2-20 per cent, and the addition of a clot to the culture. Chloramphenicol (6.1 x 10^-3 M), diphenylhydantoin (5 x 10^-4 M) and 6-mercaptopurine (1.3 x 10^-3 M), resulted in a decrease in growth.

Other experiments which consisted of adding PHA to cultures containing only erythrocytes demonstrated that PHA resulted in a firm binding of the cells to the glass, an effect not produced by agglutinating the erythrocytes by other means. It seems likely that PHA induces cell division either by its surface action, as demonstrated above, or by supplying some unrecognized substance necessary for growth to occur. The means of quantitating growth described here and the demonstrated depressing effects of various drugs indicates that PHA stimulated cultures may be useful for in vitro studies of drug resistance in leukemia.

COMPARISON OF CELL RENEWAL IN NORMAL AND LEUKEMIC STATES. Alberto Monti, Mary A. Maloney, Charlotte L. Weber, and Harvey H. Patt (Sponsored by Steven O. Schwartz), Argonne National Laboratory and the Department of Hematology of The Hektoen Institute for Medical Research of the Cook County Hospital, Chicago, Ill.

These studies are concerned with parameters of cell renewal in normal and leukemic patients, as revealed by autoradiographic analysis of samples of bone marrow and blood obtained after administration of tritiated thymidine (H3Th). Six patients have been studied thus far: three hematologically normal, one with chronic and two with acute granulocytic leukemia; each received 100 µc. per Kg. of H3Th intravenously. The initial labeling of proliferating myeloid cells was similar in the chronic leukemic and in the hematologically normal patients. This suggests that DNA synthesis time represents the same proportion of the cell generation cycle. The subsequent buildup of labeled cells in the proliferative pool and in the metamyelocytes was also the same, indicating a similar DNA synthesis time and hence, a similar turnover time. Labeled polymorphonuclear cells appeared in blood by the fifth day after H3Th injection in both the chronic leukemic and normal patients and disappeared at approximately the same rate. In the two patients with acute leukemia, the initial labeling of myeloblasts was comparable to that of normal myeloblasts. This suggests a similar turnover time of normal and leukemic myeloblasts, assuming that the DNA synthetic period is unchanged in acute leukemia, and that all leukemic myeloblasts proliferate. The validity of these assumptions, which are being explored in present studies, should permit an evaluation of the extent to which the altered proliferative capacity contributes to the hypercellularity of leukemic states.
IN VITRO DNA SYNTHESIS BY HUMAN MARROW CELLS. Clarence P. Alfrey, Jr., Joseph M. Kiely, W. Neulon Tauxe, and Charles A. Owen, Jr., Mayo Clinic and Mayo Foundation, Rochester, Minn.

The in vitro determination of the synthesis of desoxyribonucleic acid (DNA) in human marrow was measured by the uptake of tritiated thymidine. A technique was developed for accomplishing this with samples of 0.1 to 0.2 ml. of marrow. Three normal subjects were studied in this manner, as were one patient with mild anemia, one with thrombocytopenia, one with hyperglobulinemia, eight with acute leukemia, and three with multiple myeloma. The effect of addition of amethopterin, 6-mercaptopurine, nitrogen mustard, and actinomycin-D on the appearance of radioactivity in the nuclei of marrow cells also was evaluated.

Uptake of tritiated thymidine was found to be approximately linear for at least one hour; further incorporation of labeled thymidine occurred for several hours. A smaller fraction of mature cells than of immature cells synthesized DNA during the period of exposure to tritiated thymidine. In the normal persons, about half of the blast cells incorporated tritiated thymidine, whereas only 2 to 14 per cent of blast cells in patients with acute leukemia did so. Only 1 to 4 per cent of myeloma cells incorporated this radioactive label. In patients with multiple myeloma and acute leukemia, only limited correlation could be found between the percentage of cells labeled and the course of the disease, peripheral leukocyte count, concentration of hemoglobin, platelet count, or percentage of abnormal cells in the marrow.

Amethopterin, 6-mercaptopurine, nitrogen mustard, and actinomycin-D had no effect on DNA synthesis as measured by incorporation of tritiated thymidine into bone marrow cells during short term incubation. Similarly, DNA synthesis from thymidine was not inhibited by the enzyme poisons sodium azide, potassium cyanide, and dinitrophenol.

PHAGOCYTIC RETICULAR CELLS AND THEIR RELATION TO FATE AND FUNCTION OF SMALL LYMPHOCYTES IN GUINEA-PIG BONE MARROW. Dennis G. Osmond and N. B. Everett, Department of Anatomy, University of Washington, Seattle, Wash.

Tritiated thymidine and radioautography have been employed to study the DNA labeling pattern of small lymphocytes in guinea-pig bone marrow. In the course of these studies, phagocytized labeled lymphocytes have been observed in the bone-marrow reticular cells. Male guinea-pigs of the Hartley strain were administered H3-thymidine intraperitoneally (1 µc./Gm. body weight). After 25, 48, and 72 hours, smears were made of marrow suspended in homologous serum. Radioautographs were prepared by coating the smears with melted emulsion. After each time interval the cytoplasm of reticular cells was observed to contain as many as three labeled small lymphocytes, some of which evidenced partial digestion. The early appearance of these labeled lymphocytes in reticular cells and the evidence of their partial digestion as early as 25 hours after thymidine administration clearly indicates a short life span for these cells.

It is apparent that this phenomenon of lymphocyte phagocytosis does not represent an abnormal state, since small lymphocytes may be observed in reticular cells of suitably prepared bone-marrow smears from normal guinea pigs receiving no H3-thymidine. It is likely that the phagocytosis of lymphocytes by bone marrow reticular cells exists on a significant scale and may be important in providing a source of DNA or other materials necessary to the reticular cells for their metabolism and/or proliferation. This and other possible functional interpretations of the observations will be discussed.

FACTORS INFLUENCING THE FORMATION OF LE CELLS. Earl N. Metz and John Laszlo, Duke University Medical Center, Durham, N. C.

It was observed that EDTA inhibits the LE phenomenon. This paper summarizes experiments designed to elucidate the mechanisms of chelate interference, and the role of divalent cations, serum complement, and phagocytosis in the formation of the LE cell. Calcium or magnesium salts restored the LE phenomenon after it had been blocked by EDTA, although
excess amounts of Ca\(^{++}\) or Mg\(^{++}\) were inhibitory. Zinc failed to reverse EDTA inhibition. EDTA also inhibited serum complement as measured by the sheep cell hemolysis method. The LE phenomenon was inhibited when the complement of lupus serum was heat inactivated. Addition of complement restored LE cell formation. Further heating (70 C.) irreversibly inactivated the LE factor.

Phagocytosis by leukocytes also requires divalent cation, and this function was inhibited by EDTA. Phagocytosis of starch, but not of 0.8\(\mu\) latex particles, required complement as well. The formation of the LE cell had many similarities to starch phagocytosis as regards morphologic appearance and requirement of divalent cations, complement, and phagocytes. Addition of lupus serum to traumatized leukocytes resulted in increased oxygen consumption during the formation of LE cells. EDTA also blocked this enhanced respiration.

Citrate and oxalate were much less inhibitory to LE cell formation, serum complement titers, and leukocyte phagocytosis.

**SOME METABOLIC EFFECTS OF ANTILEUKOCYTE SERUM.** Julius Kritzman, and John McCarthy,
The Arthur G. Rotch Laboratory of The Boston Dispensary, Boston, Mass.

Potent antisera were produced by immunizing rabbits against guinea-pig granulocytes obtained by the artificial production of sterile, peritoneal exudates. The antisera were characterized immunologically by the production of multiple lines of precipitate on double diffusion in agar against whole-leukocyte extracts and by the precipitation of latex particles coated with extracts of lyophilized leukocytes. This latex fixation reaction was species specific and did not occur against latex particles coated with human leukocyte extracts. The leuko-agglutinin reaction was erratic and did not correlate well with the latex fixation, nor was it as reproducible. By these techniques, the antisera were shown to be potent and to contain antibodies to multiple cell constituents. The effect of these antisera on cell respiration and protein metabolism was studied. It was found that the antiserum (as compared with normal rabbit serum) caused moderate inhibition of entry of C\(^{14}\)-labeled DL-leucine into the granulocytes, but that most strikingly it caused almost 100 per cent inhibition of incorporation of C\(^{14}\)-labeled leucine into cell protein. The antisera also inhibited cell respiration, but to a considerably lesser degree. This effect was also observed with several other C\(^{14}\)-labeled amino acids. The inhibitory effect of the native sera was totally abolished at 56 C. for 30 minutes. The inhibitory effect could be restored by the addition of fresh rabbit serum. Absorption of the antisera by two volumes of packed guinea-pig granulocytes at 37 C. for one hour removed all latex-fixing properties of the serum but did not remove the inhibitory activity. Exhaustive absorption over a period of 24 hours, however, did remove inhibitory activity. These studies suggest possible mechanisms by which humoral antibodies against cells may cause their malfunction or death.

**DEMONSTRATION OF TISSUE ANTIGENS IN RABBIT BLOOD PLATELETS.** Shirley Ebbe and Mario Baldini, Blood Research Laboratory, Pratt Diagnostic Clinic-New England Center Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.

Platelet iso-agglutinins and reduced viability of transfused platelets have been observed repeatedly in humans who have received multiple transfusions of “blood group-compatible” platelets. This suggested that platelets have an antigenic structure totally or partially unrelated to red cell blood groups. The possibility that platelets may contain tissue antigens was considered. Experiments were designed, in the rabbit, to investigate the relationship between platelet antigens and skin antigens.

The influence of skin homografts on viability of subsequently transfused homologous platelets from the skin donor (“specific” platelets) or from another donor (“nonspecific” platelets) was first evaluated. Platelet viability was measured, using platelets labeled in vivo with \(^{33}P\). One to three successive skin homografts progressively reduced the survival time of “specific” platelets. It could be demonstrated that this effect was not due to the amount of blood contained in the grafts. The survival time of “nonspecific” platelets was also shortened by previous skin homografting, but to a lesser degree.
One skin homograft followed by one platelet infusion produced shortening of the survival time of subsequently infused platelets to a greater degree than was seen after only a skin graft or only a platelet infusion. This phenomenon suggested a secondary immunologic response due to similarity between the antigens of platelets and skin.

The rejection time of skin homografts was then studied in recipient animals injected previously with suspensions of homologous platelets which had been meticulously prepared free of leukocytes. In half of these experiments, rejection times of skin grafts from the “specific” platelet donors were significantly shorter than those from “nonspecific” donors. In the other half, rejection times of both types of grafts were normal.

Conclusions:
1. Blood platelets and skin have antigens in common.
2. These common antigens are not completely individual-specific.
3. The above results also indicate that blood platelets contain transplantation antigens.

CONTINUOUS IN VIVO COAGULATION, THE DETERMINANT OF PLATELET SURVIVAL. Edward Adelson, Jack J. Rheingold, Arnold A. Lear, Jenefr Isbister, James C. Kirby and Richard M. Kaufman, Department of Medicine, George Washington University School of Medicine, Washington, D. C.

If clotting is a continuous process in the normal subject, platelets must be destroyed in a random manner and platelet survival curves should be exponential. However, in some laboratories, platelet survival studies have shown linear curves which suggest that platelet destruction is primarily a process of aging. Other workers, ourselves included, report exponential curves indicating random destruction. To determine whether aging plays a significant role in platelet survival, a technique has been devised to provide a population of tagged platelets less than two days old. If aging determines platelet survival, the survival curve should form a plateau with a sharp drop at the end of the platelet life span.

The donor dog is given Na$_2$P$_{32}$O$_4$. One week later he is infused with large amounts of thromboplastin. His platelet count drops nearly to zero, where it remains for a period of days. On the second day of rise in the platelet count, the dog is phlebotomized, and the whole blood containing radioactive platelets less than two days old is transfused into the recipient dog. The resultant platelet survival curve is exponential and identical with the curve obtained when in vivo $P^{32}$-tagged platelets of mixed ages are transfused. This indicates that random destruction rather than aging determines platelet survival in the normal.

To ascertain whether the random destruction is due to in vivo coagulation, the experiment was repeated in dogs treated with heparin. This time, a plateau was obtained with a sharp drop on the tenth day of platelet life. Thus, when coagulation processes are prevented, platelet survival is a function of platelet aging.

We conclude that (1) platelets are destroyed in a random fashion in the normal subject; and (2) the process responsible for this random destruction is continuous in vivo coagulation.

PLATELET RESPONSE TO NATURAL AND FOREIGN AGENTS. T. T. Odell, Jr., T. P. McDonald, T. C. Detwiler, and F. L. Housden, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Several investigators have demonstrated that a marked depletion in platelet numbers in the blood is followed by increases to levels significantly greater than normal in a few days. The time pattern and the changes observed in the megakaryocytes suggest a stimulation of platelet production. Other results indicate that injection of serum or serum extracts can cause an increase in number of circulating platelets. We reported that injecting normal rats with serum of rats rendered platelet-deficient by bleeding or by injection of platelet-specific antiserum, caused an increase in platelet numbers to about 1½ times normal levels. Serum of untreated control rats was ineffective. Similar results have also been reported from investigations with rabbits.

We recently found that a similar platelet response can be induced by injecting various foreign agents. A solution of egg albumin, normal human plasma, or a suspension of finely
ground glass was injected subcutaneously into different groups of normal rats in the morning
and afternoon of two successive days. Platelet counts rose to 138–166 per cent of initial
counts on the fifth day when the experiment was terminated, a pattern previously seen in
rats given serum from platelet-depleted donors (active serum). White cell counts at five
days were: (a) not altered in the rats injected with active serum or normal human plasma,
(b) marginally increased by ground glass, and (c) more than doubled by higher doses (1.68
mg. total) of soluble egg albumin (relative granulocyte increase).

These results raise the question of whether the final pathway in platelet stimulation is the
same with foreign material as with the indigenous material. They also emphasize the need
for caution in experiments designed to concentrate and characterize a humoral agent with
thrombopoietic activity.

STUDIES ON THE PLATELET INCREASE INDUCED BY CITROVORUM FACTOR. Donald M. Hayes,
Charles L. Spurr, and William B. Deskins, Department of Medicine, Bowman Gray School
of Medicine of Wake Forest College, and the North Carolina Baptist Hospital, Winston-
Salem, N. C.

In the therapy of localized malignant tumors, several patients have received intra-arterial
amethopterin (40 mg. per day) and intramuscular citrovorum factor (9 mg. every four
hours). An unusual finding in these patients was a marked and sustained rise in the platelet
count while on therapy with no significant change in white blood cell counts or hemoglobin.
To investigate this phenomenon, 25 adult guinea pigs of mixed sex were divided into five
groups of five animals each.

Group A: Environmental controls; no treatment.
Group B: Drug controls; daily injections of 0.1 ml. saline.
Group C: Amethopterin only (0.5 mg. per Kg. per day).
Group D: Citrovorum factor only; (0.13 mg. per Kg. every four hours).
Group E: Amethopterin (daily) and citrovorum factor (every four hours).

Baseline platelet counts were performed daily for three days prior to beginning drug.
Injections were continued for ten days, at which time it was noted that Groups D and E
only had undergone a thrombocytosis of 50–100 per cent. These groups were followed with
platelet counts every other day for a total of 30 days before the values returned to their
baseline level. Because the platelet increase in group D was greatest, it was concluded that
citrovorum factor was probably the stimulus for this thrombocytosis. Using doses of 0.03,
0.06, 0.13, 0.20, and 0.25 mg. per Kg. every four hours, a dose response curve was derived
which revealed 0.13 mg. per Kg. to be the optimum dose, i.e., the one inducing the maximum
rise in platelet count. By using this dose and varying the time schedule, 0.13 mg. per Kg.
every four hours for seven days was found to constitute the optimum program. Although
the maximum rise in platelet count occurred during this seven day period, it was found that
this increased level could be maintained by continued drug administration for as long as
30 days. Preliminary data on a number of thrombocytopenic patients treated with citrovorum
factor in this fashion are to be presented.

AN ELECTRON MICROSCOPIC STUDY OF IN VIVO PRIMARY THROMBUS FORMATION. H. Cottier,
B. Roos, H. Rentsch and E. P. Cronkite, Institute of Pathology, University of Berne,
Switzerland, and Medical Research Center, Brookhaven National Laboratory, Upton, N. Y.

Earlier electron microscopic work on ultrastructural changes occurring in viscous meta-
morphosis of platelets and clot formation was based on imprints or sections of in vitro
preparations. Under these conditions artifacts cannot be excluded. Thus, in vivo studies are
essential.
A single, short, local irradiation of the head of rats, with high doses (8,000 r at 250
r/min.) — by damaging the endothelium and parenchyma — initiates the production of
intravascular platelet aggregates and thrombi. The high vascularity of the pituitary makes
it easy to find the preceding by electron microscopy; all stages from platelet aggregation to
clot retraction can be followed. Initially, the aggregated platelets show intact external membranes in most instances. Swelling is most marked in platelets located at the surface of the aggregate. Progressive degranulation appears early and often without visible damage to the platelet membrane.

In contrast to in vitro findings, the aggregating platelets show only moderate exocytosis of pseudopodes; do not spread out appreciably on the endothelial surface; and do not undergo coalescence of the central granules to form so-called pseudonuclei. Deposits of granular osmiophilic material sometimes arranged in short chains on the surface of or between aggregated platelets increase in number, size, and electron density as degranulation of the granulomere progresses. Some of the mitochondria retain their cristal structure over the period of degranulation, but are often swollen. Consecutively autolytic centers develop inside the platelets. In the final stage the volume of the disintegrated, "empty" platelets shrinks considerably as the fibrin increases in mass and density. The possible significance of these ultrastructural changes will be discussed.

Short, local irradiation of the pituitary with high doses provides a useful method of producing intracapillary primary thrombus formation suitable for electron microscopic studies. Possible applications of this procedure will be considered.

HEREDOFAMILIAL DEFICIENCIES OF CLOTTING FACTORS VII AND X ASSOCIATED WITH CAROTID BODY TUMORS. Arnold Kroll, Liberto Pecchet, Benjamin Alexander and Frances Cochios, Medical Research Laboratories of the Yamins Laboratory, Beth Israel Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass. (Supported by a grant (H-656) from the National Heart Institute of the National Institutes of Health, Public Health Service, U. S. Government.)

Deficiency of Factors VII (proconvertin) and/or X (Stuart) associated with carotid body tumors was frequent in five generations of one family of German-Irish extraction comprising 112 individuals including the propositus in whom an elevated prothrombin time was found prior to surgery.

Thirteen individuals (both sexes) had tumors, six documented histologically, six disclosed by history and physical, and one by history alone. Eight were siblings of the propositus (generation 3), one among her direct descendents, and three in their first cousins. Nine subjects with tumors also had coagulation defects; three had normal coagulation; the remaining one was not tested.

The coagulation disturbance also involved both sexes. Forty-two subjects showed deficient Factor X (range 10-69 per cent, in 33, less than 50 per cent of normal; in 7, below 20 per cent. Five were siblings of the propositus, and 7 were her children. Also deficient were siblings of the propositus' mother, some of their children and grandchildren, and some of the mother's first cousins.

Twenty-six were also deficient in Factor VII (range 19-85 per cent), in 14, 50 per cent of normal or less. Generally, the VII defect was milder than X, the lowest values being 10, 25, and 28 per cent respectively. Although most of those with clotting abnormality were deficient in both VII and X, 16 showed depression only in X. Conversely, in none was the defect limited solely to Factor VII. The profile of the defect was transmitted specifically to offspring. Other hemostatic and coagulation parameters were normal. No strict correlation was evident between tumors and clotting abnormalities. Although the latter was first demonstrated in generation 2, the former first appeared in generation 3.

Epistaxis, bleeding gums, easy bruisability, menorrhagia, and hemorrhage following dental extractions, were prominent in more than 40 individuals, preponderantly in generations 3 and 4.

The extreme rarity of each of the abnormalities makes the probability of their combination in a single family by chance alone, astronomically small. The biologic significance, patterns of transmission, variable expressivity, the possibilities of chromosomal cross-over, inherent chromosomal susceptibility to multiple point-mutations, etc., will be discussed.
PLASMA THROMBOPLASTIN ANTECEDENT DEFICIENCY ASSOCIATED WITH A UNIQUE TISSUE DISORDER. Robert L. Wall, Dante Scarpelli, William Molnar, and Charles Coltman, University Hospital, Columbus, Ohio.

The simultaneous occurrence of more than one inherited disorder in a family offers an interesting opportunity for the better understanding of each disorder. Inherited coagulation deficiencies rarely have been observed associated with other genetic disorders. Recently, a family was described with the combination of Factor IX deficiency and Ehlers-Danlos syndrome.

The present study describes the simultaneous appearance of plasma thromboplastin antecedent (P.T.A.) deficiency and a previously undescribed connective tissue disorder. The connective tissue disorder produces both shortening of the long bones, with resulting short stature, and multiple gross exostoses. The tissues are friable to surgical dissection. Finally, the elastic fibers of arteries are fragmented, calcified, and show redundant folding, most prominently in the pancreatic artery. This defect has resulted in fatal hemorrhage from the pancreatic artery in two sisters.

Forty members from four generations of this family have been studied. The P.T.A. deficiency varies in severity among the members of one generation and between generations. The phenotype of the connective tissue disorder, while resembling Ehler-Danlos syndrome in some respects (i.e., friability of tissues and hemorrhagic manifestations), differs radically in other respects (i.e., the absence of hyperelastic skin and hyperextensible joints, and the presence of multiple exostoses and fragmented arterial elastic fibers). This disorder also appears to be transmitted as an autosomal dominant. The possibility of monogenetic origin of the entire disease complex is suggested by its presence in one grandparent and child, and absence in a parent. Chromosomal abnormalities are under investigation.
SECOND SYMPOSIUM
Tuesday, November 28, 1961, 2:30–5:30 p.m.

SOME ASPECTS OF BONE-MARROW FAILURE
Moderator: Frank H. Gardner

IN VITRO ACTION OF CHLORAMPHENICOL AND CHLORAMPHENICOL ANALOGS ON HUMAN ERYTHROID TISSUE. Allan J. Erslev and Ioannis A. Iossifides, Cardeza Foundation, Jefferson Medical College, Philadelphia, Pa.

In order to elucidate the pathogenesis of the erythroid suppression occasionally observed in vivo after the administration of chloramphenicol, a study was made of the in vitro effect of chloramphenicol, its isomers, its known metabolic breakdown products and a number of its chemical analogs. Suspensions of human bone marrow obtained from surgically removed ribs were used to measure the rate of cellular uptake of iron; suspensions of human reticulocytes were used to determine the efficiency with which the incorporated iron was utilized in the synthesis of hemoglobin.

Results and conclusions: (1) Chloramphenicol suppresses both uptake of iron and its intracellular utilization when used in high concentrations (10 to 15 times therapeutic levels) for short periods of time. It is conceivable that a similar suppression may occur in vivo at low concentrations for prolonged periods of time. (2) The known metabolic products, with the exception of the nitrophenylamine analog, are inert in the test systems used here. Nitrophenylamine which suppresses iron uptake but not iron utilization is a compound found only in trace amounts in vivo. (3) The molecular arrangement at the site of the nitro-phenyl group of chloramphenicol is partly responsible for the inhibition of iron uptake but not for the inhibition of intracellular iron utilization. (4) The spatial configuration of the chloride atoms in chloramphenicol is partly responsible for the inhibition of intracellular iron utilization. (5) These test systems can be used for the study of the cellular action of drugs, but so far can be used only with great caution to predict the potential in vivo toxicity of a new compound. Tetracycline, for example, in concentrations of 10 to 15 times therapeutic levels, was found to suppress both iron uptake and iron utilization, although this drug in therapeutic concentrations in vivo probably is hematologically inert.

BONE MARROW PANHYPOPLASIA IN HUMANS EXPERIMENTALLY INDUCED BY VIRAL INFECTION. Donald L. Howie and William H. Crosby, Department of Hematology, Walter Reed Army Institute of Research, Washington, D. C.

It has been observed that profound marrow and lymph node panhypoplasia occurs in animals during infection with the virus of Venezuelan equine encephalitis. This virus, modified by passage through tissue culture, is being used experimentally in the treatment of patients with various lymphomas. Its effect on these patients and on healthy human volunteers has been observed. Patients with lymphomas appear to develop a more prolonged and intense viremia than do healthy subjects. They also develop more profound changes in blood and bone marrow.

Fever up to 104 F. occurs within 24 to 48 hours following inoculation. At this time there may be a slight granulocytosis. Then, within 24 to 36 hours the temperature falls, only to rise again about the seventh postinoculation day, gradually falling to normal in another two to five days. During the second period of fever, vaculated, abnormal lymphocytes appear in the blood, followed by reticulocytopenia, lymphocytopenia, thrombocytopenia and granulocytopenia, usually in this order.

At the time of maximum blood changes, panhypoplasia of marrow is observed. This hypoplasia may be extreme, with only fat, plasma cells, reticulum cells and scattered blasts...
remaining; few cells of the erythrocytic or granulocytic series beyond blast stages can be found. Lesser degrees of marrow injury may occur, especially in healthy adults.

In 3 to 14 days from the time of maximum hypoplasia complete recovery of bone marrow and circulating elements occurs. The blood cells usually reappear in reverse order of their disappearance.

The implications of this phenomenon in regard to the pathogenesis of the aplastic or aregenerative crisis will be discussed.


Experimental studies suggest that the erythroid series is not self-sustaining but is constantly repopulated from a primitive or stem cell compartment. Erythropoietine is at least one factor responsible for the differentiation of stem cells into erythroid elements. Following differentiation a series of mitotic divisions together with maturation occur before the emergence of the red cell as a reticulocyte. The number of divisions occurring before emergence is variable, as is intramarrow cell death. Intense stimulation of erythropoiesis (erythropoietine, blood loss, phenylhydrazine) is associated with the production of macrocytes, presumably due to skipped divisions; the life span of these macrocytes is shortened. Irradiation reduces cell production due to death of erythroid cells and stem cells, cells dying either immediately or as they enter mitoses. Hypertransfusion suppresses red cell formation by reducing the number of stem cells differentiating into erythroid elements.

Theoretically, the refractory anemias form three general groups: (a) Those in which there is failure of stem cell differentiation or a defect in stem cell production, the result being aplasia or hypoplasia. (b) Those in which stem cell differentiation is normal but there is increased intramarrow cellular death. In such instances there is deficient red cell production associated with a cellular marrow. When death of cells occurs early in the maturation phase, e.g., at the pronormoblast stage, the picture of "maturation arrest" is present. Reduced numbers of division in the developmental sequence might account for the macrocytosis, the variability of this phenomenon being reflected as anisocytosis. (c) Instances in which both (a) and (b) apply. The cause of these defects remains obscure. Inability to produce erythropoietine, however, does not appear to be implicated, since high concentrations of erythropoietine are present in the urine of all patients with refractory anemias, when the hemoglobin is below 7 Gm. per hundred. Patients with refractory anemia and cellular marrow respond normally to such stimuli as hypertransfusion, and in some instances may have an increased production following blood loss. The role of androgens is obscure, although they appear to exert their effect at the stem cell level.

ROLE OF THE SPLEEN AND EFFECT OF SPLENECTOMY ON BONE MARROW FUNCTION. William H. Crosby, Department of Hematology, Walter Reed Army Institute of Research, Washington, D. C.

Most of the hypersplenic syndromes seem to result from destruction of blood elements within the spleen. The bone marrow increases its production of blood cells when the spleen overactively destroys them. Hematopoiesis returns to normal when splenectomy is successful in curing the disease.

It is not certain whether the spleen has any other or any more direct effect upon marrow activity. However, the consequences of splenectomy sometimes suggest that the marrow has been inhibited or that the spleen has caused injury by some means other than sequestration and destruction of blood cells. (1) Specific hypocellularities of the marrow (megakaryocytes or plasma cells, for example) sometimes improve immediately after splenectomy. (2) In a few cases, chronic panhypoplasia has been corrected immediately after splenectomy. (3) In autoimmune disease the autoantibodies sometimes disappear immediately after splenectomy. (4) Autoantibodies may be directed against the marrow
itself, although it has not been shown that such antibodies originate in the spleen. (5) In
myelofibrosis, erythropoiesis within the marrow sometimes improves after splenectomy.
(6) In chronic idiopathic thrombocytopenic purpura, the production of platelets may be
relatively diminished. This has not been proved and it has not been related to the spleen
except by inference.

The Role of Testosterone in Bone Marrow Failure. Frank H. Gardner, Peter Bent
Brigham Hospital, Boston, Mass.

Androgen therapy has been used during the past five years in a variety of anemias
associated with bone marrow dyspoiesis. No method is available to predict which patient
will respond to testosterone. The anemias that have responded most satisfactorily are
associated with inadequate bone marrow erythroid precursors; i.e., hypoplastic anemia
(acquired and congenital), and myelofibrosis with extramedullary hematopoiesis. Favorable
responses have been noted in malignant reticulum disorders such as lymphoma and multiple
myeloma. There is no evidence to imply a hormonal effect on the neoplasia, but rather
androgens have been helpful to maintain higher blood values to allow more intensive
cytolytic therapy. No erythroid response has been obtained in azotemia, and there is no
evidence of response in myelocytic leukemia. Pharmacologic doses can increase the red
cell volume in aged men and women. The onset of the erythroid response is in part re-
lated to dosage and duration of administration. There is inadequate information to know
if erythrocytosis can be obtained in young adults with testosterone.

The mechanism for testosterone stimulation of the bone marrow is not known. The
response appears to be a nonspecific stimulation of erythroid precursors. The rate of red
cell maturation is not altered. The erythrocyte has morphologic alterations manifested by
hypochromia and a moderate decrease in the hemoglobin
concentration. The possible
alterations in hemoglobin synthesis are not proved.

Alkali-Resistant Hemoglobin in Aplastic Anemia, Acquired and Congenital Types.
Louis K. Diamond, Children's Hospital, Department of Pediatrics, Harvard Medical
School, Boston, Mass.

Infants and children with aplastic anemia treated successfully with corticosteroids and
testosterone, as described last year, were noted to have an abnormal amount of alkali-
resistant hemoglobin. When measured by the one-minute alkali denaturation method of
Singer, values ranged from 3 to 15 per cent (normal for children above three years of
age = less than 2 per cent). Agar-gel electrophoresis performed with hemolysate from
these patients revealed a benzidine-positive band which showed the same mobility as the
most intense band of normal cord blood. There was good agreement between the intensity
of this band and the concentration of the alkali-resistant hemoglobin determined chemically.
An acid hydrolysis of dried films of the peripheral blood from these patients revealed the
presence of alkali-resistant hemoglobin in a large number of the erythrocytes.
Quantitative changes in this hemoglobin were followed over a period of 6 to 36 months
in patients with aplastic anemia, both acquired and congenital types, during hematologic
remission. The alkali-resistant hemoglobin did not fall significantly in the patients with the
congenital type. However, it showed a steady decrease in those with acquired aplastic
anemia, although this was slow and an abnormal amount of alkali-resistant hemoglobin
still persisted long after the anemia was corrected. This form of hemoglobin was not
found in the parents and siblings of the patients with congenital aplastic anemia except
in one case where an abnormal amount (5 per cent) was seen in a sibling (13 year old
female) who had leukopenia and thrombocytopenia but no anemia. A year later, she
showed the fullblown picture of aplastic anemia. In one patient with the congenital form
who succumbed to hemorrhage and infection, autopsy revealed no abnormal erythropoietic
activity in the liver and spleen. All hematopoiesis was localized in the bone marrow alone.

Conclusions: (1) Alkali-resistant hemoglobin is increased in patients with aplastic anemia.
(2) This hemoglobin seems to have the same physiochemical characteristics as the fetal
hemoglobin of cord blood. (3) The concentration of this hemoglobin decreases slowly in patients with acquired aplastic anemia in remission, and tends to fluctuate without decreasing significantly in patients with congenital aplastic anemia. (4) The increase in concentration of this hemoglobin in patients with congenital aplastic anemia may precede the onset of pancytopenia. (5) The site of the production of this hemoglobin seems to be bone marrow and not the usual fetal sites.

TO BE READ IF TIME PERMITS

A SIMPLE METHOD FOR QUANTITATION OF A2 HEMOGLOBIN FRACTION. Ruth S. Hoffman and Charles C. Sprague, Department of Medicine, Division of Hematology, Tulane University School of Medicine, New Orleans, La.

A simple method of hemoglobin electrophoresis will be presented in which the A2 component was separated on paper strips using a discontinuous buffer system. Electrophoresis was carried out overnight at 150 volts in a Durrum-type cell. The paper strips were stained with bromphenol blue, and percentages of components were obtained from a densitometer—integrator trace in a manner similar to serum protein quantitation.

Percentages for A2 fractions and major components will be reported on normal persons and those with iron deficiency, thalassemia, sickle cell trait, and sickle cell thalassemia. The range of values for A2 in these groups was found comparable to those reported in the literature by other methods. It was noted in all groups, except iron deficiency where low values were obtained initially, that the value of A2 decreased in relation to the number of days which the sample had been refrigerated. Statistic analysis revealed that 24 per cent of the variance in the A2 values could be accounted for by differences in refrigeration interval. It was shown further that the progressive decrease in percentage of A2 followed a linear regression. A significant difference was observed in the value of the A2 fraction in paired fresh and frozen samples.

A description of the procedure and method of quantitation will be presented with results obtained and appropriate illustrations.

PLATELET LABELING WITH CR51 FOR LIFE-SPAN DETERMINATION. Robert C. Brown, W. Newton Tauxe and Albert B. Hagedorn, Mayo Clinic and Mayo Foundation, Rochester, Minn.

The present investigation was directed toward development of a simpler and more satisfactory method of determining platelet life span and rate of destruction than has been available. Utilizing Cr51 as a label and requiring only about 50 ml. of venous blood, the technique devised is fairly simple to perform, reliably reproducible, and quite suitable for the study of various thrombocytic disorders in the human. Particular attention was devoted to the problems of chromium toxicity to the platelets, elimination of as many potentially injurious steps in the labeling process as possible, fixation of the label, and elution thereof from the thrombocytes.

In a series of normal subjects, the life span was found to average 10.8 ± 0.8 days, with a mode of destruction resultant from a combination of linear and exponential factors. Similar results were noted in cases of thrombocytosis; however, in idiopathic thrombocytopenic purpura the effective life span was but three to four days, with a distinctly exponential mode of platelet disappearance.

ENZYMATIC FIBRINOLYSIS INDUCED IN HUMAN BLOOD BY SYNTHETIC HYDROTROPIC COMPOUNDS. K. N. von Kaulla, Department of Medicine, University of Colorado School of Medicine, and Belle Bonfils Memorial Blood Bank, Denver, Col.

Previous studies from this laboratory (Proc. Soc. Exper. Biol. & Med. 106:520, 1961) indicate that certain hydrotropic urea derivatives, 0.4 M ethyurethan, excluding urea itself, activate the fibrinolytic enzyme system upon dissolution in human plasma. The compounds are inactive in both bovine fibrin and plasma because their fibrinolysis-activating property
depends upon the presence of plasminogen plus an additional factor contained in human plasma. In this respect they resemble streptokinase.

Recent investigations reveal that hydrotropic compounds unrelated to urea or its derivatives (0.24 M 2,4-dimethylbenzenesulfonate, 0.1 M 2-naphthoate, 0.08 M p-bromo-benzoate, and others) likewise have the capacity to induce marked fibrinolytic activity in human plasma upon dissolution. This capacity seems to be closely related to the presence of a large anion in the molecule; large cations are ineffective. Amphoteric surfactants, anionic in alkaline media, induce lysis in a concentration of \(2 \times 10^{-2}\), and are among the most active compounds observed so far. Fibrinolytic activity induced by synthetic agents was measured quantitatively by progressive dissolution of preformed human plasma standard clots (Thromb. Diath. Haem. 5:489, 1961) and by a caseolytic method; and qualitatively by spontaneous dissolution of thrombin-clotted plasma containing these agents and by bovine fibrin plates on which the compounds were active only when dissolved in human plasma. The activity induced by the synthetic agents in human plasma develops more slowly but is more stable than that induced by enzymatic activators.

As a working hypothesis, it is thought that hydrophobic groups of the compounds attach themselves to an inhibitor of a plasminogen activator, whereas the hydrophilic group is directed toward the surrounding water thus solubilizing the inhibitor and releasing the activator for plasminogen activation. It is hoped that the synthetic fibrinolytic agents might be developed into versatile, nonantigenic, and inexpensive thrombolytic preparations.

**COMBINED THERAPY OF AGNOCENIC MYELOID METAPLASIA WITH TESTOSTERONE, BUSULFAN, AND PREDNISONE. Richard T. Silver, David E. Jenkins, Jr., and Ralph L. Engle, Jr., Department of Medicine, New York Hospital-Cornell Medical Center, New York, N. Y.**

Testosterone preparations have been used in the treatment of patients with agnogenic myeloid metaplasia in an attempt to stimulate erythropoesis, and busulfan or prednisone has been used in an effort to diminish hemolysis. General agreement has not been reached concerning the value of these measures.

Accordingly, nine patients with agnogenic myeloid metaplasia were treated first with testosterone enanthate, 400 to 600 mg. intramuscularly each week for three to four months. Then, if the white-blood-cell and platelet counts were elevated, busulfan, four to eight mg. daily, was given in addition to the testosterone; if the Cr51 red blood cell survival time was shortened and if the white blood cell and platelet counts were normal or low, prednisone, 40–60 mg. daily, was given in addition to the testosterone. Before and after each change in therapy, all patients were evaluated with comprehensive hematologic tests which included, in part, bone marrow biopsy, spleen biopsy, Cr51 red blood cell mass and survival studies, Fe59 plasma clearance and red blood cell utilization, and I131 plasma volume determination.

Of the nine patients studied before treatment with testosterone, all had varying degrees of anemia, and two had severe thrombocytopenia. Six patients had less than 70 per cent uptake of Fe59 in their red blood cells; two had a low normal uptake. Only three patients had a shortened Cr51 red cell survival time. After treatment with testosterone, six patients showed striking hematologic improvement, one had no response, one died soon after treatment was begun, and one has not yet been completely evaluated. One patient with an elevated white blood cell count and shortened Cr51 red blood cell survival time after testosterone, has shown no further improvement with the addition of busulfan.

These preliminary results are added evidence that testosterone is of value in treatment of agnogenic myeloid metaplasia. Further evaluation of the addition of busulfan or prednisone is in progress.

**THE EFFECTS OF BLOOD pH ON TRANSPORT OF BILIRUBIN. Arthur Sawitsky and Eli Seifter, Department of Laboratories and Hematology Service, Long Island Jewish Hospital, New Hyde Park, N. Y.**

The pH of the blood of young rabbits was varied and a metabolic type of acidosis or
alkalosis was produced. Sodium bilirubinate (40 mg. per Kg. body weight) was then injected intravenously. The concentration of bilirubin was determined in the blood and spinal fluid, and adipose tissue was extracted for pigment. In the control animals, where the blood pH was normal, no bilirubin appeared in either the spinal fluid or adipose tissue. In animals rendered acidic by prior treatment with ammonium chloride or acetazolamide, the injection of sodium bilirubinate led to death when the blood pH was below 7.1. Above this pH, the animals survived and showed spinal fluid bilirubin concentrations of 0.2 to 1.2 mg. per cent. The adipose tissue of these animals contained significant extractable bilirubin. When the rabbits were made alkalotic with sodium bicarbonate, no bilirubin was found in the spinal fluid or adipose tissue. Animals first made acidic with acetazolamide and then treated with sodium bicarbonate showed little or no bilirubin in the spinal fluid.

Respiratory acidosis was followed by the appearance of large amounts of bilirubin in the adipose tissue but only small amounts in the spinal fluid. Respiratory alkalosis on the other hand resulted in bilirubin-free adipose tissue, but spinal fluid with significant concentrations of bilirubin. Metabolic alkalosis was the only experimental condition in which no bilirubin was found in either the spinal fluid or adipose tissue.

Studies with other organic ions, such as salicylate, suggest that the transport of these ions, too, may be partially pH dependent. These results lead to the conclusion that the rate of transport of an organic acid across a membrane is in part a function of its lipid solubility; but the equilibrium concentrations in either compartment are related to the dissociation constant of the acid and the pH difference between the compartments.

MECHANISM OF THE IMMUNOHEMATOLOGIC EFFECT OF PAPAIN AND RELATED ENZYMES.
Morton D. Prager and Klara Efron, Wadley Research Institute and Blood Bank, Dallas, Texas.

Human erythrocytes treated with the thiol proteases papain, bromelin, and ficin, or the serine protease trypsin, agglutinate specifically with nonsaline-agglutinating Rh antisera: Only two minutes' incubation with enzyme is required. While not excluding covalent bond cleavage, the speed of reaction suggests that it may be ionic. Of 18 enzymes examined, only the four mentioned produced the serologic effect. They also share common specificity in catalyzing hydrolysis of arginine and lysine derivatives. However, thrombin and plasmin with the same specificity are serologically inactive.

In studies with crystalline papain as a prototype it was found that nonactivated enzyme, and enzyme in presence of p-chloromercuribenzoate, are serologically active with nonsaline-agglutinating Rh antisera: Only two minutes' incubation with enzyme is required. While not excluding covalent bond cleavage, the speed of reaction suggests that it may be ionic. Of 18 enzymes examined, only the four mentioned produced the serologic effect. They also share common specificity in catalyzing hydrolysis of arginine and lysine derivatives. However, thrombin and plasmin with the same specificity are serologically inactive.

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Susceptibility to Immune Hemolysis as Related to the Age of Human and Dog Red Blood Cells. Robert C. Griggs and John W. Harris, Western Reserve University School of Medicine Cleveland Metropolitan General Hospital, Cleveland, Ohio.

Susceptibility of human and dog erythrocytes to immune hemolysis as related to cell age was studied in three different hemolytic systems: (a) human red cells and a Donath-Landsteiner antibody from a patient with paroxysmal cold hemoglobinuria (PCH); (b) human red cells and an anti-human red cell rabbit antibody; and (c) dog red cells and an anti-dog red cell rabbit antibody. An identifiable population of erythrocytes of known age was produced by the administration of Fe59 to two humans and two dogs, previously bled to stimulate erythropoiesis. At intervals thereafter and for a minimum of 130 days samples of red cells were hemolysed in vitro by the antibody and appropriate amounts of human or canine complement. Two different hemolytic levels for each sample gave similar results. The degree of hemolysis of the whole population determined by hemoglobin released was compared to hemolysis of the tagged cells, as indicated by the Fe59 released. With random hemolysis, independent of cell age, the two figures should be identical.

The most striking changes with age were seen with human red cells hemolysed by PCH antibody. Barely detectable hemolysis of new red cells was followed by gradually increasing hemolysis that reached a maximum of approximately 75 per cent between 110 and 125 days.

Using the rabbit antibody, human red cells showed some hemolysis when very young, less hemolysis at mid-life and then increasing hemolysis to a maximum at 100 to 120 days. Dog red cells hemolysed by rabbit antibody showed gradually increasing hemolysis with age.

These findings indicate that with increasing age the erythrocytes became more susceptible to immune hemolysis; for human erythrocytes maximum susceptibility was reached at their expected life span of 120 days.

The Disappearance of Intravenously Injected Radioactive Vitamin $B_{12}$ in Pernicious Anemia in Relapse and in Remission. Eugene A. Brody, Solomon Estren and Louis R. Wasserman, The Mount Sinai Hospital, New York, N. Y.

Studies have demonstrated that the disappearance from the plasma of intravenously administered radiocobalt vitamin $B_{12}$ (0.5 $\mu$g.) is slower in all patients with pernicious anemia than in the normal. In pernicious anemia in relapse, the disappearance was considerably slower than in the normal; in complete remission, the curves approach but never reach the normal.

The variation of the pernicious anemia curve from that of the normal could not be explained by any difference in tissue uptake in urinary excretion of radioactivity in vitamin $B_{12}$ binding capacity of the serum (there is no difference between that of normal serum and that of pernicious anemia in remission), or isotope dilution factors. The difference was not due to increased binding of the injected $B_{12}$ by circulating globulin as seen in similar experiments in patients with chronic myelocytic leukemia.

When the test dose was preincubated with concentrated purified intrinsic factor before injection into normal subjects and into patients with pernicious anemia in relapse, in partial remission, and in complete remission, the resulting disappearance curves were all normal. The significance of these findings is discussed in terms of the transfer of vitamin $B_{12}$ from plasma to tissues and of the role of extragastric (e.g., plasma) intrinsic factor in this transfer. The absence of a plasma $B_{12}$ "transferring substance" is postulated as an explanation for the slow disappearance rates in patients with pernicious anemia.

The Immunologic Properties of Human Hemoglobin. Paul Heller, Vincent J. Yokults and Aaron M. Josephson, West Side Hospital, University of Illinois College of Medicine, Chicago, Ill.

Previous immunoelectrophoretic (i.e.p.) studies in our laboratory have shown that the
minor component A2 of human hemoglobin and a cathodically moving (pH 8.8) non-hemoglobin fraction which appears to consist of enzyme proteins, are highly antigenic to guinea-pigs. The main components A, S, and C were found not to be antigenic. A hemoglobin-free precipitin line in the area of A hemoglobin was found to contain catalase. Antiserum to fetal hemoglobin reacted with cord-blood hemolysate as test antigen in the expected manner, but the line extended over a wide area. The line was similar with S-hemolysate as test antigen, whereas with A the line showed two arcs which were confluent, and with C the line was shifted toward the cathode. The distribution of fetal hemoglobin on the electrophoregram appeared to depend on the position of the main component. There was no immunologic difference between fetal hemoglobin of cord blood and of thalassemic blood. These data and other supporting evidence are consistent with the hypothesis that fetal hemoglobin is immunologically homogeneous and electrophoretically heterogeneous.

The i.e.p. pattern of the reaction of whole hemolysates of A, S, or C blood with the respective antisera was basically the same, regardless of the identity of the hemolysates or the antisera. It consisted of precipitin lines to A2 and fetal hemoglobin, and the two non-hemoglobin components. Small differences concerned, as in the case of the isolated fraction, the fetal precipitin line, the position and shape of which depended upon the test antigen.

These data appear to explain the previously found lack of immunologic specificity of A-, S-, and C-hemolysates. Such specificity, however, is demonstrable for fetal hemoglobin. This phenomenon suggests lack of antigenicity of the α and β chains whereas the γ and δ chains seem to have greater species specificity. Preliminary studies indicate that the γ chain is a reactive test antigen, whereas the α and β chains appear to be immunologically inert.

INTERACTING THALASSEMIA WITH NORMAL A2 HEMOGLOBIN AND ELEVATED OR NORMAL FETAL HEMOGLOBIN LEVELS. James A. Wolff, College of Physicians and Surgeons, Columbia University, New York, N. Y.

The pedigrees of three subjects with thalassemia major, one or more of whose parents have normal values for A2 hemoglobin, have been analyzed.

In the families of two of the six parents, hereditary transmission of “classic” thalassemia with elevated levels of A2 hemoglobin has occurred. Two other parents have a type of thalassemia characterized by normal A2-hemoglobin levels and unusually elevated fetal hemoglobin levels, of which the hereditary transmission is clearcut. One parent is a member of a family showing transmission of thalassemia in association with an unusually high fetal hemoglobin and presumably Lepore hemoglobin. One parent has “morphologic” thalassemia with normal levels of both minor hemoglobin components.

Most persons with thalassemia and elevated A2-hemoglobin levels have normal fetal hemoglobin levels. When elevated, the increase does not exceed 5.0 per cent. Thalassemia characterized by a normal A2-hemoglobin level usually has an elevated fetal hemoglobin level. This increase, when present, exceeds 5.0 per cent. Thalassemia characterized by the presence of Lepore hemoglobin may also have levels of fetal hemoglobin greater than 5.0 per cent.

The four types of thalassemia described in the pedigrees of the three reported persons with thalassemia major are produced by defects of β-chain synthesis of hemoglobin alone or in combination with deficiencies of δ-chain or ε-chain and γ-chain synthesis of hemoglobin.


The most frequently used techniques for estimation of heparin levels (or protamine requirement) after open-heart surgery are protamine titrations and thrombin times, Evi-
dence will be presented that, under some conditions, the total amount of heparin present is measured, while other approaches are influenced by factors in the blood antagonistic to heparin.

Disruption of red blood cells and platelets during storage of heparinized blood or in the course of extracorporeal circulation liberates antiheparin, thromboplastic, and thrombin-accelerator activities. These substances shorten the clotting time of heparinized blood. Use of the thrombin time to estimate heparin levels will provide a falsely low answer in proportion to the amount of antiheparin and thrombin-accelerators released. Protamine titration with an endpoint taken as the minimum amount of protamine that will shorten the clotting time of heparinized blood to 15 minutes is influenced also by release of heparin antagonists during storage or trauma. On the other hand, protamine titration with an endpoint of the least amount of protamine that results in a minimum clotting time is not influenced by endogenous heparin antagonists, suggesting that protamine has a greater affinity for heparin than for the latter. Using this endpoint, heparin levels in blood are unaffected by storage for as long as six months, by any degree of trauma to blood, or by freezing and thawing whole blood.

Thus, protamine titration with a minimum clotting time as endpoint provides the best index of the amount of protamine required to neutralize all heparin present. The 15-minute endpoint or the thrombin time provides a better index of the effective level of the anticoagulant. Failure to neutralize all heparin present might provide an explanation for heparin rebound, if the remaining anticoagulant has been temporarily neutralized by endogenous antagonists with rapid clearance times. This remains to be proved.

Hematological Remissions in Acute Leukemia with Cyclophosphamide. Charlotte T. C. Tan, Jane Pho, Margaret Lyman, M. Lois Murphy, Harold W. Dargeon and Joseph H. Burchenal, Divisions of Clinical Chemotherapy, Sloan-Kettering Institute and the Departments of Pediatrics and Medicine, Memorial Sloan-Kettering Cancer Center, New York, N. Y.

Cyclophosphamide has been given to a total of 50 children with acute leukemia. Most of these children had previously been treated with either 6-mercaptopurine, methotrexate, or both, and adrenal steroids, and had become resistant to these agents. Cyclophosphamide produced remissions in 14 of 40, or 35 per cent of adequately treated patients. The oral dosage varied from 2 to 10 mg. per Kg. of body weight daily, and intravenous dosage from 5 to 15 mg. per Kg. of body weight weekly. After remission occurred, the drug was continued. If signs of hematologic depression or toxicity developed, the dosage was then reduced or discontinued temporarily.

Toxicity consisted of redness or ulceration of the buccal mucosa, nausea, vomiting, skin eruptions, alopecia and cystitis. Toxic signs usually disappeared when the drug was discontinued.

Of these 40 patients, 7 had a complete and 7 a partial remission according to the CCNSC criteria of remission in children with acute leukemia. The duration of remissions in 6 of these 14 patients was one month or less, in 4 patients it was one to two months, in 2 patients 2 to 3 months, and in 2 patients 4 and 10 months respectively. Two of these patients are still on therapy and in remission for more than one and three months respectively.

Ten children received a total dose of less than 15 mg. per Kg. of body weight in less than 10 days and were considered to be inadequately treated.

The incidence and duration of remissions were not significantly influenced by the various dosage schedules or the route of administration of the drug.
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