CURRENT BIOCHEMICAL AND GENETICAL CONCEPTS OF THALASSEMIA

Moderator: Park S. Gerald

WHAT IS THALASSEMIA? Chairman's Introduction.

The term “thalassemia” conjures to mind a number of characteristic clinical features. No single feature, however, nor combination, is so characteristic as to serve as an absolute diagnostic criterion. Nonetheless, it seems possible to advance at the present time a definition of thalassemia which will be acceptable to a significant number, though perhaps not a majority, of current investigators. The definition proposed is that thalassemia is a group of genetically determined conditions with a primary alternation in hemoglobin synthesis, resulting in diminished production, but without formation of an electrophoretically abnormal hemoglobin. During the course of the Symposium, the recently available biochemical and genetic facts relating to such possible disturbances of hemoglobin synthesis will be presented and assessed.


A unifying hypothesis is proposed which seeks to explain the occurrence of thalassemia and some of its interactions with the other hemoglobinopathies in which chemically abnormal hemoglobins are recognized. In particular, we wish to suggest that thalassemia is a mutation producing a hemoglobin with a ‘hidden’ amino acid substitution, such that the hemoglobin produced is electrophoretically normal. There are many such mutations possible. It is hoped that the hypothesis, even if proved wrong, will serve to stimulate interest and direct experiment in this field.


The early observations on individuals presumed to possess both a gene for Thalassemia and for sickling or for hemoglobin C demonstrated the occurrence of so-called interaction resulting in the virtual suppression of hemoglobin A production, enhancement of the proportion of the abnormal hemoglobin well beyond that expected for the simple heterozygous state and the clinical hematologic complex of severity roughly comparable to sickle cell anemia.

Subsequently it became apparent that such a uniform picture is not always to be expected and that in persons presumed to possess an abnormal hemoglobin gene and a gene for Thalassemia or a Thalassemia-like state might result in a relatively mild or asymptomatic hematologic state without enhancement of the proportion of abnormal hemoglobin. These findings suggested that there might be two or more Thalassemia-like syndromes caused by
different genes. One of these which has been referred to as the “classical” Thalassemia gene has since been shown to be rather consistently associated with an elevation of A₂ hemoglobin. Additional variants of this latter gene which is prevalent in Mediterranean races will be discussed. Another appears to be characterized by the production of high percentages of fetal hemoglobin in heterozygotes, transmission in a manner analogous to hemoglobin S and C genes, a normal blood picture in the simple heterozygous state and interaction-like effects in combination with other abnormal hemoglobin genes.

From statistical data not as yet backed by direct observations on pertinent pedigrees it appears that the "high A₂" or “classical” Thalassemia gene is allelic to the genes for hemoglobin S and C. Studies to be presented support the theory that there is at least one other Thalassemia-like syndrome in which the underlying gene does not cause elevated A₂ production or "interaction" either at the biochemical or at the hematologic level. This gene has been observed in Negros and appears to be non-allelic to the S-C group of genes.

**Hgb H Disease, Bart’s Hemoglobin, and Thalassemia.** P. S. Gerald, Boston, Mass.

Hgb H and Bart’s hemoglobin, both individually and in combination, are usually associated with thalassemia. Since neither of these hemoglobins possesses the α-chains found in all other hemoglobins, it is presumed that the associated thalassemia is responsible for a deficiency of α-chains—i.e., it is an α-thalassemia. One of the most outstanding characteristics of α-thalassemia, as so defined, is its normal Hgb A₂ level, in contrast to the elevated A₂ concentration found in Cooley’s anemia families. This and other features are explicable in the light of current evidence that both Hgb A₁ and Hgb A₂ possess α-chains which are under the same genetic control.


Hemoglobin A₂ is a normal, though minor, component found in the hemolysates of adult humans. Its chemical structure has been compared with that of the major adult component, hemoglobin A by “fingerprinting and other techniques. It appears that hemoglobin A₂ has two alpha-polypeptide chains in its molecule which seem to be identical in structure and genetic control with the two alpha-chains of hemoglobin A and of fetal hemoglobin. The other two peptide chains of hemoglobin A₂—the delta chains—are chemically distinct from the corresponding beta peptide chains of hemoglobin A; they are also controlled by separate genes. In many cases of thalassemia—the so called beta-chain thalassemias—the level of hemoglobin A₂ is doubled. These findings will be discussed in the light of current hypotheses of thalassemia.


Studies have been made of the hemoglobins in a family containing genes for thalassemia and hemoglobins G and S. It is evident from these studies that the inheritance of hemoglobins G and S is probably controlled by allelic genes. This is in contrast to earlier reports that the gene for hemoglobin G cannot be allelic with either the gene for hemoglobin S or thalassemia. Errors in interpretation of the previous studies on this family resulted from the presence of hemoglobin components (not fetal nor A₂) that are ordinarily found in minor amounts in normal individuals, but which often occur in greater than normal quantities in members of this family. In order to determine whether these components reflect changes associated with the thalassemia trait, a chromatographic analysis of the minor components in individuals of this family has been undertaken. These minor components appear to have no direct relationship to the occurrence of thalassemia although they do contain the primary structural alterations associated with hemoglobins G or S. The minor components seem to be derived, at least partially, by denaturation of the hemoglobins present in major amount in erythrocytes.

By applying a variation of the hypotonic squash technic and a hypotonic dried-film technic (Moorhead) to samples of human marrow immediately following aspiration it has been possible to obtain clear, spread mitotic figures. This approach has the considerable advantage that it (1) avoids the major artifacts of even short periods of culture, namely, alterations in chromosome size and by inference possible alterations in other morphologic characteristics, and selection of hardened cell types; (2) permits study of the in vivo effects on chromosomes of colchicine, cyanocobalamin, etc. unmodified by subsequent incubation.

Results revealed no intact cells in mitosis with chromosome numbers not interpretable as 46 (except for occasional, approximately tetraploid or multiploid cells) and, in general, the better the preparation, the more certain was the count 46. These results confirm the majority of recent observations using tissue culture methods. However, there were no examples of the type of markedly hypoploid cells reported by others in association with cellular maturity using different technics. Because of the present state of the technic, results should not be interpreted as showing that there are no cells with a slight variation in the chromosome number, or that rare cells with greater hypoploidy do not exist.

Results also showed a wide range in the sizes of corresponding chromosomes of different cells in the same apparent stage of mitosis. This appears to cast a possible doubt upon the statement that variation in nuclear size confirms the existence of somatic variation in chromosome number, because evidently chromosome size and by inference nuclear size may widely vary between cells with 46 chromosomes.

It is concluded that this approach to the study of the morphology of human bone marrow chromosomes is useful because it provides preparations of good quality free of the artifacts of tissue culture and adaptable to the study of the in vivo effects of various drugs.


A single Peyer's patch in the small intestine of the mouse, which contains about $5 \times 10^6$ fixed and free lymphatic cells, was protected while the remainder of the body was X-irradiated. Within 6 days the lymph nodes, spleen and thymus showed marked regeneration, although no granulocytic, erythrocytic, or megakaryocytic growth appeared in the repopulated tissues. These histologic findings were reflected in the peripheral blood counts. The lymphoid repopulation that followed Peyer's patch protection prevented the loss of immune response that accompanies massive total-body irradiation, as follows:

1. When lethally irradiated mice with the Peyer's patch protected were injected with 100 million rat or $10 \times 10^6$ homologous mouse bone marrow cells, the injected bone marrow cells were rejected and the mice died. Control sham-operated irradiated mice in which the patch was not protected survived as expected when given foreign bone marrow.

2. Low dose irradiation permits the "take" of tumors transplanted to non-specific strains of mice, but Peyer's patch protection during such irradiation led to rejection of the grafted tumor.

The Peyer's patch proved to be surprisingly resistant to X-irradiation, and dosages of 650 r or higher were required to abolish the immune response. While it is well known that less than 1 million mesenteric lymph node or adult mouse spleen cells will result in rejection of a rat bone marrow graft, mouse bone marrow and mouse fetal liver cells do not result in immediate rejection. Homologous mouse fetal liver in conjunction with rat bone marrow resulted in persistence of the rat cells, and "secondary disease" ensued.
THIRD ANNUAL MEETING

CARBOHYDRATE METABOLISM IN LYMPHOCYTIC LEUKEMIA LEUKOCYTES. E. P. Noble, R. L. Stjernholm and A. S. Weisberger, Cleveland, Ohio.

Leukocytes from patients with chronic lymphocytic leukemia were incubated with various C\(^{14}\) labeled substrates. As indicators of metabolism, glycogen of the cells and lactate of the medium were isolated, purified, and the distribution of label in each compound was determined by degradation.

C\(^{14}\) activity from labeled glycerol and pyruvate was located in the carbon 4,5 and 6 unit of glycogen glucose. The highly asymmetrical distribution of label is in accord with the transaldolase exchange reaction (Noble et al. J. Biol. Chem. 235, 1261 (1959)) and indicates a lack of net glycogen synthesis. Glycerol-1 (3)-C\(^{14}\) labeled carbon 4 and carbon 6 equally and exclusively. Pyruvate-3-C\(^{14}\) activity was primarily incorporated into carbon 6; a minor amount was found in carbon 5.

D-Ribose-1-C\(^{14}\) and D-xylose-1-C\(^{14}\) yielded glycogen labeled primarily in carbon 1 with smaller amounts in carbon 3 and carbon 6. The ratio of C\(^{14}\) of carbon 1 and carbon 3 was 2 with ribose and 4 with xylose. This observed distribution of label is in accord with the transaldolase-transketolase sequence of reactions.

About 80\% of glucose-1 or-2-C\(^{14}\) entered glycogen without randomization. The remaining C\(^{14}\) activity was primarily accounted for in carbon 6 and carbon 5 respectively. In contrast, glucose-6-C\(^{14}\) gave a glycogen with 98\% of its activity in carbon 6. These results are similar to previous studies on normal polymorphonuclear leukocytes (Stjernholm and Noble, Fed. Proceedings, 19, 82 (1960)) and further support the role of the transaldolase-exchange reaction in white cell metabolism. Lactate degradation data derived from all labeled substrates indicate that the main route for carbohydrate catabolism is through the Embden-Meyerhof pathway.

IN VITRO METABOLIC INTERACTION OF ISOLATED NUCLEAR RIBOSOMES, DNA AND HISTONE FROM LYMPHOCYTES. John H. Frenster, Vincent C. Alifrey and Alfred E. Mirsky, New York, N. Y.

The cell nucleus, isolated from calf thymus lymphocytes, remains capable of intensive protein and RNA metabolism after its isolation, mainly in its ribonucleoprotein particles (ribosomes) which are contained within the cell nucleus. Such nuclear ribosomes are believed to be present both in the nucleolus and along the chromosomes of the nucleus. These nuclear ribosomes can be extracted from the whole nucleus, they remain capable of protein and RNA metabolism in a nucleus-free system after their extraction.

Nuclear ribosomes, DNA and histone were each isolated from the cell nuclei of calf thymus lymphocytes, and their metabolic interaction studied during in vitro incubations. It was hoped to determine whether DNA and histone are metabolic antagonists as has been postulated by Stedman.

Isolated nuclear ribosomes incubated with L-leucine-1-C\(^{14}\) in a nucleus-free system display a progressive increase in the radioactivity of their contained proteins over a 60 minute period. Lymphocyte DNA added to such incubations, results in a 50-100 percent increase in this rate of protein metabolism of the nuclear ribosomes.

Addition of lymphocyte histone effectively antagonizes the stimulatory effect of added DNA, and histone produces an inhibition when added alone. DNAase added with DNA abolishes the stimulatory effect of DNA, but DNAase alone has no effect. DNA prepared from calf thymus lymphocytes, calf kidney, or wheat germ appears equally effective in stimulation. Polyethylene Sulfonate, like DNA a polyanion, by contrast produces an inhibition of protein metabolism when added to incubations of nuclear ribosomes.

These data suggest that DNA and histone are antagonists in their effects on the metabolism of nuclear ribosomes in vitro. The stimulatory effect of DNA depends upon the intactness of the DNA molecule, is general for DNA of various organs and species, and is not a general polyanion effect.

Much information has accumulated in recent years on relations between the titer of circulating antibody and the manifestations of immunity. However, little is known on the duration of antibody synthesis. In order to obtain more information on the production of various types of antibody, we injected rabbits intravenously with one ml. of a 3% solution of various protein antigens. Antibody titers were measured by the conventional precipitin test and also by the much more sensitive hemagglutination of antigen-coated red blood cells. Bis-diazotized benzidine (BDB) was used for the coupling of protein antigens with the erythrocytes. Precipitating antibodies were detectable by the 7th to 10th day after injection. Their titer declined then, and the precipitin test became negative by the 30th to 35th day. The hemagglutination titer (BDB test) reached a maximum by the 10th to 12th day and remained unchanged at the maximum value for many months. It decreased slowly, but was still distinctly positive after a year. Both precipitating and non-precipitating antibodies seem to have the same sedimentation properties in the ultracentrifuge, and the same mobility in electrophoresis. Preliminary experiments indicate, however, that the nonprecipitating antibodies are unable to sensitize the guinea-pig uterus in the Schultz-Dale reaction whereas the precipitating antibodies give a positive reaction. Our findings demonstrate that antibody, even after a single intravenous injection, circulates much longer than generally assumed. They also reveal that the high titer of the non-precipitating antibodies, after a second injection of the antigen, drops rapidly to negative values, but increases again to high values two to three days later. The rapidity and intensity of the secondary response may be due to the immediate formation of complexes between the reinjected antigen and circulating, non-precipitating, antibody.


The molecular composition of the cell, including the nucleus, has been studied by immunoelectrophoresis. Leukocytes collected from patients with leukemia were separated from erythrocytes by differential sedimentation in the presence of Dextran, then washed extensively with 0.05 M citrate. Subsequent exposure overnight to 0.1 M glycine dissolves the entire cell and gives a solution consisting mostly of non-dialyzable, non-viscous deoxyribonucleoprotein (DNA-P), which is insoluble in 0.15 M NaCl.

The DNA-P can be analyzed by electrophoresis on cellulose acetate membranes using barbital buffer pH 8.6. The identity of the bands can be shown by the Feulgen stain and the histone stain of Alpert and Geschwind. The DNA travels toward the anode as a single homogeneous band. Two major bands of histone are seen also moving toward the anode, and the more rapid one is tightly attached to the DNA band.

Antibodies to the leukocyte extracts were made by injection into rabbits in combination with Freund's adjuvant. The antisera were then used to analyze the extracts by the process of immunoelectrophoresis directly on cellulose acetate membranes. At least five antigenic components have been seen so far, and one of these is the DNA-histone complex previously mentioned. Another is probably the histone which travels free of the DNA.

When lupus serum was substituted for the rabbit serum, a variety of antibodies were revealed precipitating with the leukocyte fractions. All positive sera showed antibodies to the DNA histone complex; some showed other antibodies, probably directed against the second histone fraction.

The method is now being extended to a study of comparative immunoochemistry and a search for antigenic properties characteristic of neoplasia.

THE SEROLOGICAL DETECTION OF LEUKOCYTE AND PLATELET ANTIBODIES. Henry E. Wilson, Howard Johnson and Matthew Dodd, Columbus, Ohio.

A high coincidence of leukocyte and platelet agglutinins in transfused and non-transfused patients has been reported previously using techniques developed in this laboratory.
In a search for more sensitive tests for these antibodies a complement-fixation test for platelets has been developed which is significantly more sensitive than the platelet agglutination test.

Of 247 serum samples tested 49 sera from 32 patients have exhibited complement-fixing antibodies for platelets. Thirty-two of these sera have been tested for platelet agglutinins and leukocyte agglutinins. Fifteen of these sera contained antibodies demonstrable by all three tests, while 19 contained leukocyte agglutinins but not platelet agglutinins. Two sera contained leukocyte agglutinins but no platelet agglutinins or complement-fixing antibodies. Most sera containing antibodies were from patients who had received blood transfusions, but 5 of these were from patients who gave no history of transfusion or pregnancy.

The complement-fixation testing of 9 positive sera using as antigen platelets from floral donors has demonstrated a marked variation in the specificity of presumably normal platelets. All 9 sera were positive with platelets from at least 2 donors and 5 sera were positive with platelets from all 7 donors. There was a considerable variation in the intensity of the positive tests.

Platelet adsorption of sera positive for both platelet and leukocyte antibodies reduced the platelet complement-fixing antibody titer proportionately much more than it reduced the leukocyte agglutinin titer. These observations suggest that leukocytes and platelets are different but related antigens. Serial observations in patients who have been repeatedly transfused suggest that this complement-fixation test detects antibodies earlier than either of the other two tests.


During purification of the antibodies associated with “iso” and “auto” immune leucopenia and thrombocytopenia a previously unknown protein has been noted in normal human plasma (1), (2). This substance has profound cytotoxic action (in-vitro) against leucocytes and platelets. It is present in the pre-albumin area by electrophoresis, and has a mobility greater than albumin. Like the glycoproteins, it can be concentrated in Fraction VI, by method 10 of Cohn. Further chromatographic purification has been achieved. It can be eluted in Fraction VI-5 from DEAE columns at pH 4.4, ionic strength 0.05M. By freeze-drying of the eluate, after dialysis against water, the trace protein has been recovered as a faint white powder on the walls of the dessication vessel. It represents an estimated 70 gamma % of normal plasma protein.

The non-antibody nature of this protein is indicated by its failure to be adsorbed by the blood cells with which it specifically interacts. Its principal action is that of agglutination. The agglutination is inhibited by serum albumin or whole plasma in concentrations between 4:1 and 10:1. The inhibitory action in-vitro may indicate that the protein is inactive in vivo except in the presence of significant hypoalbuminemia.

Another protein with similar biologic action against platelets and leucocytes has been identified in close association with serum albumin. This component is on the slow-moving “face” of albumin by electrophoresis. It too, is present in Fraction VI and can be further purified by DEAE chromatography, where it appears in subfraction VI-2, pH 6.8, ionic strength 0.15M. The relative potency of these two materials is estimated as 1:10 for VI-2: VI-5.

It is postulated that Fraction VI-2 represents the carrier state of the cytotoxin, loosely bound to albumin. Physiologic studies of the material are underway.


The survival curves of small amounts of anti-hemophilic globulin (AHG) and plasma thromboplastin component (PTC) transfused into normal recipients have been studied by means of an isotope technique. Cancer patients were given injections of S35-methionine. Twenty-four hours later these patients were phlebotomized and the blood immediately
transfused into normal recipients. At intervals, samples of blood were drawn from these recipients. AHG was separated from plasma samples and PTC from serum samples by means of specific antibodies.

A specific AHG antibody was prepared by injecting partially purified AHG into rabbits. The resulting rabbit plasma was adsorbed repeatedly with plasma from a severe hemophiliac. After repeated adsorptions, a product was obtained which gave no precipitin with hemophiliac plasma, but a definite precipitin with normal plasma. By the partial-thromboplastin test, it was demonstrated that this antibody caused loss of anti-hemophiliac globulin activity from normal plasma.

By a similar technique a rabbit serum with specific activity against PTC was produced. This antibody caused loss of PTC activity from normal serum as demonstrated by the thromboplastin generation test.

When the radioactive plasma and serum samples were mixed with the specific antibodies, precipitins were obtained. These precipitins were washed repeatedly, dried, suspended by means of hyamine-10x in a toluene-fluor solution and the radioactivity was counted in a liquid scintillation counter.

For each of the proteins the survival curve has two components. During the first few post-transfusion hours, there is a rapid drop which probably represents equilibration between the intravascular and extravascular compartments. The curves then follow a more gradual drop which we interpret as the true survival. For AHG this curve has a half-life of 2.7 days. For PTC the curve has a half-life of 8.1 days.


Studies on a patient with chronic congenital thrombopenic purpura who responded repeatedly to injections of normal human plasma indicated the existence of a circulating thrombopoietic factor and prompted the search for an animal assay system. The present investigations are concerned with the stimulation of platelet formation in rats by a fraction of human plasma.

When rats were injected repeatedly with whole fresh or stored human plasma, a fall in platelet count occurred and the animals died after one week. However, when a filtrate of acidified fresh plasma boiled for 30 minutes was employed, a significant increase in platelet count occurred. In non-splenectomized rats daily intra-peritoneal injections of the filtrate (2 ml./100 gm. rat body wt.) induced a mean increase in platelet count of 600,000/cu. mm. In splenectomized rats treated similarly a mean increase of 810,000/cu. mm. occurred. Inasmuch as 4 of 6 filtrates tested produced an increase of 900,000/cu. mm. in the splenectomized animals it appeared that the filtrate dosage employed induced maximal platelet response. However, when less active filtrates were employed paired experiments demonstrated an augmented response of 50-100% in the splenectomized animals when compared with the non-splenectomized animals receiving the same dose of the same filtrate.

Studies on storage stability of the thrombopoietic factor demonstrated steady loss in activity when either whole plasma or the filtrate was stored at 40 C. or -20°C. Aged liquid and frozen plasmas demonstrated no thrombopoietic activity.

The data indicate that acute and chronic acquired ITP are not associated with lack of plasma thrombopoietic activity. However, deficiency of the platelet stimulating factor as a cause of some types of congenital thrombopenic purpura seems confirmed by these studies. The availability of an animal assay system offers the possibility for further study of humoral regulation of platelet production.


We have assayed plasma thromboplastin antecedent (PTA) activity in 67 members of 8 families each containing a propositus with PTA deficiency. We have found that PTA deficiency exists in two forms: (1) Major PTA deficiency—present in each propositus and
characterized by PTA levels of less than 20 per cent and by the threat of serious post-operative bleeding, and (2) Minor PTA deficiency—present in the parents, children, and some siblings of the propositi and characterized by PTA levels between 30 and 60 per cent and occasionally by minor post-operative bleeding.

Our data may be summarized as follows:

(1) Forty-five control normal subjects (ages 8 to 50 years) had PTA levels between 63 and 136 per cent (mean 94 per cent). The 8 propositi and 7 others discovered to have major PTA deficiency during the study had PTA levels which varied between 3 and 20 per cent (mean 8%).

(2) Fifteen children of parents with major PTA deficiency had minor PTA deficiency with PTA levels between 33 and 60 per cent of normal (mean 44 per cent). Two children had major PTA deficiency. Their parents were first cousins; one parent had major PTA deficiency and the other minor PTA deficiency. No child of a parent with major PTA deficiency had a normal PTA level.

(3) Eleven parents of patients with major PTA deficiency were studied. Their levels ranged between 37 and 93 per cent of normal (mean 53 per cent). Only 3 parents had PTA levels that overlapped into normal range (63 per cent, 73 per cent and 93 per cent).

These data fit the hypothesis that major PTA deficiency, the clinically significant form of the disorder, is an expression of the homozygous inheritance of the gene for PTA deficiency. Minor PTA deficiency is thought to represent the heterozygous state.

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A transitory hyperprothrombinemia following recovery from the usual hypoprothrombic reaction to dicoumarol was observed in the horse and human. Measurement of this “rebound” phenomenon required use of the modified 2-stage prothrombin analysis of Ware and Seegers (Am. J. Clin. Path., 19, 471 (1949)).

A 770-kg. horse, maintained on a constant diet, ingested a single 3.0-gm. dose of dicoumarol. The plasma prothrombin level decreased to 78 per cent of normal, returned to normal in four days, and subsequently increased to 20 per cent above normal. This hyperprothrombinemia lasted four days. The same animal then received 6.0 gm. dicoumarol over two successive days. Plasma prothrombin decreased to 68 per cent of normal, returned to normal in five days, and “rebounded” again to 20 per cent above normal.

A 91-kg. human male on an uncontrolled diet ingested a single 300-mg. dose of dicoumarol. A 50-per-cent reduction in prothrombin level was followed by a scant 10-per-cent rebound. Another 91-kg. human received an initial 300 mg. of the drug, with a 100-mg. dose two days later. Responses were a 45-per-cent reduction and 22-per-cent rebound of the plasma prothrombin. At these dosages, neither person’s responses to dicoumarol were reflected in the 1-stage prothrombin time, and the hematocrits were constant.

Actually, the prothrombin rebound phenomenon was first implied nineteen years ago by McGinty et al. (Science, 96, 540 (1942)), who used the original 2-stage prothrombin analysis (Am. J. Physiol., 114, 667 (1936)) to study the response of dogs to massive doses of dicoumarol. From the standpoint of protein biosynthesis, the rebound of plasma prothrombin following dicoumarol action may be analogous to plasma protein rebound following plasmapheresis (Vox Sang., 4, 70 (1959)).

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**Circulating Anticoagulant Potentiated by Normal Plasma; Clinical and Coagulation Studies of Six Cases. Robert L. Rosenthal, New York, N. Y.**

This report is based upon six patients with circulating anticoagulants of special significance because their activity was potentiated by the addition of normal plasma. The potentiation was best demonstrated in the recalcified plasma clotting time of mixtures of 1 part control to 1 part patient plasma and 1 part control to 3 parts patient plasma. Coagulation studies generally revealed moderate increases in clotting time and prothrombin time, with prothrombin concentration depressed to 20 to 30% of normal and normal values for
Factors V and VII and Stuart factor. The serum prothrombin time was unusually long (about 60 sec., normal above 25 sec.). Thromboplastin generation was normal. Extensive studies have revealed that the anticoagulant activity was increased by a combination of prothrombin and Factor VII, and manifested part of its activity as an anti-thrombin. The anticoagulant was stable to 60° c. for 30 min.; not absorbed by barium sulfate; not removed by ether extraction; present in serum; and stable on storage at −20° c. for several weeks.

Clinically, the patients, 3 males and 3 females, ranged in age from 12 to 60 years. Only 1 patient, female aged 12, revealed definite clinical and laboratory findings of systemic lupus erythematosis. The other patients showed various types of hemorrhagic manifestations and intermittent thrombocytopenia which responded to splenectomy in 2 cases. Two of the patients were mother and son. Although steroid therapy had a beneficial effect in general and decreased the hemorrhagic manifestations, there was no demonstrable effect on the potentiating anticoagulant.


The authors have reviewed the clinical and pathologic findings on 49 cases of thrombotic thrombocytopenic purpura (TTP) in the files of the Armed Forces Institute of Pathology. Two of the patients were seen clinically by the second author. An improved picture of this disease has emerged.

All cases presented an acute rapidly progressive terminal phase in which the clinical picture was strikingly similar. This was characterized by the QUINTAD of pyrexia, anemia, neurologic symptoms and signs, thrombocytopenic purpura and renal abnormalities (PAN-TR&R). The renal manifestations not previously emphasized, were found in 47 of 49 cases. Thirty had severe proteinuria and 37 had azotemia.

A prodromal phase with variable manifestations extending over a period of two months to ten years, was found in nearly two-thirds of the patients. These manifestations included recurrent fever, anemia, proteinuria, abnormal bleeding, arthralgias and hemiparesis. The appearance of the QUINTAD was often explosive and heralded the acute final phase of the disease.

Morphologic studies demonstrated a remarkably consistent widespread distribution of the characteristic occlusive vascular lesions. Detailed study of the lesion distribution revealed a consistent involvement of small vessels in specific anatomic sites adjacent to highly vascular tissue, supporting the contention that the principal localization is at arterio-capillary junctions. The vascular lesions were extremely numerous in almost every case in the myocardium, adrenals, pancreas, posterior pituitary and central nervous system. They were also found in the remaining organs and tissue in the majority of the cases, but with considerable less frequency. They were predominantly of recent type and appeared related to the acute phase. A few old lesions were present in almost every case, suggesting a previous episode.

In the kidneys there was also enlargement of glomeruli with increased cellularity, resembling a proliferative glomerulonephritis. The spleen exhibited marked entrapment of erythrocytes in the cords of Billroth and hemosiderosis, presenting morphologic findings similar to that observed in hemolytic anemias of spherocytic type.

**CHEMISTRY AND PHYSIOLOGY OF THE COBAMIDE COENZYMES. Dr. H. A. Barker, Berkeley, Calif.**

Coenzymes of the vitamin B₁₂ group, collectively called cobamide coenzymes, were discovered during studies of the decomposition of glutamate by extracts of *Clostridium tetanomorphum*. The conversion of glutamate to β-methylaspartate was found to require a new coenzyme that was ultimately identified as a derivative of pseudovitamin B₁₂. Subsequently, the coenzyme form of vitamin B₁₂ was isolated in substantial amounts as a red crystalline product from cells of propionic acid bacteria. The structure of coenzyme B₁₂ is...
similar to that of cyanocobalamin except that it lacks a cyanide group, contains an additional adenine nucleoside, and probably contains divalent cobalt. Coenzyme B_{12} differs from vitamin B_{12} in its spectrum and its electrical charge in acid solution. The coenzyme is readily converted to the vitamin by exposure to light or cyanide ion. Coenzyme B_{12} occurs in bacteria and liver of several species including man; it is much more abundant in living organisms than the B_{12} vitamins. As a nutrient for animals and microorganisms, coenzyme B_{12} behaves much like vitamin B_{12}. Its therapeutic uses are being explored; available evidence indicates it is about as effective as the vitamin. Coenzyme B_{12} occurs in bacteria and liver of several species including man; it is much more abundant in living organisms than the B_{12} vitamins. As a nutrient for animals and microorganisms, coenzyme B_{12} behaves much like vitamin B_{12}. Its therapeutic uses are being explored; available evidence indicates it is about as effective as the vitamin. Coenzyme B_{12} and its analogs are essential for two enzymatic reactions that are only known to occur in bacteria: the isomerization of glutamate to ß-methylaspartate and the conversion of ethylene glycol to acetaldehyde; and for one enzymatic reaction occurring in both animals and bacteria: the isomerization of succinyl CoA to methylmalonyl CoA. The latter reaction is involved in the oxidation of propionic acid. The coenzymes are probably required for still other enzymatic reactions, not yet identified.

**The Role of Folic Acid and Its Coenzymes—Physiologic and Pharmacologic Interrelationships.** Richard W. Vilter, Cincinnati, Ohio.

N10 formyl, hydroxymethyl, or formimino tetrahydro pteroylglutamic acid and intermediary products are the folic acid coenzymes. These compounds facilitate the transfer of single carbon units, and in this fashion are involved in the closure of the purine ring, in the methylation of pyrimidine, in the interconversion of glycine and serine as well as in the formation of methionine from homocysteine. The most important of these reactions to the megaloblastic anemia problem is the formation of thymidylate acid from deoxyuridylate acid. When this reaction is blocked by lack of folic acid coenzymes, chromatin structure is abnormal, division of cells is retarded and they take on an appearance which has been called megaloblastic. Ascorbic acid protects the reduced forms of folic acid, and vitamin B_{12} appears to favor reduction reactions necessary for the formation of the folic acid coenzymes and for the conversion of the ribosyl to the deoxyribosyl moiety. This scheme places folic acid at the center of these chemical reactions which are necessary to prevent megaloblastosis.

Most types of megaloblastic anemias will respond at least temporarily to folic acid because deficiencies of vitamin B_{12} and ascorbic acid impair the formation of the folic acid coenzymes. In the latter circumstances folic acid must be administered in 1—15 mg. doses daily to induce a hematological response. These are pharmacologic doses. When megaloblastic anemia occurs because of a dietary deficiency of folic acid, a favorable response can be obtained with 250 γ daily. These are physiologic doses because they correspond to the amounts of folic acid which can be obtained from the folic acid conjugates in the diet. Many patients with megaloblastic anemia have mixed deficiency states, either of folic acid and vitamin B_{12}, of folic acid and ascorbic acid, or of all these vitamins. Such persons will respond to various amounts of either folic acid, vitamin B_{12}, or ascorbic acid, depending on the degree of the combined deficiency states. Only by a battery of chemical, microbiological, and isotopic tests can the relationship of the various deficiencies and their causes be assessed with accuracy.

**Studies with Tritiated Folic Acid.** A. S. V. Burgen, Montreal.

Tritiated folic acid has been prepared, purified by chromatography on DEAE-cellulose, and administered intravenously and orally to normal subjects and to patients with untreated pernicious anemia and nontropical sprue. The plasma disappearance curve for normal subjects given 15 μg./Kg. (10 μc.) corresponds to that found by Chanarin, Mollin and Anderson by a microbiological assay method, using nonlabeled folic acid. The plasma disappearance was more rapid than normal in pernicious anemia and nontropical sprue, indicating an increased rate of tissue uptake of the vitamin. Urinary excretion in six hours was 15—30 per cent of the administered radioactivity in normal subjects but only 4—5 per cent in patients with untreated pernicious anemia. Five months of treatment of the latter with
vitamin B₁₂ alone caused the plasma disappearance curve of tritiated folic acid to return toward normal but did not always restore the urinary excretion value to normal. Excretion of an orally administered dose of tritiated folic acid in folic acid-preloaded normal subjects was 40–75 per cent but in preloaded nontropical sprue was only 6–13 per cent. Blood levels after an oral dose could easily be measured in normals but not in nontropical sprue.

At six hours after an IV dose, blood levels and urinary excretion in normal subjects were almost zero. The retained radioactivity (70–85 per cent of the administered dose) was stored in the cells largely as unchanging folic acid, since a 30 mg. IV carrier dose of nonlabeled folic acid displaced out into the urine a further 60 per cent of the radioactivity at 8 hours, 65 per cent of which was folic acid, the remaining 35 per cent being citrovorum factor, p-aminobenzoylglutamate, and two unidentified compounds, one of which was a pteridine. At 72 hours only 27 per cent could be displaced, indicating a slow transformation or sequestration of the stored folic acid.


Therapeutic trial: (1) Method: No therapy for 10 days, then 0.4 mg. folic acid (FA), intramuscularly, daily X 10 (Arch. Int. Med. 105:352, 1960). (2) Assets: (a) Definitive when good response occurs (if it follows control period); (b) Universally available. (3) Liabilities: (a) Therapy delayed; (b) Value proportional to degree of anemia (with slight anemia get only slight reticulocytosis); (c) May not work if complicating anemia present (renal disease, etc.).

Serum Folic Acid (FA) (L. casei) activity: (1) Method: Serum fed to L. casei, which grows in proportion to serum FA activity (Blood 15:228, 1960). (2) Assets: (a) Directly reflects FA deficiency; (b) Therapy may start immediately after serum drawn; (c) Probably earliest laboratory abnormality of FA deficiency (seems to fall to low levels within 3 months of deprivation of FA); (d) Definitive; (e) Not affected by complications patient may have. (3): Liabilities: (a) Not universally available; (b) Difficult to set up.

Formimino glutamic acid (FiGlu) in Urine: (1) Methods: Bacteriologic, enzymatic and spectrophotometric, chromatographic, electrophoretic, colorimetric. (2) Assets: (a) Usually reliable; (b) Relatively simple method now available (Lancet 2:347, 1960) (High voltage not necessary—R. Zalusky). (3) Liabilities: (a) Best method (enzymatic) is difficult and not universally available; (b) Histidine-loading usually necessary, sometimes delaying therapy; (c) Some B₁₂-deficient patients may have high FiGlu. This may represent inability to utilize FA due to B₁₂ deficiency. In such cases a diagnosis of nutritional (primary) FA deficiency may be erroneous; (d) Deficiency of formimino transferase (as with liver disease) will lead to a diagnosis of FA deficiency.

FA load test: (1) Method: 15 µg. FA/Kg. body weight given intravenously. Disappearance followed by serial venipuncture and assay with S. faecalis (Brit. J. Haemat. 4:435, 1958). (2) Assets: (a) Usually rapid disappearance when FA deficiency present. (3) Liabilities: (a) Rapid disappearance in half of B₁₂-deficient patients, hence erroneous diagnosis of primary FA deficiency may be frequent; (b) Not universally available; (c) Difficult to set up. Studies in our laboratory suggest the rapid disappearance with B₁₂ deficiency occurs only when measured with S. faecalis, not when measured with L. casei. This could be interpreted to mean rapid conversion of FA to a metabolically useful form, perhaps with inability to then use the active form due to B₁₂ deficiency interfering with FA metabolism. In FA deficiency we find rapid disappearance with both S. faecalis and L. casei.

The Absorption of Folic Acid and Vitamin B₁₂ from the Gastrointestinal Tract. Bernard Cooper, Montreal, Can.

Absorption studies in man following resection of various segments of small intestine suggest that the primary site of folic acid absorption is in the jejunum whereas that of vitamin B₁₂ is in the ileum. The absorption of PGA from the gastrointestinal tract is
reduced in idiopathic steatorrhea. The efficiency of PGA absorption from the gastrointestinal tract in pregnancy is not completely established, but determinations of urinary excretion of folic acid activity after oral and parenteral administration of PGA show no abnormality in pregnancy. The curve of serum PGA activity after oral administration of folic acid is similar to those of penicillin V and D-xylose. This suggests that the mechanism of absorption of these substances may be similar, i.e. by passive transport across the jejunum. However, the folic acid activity of food is conjugated as polyglutamates which are absorbed less efficiently from the gastrointestinal tract than is crystalline folic acid. For this reason, studies of absorption of crystalline folic acid must be interpreted with caution.

Vitamin B₁₂ probably is absorbed physiologically by: (1) successful competition for the vitamin B₁₂ in food by the gastric intrinsic factor, (2) uptake of the intrinsic factor B₁₂ complex by the intestinal wall, and (3) subsequent transport of vitamin B₁₂ across the intestine. It is suggested that the intrinsic factor B₁₂ complex is adsorbed to the intestinal wall by a physicochemical process requiring calcium ions and that free vitamin B₁₂ then is released from the intrinsic factor B₁₂ complex and transported passively across the mucosa. The delay of the B₁₂ in the intestinal wall before its appearance in the peripheral blood then could be due to the time required for: (1) transit to the ileum, (2) uptake of the intrinsic factor B₁₂ complex by the intestinal wall, (3) release of free B₁₂ from the intrinsic factor B₁₂ complex by an enzyme type of reaction, and (4) attainment of a critical concentration of free B₁₂, at, or within, the intestinal wall before passive transport can begin. These postulates, although unproved, provide a working model of vitamin B₁₂ absorption consistent with experimental observations.

STUDIES ON PYRIMIDINE METABOLISM IN MEGALOBLASTIC ANEMIAS. Lloyd H. Smith, Jr., Boston, Mass.

Assay procedures have been developed to measure the activities of five sequential enzymes in pyrimidine nucleotide biosynthesis, using circulating human erythrocytes and leukocytes. In the study of ten patients with pernicious anemia, marked elevations of aspartate carbamyltransferase and dihydroorotase were found in both erythrocytes and leukocytes, reverting to normal levels after treatment with B₁₂. The pattern of enzyme abnormality found suggests that of "pyrimidine starvation," as described in E. coli, with enzyme changes secondary to the release of negative feedback inhibition. Orotic aciduria is a rare disorder of resistant megaloblastic anemia associated with the excretion in the urine of large amounts of orotic acid. Deficiencies of orotidyl pyrophosphorylase and orotidyl decarboxylase were found in erythrocytes from the parents and two of the three surviving siblings of the deceased propositus of this syndrome. This suggests that the syndrome is transmitted as an autosomal recessive trait, the deceased patient being presumably homozygous. In a small control series, another heterozygote of orotic aciduria was discovered. In preliminary studies with anticonvulsant medications no inhibitory effect on pyrimidine metabolism has been demonstrated.

THE MECHANISM OF ACTION OF FOLIC ACID ANTAGONISTS. Arnold D. Welch, New Haven, Conn.

In the metabolic transformation and transfer of one-carbon compounds, a coenzyme, tetrahydrofolic acid (THF; 5,6,7,8, tetrahydro-pteroylglutamic acid), is essential.

The dietary and bacterial sources of this vitamin are generally adequate to maintain the needs of the body; however, with (a) unusually poor diets, (b) derangements of the bacterial population of the intestine, (c) disturbances of intestinal absorption, (d) unusual demands, as in pregnancy, (e) ingestion of certain drugs, or (f) derangements in the metabolism of folic acid, as in deficiencies of ascorbic acid or of vitamin B₁₂, ordinary dietary sources of folic acid-like compounds fail to meet the basic requirements of cells. Part of the requirement for folic acid undoubtedly is met by the reutilization of oxidized THF formed during certain coenzymic functions of the compound. For example, the methyl group of the thymine-moiety of thymidylc acid is formed with the simultaneous
separation of \(dl\)-hydrofolic acid. This partially hydrogenated form of folic acid is useless as a coenzyme and must be converted to the tetrahydro form. The transfer of the so-called formyl group leads to the separation of THF (rather than dihydrofolic acid); the coenzyme is immediately available for continuing its catalytic functions.

The enzymic conversion of folic acid to the \(dt\)-hydro form, and this, in turn, to the tetrahydrofolic acid (THF), appears to be catalyzed by a single enzyme, folic acid reductase (if two enzymes are involved, they possess almost identical physico-chemical properties).

The binding of the inhibitory analog, amethopterin, by the folic acid reductase of the liver, following the injection of the drug into animals, is so great that even extensive dialysis of the antagonist-containing fraction of the homogenized liver fails to remove the inhibitor, unless the dialysis is performed in the presence of a considerable amount of folic acid. This explains the fact that the administration of folic acid (5 formyl-5,6,7,8-tetrahydropteroylglutamic acid), if not too long delayed, can abolish the toxic effects of the folic acid antagonists. The reason for this is that folinic acid, while it does not restore folic acid reductase function, circumvents the need for it. The tremendous affinity of amethopterin for folic acid reductase, and apparently to no significant degree for any other protein, can be used in investigations of the distribution of the enzyme in various tissues, using tritium-labelled drug. Recent studies (a) of the relative requirement, during rapid reproduction in culture, of murine malignant lymphoblasts and mast cells for folic acid versus folinic acid; and (b) of the folic acid reductase activity of such cells show that such cells use folic acid extremely inefficiently, apparently because their folic acid reductase activity is very limited. As might be predicted, the growth of these cells is extremely susceptible to inhibition by amethopterin. This provides a biochemical basis for the development of amethopterin resistance in the chemotherapy of leukemia. Neoplastic cells were grown either in mice treated with amethopterin or in cultures containing inhibitory but nonlethal concentrations of the drug. Among the first group of cells to survive were genetically stable drug-resistant clones, the growth of which was inhibited to the same degree as in the parent strain, only by twice the concentration of amethopterin. It was possible to obtain progressively more highly resistant cells; a strain with a 100,000-fold increase in its capacity to resist amethopterin has been isolated. The first resistant cell to emerge, that which has been referred to as having “2-fold resistance,” and which is selected by the drug either in culture or in vivo, possesses twice the amount of folic acid reductase present in the “wild strain” of lymphoblasts. Cells with a “6-fold resistance” to amethopterin have a 16-fold increase in content of folic acid reductase.

These findings permit the speculation that, among the vast numbers of initially Methotrexate-susceptible leukemia cells present in an untreated patient, an unpredictable proportion exists with a fortuitously higher amount of folic acid reductase. If the proportion is very large, the clinical result may be barely discernible; obviously, various intermediate gradations in the proportion of mutants with high folic acid reductase may occur. At present, no biochemical leads have been obtained which would suggest a means of preventing the emergence of these fortuitously drug-resistant cells, although studies of their possible transformation to drug-sensitivity, through exposure to preparations of so-called transforming factors (nucleic acids), have been initiated. In present practice, then, the time to use a second drug to potentiate chemotherapeutic utility would be immediately following exposure to a drug that has induced a good remission.

Amethopterin passes cell membranes very poorly. In order to attain inhibition of the intracellular folic acid reductase, a high concentration of amethopterin is required in the extracellular fluid; also the compound almost completely fails to pass the blood-brain barrier. This latter fact has led to the intrathecal use of Methotrexate in cases of suspected leukemic cell involvement of the central nervous system but usually at a late stage of the disease. Perhaps the time to use amethopterin intrathecally is not in desperation, but on the very first exposure of the patient to the drug. Then, if susceptible lymphoblasts have already passed the blood-brain barrier, at least a chance will be afforded to annihilate them. Further investigation of the relation of chemical structure to membrane permeability, as well as to the capacity to inhibit folic acid reductase, could lead to a derivative of much greater clinical utility.
STUDIES ON THE HEREDITARY GAMMA GLOBULIN GROUPS IN MAN. Hugh Fudenberg and Henry G. Kunkel.

The "rheumatoid factor" present in the sera of patients with rheumatoid arthritis resembles antiglobulin (Coombs') serum in that it will produce agglutination of human cells coated with "incomplete" antibodies, such as anti-Rh or acquired hemolytic anemia antibodies. As with Coombs' serum, this agglutination is inhibited by the addition of pooled Fraction II; in contrast to Coombs' serum, proper selection of the rheumatoid serum and anti-Rh reagents permits establishment of an agglutination system which is inhibited by the \( \gamma \)-globulin of some individuals, but not of others, as demonstrated by Grubb and Laurell. The inhibitory property, termed Gm(a) is genetically determined. Use of a test system involving another reagent pair can demonstrate an inhibitor antithetical to Gm(a), termed Gm(b) (Harboe). In whites, all Gm(a) negative sera are Gm(b) positive, i.e., Gm(a-b+), and all Gm(b) negative sera are Gm(a) positive, i.e., Gm(a+b-). Heterozygotes are Gm(a+b+). These genetically determined human \( \gamma \)-globulin groups are analogous to the genetically determined antigens on the red cell and like them can be used in exclusion of paternity and in anthropologic studies.

The studies to be presented demonstrate the presence of at least 8 different, genetically determined "antigens" in human \( \gamma \)-globulin and 8 different rheumatoid factors of corresponding specificity. The studies also demonstrate that the rheumatoid factor obtained from a given individual has preferential specificity for genetic \( \gamma \)-globulin groups absent from but allelic to those present in the individuals' own \( \gamma \)-globulin. Data concerning some of the physical and chemical properties of the antigen and antibody involved in the test system will also be presented.

Phosphorus Metabolism of the Human Red Cell. Grant R. Bartlett, La Jolla, Calif.

Probably the most active metabolic process in the mature red cell involves the phosphorus of water-soluble constituents. We have been able to isolate 36 different kinds of phosphorus groups from the red cell in sufficient purity for measurement of specific radioactivity (SA) following a short (5 minute) incubation of blood with P32-labeled inorganic phosphate. The difference in the SA of these phosphate groups after brief tagging provide a flow pattern showing both the direction and magnitude of the movement of phosphorus through available metabolic channels. Some observations of special interest were: 1. The rate of P32 entry into glucose diphosphate and uridine diphosphoglucose establishes the importance of this by-pass and supports previous indications of a glycogen pathway. 2. The very high SA found in a new kind of adenine nucleotide means that another link must be added to the energy conversion chain. 3. The absence of radioactivity in DPN and TPN reveals a surprising stability for these important coenzymes. 4. The extremely rapid equilibration of the labile phosphates of the nucleotides points to the existence of potent phosphorus transferase enzymes. 5. The relatively low SA of 2,3-diphosphoglycerate rules out appreciable shunting from 1,3- to 2,3-diphosphoglycerates (so-called Rapoport cycle) during the steady state. 6. The lack of P32 incorporation into the phosphate attached to the ribose of nucleotides emphasizes the metabolic stability of this bond. 7. The inorganic polyphosphates picked up no P32 proving these compounds to be structural rather than metabolic components of the cell. These data on the normal human red cell provide a base for a more thorough examination of red cell metabolism in aberrant states.

The Kinetics of Red Cell Production. Frederick Stohlman, Jr., Bethesda, Md.

Studies on irradiated, hypertransfused, and erythropoietine treated animals have enabled us to construct a model for the kinetics of red cell production in both normal and certain abnormal states. These studies indicate that the erythroid compartment is not self-sustain-
ing but is constantly replenished from a primitive or stem cell pool. Erythropoietine promotes the differentiation of stem cells into red cell precursors. The resulting depletion of the stem cell compartment stimulates stem cell proliferation. Once the stem has differentiated into an erythroid element, non-synchronous division and maturation occurs. Small cohorts of cells may mature without dividing and others may die in the bone marrow. In some abnormal conditions the proportion of cells which mature without dividing may increase; in others the amount of ineffective erythropoiesis assumes a significant role. The previously described acceleration of red cell regeneration in the irradiated animal, following the early administration of erythropoietine, appears to be due to the removal (by differentiation) of damaged stem cells which serves as a stimulus for an earlier regeneration of the stem cell compartment. The latter results in earlier regeneration of erythroid elements. Studies on compensated hemolytic syndromes and dose response curves to erythropoietine indicate, however, that the differentiation of stem cells by erythropoietine does not constitute the sole regulant of erythropoiesis.

SOME PROBLEMS IN ANALYSIS OF HEMOPORIC DATA OBTAINED FROM H3THYMIDINE LABELING OF HUMAN BONE MARROW. T. M. Fliedner, E. P. Cronkite, V. P. Bond, S. A. Killmann, P. G. Reizenstein and J. S. Robertson, Upton, N. Y.

Problems concerned with hemopoietic cell proliferation are being attacked by means of diverse group of techniques all of which yield valuable data. However, the numbers for the time parameters of the cell generative cycle, which are extracted by analysis of the data, are highly dependent upon cytologic criteria, fundamental assumptions regarding the specific technique and the hemopoietic model used to describe the orderly flow of cells through proliferation, differentiation and maturation. A valuable autoradiographic observation is the decrease in the grain count with time for an identifiable cell type. Analyses based on decreasing count have been made for erythropoiesis and granulopoiesis. Over certain time periods grain counts diminish exponentially. For a single self-sustaining proliferative compartment the slope will be a function of the average time between successive mitoses. However, in the case of a series of cytologically identifiable compartments the rate of decrease in grain count in later compartments are highly dependent upon the grain count of entering cells and whether each cell divides while within the compartment or passes through only by further maturation. Grain count data for erythropoiesis and granulopoiesis along with difficulties encountered in interpretation will be presented in addition to the information obtained from the flow of labeled cells from the last proliferating compartment into non-proliferative marrow compartments and thence to the peripheral blood.

DISTRIBUTION OF RADIOACTIVE CHROMIUM AMONG HEMOGLOBIN COMPONENTS. Daniel Malcolm and Helen M. Ranney, New York, N. Y.

This report deals with the distribution of radiochromium among the electrophoretic and chromatographic fractions of hemoglobin obtained after incubation of whole blood with Na2Cr51O4. Electrophoretic components of hemoglobin were eluted from starch granules after electrophoresis in barbital buffer (pH 8.6); chromatographic components were obtained by elution from Amberlite XE64 resin with phosphate-citrate buffer (pH 6.3, increasing sodium concentration from 65 to 425 mEq./liter).

In normal blood the average specific activity of the electrophoretically rapid hemoglobin A3 component was 14 times that of the unfractionated hemoglobin and 40 times that of the A1 and A2 components. The first of the three chromatographic fractions of normal hemoglobin had a higher specific activity than did either unfractionated hemoglobin or the other two fractions. Studies of the distribution of Cr51 among components of hemoglobin in blood obtained from patients with sickle cell trait or hemoglobin C trait, disclosed greater specific activity in hemoglobin A than in either hemoglobin S or hemoglobin C.

In aqueous solution, hemoglobin could be labeled by incubation with either Na2Cr51O4 or Cr51Cl3. When the unbound radiochromium was removed by dialysis, the electrophoretically rapid A3 component was found to have greater specific activity than the
unfractionated hemoglobin or the other electrophoretic components. These studies indicate that hemoglobin A2 may be responsible for the binding of chromium in the normal erythrocyte. Studies of the number and types of binding sites for chromium in various hemoglobin components are in progress.

STUDIES ON THE BINDING OF CHROMIUM$^{51}$ TO HEMOGLOBIN. Howard A. Pearson and Kenneth N. Vertrees, Bethesda, Md.

In 1950, Gray and Sterling showed that intra-erythrocytic hemoglobin could be labeled by radioactive chromium (Cr$^{51}$) and this became the basis for in vivo studies of red cell volume and estimations of red cell life span.

The molecule of normal adult Hgb. A contains two pairs of polypeptide chains (α and B) and can be designated as α$_2$β$_2$. Most of the abnormal hemoglobins are altered only in B$_2$ chains. For example, Hgb. C can be represented as α$_2$B$_2$C. Hgb. H, an electrophoretically “fast” variant, contains no α chains and has been designated as B$_4$. In acid solutions the hemoglobin molecule undergoes asymmetric dissociation into free α2 and B$_2$ units which recombine randomly when the solution is neutralized. These facts have been utilized in an attempt to elucidate possible sites of Cr$^{51}$ binding to hemoglobin.

A. Distribution of Cr$^{51}$ and Fe$^{59}$ activity in hemoglobin components of a patient with Hemoglobin H disease.

A 45 year old male with iron refractory hypochromic, microcytic anemia was found to have Hgb. H disease. The patient's red cells were tagged with Fe$^{59}$ and Cr$^{51}$ as part of erythrokinetic studies and were separated by starch block electrophoresis pH 7.0. Components were then eluted and analyzed.

Results:

<table>
<thead>
<tr>
<th></th>
<th>% of total hgb.</th>
<th>cts/min Fe$^{59}$</th>
<th>% of total Fe$^{59}$ counts</th>
<th>cts/min Cr$^{51}$</th>
<th>% of total Cr$^{51}$ counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb. H</td>
<td>18</td>
<td>16</td>
<td>20.5</td>
<td>1107</td>
<td>33.7</td>
</tr>
<tr>
<td>Hgb. A</td>
<td>82</td>
<td>62</td>
<td>79.5</td>
<td>2178</td>
<td>66.3</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>78</td>
<td>100</td>
<td>3285</td>
<td>100</td>
</tr>
</tbody>
</table>

A possible explanation for the above findings would be that Cr$^{51}$ tags primarily to the B chains. To test this hypothesis, recombination experiments utilizing tagged Hgb. A and nonradioactive Hgb. C were performed.

B. Recombination experiments.

I. Hypothesis:

If Cr$^{51}$ binds only to B chains no radioactive Hgb. C would be formed by hybridization. On the other hand, if Cr$^{51}$ tags to both α and B chains, nonspecifically, a radioactive Hgb. C hybrid would be formed by recombination.

The heterogeneity of normal adult human hemoglobin is a well established fact. The present study was designed to determine if a) the main fraction (A₁), the slow component (A₂) and the non-hemoglobin fraction (unidentified component 1, U.C₁) have specific antigenicity; b) if A₂ can be demonstrated immunologically in cord blood and in the presence of S and C hemoglobin. Previously described technics (Am. J. Clin. Path., 34:28, 1960) were used for the preparation and agar gel electrophoresis of the hemolysates and for the elution of the fractions from agar gel. Antibera to A₁, A₂ and U.C₁ were prepared in rabbits. By an agar gel double diffusion technic and immunoelectrophoresis the antisera were tested against hemolysates of normal adult, cord hemoglobin, hemoglobin S, A-S, A-C, S-Thalassemia, and against separate eluates of portions of the agar gel electrophoretogram of normal, fetal, S and A-C hemoglobins.

Antibody to A₂ hemoglobin appeared earlier than to A₁ and U.C₁. A₂ was only weakly antigenic. With this technic A₁ and U.C₁ were demonstrated in whole hemolysates of hemoglobin A, S, A-S, A-C and fetal. A₂ from normal, thalassemic and cord blood were found to be immunologically identical. Anti-A₂ was immunologically specific and did not react with eluates of A₁ and the central portions of S, C and fetal hemoglobin. It did, however, react with eluates from the interspace between A and C hemoglobin.

The finding of specific antigenicity of A₂ hemoglobin is in agreement with the recently detected differences in the "finger print" between A₁ and A₂ hemoglobin. The presence of A₂ in hemoglobins other than A speaks for the specific genetic determination of this component.


These studies were designed to localize the biosynthetic defect in heme synthesis which has been described in thalassemia major. Circulating erythrocytes from patients with various acquired and hereditary anemias were washed, hemolysed, and incubated for 4 hours at 37°C, with different substrates of porphyrin synthesis. Heme synthesis was studied (1) by incubating the hemolysates with C¹⁴-tagged glycine, C¹⁴γ aminolevulinic acid (γ ALA), and C¹⁴ protoporphyrin and isolating C¹⁴-tagged heme; and (2) by incubating Fe⁵⁹SO₄ with hemolysates in the presence of the same non-tagged substrates and porphobilinogen (PBG), and isolating Fe⁵⁹-tagged heme. Free porphyrin synthesis was measured by incubating the hemolysates with glycine and γ ALA, with and without added iron. The free porphyrins were separated by solvent extraction, quantitated by fluorimetry, and identified by paper chromatography of the methyl esters.

Thalassemic cells demonstrated much poorer incorporation of glycine into heme than non-thalassemic cells. Both groups showed comparable incorporation of γ ALA, PBG, and protoporphyrin into heme determined by both C¹⁴ and Fe⁵⁹ methods. Smaller amounts of heme were apparently synthesized by thalassemic cells by the Fe⁵⁹ method than by the C¹⁴ method. This probably reflects the greatly increased iron content of these cells. Large amounts of free porphyrins accumulated in all incubates with γ ALA as substrate, and were roughly correlated with the number of immature erythrocytes (reticulocytes and normoblasts). Protoporphyrin was not synthesized in the absence of immature erythrocytes, whereas small amounts of both uroporphyrin and coproporphyrin were. Thalassemic erythrocytes synthesized a greater proportion of free protoporphyrin compared with non-thalassemic cells. All isolated porphyrins were of the type III isomeric variety.

These studies suggest that there are two probable defective steps in hemoglobin biosynthesis in thalassemia: (1) the conversion of glycine to γ ALA, and (2) at a step following the formation of protoporphyrin and heme. The second defect probably involves the formation of globin, or the combination of protoporphyrin, iron, heme, and globin to form hemoglobin.
A STUDY OF ASSOCIATED IRON DEFICIENCY AND HEMOLYTIC ANEMIA. Dorothy C. H. Ley, Toronto, Canada.

The association of a hemolytic process with iron deficiency anemia has been postulated on a theoretical basis, but only a few such cases have been reported in detail in the literature. The two patients with whom this study is concerned were both females known to be suffering from hemolytic anemia of obscure origin, and who, on detailed haematological investigation, were found to have concomitant iron deficiency. Investigation included the repeated measurement of corpuscular constants, serum iron and unsaturated iron binding protein, radioactive iron (Fe59) clearance and red cell utilization and the measurement of red cell life span using radioactive chromium. A search was made for the presence of abnormal hemoglobins, of an immune mechanism as the cause of the hemolytic process, and of abnormalities of serum proteins separated by paper electrophoresis. Iron and fat absorption were measured in both subjects. In an attempt to delineate the underlying mechanism, the survival of iron-deficient, chromium-labelled red cells was measured in three normal recipients. Red cell life span was also measured in three patients with uncomplicated iron deficiency anemia who were not bleeding.

Both patients exhibited the pattern of severe iron deficiency so far as their internal iron metabolism was concerned, but they had a shortened red cell life span. None of the iron deficient patients similarly studied had such shortening of their red cell life span. None of the iron deficient patients similarly studied had such shortening of their red cell survival. All parameters, including red cell survival times, returned toward normal on iron therapy alone. Both patients, however, had decreased absorption of oral iron even in the presence of a low serum iron. The results of these investigations will be presented in detail and in the light of our findings, a theory will be put forward to explain the association of these two phenomena.

THE DETECTION OF ABO ISOAGGLUTININS RELATED TO ERYTHROBLASTOSIS. Shaul Kochwa, Richard E. Rosenfeld and Lisa Tallal, New York, N. Y.

A method has been developed for the prenatal detection of ABO isoagglutinins that are related to erythroblastosis. All forms of erythroblastosis are dependent upon maternal isoagglutinins which pass the placental barrier and unite with the erythrocytes of the fetus, but in the case of ABO incompatibility the competitive neutralizing effects of ABO antigens found in the secretions and tissues of the fetus require equal and simultaneous consideration. ABO isoagglutinins which sediment as 7S globulins can be separated on DEAE cellulose columns. These antibodies were demonstrated to occur in equal titer in the paired sera obtained at delivery of mothers and their ABO compatible newborn. Most maternal isoagglutinins were encountered in those column fractions which contained 19S y globulins, but cord serum isoagglutinins were recovered only in 7S y globulin fractions.

Of 110 prenatal sera tested, 7S isoagglutinins were not encountered in all of 28 type A and 9 type B mothers, but were found in 53 out of 73 O mothers ten of whom possessed specific titers of 1:20 to 1:160 and delivered ABO incompatible newborn. When tested for resistance to inhibition by specific soluble blood group substances (hog A and horse B), only five of 73 type O mothers were found to have non-inhibitible 7S isoagglutinins. Four of these mothers delivered type O children, but one delivered a type B child. This was the only newborn of the series to have ABO erythroblastosis, and exchange transfusion therapy was required.

Six other women with a history of having had one or more children with severe ABO erythroblastosis, and five unselected donors who had been immunized with specific soluble blood group substances, were tested in the same fashion, and all were found to have non-inhibitible 7S isoagglutinins of the proper specificity. The column chromatographic separation of 7S isoagglutinins offers a new approach to
study isoimmunized mothers, especially of primagravida, for estimation of the severity of erythroblastosis to be expected. Theoretical considerations will be discussed.

**Modification of the Sickling Process and of In Vivo Survival of Sickle Cells by the Administration of Met-Hemoglobin-Forming Agents.** Ernest Bentler. Duarte, Calif.

Many of the symptoms and signs of sickle cell anemia cannot readily be explained on the basis of the decreased oxygen-carrying capacity of the blood. Rather, they would seem to be related to the in vivo shape changes of the erythrocytes. It is known that oxidation of all of the hemoglobin of sickle cells to methemoglobin prevents the sickling process. In the present studies it has been found that conversion of even less than 15% of the sickle hemoglobin to the oxidized form retards sickling in vitro when the cells are exposed to standard gas mixtures.

Five volunteer adult patients with sickle cell disease have been treated experimentally with the methemoglobin-forming agents, sodium nitrite and/or para-aminopropiophenone (PAPP). In two of these patients it has been possible to produce striking prolongation of red cell survival as measured by the Cr\(^{51}\) technique. In one other patient, although only a modest improvement of red cell survival was achieved through the administration of para-aminopropiophenone, a striking rise in the hematocrit, to almost double its original value was produced. Neither of the two drugs employed to produce chronic methemoglobinemia is entirely satisfactory. In some patients vascular side-effects of sodium nitrite are very troublesome. PAPP administration results in the formation of Heinz bodies and the drug has a mild hemolytic effect. Methemoglobin is not evenly distributed throughout the entire red blood cell population; more methemoglobin is formed in the older members of the red cell population than in newly produced red cells. The results of our studies do not give rise to a practical clinical approach to sickle cell disease. However, the fact that marked prolongation of red cell survival has been achieved in some patients encourages us to believe that the use of other methemoglobin-forming agents, or the formation of other pigments such as carboxyhemoglobin or cyanmethemoglobin, or the stimulation of fetal hemoglobin production may represent worthwhile approaches to management of this disorder.

**The So-Called "Physiological Anemia of Pregnancy."** Nannie de Leeuw, Louis Lowenstein, Yang-Shu Hsieh and Harry Manderson, Montreal, Can.

In normal pregnancy the peripheral blood values decrease. It has been stated by some that this is due to iron deficiency, whereas others state that it is caused by a greater increase in plasma than in red-cell volume during pregnancy. Simultaneous observations on plasma and red cell volume changes and iron stores in normal pregnant women, with and without iron therapy, have not been reported.

This investigation endeavors to determine the role of iron deficiency and hydremia in "physiological anaemia of pregnancy" and to study the effect of parenteral and oral iron administration.

Three groups of 20 normal women, aged 17–35, were studied from early pregnancy through delivery, and for one year post partum. One group of patients received no iron therapy; the second group received 1000 mg. of iron (iron-dextran) intramuscularly; and the third ingested molybdenized ferrous sulphate containing 78 mg. of elemental iron daily for 24 weeks.

Serial hematological studies included serum iron, unsaturated iron binding capacity, and red-cell and plasma volumes. Bone marrow smears at term were stained for iron. These studies showed that although hemoglobin, hematocrit, and serum iron values decreased, the red-cell mass in the control patients increased by 15% from early pregnancy to term; stainable marrow iron was absent at term and was not replenished six months later. The iron-dextran-treated patients had higher haemoglobin, haematocrit, serum iron and red-cell
volume (28% increase), than the first group. Iron stores were present at term and six months later. Results in orally treated patients were not significantly different from those in parenterally treated patients. Changes in plasma volume were identical in the three groups. Peripheral blood values in babies of iron-dextran-treated mothers, up to nine months of age, were not greater than in those of mothers receiving no iron. It is concluded that iron deficiency and/or hydremia may contribute to development of the so-called "physiological anemia of pregnancy."

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FAMILY STUDIES IN HEREDITARY SPHEROCYTOSIS. W. A. Newton, Jr. and Stella B. Kontras, Columbus.

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