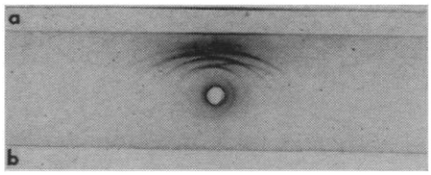

Fig. 1. Precipitin reaction in agar gel of human serum with antiserum to whole human serum (*a*) and antiserum to human collagen (*b*).

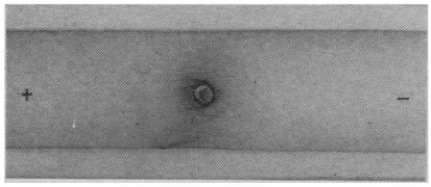

Fig. 2. Immunoelectrophoresis of human serum: precipitin reaction with antiserum to human collagen.

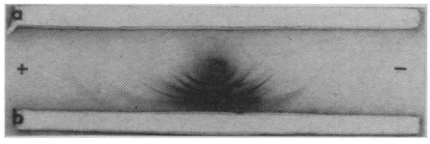

Fig. 3. Immunoelectrophoresis of human serum: precipitin reaction with (*a*) antiserum to collagen and (*b*) antiserum to whole human serum.

α_2-macroglobulin or an α- or β-lipoprotein. We (*2*) have found a hydroxyproline-bound protein localized in the α_2-β-globulins fraction; the fraction was characterized, after separation by column electrophoresis on acetolized cellulose (*3*). We have noted that antiserum to human serum gives a precipitin reaction with soluble human collagen; accordingly we have sought serum proteins showing the same antigenicity as collagen. We now report attempts to characterize such a serum fraction by immunoelectrophoresis.

We prepared antibodies from soluble human collagen; soluble collagen citrate was prepared by the method of Gallop and Seifter (*4*). Fragments of human skin, homogenized at 0°C in 0.5*M* sodium acetate in a VirTis-45 blendor were centrifuged at 5°C; the pellet was suspended in similar solution (at 4 ml per gram of tissue) for 18 hours and recentrifuged four times. The final pellet was washed four times by centrifugation from aqueous suspension. Soluble collagen was then extracted from the washed pellet by three successive 18-hour suspensions in 0.075*M* citrate buffer at *p*H 3.7 (at 4 ml per gram), each suspension being centrifuged for 1 hour at 18,000 rev/min. The final pellet was discarded. Soluble collagen was precipitated from the combined supernatants by dialysis against 0.02*M* disodium phosphate buffer. Purity of this soluble collagen was verified first by gel electrophoresis: it gave only one distinct zone. The amount of hydroxyproline in this preparation of soluble collagen was estimated to be 10.7 percent of the weight of protein.

Antiserums to collagen were prepared (*5*) by injecting, in divided doses, 2 ml of the following solution into the toe pads of each of three rabbits: equal parts of 1 percent collagen in citrate buffer and complete Freund's adjuvant. Four weeks after injection, the rabbits were bled, and the presence of antibodies to collagen was verified by the Ouchterlony technique. This antiserum gave a precipitin line when human serum was used as antigen (Fig. 1*b*); the line is comparable to the precipitin line produced by antiserum to whole human serum reacting with human serum used as antigen (Fig. 1*a*).

Electrophoretic localization in human serum of collagen-like antigen was tested by immunoelectrophoresis, according to Scheidegger's (*6*) modification of Grabar and William's procedure, with rabbit antiserum to human collagen. Study by microimmunoelectrophoresis suggests that in human serum there is a fraction that gives a precipitin reaction with specific antiserum to collagen (Fig. 2). At *p*H 8.4 this fraction has a slow rate of migration toward the anode and is situated near the starting zone.

We compared these results with those obtained by immunoelectrophoresis with rabbit antiserum to whole human serum (*7*). Antigenic, collagen-like, serum protein occurs in the α_2-β-lipoprotein zone (Fig. 3). This precipitin line is really due to collagen and not to β-lipoprotein, for it is not colored by Sudan Black BB, unlike lines given by the lipoprotein (*8*); and it is not removed after absorption of antiserum to collagen by purified β-lipoprotein prepared according to the Burstein technique (*9*). This fraction appears to be localized in the same zone as those fractions separated by cellulose-column electrophoresis.

These findings suggest that there is in human serum a collagen-like antigen that migrates electrophoretically with the α_2-β-protein fraction that contains hydroxyproline. Therefore we think that soluble collagen exists in human serum and presume that this soluble collagen is related to the collagen of connective tissue.

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23 August 1965

Uptake as a Determinant of Methotrexate Response in Mouse Leukemias

Abstract. *Methotrexate-promoted survival of mice bearing different transplantable leukemias was compared with uptake of the drug by leukemia cells in vitro. A high degree of correlation* ($r^2 = 0.88$) *showed that uptake may be a major determinant of the antitumor activity of methotrexate against transplantable mouse leukemias.*

Methotrexate is a chemotherapeutic agent which initially controls certain mouse and human leukemias. This drug is tightly bound by the enzyme dihydrofolate reductase (*1*). The resulting inhibition of this enzyme blocks reduction of folate and dihydrofolate to tetrahydrofolate, and this leads to interference with one-carbon metabolism, causing death of growing cells (*2*). The effectiveness of methotrexate as an antileukemic agent is limited by the eventual development of resistance (*3*).

Figure 1 shows the relative sensitivity of ten transplantable mouse leukemias to methotrexate as measured by drug-promoted survival of tumor-bearing animals (*4*) and the corresponding uptake of the drug by each cell line in vitro. A significant correlation ($r^2 = 0.88$) exists between these two variables, showing that the drug uptake by each cell line is an important determinant of survival of tumor-bearing animals.

Ascites fluid was collected 24 to 72 hours before the expected death of tumor-bearing animals and was diluted with ten volumes of buffer containing 10 m*M* tris *p*H 7.2, 140 m*M* NaCl, and 1 mg of EDTA (ethylene diaminetetraacetate). The cells were collected by centrifugation, erythrocytes were lysed by resuspension for 30 seconds in 35 m*M* NaCl, and isotonicity was restored by addition of concentrated NaCl solution. The cells were again collected by centrifugation and resuspended in ten volumes of incubation medium (*5*). Samples (150 μl) of this 10 percent suspension were incubated at 37°C in medium containing 0.1 μg of tritiated methotrexate per milliliter (*6*). After 30 minutes, the cell suspension was diluted with 0.8 ml of cold incubation medium, and the cells were collected by centrifugation for 30 seconds at 1000*g* in a Misco microcentrifuge. After two additional washings by resuspension and centrifugation, the intracellular methotrexate was extracted by suspending the cell pellet in 0.5 ml of 0.01*N* acetic acid and heating for 10 minutes at 60°C (*7*). Cellular debris was removed by centrifugation and a portion of the fluid was mixed with Bray's solution (*8*) for determination of radioactivity with a Nuclear-Chicago liquid scintillation counter.

The L1210 strain of mouse leukemia has frequently been used in screening antileukemic drugs (*9*). Lives of animals bearing L1210 are prolonged about twofold by ten daily intraperitoneal injections of methotrexate (1.5 mg/kg). Other cell lines—P329, 70429, P1534Ja, P815, and P288—show varying degrees of inherent sensitivity to this drug (*4*).

From leukemias L1210 and P288, two resistant sublines have been developed by exposure of cells to methotrexate. These lines, designated L1210/MTX and P288/MTX, show drug-induced or "acquired" resistance; the drug is ineffective in increasing survival of animals bearing these tumors (Fig. 1). These sublines differ from their parent strains in at least two ways, impaired uptake of methotrexate, reported here, and an eight- to tenfold increase in the level of dihydrofolate reductase, (*4*).

Leukemia P815 is moderately resistant to methotrexate. A derived cell line, P815/VLB, originally selected for acquired resistance to Vinblastine, shows "collateral" sensitivity to methotrexate (*10*)—that is, methotrexate-promoted survival is longer in animals bearing the P815/VLB line. Drug uptake is correspondingly greater than that in the P815 line.

The basis for methotrexate resistance has been investigated in many biological systems. Moderate degrees of drug-induced resistance in animal leukemias have been attributed to increase in dihydrofolate reductase (*11*). But the activity of intracellular dihydrofolate reductase has not proved a reliable index of relative drug resistance. The variation in the sensitivity in 23 mouse leukemias was not correlated with intracellular levels of this enzyme (*4*). Elevated enzyme alone could not account for the 100,000-fold resistance to methotrexate observed in a tumor line developed in tissue culture (*12*). Drug uptake, as a factor in methotrexate resistance, was studied by Fischer (*13*) in a mouse-leukemia cell line isolated in tissue culture. Drug resistance was accompanied by low drug uptake. Werkheiser *et al.* suggested the possibility of a transport impairment to account for the behavior of a drug-resistant cell line derived from leukemia P388 (*14*). No previous attempts have been reported correlating uptake with drug response in vivo.

This study shows that the rate of methotrexate uptake is an important determinant of drug response in a group of mouse leukemias. This group contained cell lines with varying degrees of natural resistance to the drug, as well as two lines with acquired resistance and one line with collateral sensitivity. In studies on human leukemias we have found that some cases of methotrexate resistance showed impaired uptake of drug (*15*).

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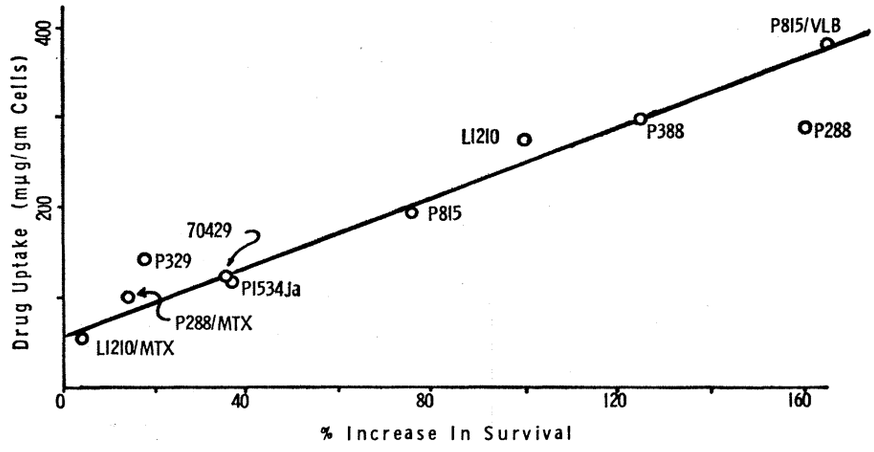

Fig. 1. Correlation between methotrexate uptake of cell suspensions and methotrexate-promoted survival time of tumor-bearing animals. Responsiveness of tumors to methotrexate in vivo was estimated from increase in survival time of tumor-bearing animals after intraperitoneal injection of methotrexate (1.5 mg/kg) from day 1 to day 10 to animals that had been inoculated with 10^6 tumor cells. The percentage increase in survival is calculated as $[100\ (T\text{-}C)/C]$; where C is the mean survival time (days) of untreated, tumor-bearing animals, and T is the mean survival time of methotrexate-treated animals. Drug uptake is determined after incubation of cell suspensions in medium containing 0.1 μg of methotrexate per milliliter for 30 minutes at 37°C.

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6. Resistant cell lines showed low drug uptake when compared with drug sensitive leukemias as the external drug level was varied from 0.1 to 100 μg/ml. The uptake of radioactive drug was linear in all experiments for at least 45 minutes. Tritiated methotrexate (9170 mc/mmole) was obtained from the Nuclear-Chicago Corp.
7. At least 96 percent of the radioactivity in the cell pellet was extracted by this procedure. Chromatography (descending, Whatman No. 1 paper; 0.01*M* phosphate buffer at *p*H 7, or

0.5 percent aqueous Na_2CO_3) showed that more than 90 percent of the radioactivity extracted chromatographed as methotrexate.
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Hageman Factor: Alterations in Physical Properties during Activation

Abstract. *Highly purified preparations of Hageman factor, a potent clot-promoting agent in normal mammalian plasma, had a sedimentation coefficient of approximately 5S before activation. After activation, the Hageman factor behaved as a much less soluble or larger molecule during ultracentrifugation and gel filtration. No significant change in sedimentation behavior was noted when the Hageman factor in plasma deficient in plasma thromboplastin antecedent was activated. The altered sedimentation behavior of purified activated Hageman factor probably reflects its decreased solubility in aqueous media.*

Blood readily clots in vitro upon contact with certain adsorbent surfaces (*1*). Generation of clot-promoting activity by surfaces depends partly upon the activation of Hageman factor, a plasma protein deficient in persons with Hageman trait (*2*). Once activated, Hageman factor behaves as an enzyme and initiates a series of enzymatic reactions culminating in the generation of thrombin and the formation of fibrin (*3*).

The experiments we report here demonstrate that highly purified Hageman factor undergoes a change in its physical properties during activation. Hageman factor had a sedimentation coefficient of approximately 5*S* before activation. In contrast, activated Hageman factor was associated either with a macromolecule or with an insoluble substance.

Hageman factor was purified 3000- to 5000-fold with respect to human serum (*4*). Although this preparation was originally thought to be activated Hageman factor (*4*), it has been shown to contain only 5 to 10 percent of activated material (*5*). The term "Hageman factor" refers to the precursor, or unactivated form, whereas "activated Hageman factor" refers to the form which has enzymatic activity and accelerates clotting.

The preparation of purified Hageman factor was dissolved and applied to linear sucrose density-gradient mixtures (5 to 20 percent) and ultracentrifuged (*6*). The Hageman factor and sucrose were each dissolved in 0.05*M* tris buffer, *p*H 7.4. After centrifugation in a Spinco model L-2 ultracentrifuge at 100,000 or 105,000*g* for 24 hours at 0°C in an SW-39 head, the gradient mixtures were fractionated. Usually, successive 0.2-ml portions were removed from the upper surfaces of the gradient mixtures, and added to 0.5 ml of buffered 1 percent bovine serum albumin. These diluted fractions were then tested for their Hageman factor content (*4, 5*). Hageman factor isolated from gradient fractions shortened the abnormally long recalcified clotting time of plasma from a person known to be deficient in Hageman factor. This assay was performed in the presence of kaolin (a substance which activates Hageman factor) and a crude phospholipid ("cephalin") (*7*). Activated Hageman factor in fractions shortened the recalcification time of the same plasma in the presence of "cephalin" alone in silicone-coated test tubes. Plasma was prepared in silicone-coated apparatus.

Table 1. Clot-promoting action of purified Hageman factor upon Hageman plasma deficient in Hageman factor. A mixture of 0.1 ml of test material, 0.1 ml of "cephalin" (in barbital-saline buffer, 0.06 mg/ml, *p*H 7.4). and 0.1 ml of plasma deficient in Hageman factor was incubated in silicone-coated Pyrex tubes (10 by 75 mm) for 8 minutes. Then 0.1 ml of 0.025*M* calcium chloride was added, mixed, and the time required for visible fibrin formation at 37°C was measured.

Hageman factor	Clotting time (sec)
Untreated	>400
Ellagic acid-activated	79
Barium carbonate-activated	70

After purified Hageman factor was subjected to sucrose gradient ultracentrifugation, this activity was located in fractions containing proteins with sedimentation coefficients of 4.5 to 5.5*S* (Fig. 1).

The same preparation of Hageman factor was activated by solutions of ellagic acid or by barium carbonate (Table 1), and subjected to ultracentrifugation in the same way as Hageman factor. Ellagic acid (4,4′,5,5′6,6′-hexahydroxydiphenic acid 2,6:2′,6′-dilactone), dissolved in concentrations as low as $10^{-8}M$, activated Hageman factor by an unknown mechanism (*5*).

The ellagic acid was synthesized (*8*) by the method of Perkin and Nierenstein (*9*) by oxidative coupling of gallic acid (*10*). It was dissolved at a concentration of $2 \times 10^{-4}M$ in tris buffer and carefully centrifuged to remove any insoluble material before mixing it with Hageman factor. After exposure to Hageman-factor solutions, barium carbonate was removed by dialysis after its conversion to soluble acetate by the addition of equivalent amounts of acetic acid.

Once activated by either technique, coagulant properties attributable to activated Hageman factor resided in density-gradient fractions containing the heaviest or least soluble materials (Fig. 1). In other experiments, Hageman factor activated by ellagic acid was centrifuged at 100,000*g* for only 100 minutes, or 31,000*g* for 120 minutes. Here too, the activated Hageman factor was located principally in fractions at the bottom of the gradient mixture. Activated Hageman factor was also separated from Hageman factor during column chromatography by Sephadex G-200 gel filtration. During elution with buffer composed of 0.025*M* barbital in 0.0125*M* saline at *p*H 7.4, activated Hageman factor was found in fractions known to contain the heaviest materials. In contrast, Hageman factor was in effluent fractions known to contain 4.5*S* materials.

The preparations of purified Hageman factor tested invariably contained a small proportion of activated en-