(5) The kinetics of the uptake of BSA and BGG was studied by a modification of the incubation technique in order to measure very short incubation times from 10 sec up to 60 min. 3×10^7 spleen cells were incubated at 37 °C with 1.0×10^{-6} g BSA. After 10 sec 2500 cpm were counted on the washed spleen cells or 0.5×10^{-8} g BSA. After 30 sec 3500 cpm and after 120 sec 4700 cpm were counted. This means that in 2 min nearly the whole amount of BSA is taken up by the cells, since the amount after 30 min incubation time is 4750 cpm. This amount remains constant throughout 2 h incubation. This result indicates that the BSA molecule is absorbed in seconds on the cell surface (Figure 2).

All our experiments showed after 10–20 min incubation an excess of uptake which disappeared a few minutes later. This excess of uptake is not observed if the cells are incubated with brucella antigen (Figure 2).

Zusammenfassung. 6×10^{-10} g bis 10^{-4} g J 125 Rinderserum-Albumin (BSA) bzw. $1{,}0 \times 10^{-7}$ bis $1{,}0 \times 10^{-6}$ g J 125 Rinderserum-γ-Globulin (BGG) werden 3×10^7 Milzzellen normaler Meerschweinchen in vitro angeboten. Unabhängig von der Höhe des Angebotes beträgt die Menge des aufgenommenen BSA 1%, die des aufgenommenen BGG 10% des Angebotes. Durch 4 °C Inkubationstemperatur wird die Aufnahme z.T. behindert. Die Aufnahme findet nach wenigen Sekunden Inkubationszeit statt und ist nach etwa 2–3 min abgeschlossen.

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Distribution of Procoagulant Activity in the Subcellular Fractions of Human Granulocytes

Procoagulant activities in the leucocytes were found by several authors[1,2]. Lisiewicz[2] stated that this activity has similar features to that of platelet factor 3. The granulocytes possess also an anticoagulant activity[3,4]. Saba et al.[4] demonstrated that this activity is connected with lysosomal cationic proteins. The purpose of this work was to study the distribution of procoagulant activity in the subcellular fractions of human granulocytes.

Material and method. The granulocytes were obtained from human purulent pleural exudate. The Cohn and Hirsch method[5] was used to obtain the following subcellular granulocyte fractions: I, sediment after 400 *g*; II, sediment after 8200 *g*; III, supernatant after 8200 *g*. Fraction I contains nuclei, membranes and cell debris, large specific granules; fraction II, specific granules (lysosomes); fraction III, smaller cytoplasmic alements: small specific granules, mitochondria, ribosomes, endoplasmatic reticulum and Golgi apparatus fragments.

All granulocyte fractions and granulocyte homogenates were suspended in a solution of 0.34 *M* saccharose. They were frozen and thawed 6 times, homogenized 1–2 min using a Potter homogenizer. All the preparations were dialysed against a phosphate buffer (0.025 *M*; pH 7.65; +4 °C; 18 h). Human plasmas deficient in factor VIII, IX and XII were obtained from patients with hereditary deficiencies of these factors. Factor VIII, IX and XII were determined according to the method of Soulier and Larrieu[6], slightly modified by Niewiarowski et al.[7].

In order to compare the procoagulant activity of human plasma with that of the granulocyte suspension, the granulocyte suspension was diluted to a concentration of 6000 cells/μl. The procoagulant activity was tested simultaneously in the granulocyte suspension and in the plasma and the dilution curves were made. Each clotting factor activity in the granulocyte suspension was expressed as % of the activity of the same factor, found in the equal volume of plasma (accepted as 100%). Furthermore, the specific activity of each clotting factor was calculated according to the formula: $1000/(t \times p)$ where t = clotting time in sec, p = protein concentration (mg/ml).

Result and discussion. The results are summarized in the Table. It can be seen that the relative procoagulant activity of the homogenate and of subcellular fractions is of the same order of magnitude as that of normal plasma. The specific procoagulant activity of the homogenate is considerably higher than of plasma. It is higher in all isolated subcellular fractions, particularly in fraction II and III, than in the whole homogenate. This fact may be explained by the presence of anticoagulant activity which is diminished by dilution during fractionation. Factor XII-like activity can be adsorbed partially

Distribution of procoagulant activity in subcellular fraction of human granulocyte[a]

Granulocyte fraction		Substrate plasma						
		Factor VIII deficient		Factor IX deficient		Factor XII deficient		
		A	B	A	B	A	B	C
Total homogenate		116%	11.8	51%	8.1	43%	7.1	–
Sub-fractions	I	112%	20.4	40%	12.6	46%	11.7	–
	II	77%	43.7	41%	36.9	49%	36.4	17.8
	III	190%	147.2	26%	56.6	38%	59.0	17.0
Human plasma		100%	1.8	100%	2.4	100%	1.7	–

[a] Mean values from 5 determinations. A, relative activity as compared with normal plasma; B, specific activity $1000/(t \times p)$; C, specific factor XII-like activity after adsorption of tested material on kaolin and elution at alkaline pH.

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from the granulocyte subfractions II and III by kaolin and eluted at alkaline pH.

At the present state of experimental evidence, it is not possible to reach a conclusion as to whether the granulocyte and plasma-clotting factors are identical. RAPAPORT and HJORT[8] found that rabbit neutrophils do not bind plasma-clotting factors. On the other hand, there is a certain possibility that some plasma-clotting factors may originate from granulocytes. The experiments with adsorption of procoagulant activity by kaolin from granulocyte subfractions and elution at alkaline pH provides further experimental evidence that factor XII (Hageman) may be localized in granulocytes. The specific procoagulant activities are much more concentrated in leucocytes than in plasma. For this reason, granulocytes, as well as platelets, may be considered as active centers in the formation of hemostatic plug.

It is of interest to note that procoagulant activity is mainly localized in the supernatant after 8200 *g* and in the sediment at 8200 *g*. The enzymes forming kinins were also found in the same fractions[9]. Since the work of MARGOLIS appeared[10], it is known that the formation of kinins may be initiated by the active Hageman factor. These facts could indicate the role of granulocyte factor XII for the generation of kinins in synovial fluid of patients with rheumatoid arthritis[11]. It has been suggested that the procoagulant activity of the leucocytes may be involved in the formation of fibrin in inflammatory exudates and in the intravascular clotting[12]. The present communication confirms this point of view.

Résumé. Les extraits granulocytaires racourcissent le temps de coagulation de plasmas déficients en facteurs VIII, IX et XII. Une activité paracoagulante particulièrement élevée se manifeste dans les subfractions de granulocytes contenant des lysosomes et d'autres structures cytoplasmatiques.

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The Positive Direct Coombs Test of Cephalothin-Treated Blood

MOLTHAN et al.[1] have recently reported that the antibiotic cephalothin, incubated in vitro with normal blood under suitable experimental conditions, produces a positive direct Coombs test; moreover, they observed that the antiglobulin test was slightly stronger when the blood of 4 azotemic patients was used instead of normal blood. According to the authors, the erythrocytes from patients with poor kidney function could be more sensitive to cephalothin than normal cells. We studied the in vitro action of cephalothin on the blood of 14 healthy subjects, 14 azotemic patients and 13 patients suffering from classical (portal) cirrhosis of the liver. Four volumes of blood, drawn with acid-citrate-dextrose, were incubated with one volume of cephalothin (Keflin Lilly) dissolved in saline at different concentrations (final concentration of the mixtures = 0.625, 1.25, 2.5, 5, 10, 20 and 40 mg/ml). The incubation was carried out in a waterbath at 37 °C for 3 h, the suspensions being gently mixed approximately every 30 min. Direct antiglobulin tests were carried out with the subjects' red cells and ortho 'broad-spectrum' antiglobulin reagent.

The sensitivity of red cells to cephalothin (i.e. the minimum concentration of the drug [mg/ml] sufficient to produce a positive direct Coombs test) varied from case to case (Figure 1). However, the statistical analysis of the data, performed with the MANN-WHITNEY U test[2], indicated that there was no difference between the groups of normal and azotemic subjects; on the contrary, the minimum sufficient concentration of the drug was significantly lower in the group of cirrhotic patients than in that of normal subjects ($p < 0.02$).

We then investigated the relationship between the sensitivity of red cells to cephalothin and some biochemical indices of the blood. For this purpose, a multiple regression 'step-wise' analysis was carried out, challenging the minimum sufficient concentration of cephalothin in turn with hematocrit, BUN, serum total protein level and concentration of protein fractions (albumin, α_1-, α_2-, β- and γ-globulins). The red cell sensitivity to the drug appeared to depend on the level of serum γ-globulins ($F = 10.11$, $p < 0.01$), the regression coefficient being negative (Figure 2).

We could not observe the relationship between the sensitivity of red cells to cephalothin and serum albumin concentration reported by LO BUGLIO[3].

The mechanism by which cephalothin produces a positive direct Coombs test is still uncertain: at present it seems likely that both some properties of the plasma and an alteration of the red cell play a role. The data presented here show that the level of serum γ-globulins influences the sensitivity of the blood to cephalothin. In so far as the erythrocyte alteration is concerned, previous experiments by MOLTHAN et al.[1], as well as results obtained in this laboratory, have shown that a positive direct Coombs test can be obtained not only by incubating cephalothin with whole blood, but also when washed erythrocytes are exposed to the drug, if suitable antiglobulin reagents are used. In the latter experimental condition, according to MOLTHAN et al.[1], the drug alters the α- and β-globulins of the cell membrane, so that these

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