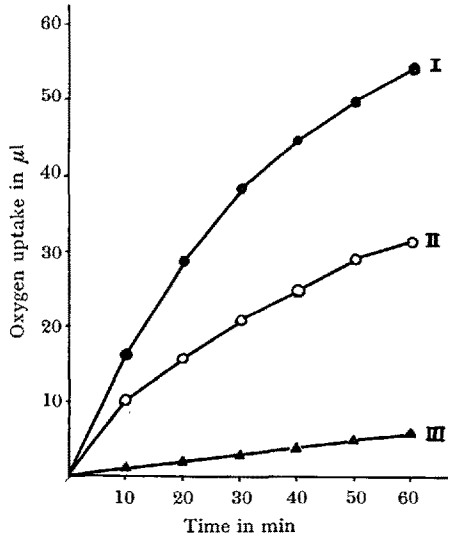
Inhibition of DOPA oxidation by various substances.

Inhibitors	Final concentration (Mol)	Inhibition % (in 60 min)	
Sodium diethyldithiocarbamate	$0{\cdot}25 \times 10^{-2}$	96	Enzyme (I)
Cyanide	$0{\cdot}25 \times 10^{-2}$	38	DOPA conc.,
Phenylthiourea	Saturate/4	84	$0{\cdot}125 \times 10^{-2} M$
95% CO with 5% O_2	in light	73	
p-Nitrophenol	$0{\cdot}5 \times 10^{-3}$	10	
Phenylthiourea	Saturate/4	95	Enzyme (II)
Salicylaldoxime	$0{\cdot}25 \times 10^{-2}$	20	DOPA conc.,
p-Nitrophenol	Saturate/4	31	$0{\cdot}125 \times 10^{-3} M$
8-Oxyquinoline	Saturate/4	20	

The activity of the enzyme was determined by measuring oxygen uptake in the Warburg apparatus at 35° C and pH 7·3, taking 1·0 ml of the enzyme preparation, 1·0 ml of inhibitor and others in the main chamber; 0·5 ml of DL-dihydroxyphenylalanine solution in the sidearm of a flask, the total volume of liquids being made to 4·0 ml. The center well contained 0·3 ml of 10% KOH; the gas phase was air.

Salicylaldoxime[5], *p*-nitrophenol[6] and 8-oxyquinoline[7] are known as sensitive reagents against some copper enzymes which are responsible for the terminal step in the biological oxidation, but inhibition by these inhibitors is not very extensive.

Fig. 2.–Inhibition of DOPA oxidation by thioglycolate.



Curve I. DOPA only (final concentration $1{\cdot}25 \times 10^{-3}$ Mol).
Curve II. DOPA plus thioglycolate (final concentration $0{\cdot}25 \times 10^{-3}$ Mol).
Curve III. DOPA plus thioglycolate (final concentration $0{\cdot}25 \times 10^{-2}$ Mol).

According to FLESCH[8] the formation of melanine in the animal is inhibited by sulfhydryl compounds, the extent of inhibition being dependent on the concentration of the inhibitor.

[5] F. KUBOWITZ, Biochem. Z. *292*, 221 (1937).
[6] J. BONNER and S. G. WILDMAN, Arch. Biochem. *10*, 497 (1946).
[7] E. STOTZ, C. J. HARRER, and C. G. KING, J. biol. Chem. *119*, 511 (1937).
[8] P. FLESCH, Proc. Soc. exper. Biol. Med. *70*, 136 (1949).

In the present experiment the oxidation of DOPA by the polyphenolase (I) was inhibited by thiosulphate and thioglycolate. The former is the simplest of sulfhydryl compounds[9]. Its inhibitory effect, as is shown in Figure 1, supports the conclusion by FLESCH. The effects of thioglycolate are shown in Figure 2.

Copper ions are known to be sensitive to thiol enzymes. The inhibition by thiosulphate and thioglycolate, therefore, might be concerned with the prosthetic group of the polyphenolase.

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Zusammenfassung

An einer aus *Scopolia japonica* isolierten Polyphenolase wurde die Wirkung verschiedener Hemmstoffe studiert. Cyanid und Salicylaldoxim wirkten relativ schwach, Diäthyldithiocarbamat, Phenylthioharnstoff und Kohlenoxyd hemmen die Oxydation stark. Der Ablauf der Hemmwirkungen von Thiosulfat und Thioglycolat wird kurvenmässig dargestellt.

[9] J. HIRAIDE, *Progressive Study of SH Group* (in Japanese) (Tokyo 1954).

Further Data on the Evaluation of Platelet Ac-globulin and its Plasmatic Origin

In the past few years, many authors have described a platelet accelerator factor which has been called Platelet Ac-globulin (or factor I). The action of this accelerator is similar to that of Plasma Factor V, and accelerates the speed of transformation of prothrombin and thrombin. Such a factor, according to some authors[1], is present in the platelets in a form which is already active, and acts in a way similar to the Serum Ac-globulin (or factor VI). Also, considering some of the physical chemical characteristics, there is an analogy between Platelet Ac-globulin and Plasma Ac-globulin. It is destroyed by heat at 56° C for 30 min, and is not dialyzed. It has been prepared in concentrated form and it has been shown to act like a protein. The activity of this accelerator has been established to be less than that of the Plasma Ac-globulin, and even though qualitatively equal, it seems that it is quantitatively equivalent to $^1/_8$–$^1/_{16}$ of the accelerator activity of Plasma Ac-globulin: such values vary considerably according to different authors. According to OWREN, the platelets possess only 6% of the accelerator activity of plasma. Recent researches[2] suggest that the accelerator factor of the platelets is adsorbed Plasma Ac-globulin (or factor V). The platelets of a para-haemophilic patient do not contain accelerators, but can acquire such an activity after having been in contact with normal plasma. Trypsin destroys almost completely Platelet Factor I, without altering platelets. If the platelets thus treated are placed in contact with a normal plasma, they recover all of the lost activity.

[1] A. G. WARE, J. L. FAHEY, and W. H. SEEGERS, Amer. J. Physiol. *154*, 140 (1948).
[2] P. HJORT, S. I. RAPAPORT, and P. A. OWREN, Blood *10*, 1139 (1955).

During the past few years, we have tried to elaborate a simple method for the quantitative determination of the Platelet Ac-globulin, according to procedures also used for the determination of Plasma Ac-globulin[3]. The results obtained by OWREN, have suggested a revision of the problem, so as to be able to determine the Ac-globulin contained in the platelets, whatever its origin may be. We used a technique obtained by modifying the Thromboplastin Generation Test of BIGGS and DOUGLAS[4].

In order to generate an active thromboplastin, there must be present an optimal quantity of platelets, AHG, PTC, PTA, Factor V, Factor VII, and calcium. All these factors contribute to produce an active thromboplastin similar to that which is generated during the normal processes of coagulation. When one of them is diminished or absent, generation of thromboplastin is incomplete and insufficient[5].

The following reagents are necessary:

(1) The platelets have been isolated with a technique previously described by employing siliconized glassware. The blood is withdrawn rapidly, using sequestrene in the proportion of $^1/_9$, then centrifuged at slow speed for 10 min. The supernatant plasma is separated and placed in a centrifuge tube containing triton in proportion of $^1/_9$, then centrifuged for 10 min at 3000 rpm. The platelets sediment is resuspended in saline to a concentration of 400,000/cm^3, and is stored at + 4°C until used. It is advisable to make the determinations within the first hour after preparation.

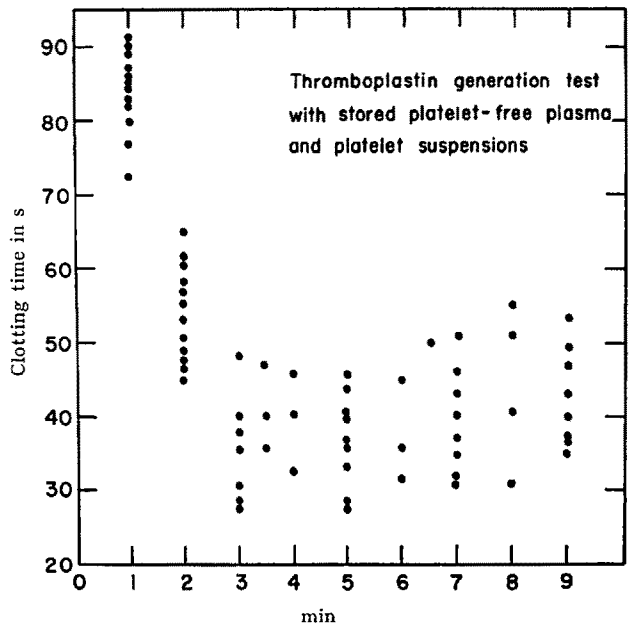
(2) The plasma used in this test is prepared in the following way: The plasma of different subjects with normal recalcification and prothrombin times is mixed. This plasma is then kept at 37°C for 24 h until the prothrombin time has been prolonged to about 40 s. In such conditions Plasma Factor V has been shown to be almost absent. The plasma is then deprothrombinized with barium sulfate, and is placed in a refrigerator at – 20°C until used. The conservation time of this plasma should be not longer than 5–6 days.

(3) The serum is prepared by mixing many normal, old sera with no residual prothrombin; this preparation is kept at – 20°C until used and lasts for a few weeks.

(4) The platelet-free plasma is prepared by centrifuging a normal plasma for 30 min at 3000 rpm in glassware accurately siliconized. After this treatment the plasma must have a recalcification time which varies between 5–9 min.

(5) The calcium chloride solution is prepared to a molar solution of $^1/_{40}$.

The technique for the study of the accelerator activity of the platelets is as follows: in a test tube kept in a water bath at 37°C, 0·3 cm^3 of plasma are placed which contains a small quantity of prothrombin and factor V, diluted $^1/_5$ in saline; 0·3 cm^3 of platelet suspension to be examined; 0·3 cm^3 of serum diluted $^1/_{10}$ in saline, and finally 0·3 cm^3 of calcium chloride. At the time of adding the calcium chloride, a stopwatch is started, and at each successive minute, 0·1 cm^3 of the mixture is removed and is placed rapidly in a test tube previously prepared and ready in the water bath at 37°C, and which contains 0·1 cm^3 of the plateletfree plasma. At the same time, 0·1 cm^3 of the calcium chloride solution is added to this mixture and a second stopwatch is started. The coagulation time is measured and the results can be transferred to a coordinate system. Since there is a determination every minute, the phenomenon can be followed up till 8–10 min, and it is possible to prepare a curve, which, when compared to a standard curve, allows us to determine the percentage of thromboplastin formed. Thromboplastin formation is not detectable in the absence of factor V (Plasma Ac-globulin). Since factor V has been removed from the plasma, the quantity of thromboplastin formed should be in relation to the percentage of Ac-globulin contained in the platelets examined. We have performed a series of determinations with the above technique on isolated platelets in a group of 12 individuals and we have ascertained that the activity of the Ac-globulin contained in the platelets is similar to that of the Plasma Ac-globulin, but is quantitatively reduced.



The generation curves of the thromboplastin vary within the limits of 10 s; this finding is referable to the different quantity of Ac-globulin adsorbed by the platelets, which therefore carry different quantities of the accelerating factor. On the basis of some researches now in progress, it seems that the content of the Ac-globulin in the platelets may be subordinated to its plasmic concentration and *vice versa*. The data of OWREN indicated that in cases of plasma Ac-globulin deficiency, the platelets are devoided of such factor, i.e. that they were not able to absorb Ac-globulin. Some of our recent preliminary researches have suggested that some cases of marked platelet deficiency may be associated with a deficient Plasma Ac-globulin, possibly in connection with a possible function of the platelets as carriers of Ac-globulin[6].

R. TURPINI

Department of Internal Medicine, University, Pavia, December 18, 1955.

[3] P. DE NICOLA, R. TURPINI, and F. WEBER, Z. ges. inn. Med. Grenzgeb. (in press).

[4] R. BIGGS and A. S. DOUGLAS, J. clin. Path. *6*, 23 (1953).

[5] C. L. SPURLING and P. D. W. KING, J. Lab. clin. Med. *44*, 336 (1954).

[6] At the time of printing we were informed by the Editor of Experientia about the just appeared paper by HÖRDER and SOKAL Acta haematol. *14*, 294 (1955).

Zusammenfassung

Die Eigenschaften des Plättchen-Ac-Globulins wurden mit Rücksicht auf die Thromboplastinbildung untersucht.

Investigations on the ACTH-Protamine complex

One of the authors reported that protamine sulphate could, to a high degree, inhibit the action of ACTH on adrenal ascorbic acid and on the involution of the thymus[1]. In the paper concerned it was mentioned that to clarify the mode of action of protamine in this respect, the authors have carried out electrophoretic and paper chromatographic investigations. The results of these investigations are as follows:

Methods. (1) In the electrophoretic investigations the materials were run on Macherey-Nagel paper No. 214 with a stabilized direct current of 4–10 V/cm voltage and of 2–4 mA intensity for 8–16 h. An acetate buffer of pH 4·7 and of 0·1–0·03 ionic strength and a borate buffer of pH 8·6 and of 0·1 ionic strength was used. The paper strips were dyed with 0·2% acid fuchsine diluted in absolute alcohol containing 10% acetic acid. The differentiation was carried out with methanol containing 10% acetic acid and with 10% acetic acid.

(2) The paper chromatographic investigations were carried out in the case of the polypeptides in a phenol-water system, in the case of the hydrolysates in a phenol-water system or in a butanol-acetic acid-water system. The polypeptides were indicated with acid fuchsine, the amino acids with ninhydrine.

(3) The frog melanophore investigations were carried out on frogs decolorized with light, according to a method modified by the authors[2]. The sensitivity of the method renders it possible to measure the quantities of the materials eluted from the chromatograms. The main point of the method is that the darkening of the frogs (resulting from the action of ACTH or from the melanophore content of ACTH) is measured by comparison with a fixed scale of 1–10. Each determination was carried out at 3 dose levels on 10 frogs per dose. The experiments were evaluated by repeated comparison with a standard (cross-over test).

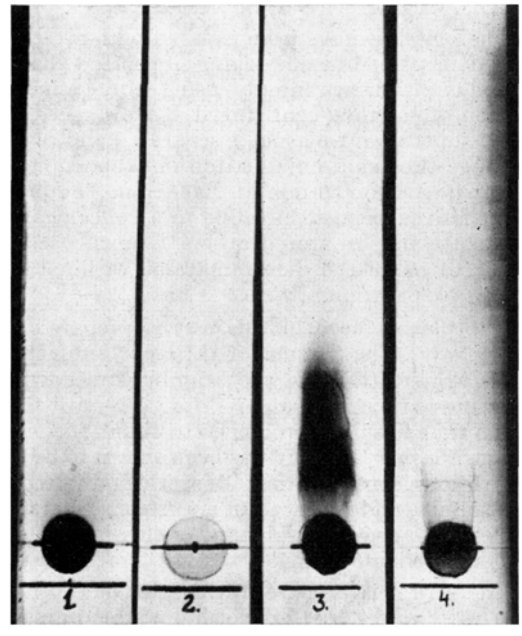
(4) The ACTH of protein type was produced according to SAYERS, WHITE and LONG[3], the peptide type material was produced by acidic hydrolysis of a material prepared according to ASTWOOD, PAYNE and RABEN[4].

(5) Pure protamine sulphate was prepared by purification of commercial protamine sulphate with oxycellulose and by precipitation with hot water. Its purity was checked by chromatographic investigations of the hydrolyzed product.

Results. In the electrophoretic investigations the diverse ACTH products split up—according to their types—into different components. Purified protamine sulphate proved to be homogenous.

In case of simultaneous running of both materials a new component appeared. A complete separation of the latter and its investigation was not possible however, since it could not be delimited from the components of ACTH.

In chromatographic investigations it was established that ACTH progressed together with the solvent, and protamine sulphate appeared as a homogenous fixed spot. In the case of simultaneous running of both materials the appearence of a new component in the form of a well defined spot was observed. The investigation of the complex-forming capacity of different ACTH types showed that in the case of materials of the protein type the greater part of the ACTH activity, in the case of hydrolyzed products only a small part of it was incorporated in the complex (Figure).



Ascending chromatograms. *1* Protamine sulphate. *2* ACTH. *3* Protein ACTH + Protamine sulphate. *4* Peptide ACTH + Protamine sulphate.

This phenomenon was indicated on the one hand by the size of the spot, on the other hand by the melanophore assay of the material eluted from the spots. By elution of the complexes with n/100 sulphuric acid it was established that the amino acids characteristic for ACTH could be found in their hydrolysates, and on the other hand the great quantity of arginine indicated the presence of protamine.

The different abilities of different ACTH preparations to form protamine complexes were also studied in investigations where the depletion of adrenal ascorbic acid (Sayers test[5]) was found to be influenced by protamine; in the case of protein type materials the effect of eightyfold quantities of ACTH could be inhibited by pretreatment with protamine, in the case of peptide type materials by only a two-threefold quantity[6].

The observations resemble those of KREBS who could —by application of salmine—essentially diminish the

[1] GY. FEKETE, Acta med. Acad. Sci. Hung. *8*, 81 (1955); Exper. *11*, 310 (1955).

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[5] G. SAYERS, M. A. SAYERS, and L. G. WOODBURY, Endocrinology *42*, 379 (1948).

[6] GY. FEKETE, J. NURIDSÁNY, and A. HEGYELI (in press).