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CHROMATOGRAPHY OF HUMAN URINARY ERYTHROPOIETIN AND GRANULOCYTE COLONY-STIMULATING FACTOR ON INSOLUBILIZED PHYTOHAEMAGGLUTININ

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Summary

Human urinary erythropoietin was adsorbed to phytohaemagglutinin coupled to agarose or porous glass and quantitatively eluted by a saturated solution of $MgCl_2$. This method provides a means of separating erythropoietin from several of its contaminants, presumably on the basis of its carbohydrate side chains. Erythropoietin which had been purified by chromatography on insolubilized phytohaemagglutinin was sufficiently free of toxicity to be assayable in tissue culture even when crude urine was used as a starting material.

Introduction

There is an increasing demand for erythropoietin preparations that are free of toxic substances and interfering activities (such as the granulocyte colony-stimulating factor [1]) and therefore suitable for in vitro studies on its mode of action. Apart from the problems arising from the low initial concentration of the hormone in serum or urine, purification of erythropoietin is quite difficult to achieve by conventional separation techniques, because erythropoietin and presumably several of its contaminants are heterogeneous with respect to charge [4,5] and size [6]. The successful separation of human urinary erythropoietin and human urinary granulocyte colony-stimulating factor (two glycoproteins of very similar size and charge) on the basis of their differential adsorption to concanavalin A-Sepharose [7] suggested that affinity chromatog-

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Abbreviations: epo U, erythropoietin unit: 1 epo U being defined as the amount of material stimulating the proliferation of erythroid progenitors in vitro [2] to the same extent as 0.5 mg of the Second International Reference Preparation of Erythropoietin [3]; Tris, tris(hydroxymethyl)-aminomethane.

raphy on other immobilized lectins with different sugar specificities might also prove useful in the isolation and characterization of erythropoietin. This communication reports the partial purification of human urinary erythropoietin by chromatography on insolubilized phytohaemagglutinin, the lectin from the red kidney bean *Phaseolus vulgaris*.

Materials and Methods

Preparation of insolubilized phytohaemagglutinin. (a) Coupling to agarose: 180 mg of Bacto-phytohaemagglutinin-P (Difco) were coupled to 7.5 g of CNBr-activated Sepharose 4B (Pharmacia) in 30 ml of 0.1 M $NaHCO_3$/0.5 M NaCl at room temperature [8]. Unreacted groups were blocked after 20 h by the addition of 1 M ethanolamine pH 8. After several alternate washes with 0.1 M sodium acetate pH 4.0/1 M NaCl, and 0.1 M sodium borate pH 8.5/1 M NaCl, the derivatized Sepharose was either used for agglutination assays or poured into a 1.6 × 9 cm column and equilibrated in 0.05 M Tris · HCl pH 7.4/0.5 M NaCl.

(b) Coupling to porous glass: 15 g of porous glass beads (CPG-10, 120—200 mesh, 363 Å pore diameter; Serva, Heidelberg, G.F.R.) were washed with concentrated nitric acid, distilled water, ethanol and diethyl ether and subsequently dried under vacuum (20 torr) at 75° C. These cleaned beads were then immersed in a 2% solution of aminopropyltriethoxysilane (Serva) in acetone and allowed to stand at 45° C overnight [9]. The aminoalkylsilane glass was rinsed with distilled water and reacted with a 1% solution of glutaraldehyde in water at 5° C. 30 min later the porous glass beads were rinsed with distilled water and mixed with 270 mg of phytohaemagglutinin-P dissolved in 50 ml of cold phosphate buffered saline. After a 2 h incubation at 5° C the beads were washed extensively with 1 M NaCl to remove unbound material, poured into a 1.6 × 11 cm column and equilibrated in 0.05 M Tris · HCl pH 7.4/0.5 M NaCl.

Chromatography of erythropoietin and granulocyte colony-stimulating factor on insolubilized phytohaemagglutinin. Unless otherwise specified, erythropoietin and the colony-stimulating factor were applied in 0.05 M Tris · HCl pH 7.4/0.5 M NaCl and eluted by the buffers listed in Table I. All operations were carried out at 5° C.

Preparation of erythropoietin. Erythorpoietin was initially extracted from pooled urines of severely anemic patients by adsorption to benzoic acid [10]. This material was further purified by ion exchange chromatography on DEAE-cellulose [11], and, where indicated, by further affinity chromatography on concanavalin A-Sepharose [2], gel filtration on Sephadex G-100 [2] and chromatography on calcium phosphate [12]. All preparations contained granulocyte colony-stimulating factor as a contaminant unless the concanavalin A purification step was included. Buffers used for chromatography on concanavalin A-Sepharose and insolubilized phytohaemagglutinin were toxic for cultures of bone marrow cells and were therefore removed by exhaustive dialysis against phosphate buffered saline or Dulbecco's modified Eagle's medium prior to the bioassay.

Protein concentrations were determined using bovine serum albumin as a standard [13,14]. Homogeneity of purified erythropoietin was evaluated by

TABLE I

CHROMATOGRAPHY OF ERYTHROPOIETIN ON PHYTOHAEMAGGLUTININ-SEPHAROSE

Elution conditions: peak I: 0.05 M Tris · HCl pH 7.4/0.5 M NaCl; Peak II: 0.05 M Tris · HCl pH 7.4/0.5 M NaCl/0.1 M D-galactose; peak III: saturated solution (at 5°C) of $MgCl_2$ [18]; flow rate: 10 ml/h.

Starting material	Erythropoietin, DEAE-purified			Erythropoietin added to normal urine		
	Activity (epo U)	Protein (mg)	Purification factor	Activity (epo U)	Protein (mg)	Purification factor
Loaded	23	5.18		26	6.36	
Recovered in						
peak I	0.6	4.74		0.7	5.29	
peak II	n.d.	n.d.		0.9	0.04	
peak III	20.9	0.40	11.8	25.5	0.91	6.9

electrophoresis on 10% polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate [15]. Heavy meromyosin from rabbit muscle (mol. wt. 204 5000) (courtesy of Dr. B. Jockusch, Biozentrum, Basel), β-galactosidase from *E. coli* (mol. wt. 130 000), bovine serum albumin (mol. wt 67 000), ovomucoid (mol. wt. 43 000), carbonic anhydrase from bovine erythrocytes (mol. wt. 29 000) and lysozyme from egg white (mol. wt. 13 700) served as markers. Proteins were localized on the gel by staining with Coomassie Brilliant Blue.

Bioassay for granulocyte colony-stimulating factor and erythropoietin. The bioassays for the colony-stimulating factor and erythropoietin were based on colony formation by proliferating granulocyte/macrophage progenitors and late (and, where indicated, also early) red cell progenitors in freshly explanted mouse bone marrow as described previously [2,11].

Agglutination assay for phytohaemagglutinin. Functionally active phytohaemagglutinin was identified by its capacity to agglutinate L 1210 leukemia cells [16].

Results

Characterization of phytohaemagglutinin-Sepharose

L 1210 leukemia cells adhered to phytohaemagglutinin-Sepharose beads (Fig. 1a) and adherence of these cells to the beads could be specifically prevented by a 10 min preincubation of the derivatized beads with the hapten inhibitor *N*-acetyl-D-galactosamine (final concentration 0.05—0.1 M) (Fig. 1b), indicating that the derivatized Sepharose contained functionally active lectin. Non-derivatized beads did not bind L 1210 cells, and cells which did not come into direct contact with derivatized beads did not agglutinate among themselves. The total amount of coupled ligand and the relative concentration of active lectin were not determined. The glycoprotein binding capacity of phytohaemagglutinin-Sepharose was found to be 0.11 mg [14] per cm^3 of packed gel when a mixture of human urinary glycoproteins was used as a standard.

Ligands which have been coupled to a solid support by the cyanogen bromide method are known to detach slowly from their matrix [16]. However, the amounts of phytohaemagglutinin contaminating the eluates from the phytohaemagglutinin-Sepharose column were too small to interfere with the bioassay.

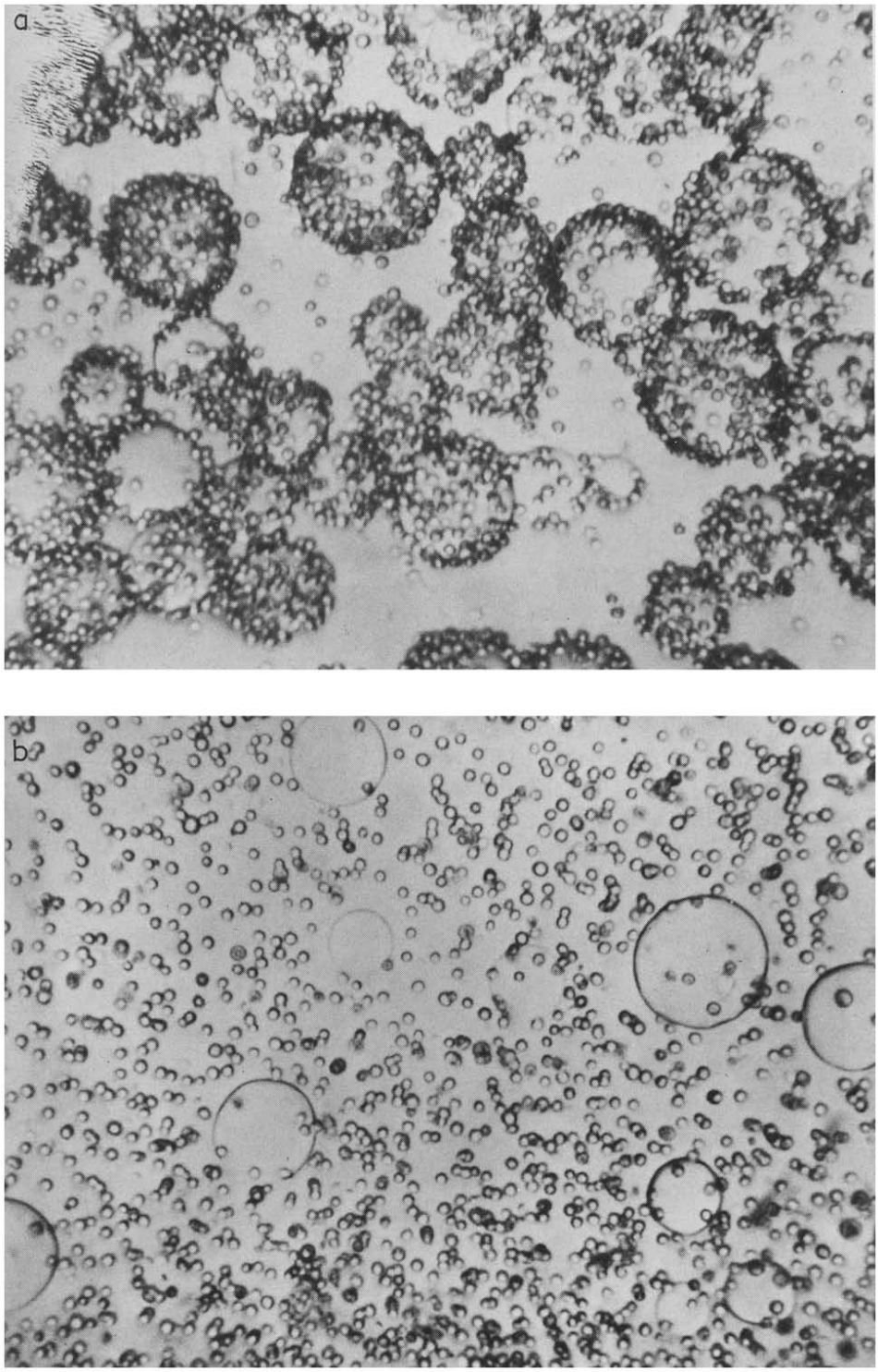

Fig. 1.(a) Adherence of L 1210 leukemia cells to phytohaemagglutinin-Sepharose beads. (b) Non-adherence of L 1210 leukemia cells to phytohaemagglutinin-Sepharose beads after a 10 min preincubation of the beads with 0.05 M *N*-acetyl-D-galactosamine.

Experiments with small amounts (4—160 μg/ml) of phytohaemagglutinin added to the culture medium showed neither a stimulation of erythorpoietin-independent colony growth nor an enhancement of the effect of a given dose of erythropoietin. Inhibition of erythroid colony growth was not observed unless the concentration of phytohaemagglutinin in culture exceeded 40 μg/ml.

Chromatography of erythropoeitin on insolubilized phytohaemagglutinin

When a DEAE-cellulose-purified sample of human urinary erythropoietin was chromatographed on phytohaemagglutinin-Sepharose (Table I, left panel) virtually all the activity was bound. Almost quantitative elution was then achieved with a saturated solution of $MgCl_2$ [18], yielding material with 11.8 times increased specific activity.

When a sample of human urinary erythorpoietin which had already been purified by adsorption onto benzoic acid and chromatography on DEAE-cellulose, concanavalin A-Sepharose, Sephadex G-100 and calcium phosphate was chromatographed on phytohaemagglutinin-Sepharose, a further 6.7-fold increase in specific activity was achieved (Table II). This highly purified material (specific activity 4240 epo U/mg protein) was concentrated by lyophilization and aliquots of it (5—20 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Subsequent staining with Coomassie Brilliant Blue revealed three broad bands with apparent molecular weights of 49 000, 44 000 and 32 000 and one sharp band at the front (mol. wt. ⩽15 000). Occasionally very faint bands corresponding to molecular weights of 65 000, 86 000 and 96 000 were also observed. None of these bands could be unambiguously attributed to erythropoietin: homogeneous preparations of erythropoietin which would permit identification of sodium dodecyl sulfate-treated erythropoietin as a distinct protein band on the gel have not yet been obtained. Sodium dodecyl sulfate-treated erythropoietin could also not be identified by its biological activity in the in vitro colony assay because of its toxicity for bone marrow cell cultures. Attempts to determine the position of erythropoietin on sodium dodecyl sulfate-polyacrylamide gels by assaying its biological activity in vivo have produced contradictory results, differing by a

TABLE II

PURIFICATION OF HUMAN URINARY ERYTHROPOIETIN

Purification step	Activity (epo U)	Protein (mg)	Specific activity (epo U/mg)	Recovery (%)	Purification factor
Water-soluble extract of 5.31 g benzoic acid precipitate derived from 23 l urine	12 690	534	24	100	1
DEAE-cellulose	15 420	203	76	122	3.2
Concanavalin A-Sepharose	12 740 *	66	193	100	8.1
Sephadex G-100	12 520	29	432	99	18
Calcium phosphate	7 000	11	636	55	27
Phytohaemagglutinin-Sepharose	9 210	2.17	4240	73	179

* Activity determined by its capacity to stimulate colony formation by early erythroid progenitors which are less susceptible to the small amounts of concanavalin A present in the column eluates than late progenitors (Sieber, F., unpublished results).

factor of two or more (mol. wt. of 23 000 [19] or mol. wt. of 45 800—51 300 [20]). The three main bands with apparent molecular weights of 49 000, 44 000 and 32 000 were very similar to the banding pattern of the phytohaemagglutinin preparation used for the derivatization of the Sepharose beads, suggesting that a major fraction of the most purified erythropoietin was phytohaemagglutinin released from the column. A contamination of the hormone by phytohaemagglutinin was also indicated by the observation that the erythropoietin preparation at a final concentration of 100 μg/ml agglutinated L 1210 cells to the same extent as phytohaemagglutinin at a final concentration of 5 μg/ml. This result did however not allow a quantitative determination of the contamination by phytohaemagglutinin, because the hormone preparation was very likely to contain competitive inhibitors of phytohaemagglutinin.

A method capable of recovering erythropoietin directly from crude urine in a form sufficiently free of toxicity to be assayable in culture would be extremely useful, especially in the view of a clinical assay for erythropoietin based on the in vitro colony method. Since urine itself is too toxic for assay in culture directly, the potential efficiency of chromatography on insolubilized phytohaemagglutinin was tested using 50 ml of dialyzed normal urine (known to contain only about 0.001 epo U/ml; [21]) to which a known amount of partially purified erythropoietin (specific activity 130 epo U/mg) had been added (Fig. 2; Table I, right panel). In an attempt to selectively remove weakly adsorbed material from the column, the $MgCl_2$ wash in this experiment was preceeded by elution with 0.1 M D-galactose, a weak hapten inhibitor of phytohaemagglutinin. Most of the activity was again recovered (with 6.9 times increased specific activity) in the $MgCl_2$ eluate.

After several alternate elutions with buffers of low and high ionic strength the flow rate of the phytohaemagglutinin-Sepharose column decreased markedly. For the routine use of insolubilized phytohaemagglutinin in a clinical assay, where the necessity for frequent repacking of the column would be an obvious disadvantage, a mechanically more resistant matrix would therefore be highly desirable. Erythorpoietin was accordingly also chromatographed on

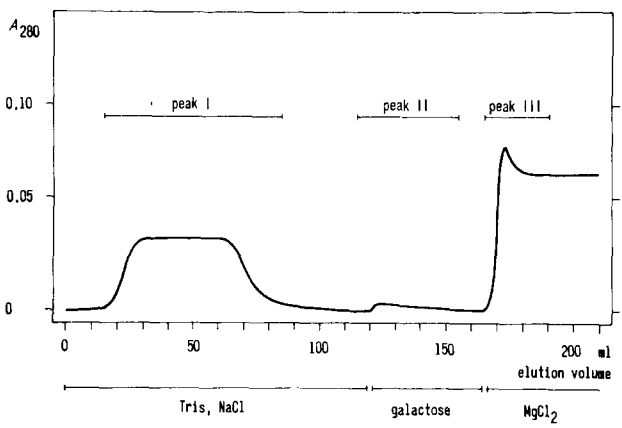

Fig. 2. Chromatography on phytohaemagglutinin-Sepharose of 50 ml of dialyzed normal urine containing 0.2 mg (26 epo U) of partially purified erythropoietin. The elevated baseline after addition of $MgCl_2$ is due to the high ultraviolet absorption of the saturated $MgCl_2$ solution.

TABLE III

CHROMATOGRAPHY OF GRANULOCYTE COLONY-STIMULATING FACTOR ON PHYTOHAEMAGGLUTININ-SEPHAROSE

Chromatography of granulocyte colony-stimulating factor (purified by affinity chromatography on concanavalin A-Sepharose and essentially free of erythropoietin) on phytohaemagglutinin-Sepharose. Relative distribution of activity in the column eluates as described in Table I.

	Concanavalin A-purified material (%)	Rechromatography of "A" (%)	Rechromatography of "C" (%)
Peak I	77 ("A")	87	6
Peak II	5	8	7
Peak III	18 ("C")	5	86

phytohaemagglutinin which had been coupled to porous glass. This column could be run at 3 times higher flow rates and the flow rates stayed high even after prolonged use. Its glycoprotein binding capacity was almost 10 times higher (1.02 mg/cm^3) than the capacity of the phytohaemagglutinin-Sepharose column. The amounts of ligand which were slowly released from the glass matrix did not interfere with the bioassay. Chromatography on phytohaemagglutinin coupled to porous glass was used to extract erythropoietin directly from crude urine from a severely anemic patient. The yield of erythropoietin obtained by this procedure was essentially the same as the yield of erythropoietin obtained by dialysis and ion exchange chromatography on DEAE-cellulose.

Chromatography of granulocyte colony-stimulating factor on insolubilized phytohaemagglutinin

Low amounts of granulocyte colony-stimulating factor were found in the Tris-NaCl, the galactose and the $MgCl_2$ eluates, a result confirmed quantitatively when higher amounts of a Con A-purified granulocyte colony-stimulating factor preparation were chromatographed on PHA-Sepharose (Table III, left panel). It was of obvious interest to know whether this apparent heterogeneity reflected a true heterogeneity in the granulocyte colony-stimulating factor or simply a low affinity of this factor for the phytohaemagglutinin-Sepharose. Results obtained by rechromatographing unretained and bound material separately (Table III, middel and right panel) suggested that the heterogeneity as reflected by its interaction with the column was the most important factor in determining its distribution on phytohaemagglutinin-Sepharose.

Discussion

The results described here indicate that chromatography on insolubilized phytohaemagglutinin is a useful method for the purification of human urinary erythropoietin. The degree of purification compared favourably with that obtained by conventional separation techniques (ref. 22 and Table II of this communication) and yields were essentially 100%. Insolubilized phytohaemagglutinin also seems to be able to extract erythropoietin from crude (undialyzed) urine quantitatively and in a form sufficiently free of toxicity to be

assayable in tissue culture. Since the method is also simple, fast, easily reproducible and inexpensive it should be applicable in a clinical assay for erythropoietin based on the in vitro colony assay. Further, chromatography on insolubilized phytohaemagglutinin gave a considerable increase in specific activity even when material was applied to the column which had already been purified by adsorption to benzoic acid, ion exchange chromatography on DEAE-cellulose, affinity chromatography on Con A-Sepharose, gel filtration on Sephadex G-100 and chromatography on calcium phosphate. The material with the highest specific activity contained phytohaemagglutinin as one if not the major contaminant. The use of matrices and coupling procedures which reduce or eliminate release of coupled ligand should therefore even further improve the efficiency of the method.

The observed binding of human urinary erythropoietin and a fraction of the granulocyte colony-stimulating factor to insolubilized phytohaemagglutinin did not give definitive evidence as to the mechanism of interaction between the hormones and the column, because the phytohaemagglutinin preparation used in this study was not a well defined entity and elution of adsorbed material was achieved non-specifically by high concentrations of $MgCl_2$. An interaction of the insolubilized lectin with the carbohydrate moiety of the glycoprotein hormones was likely, but ion exchange effects could not be excluded. Competition experiments with defined inhibitors should prove useful in clarifying this point and eventually also in distinguishing between the several slightly different oligosaccharides which are known to react with phytohaemagglutinin [23].

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References

1 Robinson, W.A., Stanley, E.R. and Metcalf, D. (1969) Blood 33, 396—399
2 Iscove, N.N., Sieber, F. and Winterhalter, K.H. (1974) J. Cell Physiol. 83, 309—320
3 Annable, L., Cotes, P.M. and Mussett, M.V. (1972) Bull. W.H.O. 47, 99—112
4 Dorado, M., Langton, A.A., Brandan, N.C. and Espada, J. (1972) Biochem. Med. 6, 238—245
5 Espada, J., Brandan, N.C. and Dorado, M. (1974) Biochim. Biophys. Acta 359, 369—378
6 Kawakita, M., Miyake, T. and Kishimoto, S. (1974) Abstract, International Symposium on Erythropoiesis, Tokyo, August 1974
7 Sieber, F., Iscove, N.N. and Winterhalter, K.H. (1973) Experientia 29, 758
8 Axén, R., Porath, J. and Ernback, S. (1967) Nature 214, 1302—1304
9 Robinson, P.J., Dunnill, P. and Lilly, M.D. (1971) Biochim. Biophys. Acta 242, 659—661
10 Espada, J. and Gutnisky, A. (1970) Biochem. Med. 3, 475—484
11 Iscove, N.N. and Sieber, F. (1975) Exp. Hemat. 3, 32—43
12 Sieber, F. (1976) Thesis Nr. 5678, Swiss Federal Institute of Technology, Zürich, Switzerland
13 Lowry, O.H., Rosebroughs, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Ehresmann, B., Imbault, P. and Weil, J.H. (1973) Anal. Biochem. 54, 454—463
15 Laemmli, U.K. (1970) Nature 227, 680—685
16 Burger, M.M. (1974) in Methods in Enzymology Vol. XXXII (Fleischer, S. and Parker, L., eds.), pp. 615—621, Academic Press, New York

17 Tesser, G.I., Fisch, H.-U. and Schwyzer, R. (1974) Helv. Chim. Acta 57, 1718—1730
18 Brennessel, B. and Goldstein, J. (1974) in Biology and Chemistry of Eukaryotic Cell Surfaces (Lee E.Y.C. and Smith, E.E., eds.), p. 364, Academic Press, New York
19 Dorado, M., Espada, J., Langton, A.A. and Brandan, N.C. (1974) Biochem. Med. 10, 1—7
20 Shelton, R.N., Ichiki, A.T. and Lange, R.D. (1975) Biochem. Med. 12, 45—54
21 Alexanian, R. (1966) Blood 28, 344—353
22 Espada, J. and Gutnisky, A. (1970) Acta Physiol. Latinoam. 20, 122—129
23 Kornfeld, R. and Kornfeld, S. (1970) J. Biol. Chem. 245, 2536—2545