Studies with ^{51}Cr-tagged red cells belonging to G6PD-deficient subjects, transfused into normal recipients receiving L-DOPA, Primaquine, DDS

Donor	Sex	G6PD U/min/g/Hb	Recipient	Sex	Drug	Dose (mg)	Days of administration	^{51}Cr T/2 before drug administration (days)	^{51}Cr T/2 after drug administration (days)
C.A.	♂	0.00	R.A.	♀	DOPA	50	7	20	20
						50	7		
			C.A.	♀	DOPA	75	6	21	21
			O.A.	♂	Primaquine	45	1	18	6
B.M.	♂	0.00	S.G.	♀	DOPA	100	9	20	20
			B.S.	♀	DOPA	100	9	18	18
			S.R.	♀	DDS	50	9	19	8

administer it to G6PD-deficient patients with neurological diseases. This may occur in areas with high incidence of the enzymatic defect of the red cells[4,5].

The transfusion of ^{51}Cr-labelled G6PD-deficient red cells into normal subjects who then receive primaquine or other oxidating substances constitutes a valuable and well-established method in order to test the haemolytic effect of drugs[6,7] on G6PD-deficient red cells. This method has been utilized in an attempt to study the possible haemolytic effects of L-DOPA on G6PD-deficient red cells.

^{51}Cr-tagged, G6PD-deficient red cells belonging to 2 Sardinian male patients, one of whom had a past history of favism, were transfused into 4 normal recipients with compatible blood groups. After the baseline assessment of the ^{51}Cr half time, the 4 subjects received, in a fasting state, daily intravenous infusions of physiological saline containing 50, 75, or 100 mg of L-DOPA (Hoffmann-La Roche[8]) for periods ranging from 7–13 days. The infusion rate was of 1.6, 2.5, 3.3 mg/min respectively. No changes in the slope of blood radioactivity could be observed after the administration of L-DOPA (Table). The ^{51}Cr-tagged red cells belonging to the same two G6PD-deficient donors, transfused into normal recipients who received thereafter primaquine or diaminodiphenylsulfone (DDS), were rapidly destroyed.

In the study outlined above, the in vivo use of L-DOPA in quantities recommended for the treatment of Parkinson's disease does not impair the normal survival of G6PD-deficient red cells. It should be noted, however, that L-DOPA might possibly undergo a different metabolic breakdown in subjects affected by favism[9].

Riassunto. Eritrociti G6PD deficienti, marcati con Cr 51, sono stati trasfusi in soggetti normali cui venivano somministrate giornalmente dosi di L-DOPA per via venosa. La L-DOPA viene usata per la terapia del morbo di Parkinson, e causa in vitro una perdita di GSH dagli eritrociti G6PD deficienti. Non si sono osservate modificazioni nella curve della radioattività ematica come conseguenza della somministrazione della L-DOPA. Si conclude pertanto che tale farmaco non ha effetti emolizzanti sugli eritrociti con carenza enzimatica di G6PD.

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Platelet Protein Synthesis Studied in a Cell-Free System

The ability of circulating blood platelets to synthesize proteins has been well established[1,2]. Since platelets do not contain DNA[3] it is to be expected that the presence in them of a functioning messenger RNA may be limited in time resulting in a progressive decrease of protein synthesis in aging platelets. This indeed could be shown by comparing the rate of protein synthesis in populations of platelets of varying age[4].

Thrombosthenin, a contractile, actomyosin-like protein is abundant in blood platelets[5,6]. It is also the protein that is most actively synthesized by the circulating platelets[7]. There exist differences of chemical and biophysical nature between thrombosthenin of platelets and actomyosin of other cellular origin[6]. Although its exact function still remains to be elucidated, the occurrence of a contractile protein in platelets may be of special significance for their hemostatic function.

Polysomes, the visible evidence for active protein synthesis in cells, have never been unequivocally demonstrated in platelets by electronmicroscopy while single ribosomes have. In the present investigation, these necessary templates for protein synthesis were indeed

shown to exist in these cells since they were isolated from human platelets and their activity was studied in a cell-free system. Furthermore, evidence is presented for the individual synthesis of actin- and myosin-like proteins by monocistronic messenger RNA's similar to their synthesis in skeletal muscle cells.

Platelets were isolated from 1500–5000 ml of fresh human blood collected with ACD anticoagulant. Red and white cell contamination was virtually eliminated by repeated differential centrifugations. Contamination by red cells was kept below 1 per 15,000 and for white cells, 1 per 45,000 platelets. Platelets were lysed with digitonin (0.1%)[6] and a microsomal fraction was prepared which had an optical absorbance ratio at the wave lengths 260/280 varying between 1.2 and 1.3. This ratio increased to 1.5–1.7 following treatment of the microsomal suspension with deoxycholate indicating that some of the ribosomes were membrane-bound in the platelets. The microsomal extract was then fractionated by sucrose gradient centrifugation. A characteristic distribution of peaks could be obtained (Figure 1). There were two classes of large, rapidly sedimenting particles later shown to be polysomes. The sedimentation coefficients of these two types of polyribosomes were about 650 S and 340 S respectively. Assuming a degree of messenger loading similar to that observed in hemoglobin synthesis[9] the larger class of polysomes, class A, was estimated to contain 50–60 ribosomes whereas the smaller one, class B, consisted of 15–20 ribosomes. There was a very large peak of 75 S ribosomes which agrees well with the electronmicroscopic finding of a relative abundance of such single ribosomes in platelets. The recovery of these two species of polysomes was significantly enhanced by extracting and separating platelet microsomes at high salt concentrations (0.25 *M* KCl). It has been shown by HEYWOOD et al.[10] that polysomes from embryonic skeletal muscle cells coprecipitate with myosin at low salt concentrations and this can be prevented by increasing the ionic strength of the extracting solution.

Microsomes isolated from platelets showed a small but significant incorporation of ^{14}C-amino acids into acid-precipitable material in a cell-free system (Table). Puromycin was an effective inhibitor of this incorporation. Various fractions of the microsomal suspension separated by centrifugation were tested for their ability to carry out protein synthesis in a cell-free system. Fractions A and B were the most active in incorporating ^{14}C-amino acids into acid-precipitable material (Table). These two fractions coincided with the identifiable peaks of polysomes isolated from blood platelets.

In an effort to determine the nature of the polypeptides synthesized by the polysomes of fractions A and B, the material released from the respective messenger RNA's was analyzed by acrylamide gel electrophoresis in 12 *M* urea at 45 °C[13]. Two distinct bands could be observed and were found to migrate identically to myosin and actin isolated from human skeletal muscle. These findings could be confirmed and extended by subjecting the newly synthesized polypeptides to column chromatography.

The product of the cell-free synthesis of the polysomes of fraction A was dialyzed against 5 m*M* *Tris*-maleate buffer, pH 7.5 containing 5 m*M* ATP. To this dialysate were added 10 mg native myosin, prepared similarly, and the mixture was then applied to a DEAE cellulose column and eluted according to the method of ASAI[14]. The peak of radioactivity coincided with that of the protein (determined by LOWRY's Method[15]) and thus suggested similarity of labelled and nonradioactive protein species. The polypeptides released by the polysomes of fraction B were dialyzed against 1 m*M* *Tris*-HCl buffer, pH 7.8 containing 0.2 m*M* ATP and 0.2 m*M* ascorbic acid. The dialysate was then applied to an acrylamide gel (P-100) column equilibrated with *Tris*-ATP-ascorbate buffer. The peak of radioactivity eluted with an apparent molecular weight of 58,000 which is close to the reported molecular weight of monomeric G-Actin. These results were highly suggestive but not conclusive

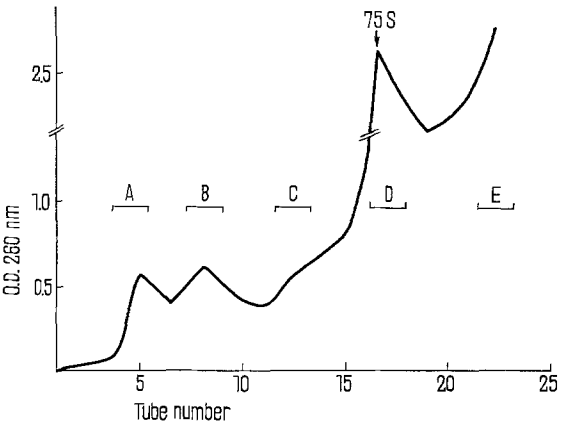

Fig. 1. Sucrose gradient analysis of platelet microsomes treated with sodium deoxycholate. 10 g of platelets (wet weight) were washed once with 0.85% NaCl and then suspended in 25 ml of cold buffer (0.25 *M* KCl, 0.01 *M* $MgCl_2$, 0.01 *M* *Tris*-HCl, pH 7.4). Digitonin was added to a final concentration of 0.1% and the suspension allowed to stand for 10 min in the cold. After complete platelet lysis, verified by phase microscopy, the suspension was spun at 15,000 × *g* for 20 min and to the resultant supernatant solution deoxycholate was added (final concentration 0.8%). This post-mitochondrial fraction was layered over a discontinuous sucrose gradient[8] and spun at 40,000 rpm for 3 h in a Spinco SW-40 rotor. The ribosomal pellets were rinsed and suspended in buffer and aliquots of 0.5 ml each of this solution were layered on a 14 ml, 15–40% linear sucrose gradient containing the buffer and centrifuged at 24,000 rpm for 120 min in a Spinco SW-40 rotor. Material was collected from 5 gradients and corresponding fractions A–E were combined and used for cell-free protein synthesis.

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evidence for the synthesis of myosin- and actin-like proteins by the polysomes isolated from platelets. It was necessary to utilize an alternative method to identify the products of cell-free synthesis. This was accomplished by adding small quantities of unlabeled native actin or myosin to the individual incubation mixtures of fractions A through D followed by reisolation of the proteins through a series of purification steps. Continual reisolation of the native protein at constant specific activity is considered to be an indication that a particular protein variety has been synthesized by the fraction of polysomes used in the original cell-free incubation. In the purification of myosin, fraction A showed the highest constant specific activity whereas fraction B, and to a lesser degree also C predominated in the purification of actin (Figure 2). These results indicate that the added unlabeled carrier protein has similar chemical properties as the newly synthesized protein of platelet polysomes. Although differences in the actomyosin-like protein isolated from muscle and blood platelets have been described[6] these were limited to the rates of hydrolysis of ATP and to slight solubility changes. Complete chemical identity of the synthesized proteins with those from skeletal muscle, however, cannot be implied from these experiments since differences in the chemical structure may exist that are too small to be detected by the purification procedures employed in this study.

A comparison of the size of the polysomes in relation to that of the polypeptide chains synthesized on them should give an indication whether these proteins are synthesized monocistronically or not. On the assumption that the polysomes isolated from platelets have similar loading characteristics of their messenger RNA's with ribosomes as those active in hemoglobin synthesis, the size of the polypeptide chains which such polysomes are capable of synthesizing could be expected to be 170,000

Characteristics of amino acid incorporation in cell-free system from human platelets

Incubation mixture	CPM
Complete System	525
— Ribosomes	41
— pH 5 Enzyme Fraction	92
— ATP and ATP Generating System	10
+ Puromycin ($1 \times 10^{-4} M$)	84
+ Ribonuclease (50 μg)	8
Ribosome Fraction	**CPM/0.4 O.D. (260 nm)**
A	1745
B	434
C	186
D	70
E	21

One ml of final incubation volume contained 0.15 *M* KCl – 0.005 *M* $MgCl_2$ – 0.01 *M* *Tris*-HCl, pH 7.4, 6 m*M* 2-mercaptoethanol or 3 m*M* dithiothreitol, 2 m*M* ATP, 0.5 m*M* GTP, 10 m*M* phosphoenolpyruvate, 50 μg pyruvate kinase, 0.2 mg soluble RNA from calf liver, 0.4 mg pH 5 fraction, prepared essentially after the procedure of HOAGLAND and ASCONAS[11] from post-microsomal supernatant of platelet lysate, 5 nmoles each of 20 amino acids (containing 0.5 μCi of a uniformly labeled L-^{14}C-amino acid mixture) and 0.35 mg ribosomes. The mixture was incubated at 37 °C for 60 min. Pancreatic ribonuclease, 50 μg was then added, the mixture cooled to 0 °C and the amino acid incorporation into acid-precipitable polypeptides determined[12]. Pooled ribosomes from fractions A–E obtained by scurose gradient analysis were used in a cell-free amino acid incorporating system similar to the one above.

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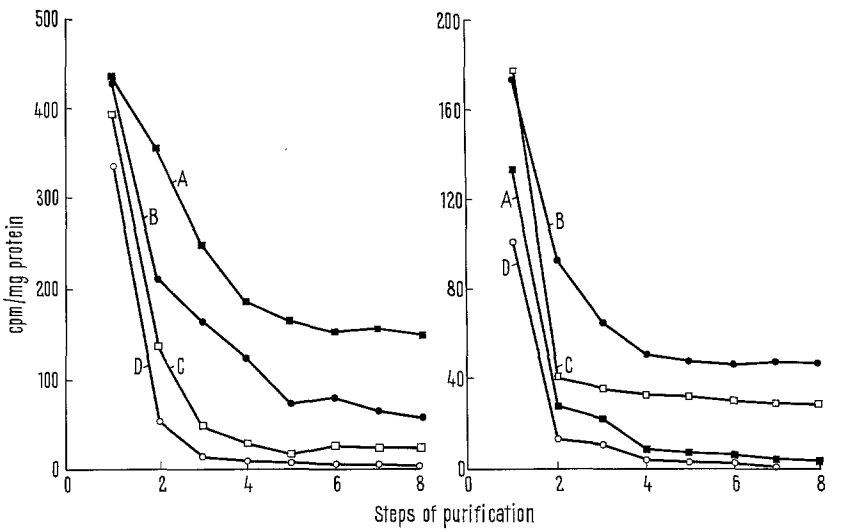

Fig. 2. Specific activity of proteins synthesized by various ribosomal fractions (A–D) in a cell-free system. Amino acid incorporating system and incubation were as described in legend to Figure 1. At the end of the incubation period pancreatic ribonuclease, 50 μg, was added to each flask and the mixtures cooled to 0 °C. 8 mg of native myosin and actin, prepared from human skeletal muscle[16,17] was added to each incubation flask and the proteins were subjected to a series of purification steps[18]. Specific activity was measured at each step.

to 200,000 for the heavier, and 50,000–70,000 for the lighter polysome fraction. A molecular weight of 200,000 has been estimated for the subunit of myosin by chemical techniques[19,20] and estimates for the molecular weight of actin[21,22] are in close agreement with the calculated polypeptide chain size that polysomes of fraction B should be capable of synthesizing. It is thus believed that both myosin and actin-like proteins of platelets are synthesized monocistronically[23].

Zusammenfassung. Zwei Klassen von Polyribosomen wurden von menschlichen Thrombozyten isoliert. Diese Polysome synthetisierten myosin- und actinähnliche Proteine in einem zellfreien System. Isolierung und Reinigung dieser Eiweisskörper zeigten deren Bildung in einer nativen Form an. Analyse der polysomalen Grösse indizierte die Synthese dieser Proteine an monocystronischen RNA-Molekülen.

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Prediction of Carcass Fat, Water and Lean Body Mass from Lee's 'Nutritive Ratio' in Rats with Hypothalamic Obesity[1]

Ventromedial hypothalamic lesions (VMNL) in mature rats result in hyperphagia and obesity[2]. Weanling rats with VMNL do not show hyperphagia and excessive weight gains[3–5] but have a higher carcass fat content than their controls[6,7].

It has been suggested that the obesity status of a rat may be assessed by dividing the cube root of the body weight by the naso-anal length. Lee[8] referred to this value as 'nutritive ratio' and considered values above 300 as indicating obesity. Several authors have used this expression to gauge hypothalamic obesity in rats with VMN lesions[9,10,4,5,11].

For the preliminary screening of weanling VMNL rats in our laboratories the above index, also referred to as 'Lee Index' by Szentagothai et al.[10], has been used extensively prior to histological verification of the hypothalamic lesions and before performing costly and time-consuming endocrine-metabolic analyses[12,7,13–16].

Analysis of our data accumulated during the last 5 years show that high correlations exist between the 'Lee Index' and carcass fat, carcass water and lean body mass in both weanling and mature rats with VMNL.

Weanling male and female rats received bilateral electrolytic VMN lesions sparing the median eminence. Sham-operated rats served as controls. The coordinates had been previously established[17]. The lesions were produced with a stereotaxic instrument using 0.25 mm stainless steel electrodes that were spar-varnish-coated and bared at the tip. An anodal current of 1.5 mA was allowed to flow for 10 sec. The animals were maintained under standard conditions and killed 2, 3, 4 and 7 weeks postoperatively. The brains were treated in a standard manner[18] and the lesions were localized using the atlas of De Groot[19]. Rats with asymmetrical lesions or with lesions exceeding beyond the ventral border of the hypothalamus were excluded from statistical analysis. Mature rats (229–251 g body weight) were treated similarly and killed 5 weeks after the operation. In the latter rats, histological verification of the hypothalamic lesions was not deemed necessary because mature rats fail to become obese when lesions are outside the VMN or are asymmetrical.

Carcass fat and water were determined according to a modification of a previously described method[20]. Lean body mass was computed from the above 2 parameters. Coefficients of correlation (r) and linear regression lines (y') were computed and are tabulated in the Table.

The Table shows that the 'Lee Index' correlates well with and allows a fairly accurate prediction of carcass fat, water and lean body mass in rats with hypothalamic obesity. Sham-operated controls, however, show poor and, except for carcass water, insignificant correlations.

The fact that significant correlations exist only in rats with hypothalamic lesions and not in controls is likely

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