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THE GLOBULIN OF CALF THYMUS NUCLEI AND THE *IN VITRO* INCORPORATION OF [^{14}C]ADENOSINETRIPHOSPHATE INTO GLOBULIN-RNA

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SUMMARY

Investigation of the globulin fraction of isolated thymus nuclei has shown that the globulin contains RNA and is a polydisperse ribonucleoprotein complex. The globulin fraction is able to incorporate [8-^{14}C]ATP into its RNA moiety. Partial fractionation of the globulin reveals that the fractions have different rates of [^{14}C]ATP incorporation.

INTRODUCTION

The ability of isolated cell nuclei to incorporate nucleic acid precursors *in vitro* has been demonstrated by a number of investigators in calf thymus, and in rabbit liver and appendix[1–4]. These precursors have been shown to be incorporated into the nucleic acids and are closely related to protein synthesis[1]. Recently a "pH 5 enzyme" has also been found in isolated nuclei from calf thymus and liver[5,6], and chicken kidney[6] which catalyzes pyrophosphate–ATP exchange in the presence of amino acids, thus being similar to the "pH 5 enzyme" prepared from cytoplasmic supernatant by ZAMECNIK and others[7,8]. These findings, therefore, substantiate the belief that RNA plays an important role in the metabolic processes of the cell nucleus. In the light of these studies, further examination of the relationship between nuclear RNA and nuclear protein fractions would appear to be of interest.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; AMP, adenosine monophosphate; CTP, cytosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; RNase, ribonuclease.

RNA has been found to be present in a number of protein fractions isolated from cell nuclei. ZBARSKII AND GEORGIEV reported that in isolated rat liver nuclei both globulin and lipoprotein contain RNA[9]; from thymus nuclei, ALLFREY AND MIRSKY obtained a pH 7.1-soluble fraction containing RNA which was metabolically active[10]; in addition, there is the "pH 5 enzyme" prepared by HOPKINS *et al.*[5,6]. Since lipoprotein can only be prepared from an alkaline extract of the nuclei[11], it seems that the globulin, which is prepared by mild procedure[12], would be best suited for investigation. This communication summarizes the results obtained from the studies of the nuclear globulin, demonstrating that the RNA moiety is an integral part of the globulin. It will also show that the globulin fraction incorporates ATP into its RNA moiety.

MATERIALS AND METHODS

Fresh calf thymus was obtained from a local packing house immediately after the animal was killed and nuclei were prepared according to ALLFREY[13]. Microscopic examinations showed that the preparations were quite clean, with very little non-nuclear contamination.

For the preparation of the globulin, the nuclei were extracted with 0.14 *M* NaCl which had been adjusted to pH 7.0. The extraction was carried out by first homogenizing the nuclei with 0.14 *M* NaCl in a Servall Omni-mixer for 15 sec in an ice–water bath, and then by stirring it for 1 h in the cold. After centrifuging the homogenate at 15,000 × *g* for 15 min, the supernatant was collected. The extraction procedure was repeated twice, the combined extract was subjected to 20,000 × *g* for 30 min, and the supernatant collected. Acidification of the opalescent solution to pH 5.0–5.2 with *N* acetic acid gave a precipitate which was then dissolved in 0.05 *M* Tris–0.14 *M* NaCl, pH 8.5.

Purification of the globulin was accomplished by repeating the isoelectric precipitation three times. For incubation studies, the precipitate was dissolved in Medium A according to HECHT *et al.*[8]. After three more times of reprecipitation, the preparation in Medium A was stored frozen, until use.

[8-^{14}C]ATP was obtained from Schwartz Laboratory. ATP, CTP, phosphoenolpyruvate and pyruvic kinase were purchased from Sigma Chemical Company.

RNA was prepared from the globulin by phenol extraction[14]. The nucleotide composition of RNA was analyzed by alkaline hydrolysis and paper electrophoresis as described by MARKHAM AND SMITH[15]. RNA content was measured by the orcinol method[16] and u.v. spectrophotometry; the two procedures gave results that checked to within 3 %. To test for DNA, the diphenylamine method[16] was used, in addition to the test for thymidylic acid from its nucleotide composition. Protein concentration was determined by Folin procedure[17].

Sedimentation patterns were taken with a Spinco Model E Ultracentrifuge.

The incubation conditions are given in the legends to the tables and figures. Reactions were stopped by chilling the incubating mixture in an ice–water bath, and an equal volume of cold 20 % TCA was added. The precipitate, after centrifugation, was washed 5–7 times with 7-ml portions of cold 10 % TCA, twice with cold 95 % ethanol, and twice with ether. The dried precipitate was taken up in NH_4OH and plated on stainless steel planchets for counting in a Tracerlab windowless gas flow counter.

RESULTS

Globulin as a ribonucleoprotein

The globulin preparations obtained from isolated calf thymus nuclei always showed an absorption spectrum characteristic of a nucleoprotein. Fig. 1 is such an absorption spectrum which shows maximal and minimal absorptions at 259 mμ and 243 mμ, respectively. The preparations were found to be diphenylamine negative and free of thymidylic acid, indicating the absence of DNA. Determinations of RNA and of protein gave an RNA content of about 10 %. Analyses of the RNA revealed only adenylic, cytidylic, guanylic and uridylic acids as its constituents (Table I). These results confirm that the nucleic acid present in the globulin is RNA. Furthermore, the RNA content was quite constant from preparation to preparation, and was not affected by further repeated isoelectric precipitation.

The RNA present in the globulin was attacked by pancreatic RNase. Digestion

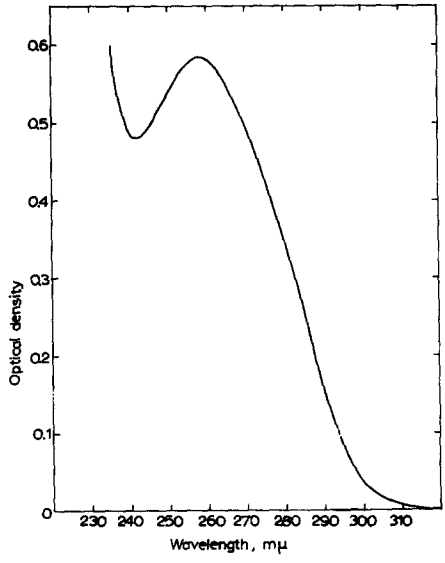

Fig. 1. U.V. absorption spectrum of nuclear globulin in saline-Tris buffer, pH 8.6.

TABLE I

COMPOSITION AND CONTENT OF RNA FRACTIONS OF CALF THYMUS NUCLEI

RNA fractions	*Molar nucleotide composition (Relative to adenylic acid as 10)*				*% of RNA*
	Adenylic	*Cytidylic*	*Guanylic*	*Uridylic*	
Globulin	10	18.3	15.6	12.2	10.8 ± 0.36(6)*
(Molar ratio)	17.8 ± 0.52	32.4 ± 0.98	27.8 ± 1.0	21.8 ± 0.43(5)*	
Whole nuclei**	10	14.2	16.2	8.5	
pH 7.1-soluble***	10	16.3	16.7	13.1	
"Nucleolar" ***	10	15.2	16.7	12.9	

* Figures in parentheses indicate number of analyses on different preparations.
** Taken from V. G. ALLFREY AND A. E. MIRSKY[9].
*** Taken from V. G. ALLFREY AND A. E. MIRSKY[10].

of the whole globulin with RNase in the cold (4°) released about 75 % of the RNA as dialyzable degradation products. The action of RNase also precipitated irreversibly approx. 66 % of the protein. It appears that the protein moiety of the globulin maintains its secondary structure by conjugation with the RNA. The destruction of the polynucleotide structure apparently caused the collapse of the protein configuration, strongly suggesting that most of the globulin is ribonucleoprotein in nature.

The globulin is complex not only in that it contains RNA, but there is also considerable molecular heterogeneity. Fig. 2a shows an ultracentrifuge diagram of a supernatant solution of the globulin after centrifuging at 40,000 × *g* for 30 min.

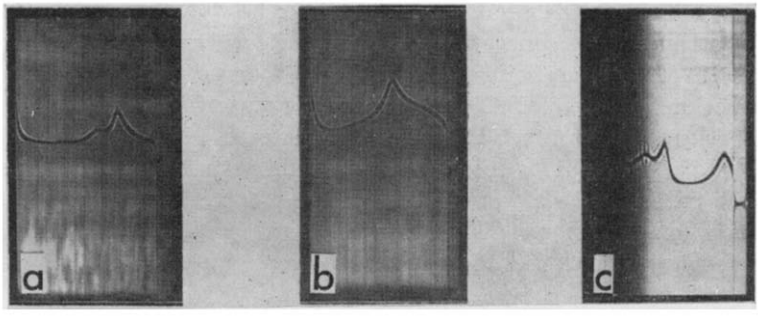

Fig. 2. Ultracentrifuge patterns of nuclear globulin in 0.14 *M* NaCl–0.05 *M* Tris, pH 8.5. (a) 40,000 × *g* supernatant of globulin solution, 96 min after attaining speed of 59,780 rev./min at 6.1°; bar angle 70°. (b) 40,000 × *g* supernatant of globulin solution, 170 min after attaining speed of 59,780 rev./min at 7.6°; bar angle 70°. (c) 40,000 × *g* pellet, 80 min after attaining speed of 24,630 rev./min at 6.9°; bar angle 65°.

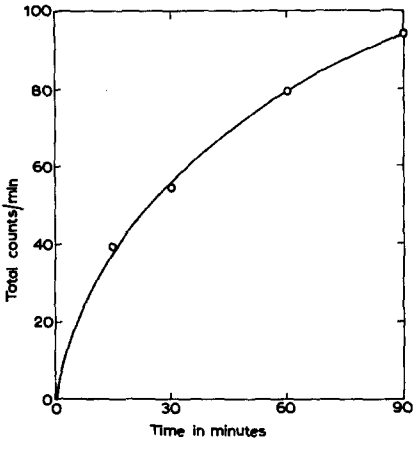

Fig. 3. Time course of incorporation of [8-^{14}C] ATP into globulin. The system contained: 6.5 mg of globulin; 2.5 μmoles [8-^{14}C]ATP ($3.4 \cdot 10^5$ counts/min/μmole); 25 μmoles phospho-enol-pyruvate; 75 μg pyruvic kinase; 2.5 μmoles CTP; 10 μmoles $MgCl_2$; 100 μmoles Tris, pH 7.5; and 750 μmoles sucrose; in a final volume of 2.5 ml. Aliquots of 0.5 ml were taken after incubation without shaking at 37° for the periods of time as indicated.

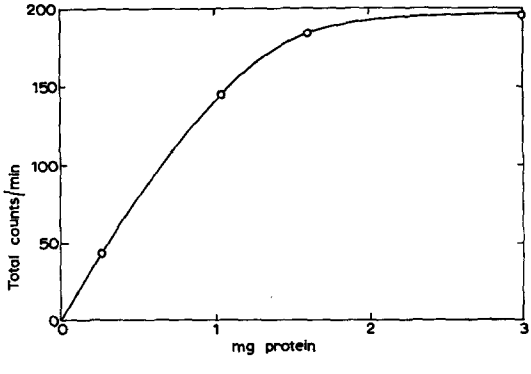

Fig. 4. Effect of protein concentration on the incorporation of [8-^{14}C]ATP into globulin. The system contained: 1 μmole [8-^{14}C]ATP ($3.4 \cdot 10^5$ counts/min); 10 μmoles phospho-enol-pyruvate; 30 μg pyruvic kinase; 1 μmole CTP; 4.0 μ moles $MgCl_2$; 40 μmoles Tris, pH 7.5; 250 μmoles sucrose; and amounts of globulin as indicated; in a final volume of 1 ml. The system was incubated without shaking for 90 min at 37°.

The slow component, after prolonged ultracentrifugation, shows further splitting as can be seen in Fig. 2b. The 40,000 × *g* pellet, when dissolved in saline-buffer, shows the rapidly sedimenting components (Fig. 2c). The use of 40,000 × *g* to clarify the solution was arbitrary; it simply makes the sedimentation pattern clearly visible. The effect of lack of transparency in the solution is apparent in Fig. 2c. It is obvious that the whole globulin fraction is a highly polydisperse system. However, the possibility cannot be ruled out that association–dissociation may have occurred during the course of preparation.

Incorporation of [8-^{14}C]ATP by globulin RNA

Figs. 3 and 4 show the uptake of [^{14}C]ATP by the globulin as a function of time of incubation and of the concentration of the protein. During the early incubating phase the rate of incorporation is rapid, and then decreases, but it does not level off. The relationship between [^{14}C]ATP uptake and concentration of the globulin is linear at low concentrations and thereafter reaches a plateau.

The uptake of [^{14}C]ATP does not require either the ATP-generating system or CTP. However, as can be seen from Table II, the presence of either or both enhances the incorporation. Omission of CTP reduces the uptake by 34 %, and the absence of an ATP-generating system causes the uptake to decrease by 40 %.

TABLE II

INCORPORATION OF [8-^{14}C]ATP INTO GLOBULIN RNA

The system contained: 3.2 mg of globulin; 1 μmole [8-^{14}C]ATP ($3.99 \cdot 10^5$ counts/min); 10 μmoles phospho-enol-pyruvate; 30 μg pyruvic kinase; 1 μmole CTP; 4.0 μmoles $MgCl_2$; 40 μmoles Tris, pH 7.5; and 250 μmoles sucrose; in a final volume of 1.0 ml. The system was incubated without shaking for 90 min at 37°.

	Total counts/min
Complete system	353
Complete system minus CTP	232
Complete system minus ATP-generating system	197
Complete system plus 8 μg RNA	330
Complete system plus 80 μg RNA	303
Complete system plus 160 μg RNA	289

The incorporated [^{14}C]ATP enters exclusively into the RNA moiety of the globulin. The radioactivity in RNA prepared from the globulin after incubating with [^{14}C]ATP accounted for all the counts taken up by the total system. Alkaline hydrolysis of the labelled RNA to its mononucleotides showed that only adenylic acid was radioactive. Under the conditions as described in the legend to Table II, the labelled adenylic acid represents 1.1 moles/1,000 moles of total AMP in the RNA.

It is apparent that the incorporation of [8-^{14}C]ATP is closely related to the RNA of the globulin. However, extra RNA, prepared from the globulin and added to the incubation system has no effect on the uptake of [^{14}C]ATP. This may also be a result of the close association of the RNA and protein portions of the globulin. Higher concentrations of added RNA even appear to have an inhibitory effect on the incorporation.

DISCUSSION

The nuclear globulin, prepared as described here, is not homogeneous but contains an almost continuous spectrum of molecular species. Centrifugation at 20,000 × *g* for 30 min was chosen only as an arbitrary standard to define the globulin fraction. It produces a preparation with a constant content of RNA. There also seems to be little doubt that the globulin contains RNA bound to protein. From the present study one cannot conclude, however, that all the molecular species in the globulin fraction are RNA-containing proteins. In fact, the RNA content must vary from one component to another, since it is found that the 40,000 × *g* pellet of the globulin has more RNA than the supernatant. It follows, therefore, that the nucleotide composition of the globulin RNA is merely an average of all the RNA components present in the globulin fraction. Nevertheless, it may be interesting to compare the composition of globulin RNA with that of the whole nuclei and other nuclear RNA fractions as listed in Table I. All the nuclear RNA fractions so far reported, including the globulin, have higher cytidylic and uridylic acid contents than the RNA of whole nuclei. The differences must be due to nuclear RNAs other than those from the nuclear fractions isolated so far. Thus, a complete enumeration of the RNA components present in nuclei must await further fractionation of the total RNA content.

It is clear from the above that the time course of [8-^{14}C]ATP incorporation must be considered as an average over the various components in the globulin fraction, since, after centrifuging the globulin at 40,000 × *g* for 30 min, the pellet has a relatively higher percentage of RNA than the supernatant. Furthermore, both fractions incorporate [^{14}C]ATP (Fig. 5). The pellet material incorporates [^{14}C]ATP for a longer time than the supernatant material. The pellet, thus, not only contains more RNA in proportion to protein, but, also is responsible, at least, for the major features of the time course curve shown in Fig. 3. Fig. 5 also demonstrates a qualitative difference between the supernatant and the pellet, since, in this experiment, the con-

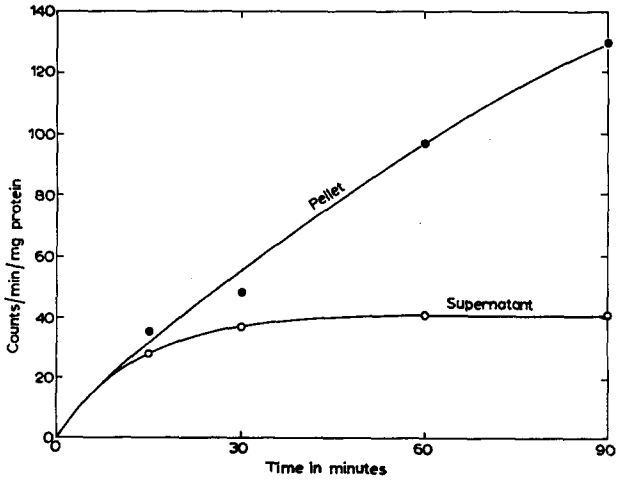

Fig. 5. Conditions of the system were the same as described in Fig. 3 except that the pellet material contained 8.7 mg protein and the supernatant contained 16.7 mg protein; both pellet and supernatant contained approx. 1.4 mg RNA.

centration of RNA was maintained about equal in both fractions. Although the supernatant contained more protein than the pellet, the specific activity of the pellet is much higher than that of the supernatant.

The complexity of the process of [^{14}C]ATP uptake can also be seen from the enhanced incorporation in the presence of a CTP or ATP-generating system (Table II). HECHT *et al.*[8] have shown that AMP can be added proximally to the RNA chain provided the terminal CTP has an unsubstituted 3′ (or adjacent 2′)-hydroxyl group. The heterogeneity of the globulin fraction may indicate that some RNA chains have such a terminus, while other RNA chains may accept the sequential addition of CTP followed by ATP. On the other hand, the incorporation of ATP could also mean two different mechanisms, one of which is CTP-dependent.

It is not known whether the globulin equates or constitutes part of the "pH 5 enzyme" found by HOPKINS *et al.* from isolated thymus nuclei. Should this be the case, the globulin, with its RNA, may, like the "pH 5 enzyme", play a role in nuclear protein synthesis. More information is needed to evaluate the biochemical role of the globulin in isolated nuclei.

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