Lability of Rat Liver Polyribosomes and Cell Sap Factors in an *in Vitro* Amino Acid Incorporation System (37413)

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(Introduced by H. E. Sauberlich)
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It was previously reported that *in vitro* incorporation of amino acids into trichloroacetic acid-precipitable protein by a cell-free system (polyribosomes plus cell sap enzymes) from rat liver was linear for only 2 min (1). This suggested that some component of the system was labile. Further evidence for this lability was the finding that fewer amino acid residues were incorporated when polyribosomes and/or cell sap enzymes had been frozen or permitted to stand in ice for several hours before testing for incorporating activity. The requirement for sulfhydryl-protective reagents in the extraction medium (1) suggested the presence of one or more labile sulfhydryl groups in the system. These observations prompted experiments designed to learn more about the lability of this system.

Materials and Methods. Male Holtzman rats (190–210 g) were fasted overnight to deplete liver glycogen stores; the livers were pooled, minced with a Harvard[3] tissue press and 1 part liver was homogenized with 2 parts (w/v) of cold (4°) buffer for the preparation of PMS[4] as described earlier (1). Cell sap was isolated in a medium ($SHKM_1$) containing 250 m*M* sucrose, 50 m*M* HEPES buffer, 50 m*M* KCl, 3.75 m*M* $MgCl_2$, 3 m*M* glutathione and 25 m*M* KOH used to adjust final pH to 7.6 at 37°. An aliquot was adjusted to pH 6.4 for use in cell sap isolation while the remainder was adjusted to pH 7.3 for use in Sephadex G-10 filtration of cell sap. The medium used to isolate polyribosomes ($SHKM_2$) was identical to $SHKM_1$ except that 5 m*M* $MgCl_2$ was used and final pH was 7.6.

Cell sap PMS and deoxycholate-treated PMS for polyribosome preparation were then centrifuged over discontinuous sucrose gradients at 321,000*g* for 1 hr 45 min (1). The former was filtered through Sephadex G-10 columns to remove cold amino acids unless stated otherwise while polyribosomes were suspended in $SHKM_2$ buffer minus sucrose. Protein analyses were performed so that cell sap and polyribosomes could be added to the incubation system in a 45:1 ratio (1).

In vitro incorporation was assayed in a 1 ml volume employing 0.2 μCi each of L-[$^{14}C_6$] leucine, L-[$^{14}C_6$] lysine, L-[$^{14}C_6$] phenylalanine, L-[$^{14}C_5$] valine and a buffer described in detail elsewhere (1). The incubations were initiated by addition of polyribosomes, carried out for 2 min (37°) in a shaking water bath and terminated with cold trichloroacetic acid. The precipitate was washed, solubilized and counted in a scintillation counter (1).

The values in Tables I–IV represent an average of duplicate and, in some cases, triplicate observations on incorporation rates. Each value represents only ^{14}C-amino acids incorporated into TCA-precipitable protein. Control incubations were performed in each case with polyribosomes left out and the dpm (range 200–300) were subtracted from average values obtained with the complete incorporation system. The values reported for incorporation rates in this report had a

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[4] Abbr: PMS = postmitochondrial supernatant; PCMB = *p*-chloromercuribenzoate; HEPES = N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid, Calbiochem, Los Angeles, CA.

±5% standard error.

Results and Discussion. To ascertain which was more labile, polyribosomes and cell sap enzymes were preincubated either separately or together at 37° for various lengths of time prior to their use in the *in vitro* incorporation system (Table I). Polyribosomes lost 10% of their ability to incorporate amino acids after 32 min of preincubation at 37°, but incorporation was reduced by 70% when cell sap enzymes were preincubated alone for 32 min. Mixing polyribosomes and cell sap enzymes together in the absence of an energy generating system and preincubating for 32 min prior to assay led to a 90% loss of incorporating activity. It was reported that polyribosomal profiles are altered following their incubation in the presence of unfractionated cell sap, but not when pH 5 enzymes or purified transfer factors I and II are used (2) indicating that some factor responsible for polyribosomal breakdown was lost in the fractionation processes. Our results also demonstrate a greater lability of polyribosomes incubated in the presence of cell sap, but cell sap enzymes are even more labile and the loss in activity is accentuated when both components are preincubated together prior to their use in the incorporation system.

TABLE I. The Influence of Preincubating Polyribosomes and Cell Sap Enzymes Separately or Together on ^{14}C-AA Incorporation into Polyribosomal[a] Protein.

Pre-incubation[c] period (min)	Preincubated component(s)[b]		
	Cell sap	Poly-ribosomes	Cell sap + poly-ribosomes
None	63,350	64,000	60,620
2	60,960	66,450	54,420
4	55,670	63,080	49,510
8	42,180	59,670	33,460
16	29,350	60,590	21,340
32	17,890	57,280	6260

[a] Fresh polyribosomes (no preincubation) were used with preincubated cell sap and vice versa. Data in the last column comes from first combining the two components, preincubating for the prescribed time period, then adding the incubation buffer. Livers from 5 animals were pooled and material was isolated for this experiment.

[b] Data expressed in dpm/2 min incubation/mg polyribosomal protein.

[c] Cell sap and polyribosome suspensions were warmed from 0 to 37° within 1 min. Zero time incubations were run with suspensions immediately after they reached 37°. Aliquots were drawn at the above specified times for use in the complete incubation system.

Several investigators have reported the use of dialyzed cell sap preparations or enzyme fractions in their incorporation systems (3–6). Since this is time consuming and may lead to depressed enzyme activity over extended periods normally used for dialysis, a comparison was made between cell sap samples prepared by dialysis and gel filtration. A cell sap preparation was divided into four aliquots; one was dialyzed only, two were filtered through Sephadex G-10 and the fourth was untreated. One of the G-10-treated aliquots was treated further with dialysis to see if this would improve the already highly active gel-filtrated material. The two aliquots not undergoing dialysis were stored at 3° for 22 hr. A direct comparison of the four aliquots was made the following day when fresh polyribosomes and cell sap were again prepared. Half of the fresh cell sap was untreated and half was passed through a column of Sephadex G-10 since its superiority for gel filtration purposes was previously demonstrated (1). All six cell sap samples were assayed with the fresh polyribosomes in the *in vitro* incorporation system (Table II). The data in Table II indicate that gel-filtrated fresh cell sap was more active than all other cell sap preparations. *In vitro* incorporation was 50% lower when cell sap enzymes had been permitted to stand in ice or were dialyzed for 22 hr, a finding similar to earlier work (2) on fractionated cell sap. Since the major function of dialysis or Sephadex gel filtration is to remove endogenous amino acids, it is advantageous to use the latter since it can be accomplished in less than 1 hr (column diameter, 1.5 cm; gel bed height, 13 cm). The superiority of unfractionated cell sap as opposed to dialyzed preparations cannot be disputed (Table II) and was reported earlier (6). However, a higher incorporation rate for nontreated cell sap stored 22 hr

TABLE II. The Influence of Dialysis, Gel Filtration and Storage at 3° on the Activity of Cell Sap Enzyme Preparations.[a]

Treatment of cell sap	dpm/2 min incubation/mg polyribosomal protein
Dialysis[b] for 22 hr at 3°	27,740
Sephadex G-10 gel filtration; stored at 3° for 22 hr	29,460
Sephadex G-10 gel filtration; dialyzed for 22 hr at 3°	22,630
Nontreated cell sap stored at 3° for 22 hr	34,720
Nontreated cell sap; freshly prepared	41,460
Sephadex G-10 gel filtration; freshly prepared	59,770

[a] Freshly prepared polyribosomes were employed with all 6 cell sap preparations. Livers from 4 rats were pooled to isolate material used in the first 4 samples, and 4 different animals were used the following day to obtain material for the last 2 samples.

[b] Dialysis tubing was boiled in water prior to use. Approximately 2-3 ml of cell sap was dialyzed in 3 changes (500 ml each) $SHKM_1$ buffer.

compared to dialyzed cell sap or cell sap after gel filtration and stored at 3° over the same time period cannot be explained. It can only be suggested that some factor which lends stability to the cell sap was removed during gel filtration and dialysis.

The effect of freezing a cell sap preparation after gel filtration is shown in Table III (Expt A). Over 20% of the activity was lost during the 24-hr freezing period (—70°), whereas cell sap stored for 24 hr in ice lost 65% of its activity. When polyribosomes were frozen overnight (—70°) and assayed the following day with fresh cell sap enzymes, incorporation was decreased approximately 10% (Expt B). In Expt C, PMS was frozen for 24 hr, thawed the following day, and used for the preparation of polyribosomes and cell sap enzymes. The activity of polyribosomes was not affected appreciably, but incorporation was reduced 45% when cell sap prepared from the frozen PMS was used with fresh polyribosomes. Hence, cell sap can be best preserved when taken through the ultracentrifugation step and frozen rather than storing crude PMS at —70° for later use. However, the loss of polyribosome activity was similar in Expt B and C indicating that crude PMS may be stored at —70° for later polyribosomal isolation. These experiments also emphasize the importance of using freshly prepared components.

Although some investigators have reported the use of frozen polyribosomes and/or cell sap preparations (5, 7–10), it seems likely that other investigators have employed preparations allowed to stand several hours in the cold. This is likely because of the long periods of centrifugation required in older

TABLE III. The Influence of Freezing on *in Vitro* Amino Acid Incorporation by Polyribosomes and Cell Sap.[a]

	dpm/2 min incubation/mg polyribosomal protein
Expt A[b]	
Cell sap was:	
a. frozen 24 hr	93,680
b. on ice 24 hr	41,640
c. fresh	118,020
Expt B[c]	
Polyribosomes frozen 24 hr	56,650
Fresh polyribosomes	64,000
Expt C	
Prepared from PMS frozen 24 hr:[d]	
Polyribosomes and cell sap	37,504
Polyribosomes only	66,868
Cell sap only	40,074
Polyribosomes and cell sap from fresh PMS	72,045

[a] Freezing was effected by placing sample tubes in a solid CO_2–ethanol bath. Samples were stored at —70°. Material used for frozen samples was isolated from a pooled liver sample of 5 rats while fresh material was isolated from a second pooled liver sample (5 rats) the next day.

[b] Fresh polyribosomes were incubated with each cell sap preparation.

[c] Fresh cell sap was used in the incubation system.

[d] Polyribosomes were assayed using fresh cell sap; cell sap was assayed using fresh polyribosomes.

model ultracentrifuges. More recently it is possible to obtain forces exceeding 400,000*g* which allows a considerable reduction in preparation time. As previously described (1) the polyribosomes and cell sap preparations employed in our experiments were centrifuged 1.75 hr at 321,000*g*. Thus, is was possible to homogenize fresh liver, sediment polyribosomes with ultracentrifugation, resuspend the polyribosomes, prepare cell sap (centrifugation and gel filtration), determine protein contents of polyribosome and cell sap preparations, dilute both preparations to the desired protein content, and complete the *in vitro* incorporation of amino acids within 6–7 hr following decapitation of the rats. Cell sap preparation through the gel filtration step can be completed in 5–6 hr after decapitation. We prefer using it as soon after the completion of this step as possible; however, it appears relatively stable with no appreciable loss in activity noted following 1–2 hr of storage on ice. From the data presented, the importance of completing the incorporation as rapidly as possible can be seen.

As previously reported (1), sulfhydryl-protective reagents (3 m*M* glutathione) added to the extraction medium led to enhanced cell sap enzyme activity. Amino acid incorporation was almost completely inhibited when 1 m*M* *p*-chloromercuribenzoate was added to the assay medium (Table IV).

TABLE IV. The Influence of *p*-Chloromercuribenzoate (PCMB) on *in Vitro* Amino Acid Incorporation by Polyribosomes and Cell Sap.[a]

PCMB concn (m*M*)[b]	dpm/2 min incubation/mg polyribosomal protein
0	69,310
0.05	71,480
0.10	69,920
0.50	41,660
1.00	285
5.00	0

[a] Livers from 5 rats were pooled and used to isolate cell sap enzymes and polyribosomes.

[b] The desired concentrations of PCMB were obtained by adding an aliquot directly to the assay media prior to additions of cell sap and polyribosomes.

The concentration of glutathione in the reaction media was 0.09 m*M* requiring approximately equimolar concentrations of PCMB before the effect on cell sap enzyme activity was manifested via decreased ^{14}C-amino acid incorporation rates. This suggests that free sulfhydryl groups are involved in the incorporation of amino acids into protein and that anything leading to their destruction may contribute to the lability of cell sap factors.

The involvement of sulfhydryl groups in protein synthesis was reported earlier when Sutter and Moldave (11) showed that transferase II is a sulfhydryl-dependent enzyme. Others (12) showed that it was a sensitive site on reticulocyte ribosomes, and not a soluble component, which was adversely affected by sulfhydryl inhibitor-reagents. McAllister and Schweet (13) reported that such inhibitors did not seem to impair the ability of reticulocyte ribosomes to bind polyuridylic acid, but the binding of transfer RNA to the ribosomes was inhibited. The site of inhibition was shown to be the ribosomal protein fraction and not the RNA of the ribosome.

Apparently some factor was missing from the *in vitro* system which was required for resynthesizing polyribosomes once the ribosomes were released from the messenger RNA. Baliga, Pronczuk and Munro (4) reported a similar breakdown of polyribosomes during incorporation. They concluded that the system finally ceases to incorporate in the presence of an adequate supply of amino acids due to the lack of chain-initiating factor required for the interaction of free ribosomes with messenger RNA. They further concluded that amino acid supply is one factor regulating attachment of free ribosomes to messenger RNA and that, to obtain polyribosomes, conditions compatible with peptide synthesis must be provided. Since an adequate supply of L-amino acids was provided in our *in vitro* system, the former explanation seems more plausible than the latter.

Summary. Cell sap enzymes were more susceptible to heat destruction (37°) than polyribosomes whereas the latter appear relatively stable for periods up to 32 min. However,

preincubating the two components together led to a more rapid disappearance of incorporating activity than did heating the components separately. Dialysis for 22 hr decreased cell sap enzyme activity 50% compared to fresh cell sap. Gel-filtrated cell sap lost a similar amount of activity when stored (3°) for 22 hr. Cell sap loses 20–25% of its activity within 24 hr during cold storage (—70°). However, polyribosomes are less susceptible losing approximately 10% of their activity. Freezing PMS (—70°) and isolating cell sap and polyribosomes 24 hr later led to a reduction in activity of 45 and 8%, respectively. PCMB lowered amino acid incorporation in the reaction media at concentrations above that of free sulfhydryl supplements.

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