IMPORTANCE OF CENTRIFUGATION TIME AND HOMOGENATE CONCENTRATION FOR ISOLATING RAT LIVER FREE POLYSOMES: EFFECT OF FASTING

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1. Introduction

Recently a clear picture of the biosynthetic functions of free and membrane-bound polysomes has emerged. According to observations [1, 2], which soon received independent support [3–7], the two classes of polysomes from rat liver synthesise different, non-overlapping families of proteins. This is disputed in one laboratory [8]. Studies in this area are often severely hindered by the low yields of free polysomes obtained with the use of discontinuous sucrose gradients. The present report shows that the yield of free polysomes from rat liver is strongly influenced by the nutritional status of the animal, the duration of centrifugation, and the concentration of post-mitochondrial supernatant (PMSN). Conditions are defined for obtaining a complete yield of free polysomes from fasted rats by centrifugation for 20–21 hr. With unfasted rats reliable conditions could not be found. Possible reasons for this are discussed.

2. Methods

Male hooded rats of 150–200 g were either fed *ad libitum* (on "Small Laboratory Animal Diet, autoclaved"; Spillers Ltd.), or fasted for 16–20 hr before killing by cervical dislocation, usually in the middle of the day. Some animals were injected intraperitoneally with 2 μC of [6-^{14}C]-orotic acid 24 hr beforehand. Livers were homogenized in 0.25 M sucrose in "medium A" (0.05 M tris-HCl, pH 7.6, 0.025 M KCL, 0.005 M $MgCl_2$) [2]. Free polysomes were isolated by a method modified from that of Bloemendal et al. [9], by centrifuging 7.5 ml of PMSN (10,000 $\times$ $g_{av.}$ for 10 min) through a discontiniuous gradient of 4 ml of 1.5 M sucrose over 6 ml of 2 M sucrose medium A, in the 8 $\times$ 25 rotor of the M.S.E. Superspeed 50 Ultracentrifuge, at 40,000 revs/min (105,000 $\times$ $g_{av.}$), for various lengths of time. The pellet of free polysomes was resuspended for assay after carefully wiping away membranes or soluble proteins adhering to the walls of the tube. Temperature was maintained at 0–5° throughout. RNA was determined by the method of Fleck and Begg [10] and DNA by a modification of the method of Burton [11]. Radioactivity was measured in a liquid scintillation counter with internal standards.

3. Results and discussion

Two centrifugation times were compared, 4–4½ hr and 20–21 hr. The shorter time has been used by Webb et al. [12], Bloemendal et al. [9] and Tata [13]. The longer time should result in sedimentation of all the free polysomes, according to Blobel and Potter [14] and Loeb et al. [15]; both groups used fasted rats.

Table 1 gives comparative values for the yields of

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Table 1
Yields of free polysomes from liver postmitochondrial supernatant (PMSN) from unfasted and fasted rats after different times of centrifugation; effect of PMSN concentration on yield.

	Expt.	% homogenate from which PMSN is derived	Unfasted rats		Fasted rats	
Time of centrifugation (hr)			4 – 4.5	20 – 21	4 – 4.5	20 – 21
Polysome yield, μg RNA per g weight of liver	1–4	25	60.8 ± 16	1310 ± 320	486 ± 107	1846 ± 396
	5a	17	191	1200		
	5b	4.3	445			
	5c	1.7	1180			
Polysome yield, μg RNA per mg DNA	1–4	25	28.7 ± 8.5	604 ± 148	147 ± 38	559 ± 124
Yield as percentage of appropriate unfasted or fasted 20–21 hr value	1–4	25	4.8 ± 1.5	100	28.4 ± 10.1	100
	5a	17	16	100		
	5b	4.3	37			
	5c	1.7	98			

Average values are expressed ± one standard deviation.

PMSN was centrifuged for 4–4.5 hr or for 20–21 hr and the pellets were analysed for RNA, in experiments 1, 2, 4 and 5 chemically and in experiment 3 by counting radioactivity after 24 hr labelling with [^{14}C]-orotic acid. Counts were converted to μg RNA by determining the specific activity of the 21-hr pellet RNA.

free polysomes at these two times from unfasted and fasted rats, with various concentrations of PMSN. The PMSN used in most experiments is 25% PMSN derived from a 25% homogenate. After centrifugation for 20–21 hr the yield of free polysomes from fasted rats is greater than that from unfasted rats when expressed as μg RNA per g liver; however, this difference largely disappears when the results are expressed per mg DNA, since this method of expression represents the yield of free polysomes per average cell. On the other hand the yield after 4–4.5 hr centrifugation is much lower and there is a striking difference between fasted and unfasted rats: the average yield of free polysomes from fasted rats is 28% of the 20–21 hr value, whereas that from unfasted rats is only 5%. This low yield was obtained repeatedly with PMSN derived from 25% homogenates regardless of whether it was estimated by determining RNA chemically [10] or by counting radioactivity in samples of resuspended pellets labelled with orotic acid without acid precipitation or washing. The latter method excludes underestimation of polysomes due to interference by glycogen with the precipitation of RNA during its determination. Substitution of 0.5 M for 1.5 M sucrose in the upper sucrose layer has virtually no effect upon the 4 hr [16] or 20 hr yields. However if the concentration of sucrose in the lower layer is reduced to less than 1.8 M there is a large increase in contamination of the free polysomes by membranes, probably due to penetration of the lower layer by bound polysomes [17]. The opposite difficulty is encountered with bound polysomes, which after 4 hr centrifugation on 1.5–2.0 M sucrose are still contaminated by 10–30% of the free polysomes.

When they plotted yield of free polysomes from fasted rats against time of centrifugation, Blobel and Potter [14] obtained a curve reaching a plateau at 10 hr. Under our conditions with fasted rats, recovery of free polysomes requires more than 10 hr centrifugation, although it is complete in 20–21 hr (fig. 1). However, with unfasted animals even centrifugation in excess of 20 hr sometimes does not cause complete sedimentation of free polysomes (fig. 1). With such prolonged periods of centrifugation consideration must be given to the possibility of contamination of

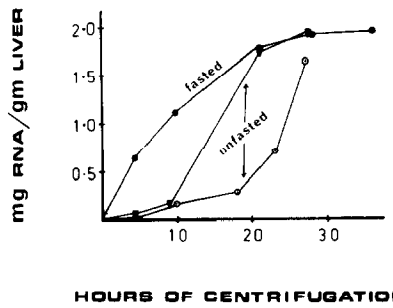

Fig. 1. Sedimentation of free polysomes from postmitochondrial supernatant of unfasted and fasted rats as a function of time of centrifugation. Centrifugation was through a discontinuous 1.5 M/2 M sucrose gradient for various lengths of time, and the pellet analysed chemically for RNA. An experiment illustrating the pattern for fasted rats is denoted —•—. Patterns obtained for unfasted rats are denoted —○— and —△—, the latter pattern being more characteristic.

the free polysomes by bound polysomes released in some time-dependent fashion from the reticulum. However there is virtually no increase in yield of free polysomes from fasted rats from 21 to 36 hr, and the radiochemical evidence also argues against release. Thus bound polysomes are a site of active incorporation of radioactive glucosamine into glycoproteins; free polysomes, when completely purified from membranous contaminants, are inactive in this respect [1, 2]. This inactivity for glucosamine incorporation is exhibited equally by free polysomes sedimented in 4 hr or 24 hr [2], indicating that there is no significant detachment of bound polysomes into the free polysome fraction during 24 hr centrifugation.

With unfasted animals the unsedimented particles might represent particulate fragments arising from polysome breakdown, or else especially large polysomes reversibly entangled with the membrane. In some experiments we have in fact obtained evidence that the unfasted animals give bigger polysomes. Moreover, one would expect tangling to be less with lower concentrations of PMSN, and the results in table 1 for unfasted rats indeed show that with 1.7% PMSN – a concentration admittedly ill-suited to preparative work – the sedimentation of free polysomes is virtually complete in 4 hr.

It is recommended that free polysomes be prepared from fasted animals by overnight centrifugation. Where unfasted animals are used the dangers of incomplete sedimentation should be recognized.

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