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Short Communication

A Simple Fractionation Procedure for Studying Incorporation of Radioactively Labelled Precursors into Mammalian Main Cell Components *

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For studying cell metabolism in-vivo and in-vitro, especially in embryonic tissue, we have been interested in developing a method that allows to follow the incorporation of radioactively labelled precursors into several acid-insoluble main cell components.

Methods used for such studies are mostly based on the fractionation procedures developed by Schmidt and Tannhauser (1945) or Ogur and Rosen (1950). But if cell homogenates from mammalian species are used, the Ogur and Rosen method suffers from the disadvantage of an incomplete extraction of RNA. Depending on the tissue studied 30—50 % of the RNA—mostly ribosomal RNA—is found in the "DNA-fraction". This difficulty can be avoided when the RNA is extracted with alkali according to the Schmidt and Tannhauser procedure. But although with this modification the RNA is quantitatively extracted within the alkali fraction, a separation of RNA from all the other cell components is not achieved and the fraction may be highly contaminated with sulfurated glycosaminoglycans.

While developing a biochemical "screening test" for studying the effect of embryotoxic substances in mammalian species, we tried to get an overall picture of cell metabolism using "unspecific" precursors and following the incorporation into a variety of acid-insoluble cell components. For this purpose a procedure was necessary that allowed the separation of rather pure fractions containing lipids, RNA, glycosamino-

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glycans, other high-molecular carbohydrates, DNA and proteins. In this paper we describe a procedure suitable for the separation of these fractions with a reasonable degree of purity that will be sufficient for many screening tests. Most data have so far been obtained using embryonic tissue in different stages of development, but many other mammalian tissues may be used as well.

Method

About 10—250 mg tissue are homogenized directly in ice cold 2% perchloric acid (PCA) or the tissue may be homogenized in a sucrose medium or any other suitable medium followed by precipitation of the acid-insoluble components with PCA. After centrifugation the sediment is washed several times with 2% PCA; this is followed by an extraction of the sediment with 5 ml of ethanol-ether (3 + 1) three times ("lipid-fraction") and once with ether.

The dry powder is suspended in 2 ml 0.1 M Tris-HCl, pH 7.5 containing 200 μg crystalline RNase (Boehringer Mannheim, heated to 80° C for 10 min) and the suspension is frozen and thawed three times using liquid nitrogen or a mixture of aceton and dry ice for freezing and ice water for thawing. This treatment may be performed within 45 min and results in a disruption of the majority of ribosomes. Subsequently the suspension is incubated at 0° C for 15 min followed by the addition of 2 ml 20% PCA and centrifugation. The supernatant is called "RNA-fraction".

The sediment is dissolved in 3 ml 0.3 N NaOH and incubated at 37° C for 1 h. After cooling to 0° C in ice-water and acidification with 1 ml ice-cooled 20% PCA and centrifugation the supernatant contains the sulfurated glycosaminoglycans. After the alkaline hydrolysis the solution is acidified. This has to be done very quickly and by thouroughly mixing. In some cases we couldn't get a fine dispensed sediment but a tough combined product which was very difficult to be handled. This can be overcome by dissolving the product a second time in NaOH and precipitating with PCA by blowing the acid out of the pipette. The precipitation then should be sedimented by reduced speed in the centrifuge (3000 rpm in a Christ Junior KS).

The sediment is resuspended in 0.1 N Na-acetate buffer pH 4.9 (the final pH should be 4.6) and 1 mg Amylo-α-1,4-1,6-glucosidase (Boehringer Mannheim) is added, followed by an incubation at 25° C for 30 min. After acidification with 1 ml of 20% PCA and centrifugation the supernatant is called "carbohydrate-fraction", most of the acid-precipitable glycogen is found in this fraction.

The sediment is washed with 5 ml 2% PCA, centrifuged and the sediment resuspended in 2 ml 5% PCA. The suspension is incubated for 15 min at 75° C. If high amounts of DNA are expected the sediment is hydrolized a second time with 1 ml 5% PCA. After centrifugation the precipitate is taken up in 0.5 ml 2.5 N NaOH. This residue represents the "protein-fraction".

Results and Discussion

32 samples may easily be handled in one experiment by one person and the whole extraction procedure for 32 samples needs about 20 h.

If the washing procedure is carefully performed the results of different samples containing the same homogenate do not differ by more than ± 5% from each other. After each fractionation step the procedure may be interrupted if the sediment is kept cool (0° C).

The procedure was checked by measuring the RNA and DNA content in different fractions and by studying the distribution of some rather specific precursors among the different fractions. The results of such studies are given in the Table. It may be seen that DNA is localized to about 90% within the "DNA-fraction". This is confirmed by experiments in which ^{3}H-thymidine was used. 5—6% of the labelled compound is present in the lipid-fraction and about 6% in the glycosaminoglycan fraction. The relative high amount in the lipid fraction may be due to non-incorporated thymine or thymidine which is extracted by the organic solvents. The 6% in the alkaline fraction may be derived from non-precipitated DNA.

Table. *Distribution of radioactivity on different fractions of acid-insoluble material of rat embryos after labelling with "specific" precursors*

The radioactively labelled substances were dissolved in saline and applied intravenously

Precursors	% Distribution					$\Sigma = 100\%$ in dpm/μg DNA	n
	Lipid	RNA	sulf. glyc. glyc.	DNA	Protein		
^{3}H-Thymidine	5	<1	6	88 ± 3	1	20.1	4
^{3}H-Uridine	6.6	71 ± 5		16 ± 3	7	84.5	3
^{14}C-Orotic acid[a]	<1	96 ± 2	3	<1	<1	50.3	4
^{3}H-Phenylalanine	7	2	9	5	77	58.7	5
$^{35}SO_4^{--}$	<1	<1	86 ± 2	7	5	10.3	4

[a] Liver tissue was used since orotic acid is only very poorly incorporated into embryonic tissues.

n = number of experimental sets. Mean ± standard deviation.

With more than 90% the recovery of RNA within the "RNA-fraction" was satisfactory, but some 5% of the RNA were found within the alkaline fraction using liver tissue for this experiment and orotic acid as a precursor. When ^{3}H-uridine (6) was used as RNA precursor in embryonic tissue, some 70% of the acid-insoluble radioactivity was found in the RNA-fraction and 16% in the DNA fraction after 3 h incorporation time. But as the isotope ^{3}H is not as specific as ^{14}C for such studies and as uridine is metabolized to DNA precursors, the real percentage of RNA may be higher in this fraction. Nevertheless, in our hands this distribution is satisfactory unless RNA is very highly labelled compared with glycosaminoglycans. In this case the values for sulfurated, protein-bound glycosaminoglycans are not reliable.

Acid-insoluble, sulfurated glycosaminoglycans are almost exclusively (86 %) found within the "mucopolysaccharide-fraction" as judged from incorporation studies with ^{35}S-sulfate.

^{14}C-phenylalanine is to 77 % recovered within the "protein-fraction". For these experiments we have used uniformly labelled phenylalanine. Since we could not exclude that some part of the alanine might have been metabolized and then incorporated into the other fractions, we have repeated these experiments with ^{3}H-ring-labelled phenylalanine. But we got the same proportions in the different fractions. It, therefore, may be concluded that the "lipid-fraction" contains some protein chains attached to the lipid components. It is, furthermore, interesting that some radioactivity (9 %) is found together with glycosaminoglycans. It is known that glycosaminoglycan-proteincomplexes are split by alkali and the results could indicate that a small portion of the polypeptides remains attached to the glycosaminoglycans and that some of the polypeptides split from the complex are not acid-precipitable.

The results of these studies indicate a fairly good separation of the main cellular components which seems to be sufficient for many incorporation studies. But we would like to stress the point that the method is found not to be applicable, if the specific activity of one fraction exceeds that of an other adjacent fraction by one order of magnitude or more. In such a case—as it was found to be with ^{3}H-labelled alkylating agents in proteins versus DNA—reliable results can only be obtained after a phenol-extraction of the DNA.

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