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Studies in Rapidly Labelled Ribonucleic Acid in Cell Fractions and in Tumour Tissues

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1. Twenty minutes after injection of [^{3}H]orotic acid into rats the rapidly labelled RNA from the liver is mainly associated with the nuclear fraction and little with the ribosomal cytoplasmic fraction. 2. The thermal denaturation of RNA from the fractions was not as reversible as that of the RNA extracted from whole liver. 3. Rapidly labelled RNA is synthesized by cells from a transplantable hepatoma when incubated in the presence of [^{3}H]uridine and, after extraction and centrifugation, the label is present in three main fractions: one which sediments to the bottom of a gradient and is associated with DNA, a second which sediments to the heavy side of the 28s RNA, and a third which has a peak of activity between 28s RNA and 18s RNA and is associated with DNA. 4. After labelling and extraction of the RNA from Ehrlich ascites cells the distribution of radioactive components is similar to that of the material from the hepatoma cells. 5. The difference between the tumour cells and liver is due to some extent to the method of homogenizing the tissues and the nature of the components is discussed.

Rapidly labelled RNA is present in small amounts and is usually isolated in association with DNA or ribosomal RNA (e.g. Hiatt, 1962; Scherrer & Darnell, 1962; Monier, Naono, Hayes, Hayes & Gros, 1962), although some separation has been achieved by Georgiev & Mantieva (1962).

Rapidly labelled RNA was isolated with DNA in some earlier studies and, by using counter-current distribution, differences were found in the rapidly labelled RNA after injection of hormones (Kidson & Kirby, 1964*a*) or feeding with a carcinogen in the diet (Kidson & Kirby, 1965). A disadvantage of the association of rapidly labelled RNA with DNA is the possibility of formation of complexes, particularly after heating or degradation; moreover, hybrids of this RNA with DNA are more easily studied if it is first isolated with the ribosomal RNA.

An extraction technique that enabled the rapidly labelled RNA to be isolated with the ribosomal RNA and freed from DNA has yielded very stable RNA (Kirby, 1965). We have examined the distribution of this material in cellular fractions from liver and the action of actinomycin on its biosynthesis as already reported by Revel & Hiatt (1964). Our results are basically in agreement with theirs, but some differences in the distribution, on centrifugation, of rapidly labelled RNA from some tumour cells compared with that from liver have been found.

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MATERIALS

[^{3}H]Orotic acid (5000mc/m-mole), [^{3}H]uridine (500–5000mc/m-mole), [^{3}H]thymidine (500–5000mc/m-mole) and [^{3}H]leucine (5000mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Ribonuclease was obtained from Armour and Co., Eastbourne, Sussex, and deoxyribonuclease (twice crystallized) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

The hepatoma was obtained by transplanting a liver tumour, induced by feeding rats with 4-dimethylamino-4′-fluoroazobenzene (O'Sullivan & Kirby, 1964).

Rats of both sexes of the Wistar strain were used, generally about 3 months old (150g.).

The tissue culture fluid was prepared as described by Fischer (1958).

Actinomycin D was a gift from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A.

METHODS

Sucrose-density-gradient centrifuging. Sucrose density gradients were linear and were analysed after centrifugation by puncturing the bottom of the tube and collecting about 30 fractions. Water (1ml.) was added to each fraction and the extinction read at 260mμ. Extraction of radioactive material from the gradients was carried out by adding to

each tube 1 ml. of a mixture of butan-1-ol (100 ml.), 2,2′-diethyldihexylamine (11·7 ml.) and acetic acid (2 ml.) (Kidson, Kirby & Ralph, 1963). After shaking and allowing the phases to separate 0·5 ml. of top phase was removed and mixed with 10 ml. of scintillation fluid for determination of the radioactivity.

Isolation of nuclei. Twenty minutes after injection of [^{3}H]orotic acid (200 μc/rat) the rats were anaesthetized, the liver was perfused through the portal vein with a solution (2°) of 0·25 M-sucrose, 2 mM-$CaCl_2$, 1 mM-magnesium acetate, 50 mM-tris–HCl buffer, pH 7·6 (10 ml.). The livers were removed, dropped into 50 ml. (about 2 vol.) of the medium used for perfusion and homogenized for 25 sec. in a blender (only the lower two blades were covered). Operations were carried out at 2° and the mixture was filtered through eight layers of surgical gauze; an equal volume of 0·75 M-sucrose containing the same salts and buffer as above was added with gentle stirring and this was poured through a funnel containing a small pad of cotton wool. The pad was finally squeezed gently and the suspension was centrifuged at 1000 rev./min./10 min./5°. The supernatant fraction was discarded, the pellet resuspended in the same buffer–salt solution (without sucrose) and centrifuged at 250 rev./min./5 min./5°. The supernatant fraction was withdrawn and the pellet containing predominantly whole nuclei, as judged by microscopic examination after staining with aceto-orcein, was used for the extraction of nuclear m-RNA/RNA.

Isolation of microsomes and ribosomes. Rats were starved for 18 hr., killed by breaking their necks, the liver was removed and dropped into a cold solution of 0·44 M-sucrose, 50 mM-tris–HCl, pH 7·6, 60 mM-KCl, 5 mM-NaCl, 0·1 mM-magnesium acetate (2 vol. of this was just sufficient to cover the lower two blades of the MSE Ato-Mix blender) and homogenized at half speed for 20 sec. The mixture was centrifuged at 12000g/10 min./5° and the supernatant fraction carefully removed. Portions (8 ml.) were each layered over 2 ml. of the same medium as originally used, but with 1·0 M-sucrose, and these were centrifuged in the angle head of Spinco model L centrifuge at 105000g/3 hr./5°. The supernatant fractions were discarded, and the pellets rinsed with the original medium and used for the preparation of RNA. When ribosomes or RNP* particles (cf. Campbell, Cooper & Hicks, 1964) were required the supernatant fraction after the first centrifugation was mixed with 1/9 vol. of 5% (w/v) sodium deoxycholate in 50 mM-tris–HCl buffer, pH 7·6, and stirred for 10 min. The mixture was layered over 2 ml. of the buffer–salt solution containing 1·0 M-sucrose and then centrifuged at 105000g/3 hr./5°. The pellets were carefully washed free from contaminating material (probably lipoprotein) and the tubes rinsed several times. The pellet was used for preparation of the RNA.

Isolation of RNA. The method was essentially that described by Kirby (1965), method II: nucleic acids were extracted with 4-aminosalicylate and phenol–cresol mixture and DNA, glycogen and s-RNA were removed from the precipitated material by extraction with 3 M-sodium acetate (pH 6·0). RNA was prepared from the nuclear pellet by mixing with an equal volume of 12% (w/v) sodium 4-aminosalicylate. The final volume should be twice that of the weight of the original liver used.

When method I (Kirby, 1965) was used to prepare RNA from nuclei, very little radioactivity was found associated with the RNA.

When RNA was prepared from microsomes or ribosomes a volume of cold 6% (w/v) sodium 4-aminosalicylate equal to twice the weight of the original liver was used.

A preparation of RNA was also made from the cytoplasmic material after sedimenting the nuclei. Solid 4-aminosalicylate was added to the supernatant fraction before extraction with the phenol–cresol mixture. The yield of RNA from nuclei was about 1 mg./g. of liver (8×10^3–10×10^3 counts/min./mg.); from microsomes it was about 2 mg./g. of liver.

RNA was prepared from hepatomas after freezing the excised tissue in liquid N_2 and from a cell suspension and from Ehrlich ascites cells after centrifuging and washing the cells.

The effects of nucleases were studied by incubating the RNA (1 mg.) with deoxyribonuclease (100 μg.) in 0·1 M-sodium acetate (1 ml.) or ribonuclease (100 μg.) for 2 hr. at 25° before layering on the sucrose gradient.

The action of actinomycin D was studied by incubating cells (37°) for 7 min. in the presence of [^{3}H]uridine (100 μc), and subsequently for 3 min. in the presence of actinomycin D (200 μg.), after which the cells were cooled (5°), centrifuged and the RNA was extracted.

The effect of non-radioactive uridine was studied by incubating cells (37°) with [^{3}H]uridine (100 μc) for 2 min. and subsequently with uridine (2 mg.) for 8 min. Cells were cooled (5°), centrifuged off and the RNA was extracted.

A cell suspension of two hepatomas was made by cutting up the tissue with scissors (10–12 days after transplantation) and dropping into cold medium (Fischer, 1958). The cells were separated by pressing through a tissue sieve (wire gauze, 4 cm. diam.) in 10 vol. of medium at 2° and were separated by centrifuging at about 500g and washing in the same medium (twice). The loose pellet was dispersed in 4 vol. of cold medium and then incubated in the presence of [^{3}H]uridine (100 μc)/37°/10 min. The cells were then centrifuged off, quickly frozen (liquid N_2), weighed to determine the volume of 4-aminosalicylate solution and phenol for the extraction, which was carried out as before. [^{3}H]Thymidine labelling was effected by injecting the animals with 3×200 μc of [^{3}H]thymidine intraperitoneally 72, 48 and 20 hr. before removal of the tumour and preparation of the cell suspension. Although very little labelling of the cytoplasmic fraction was apparent after the rats had been injected with 200 μc of [^{3}H]orotic acid, some label could be detected after injecting 600–800 μc of [^{3}H]orotic acid.

Ehrlich ascites cells were labelled by injecting each mouse intraperitoneally with [^{3}H]uridine (200 μc) 10 min. before death or 2×200 μc of [^{3}H]thymidine 20 and 12 hr. before death. The cells were removed, centrifuged and washed and the nucleic acids extracted as before.

RESULTS

Rat liver. The base compositions of the RNA samples (determined as described by Kirby, 1956) that remained insoluble in 3 M-sodium acetate after extracting the liver, the nuclear material and microsomal fraction are shown in Table 1.

The extinction and radioactive labelling patterns

* Abbreviation: RNP, ribonucleoprotein.

Table 1. *Base compositions of RNA extracted from rat liver nuclei and from cytoplasmic ribosomes by methods I and II (Kirby, 1965)*

Ratios are expressed as moles/100 total moles.

Method of extraction	Source of material	Guanine	Adenine	Cytosine	Uracil
1	Liver	32·7	19·7	31·1	16·5
1	Nuclei	32·8	19·9	30·4	16·9
1	Cytoplasm	33·6	19·1	30·2	17·1
2	Nuclei	34·0	19·7	29·8	16·5
2	Cytoplasm	34·0	19·0	30·9	16·1

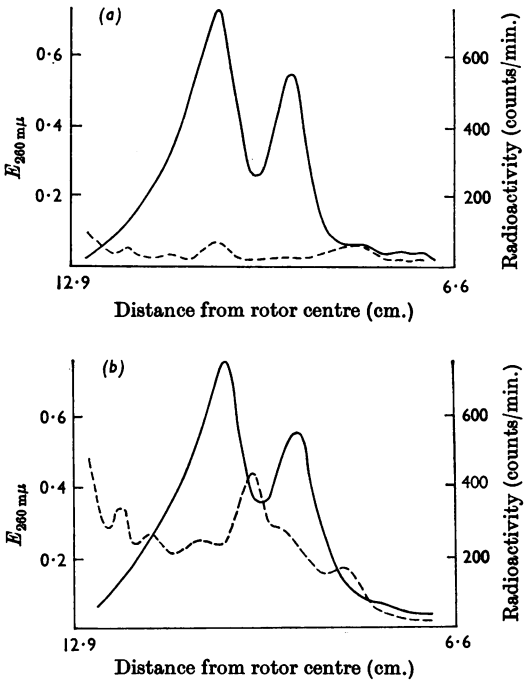

Fig. 1. Centrifugation pattern in sucrose-density gradient (5–20% sucrose in 10mM-sodium acetate, pH5·2) of rat liver ribosomal RNA. Centrifugation was at 24000 rev./min. for 16hr. in the Spinco model L centrifuge (SW25.1 rotor). ——, $E_{260\,m\mu}$; – – – –, radioactivity. (*a*) RNA from cytoplasmic fraction; (*b*) RNA from nuclear fraction.

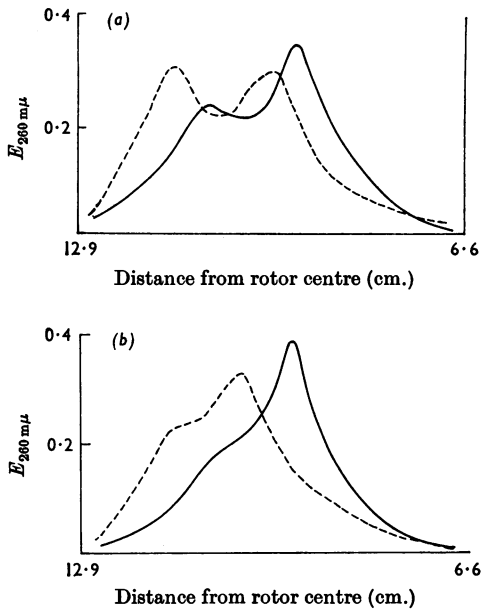

Fig. 2. Centrifugation patterns in sucrose-density gradients (5–20% sucrose) of rat liver RNA. All curves are extinction readings at 260mμ. ——, RNA heated at 70° for 10min. with gradient made up in 10mM-sodium acetate, pH5·2; – – – –, RNA heated at 70° for 10min. with gradient made up in 200mM-sodium acetate, pH5·2. Centrifugation was as in Fig. 1. (*a*) RNA from cytoplasmic fraction; (*b*) RNA from nuclear fraction.

of the RNA from the two cell fractions after centrifugation in a sucrose density gradient are shown in Fig. 1 (*a* and *b*).

The radioactivity in the nuclear fraction was absent when the rats had received 1hr. previously an injection of 150μg. of actinomycin D. The effect of heat on the two fractions in 0·01M-sodium acetate and recentrifuging in 0·2M-sodium acetate is shown in Fig. 2 (*a* and *b*). RNA from whole liver can be denatured and almost completely renatured after heating at 70°/10min., cooling and then centrifuging in 0·2M-sodium acetate (Kirby, 1965).

Hepatoma tissue. Whole tissue: the incorporation of [^{3}H]uridine into the RNA of hepatomas was very low. The RNA gave a typical two-peak pattern (extinction) on centrifugation in a sucrose-density gradient and a high proportion of the radioactivity incorporated had sedimented near the bottom of the gradient.

A good incorporation was achieved with a cell suspension isolated from the tumour and incubated

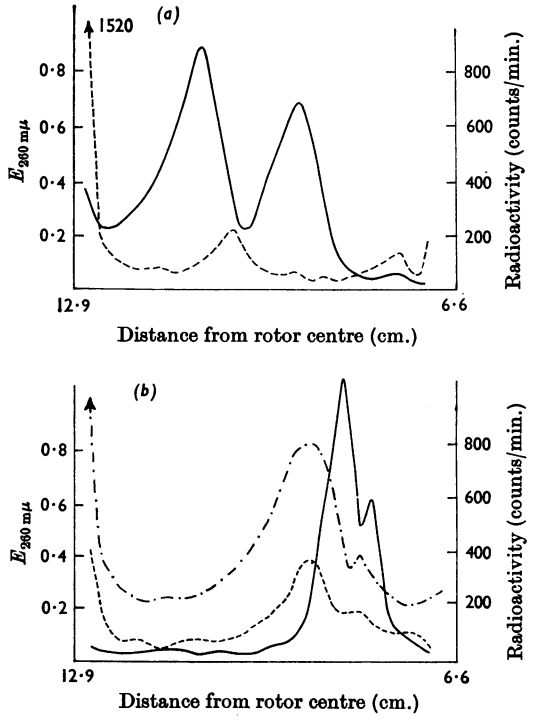

Fig. 3. Centrifugation patterns in sucrose-density gradients (5–20% sucrose in 10mM-sodium acetate, pH5·2) of RNA from hepatoma cells labelled with [^{3}H]uridine. ——, Extinction at 260mμ. (*a*) Centrifugation was at 24000 rev./min. for 16hr. in the Spinco model L centrifuge (SW25.1 rotor). – – – –, Radioactivity. (*b*) Centrifugation was at 15000 rev./min. for 16hr. in the Spinco model L centrifuge (SW25.1 rotor). –·–·–, Radioactivity; – – – –, superimposed pattern of radioactivity after incubating cells for 2min. with [^{3}H]uridine and for 8min. with uridine.

for 10min. in the presence of [^{3}H]uridine. The extinction and radioactive patterns after centrifugation at 24000 and 15000 rev./min. sucrose-density gradients are shown in Fig. 3 (*a* and *b*).

The ratio of the radioactivity associated with the material which sedimented towards the bottom of the gradient compared with that sedimenting faster than the 28s RNA varied somewhat from one batch of cells to another, but the same distribution of radioactive material was found every time. The same pattern of labelling was observed when cell suspensions from two transplantable sarcomas were incubated with [^{3}H]uridine and the RNA was extracted and centrifuged on a sucrose-density gradient.

The presence of DNA in the mixture was shown by isolation of the RNA after injection of [^{3}H]-thymidine, and the pattern of labelling is shown in Fig. 4.

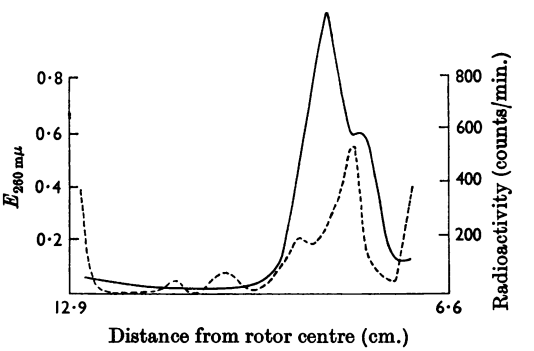

Fig. 4. Centrifugation in sucrose-density gradient (5–20% sucrose in 10mM-sodium acetate, pH5·2) of RNA from hepatoma cells labelled with [^{3}H]thymidine. Centrifugation was at 15000 rev./min. for 15hr. in the Spinco model L centrifuge (SW25.1 rotor). ——, $E_{260\,m\mu}$; – – – –, radioactivity.

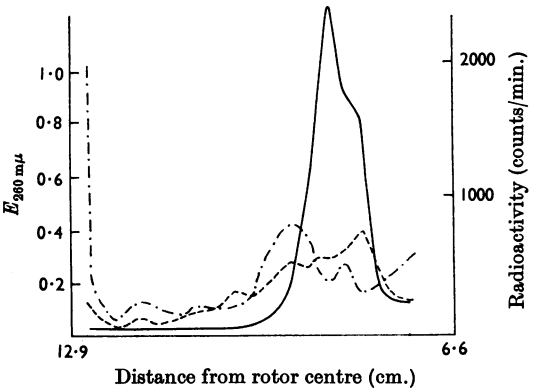

Fig. 5. Centrifugation in sucrose-density gradient (5–20% sucrose in 10mM-sodium acetate, pH5·2) of RNA from hepatoma cells labelled with [^{3}H]uridine in the absence and presence of actinomycin D. Centrifugation was at 15000 rev./min. for 15hr. in the Spinco model L centrifuge (SW25.1 rotor). ——, $E_{260\,m\mu}$; –·–·–, radioactivity in the absence of actinomycin; – – – –, radioactivity after labelling and addition of actinomycin.

All the material which had incorporated [^{3}H]-thymidine was degraded by deoxyribonuclease to material <4s and most of the material incorporating [^{3}H]uridine was degraded by ribonuclease to material <4s. However, with the last enzyme about 5% of the radioactivity sedimented to the bottom of the gradient.

The results of experiments designed to show any protein contamination were inconclusive, as, despite repeated injection of [^{3}H]leucine, on centrifuging and extracting the radioactive material, the

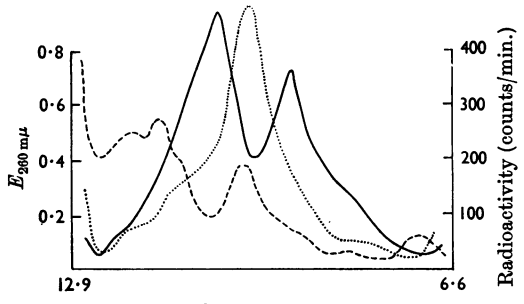

Fig. 6. Centrifugation in sucrose-density gradient (5–20% sucrose in 10mM-sodium acetate, pH5·2) of RNA from Ehrlich ascites cells labelled with [^{3}H]uridine and [^{3}H]-thymidine *in vivo*. Centrifugation was at 24000 rev./min. for 15hr. in the Spinco model L centrifuge (SW25.1 rotor). ——, $E_{260\,m\mu}$; – – – –, [^{3}H]uridine activity; ····, [^{3}H]-thymidine radioactivity (performed on parallel gradient).

only significant radioactivity above the background was in the fraction at the bottom of the gradient.

The results of experiments designed to show the effects of actinomycin D and addition of non-radioactive uridine to the cell suspension after incorporation of [^{3}H]uridine are shown in Figs. 5 and 3*b*.

Ehrlich ascites cells. The extinction and radioactive patterns obtained after labelling Ehrlich ascites cells with [^{3}H]uridine and [^{3}H]thymidine and extraction of the nucleic acids are shown in Fig. 6.

DISCUSSION

The method described for the preparation of nuclei does not give pure nuclei, but is very rapid so there is minimum chance of degradation of the RNA. Comparison of Figs. 1(*a*) and 1(*b*) shows that no rapidly labelled RNA has escaped into the cytoplasmic fraction.

The RNA samples from nuclei and cytoplasm can be prepared by application of the 4-amino-salicylate and phenol–cresol method, and in both cases RNA with two characteristic peaks (of about 28s and 18s) on centrifugation were obtained. Although the thermal stability of the RNA was good, in that it was not fragmented into sub-units of 4–6s, the reversibility of the patterns on re-centrifuging in 0·2M-sodium acetate was not as good as that of RNA extracted directly from whole liver, although the material from the microsomes was somewhat better than that from the nuclei. The base composition of the two fractions was similar (Table 1), although RNA prepared by method 2 had a slightly higher guanine content. It is possible there may have been some slight denaturation during the isolation. The identity or non-identity of ribosomal RNA from nuclei and from cytoplasm remains to be demonstrated clearly. The presence of the rapidly labelled RNA in the nuclear fraction and the action of actinomycin are in accord with the findings of Revel & Hiatt (1964).

Differences were found in the countercurrent distribution patterns of rapidly labelled RNA, isolated with the DNA, from livers and from transplantable hepatomas (of spontaneous origin) of mice which had been injected with [^{3}H]uridine (Kidson & Kirby, 1964*b*). We have used a transplantable rat hepatoma (induced by feeding with 4-dimethylamino-4′-fluoroazobenzene; O'Sullivan & Kirby, 1964) and found that the RNA could not be labelled by [^{3}H]orotic acid and only minimally with [^{3}H]uridine. However, if the tumours were removed, the cells separated from connective tissue (a particularly easy procedure) and incubated in tissue culture medium (Fischer, 1958), [^{3}H]uridine could be incorporated quickly and efficiently into the RNA. On centrifugation at 24000 rev./min. in sucrose-density gradients most of the labelled RNA sedimented faster than the 28s RNA and a better differentiation of the labelled material was found by centrifuging at 15000 rev./min. At this speed the separation of the two ribosomal components is not so good, but the rapidly labelled RNA sediments in three main fractions: one near the bottom of the gradient, one somewhat faster than the 28s RNA and a third, smaller fraction, in between the 28s and 18s RNA samples. It seemed possible that the heaviest material may be a DNA–RNA complex and labelling with [^{3}H]thymidine indicated that DNA was present in this heaviest fraction, but it was surprising to find a peak of activity coincident with the [^{3}H]uridine activity in between the 28s and 18s RNA. Isolation of the material after injection of [^{3}H]leucine showed only minimal labelling in the heaviest fraction. This was in accord with greater deproteinizing ability of phenol–cresol mixture compared with phenol. It seems likely then that this heaviest material is DNA of high molecular weight, associated with rapidly labelled RNA. We have evidence in the preparation of bacterial DNA without using a blender and with the phenol–cresol mixture that a fraction of high molecular weight can be obtained which swells but does not dissolve easily in 3M-sodium acetate. This DNA remains with the ribosomal RNA and the rapidly labelled RNA is probably trapped in the gel particles which are easily sedimented.

The fraction which sediments faster than the 28s RNA is probably a mixture but is likely to be similar to the rapidly labelled RNA of Scherrer &

Darnell (1962) and the RNA of Steele, Okamura & Busch (1964) of 35–45s and which Scherrer & Darnell (1962) believed contained precursors of ribosomal RNA. We have found (Hastings, Parish, Kirby & Klucis, 1965) this type of material in RNA from rat liver and from *Escherichia coli*, and it has been shown to contain the two ribosomal RNA samples associated with the rapidly labelled RNA.

It is possible that the uridine and thymidine labelling in the fraction appearing between the 28s and 18s RNA is coincidental, but it seems reasonable to assume that it contains segments of the heaviest material that have been broken by shearing during the preparation.

The action of actinomycin was to reduce almost completely the incorporation of [^{3}H]uridine into the heaviest fraction, and there was some evidence of degradation of the RNA (Fig. 5), although the method of extraction would ensure that the smaller segments were removed. Incubation of the hepatoma cells with [^{3}H]uridine followed by incubation with non-radioactive uridine led to a change in ratio of incorporation of [^{3}H]uridine into the heaviest fraction compared with that in the peak of the 35–45s fraction: from 1·8 to 1·3. This is an indication that the [^{3}H]uridine label is first associated with the DNA and later with the two ribosomal RNA samples. It remains to be determined whether this association with the ribosomal RNA samples occurs in the cell or is a result of the method of preparation.

An identical distribution of rapidly labelled components was found when cells from a transplantable sarcoma were labelled in a similar manner and, to confirm that this labelling pattern was not due to cell damage during the isolation, Ehrlich ascites cells were labelled with [^{3}H]uridine and [^{3}H]thymidine by intraperitoneal injection. After extraction of the RNA and centrifugation the radioactive patterns were very similar to those found by incubation of tumour cells *in vitro*, and again two peaks of [^{3}H]thymidine labelling were apparent although the relative amounts of these were different from those of the hepatoma cells (Figs. 4 and 6).

To some extent the difference in labelling patterns of these cells compared with rat liver may be due to fragmentation of DNA of the latter by action of the blender. DNAs with sedimentation constants < 20s are much more easily soluble in 3 M-sodium acetate than DNA of higher molecular weight. However, it should be noted that some label was incorporated into the heaviest material separated from hepatomas which had been disintegrated by the blender. Further work to clarify the relationship of the various components is in progress.

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