Evidence for Two Metabolically Distinct Types of Ribonucleic Acid in Chromatin and Nucleoli*

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ABSTRACT

Patterns of radioisotope incorporation are useful characteristics in describing cellular RNA fractions, and have indicated a distinctive "nuclear" RNA.

In order to characterize the RNA fractions of the two nuclear components, nucleoli and chromatin, and to determine thereby the precise localization of the RNA typical of isolated nuclei, time-courses of P^{32} incorporation into nucleolar, chromosomal, and cytoplasmic RNA of *Drosophila* salivary glands have been determined from autoradiograms. Two experiments are reported which cover 12 and 18 hour periods, including an initial 2 hour feeding on P^{32}. Concentrations of RNA-P^{32} (identified by ribonuclease digestion) were determined by grain counts.

After 1 hour only the nucleolar RNA is labelled. Activity is detectible in chromosomal and cytoplasmic RNA after the 2nd hour. The nucleolar fraction reaches its maximum activity shortly after transfer of the larvae to non-radioactive food, the other fractions several hours later. Maximum activities persist in the chromosomal and cytoplasmic fractions; nucleolar activity decreases after the 9th hour.

The observed differences in times at which incorporation begins and maximum activities are reached, and in maintenance of maximum activities indicate that chromosomal and nucleolar RNA are distinct fractions. The metabolic characteristics which have been ascribed to "nuclear" RNA apply only to the nucleolar fraction.

Studies of isolated cell fractions have demonstrated that nuclear RNA differs from cytoplasmic RNA in chemical composition and characteristic pattern of radioisotope incorporation. Further fractionation of the cytoplasm has revealed no striking differences, in composition or incorporation, among the RNA fractions of the supernatant, mitochondria, and microsomes (1, 2). Techniques for isolating the two structural components of nuclei, nucleoli and chromatin, have not yet been perfected; whether nucleoli, or chromatin, or both structures contain RNA of the type that has been described from studies of isolated nuclei has not been clarified.

The autoradiographic technique for demonstrating radioactivity in cytological preparations has been applied, in the work reported here, to this problem of characterizing the nuclear RNA fractions. The patterns of P^{32} incorporation into chromosomal, nucleolar, and cytoplasmic RNA have been determined for salivary glands of *Drosophila repleta*, and indicate that nucleolar and chromosomal RNA are metabolically distinct from each other.

Materials and Methods

The foods used for growing the larvae have been described previously (3). The larvae were raised at 20°C. and were used on the 2nd day of third instar. Larvae of about the same age were obtained in two different ways. In the first experiment, larvae which entered third instar over a 6 hour period were selected for the experiment; in later experiments, egg deposition has been limited to a maximum of 3 hours.

P^{32} was administered to the larvae in the following food: 150 μc. of P^{32} (as inorganic phosphate) per 100 mg. low phosphate yeast, 12 mg. agar, and 1 cc. of

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mineral solution (without phosphate). Two hours after they were placed on the food, the larvae were transferred to another vial of the same food, but without P^{32}, for the rest of the experiment. In one case half the larvae were left on the radioactive food throughout the period of study.

Material was fixed by the freeze-substitution method described by Freed (4). Whole larvae were frozen in isopentane which was held near its freezing point by a liquid nitrogen bath, dehydrated in absolute alcohol at −45°C. for a week, and then fixed in 80 per cent alcohol at 60°C. for 2 hours, and embedded in paraffin. Cross-sections of the larvae were cut at 4 μ and mounted serially on two slides, with every other row of 15 to 20 sections being placed on one slide. After removal of paraffin, the slides were treated with boiling alcohol-ether (3:1 for 5 minutes) and cold trichloracetic acid (5 per cent, for 5 minutes at 2–4°C.). One slide of each pair was further treated with ribonuclease (0.2 mg./cc., pH 6.0 to 6.5, for 2 hours at 37°C.). Film was applied, exposed, and developed as described previously (5).

Under the conditions used, the number of grains per unit area of film is directly proportional to exposure up to a density of 2 grains per square micron (3). Since all slides for one experiment were exposed the same length of time, or corrected to the same exposure time (for the continuous feeding experiment), the grain density above a given structure at various times after P^{32} administration should reflect the relative concentrations of P^{32} in the structure at the various times.

After the method of fixation used, the cellular structures of the salivary gland are similar in appearance to those of the living cell, as observed with the phase microscope. Nucleoli, cytoplasm, and nuclei have a homogeneous appearance in early third instar. In late third instar, granules appear in the cytoplasm, and the chromosomes become visible in the nucleus. In the larvae used in both of these experiments, the cytoplasm was found to contain granules in the samples collected 24 hours after the beginning of the experiment, but not in earlier samples. The data from these older larvae are not presented here; changes during granule formation will be discussed in a later paper.

Since the distal part of the salivary gland appears more advanced than the proximal part, incorporation

TABLE I

Grain Densities, per 40 μ^2, Found above Nucleoli (Nu), Cytoplasm (Cy), and Chromatin (Chr) in Salivary Glands

Values represent average counts for individual larvae of the first experiment. Corrected counts for nucleoli (nu*) were calculated from the diameters shown in the last column.

Time	Total P^{32}				RNAase-resistant P^{32}			Diameter
	Nu	Nu*	Cy	Chr	Nu	Cy	Chr	
hr.								μ
	0.6	0.7	−0.6	−0.2	−0.4	−0.6		6.8
1	3.0	4.1	−0.6	0.8	0.2	−0.6		6.4
	11.3	11.6	0.6	0.0	−0.2	−0.6		7.5
	25.4	36.4	5.0		3.3	1.3		6.1
2	17.2	22.0	1.0					6.6
	33.2	34.4	5.6	9.8	1.4	1.9	1.0	7.4
	34.1	45.5	14.7		3.8	2.1		6.0
3	21.8	37.7	5.3		3.3	1.9		5.5
	25.3	30.6	11.7		2.2	0.2		6.4
	47.9	51.1	19.2	24.4	8.4	3.4	15.8	7.0
4	40.0	40.0	17.5	18.6	8.7	2.6	5.6	8.1
	39.4	48.9	11.0					6.5
	35.7	41.3	17.5		3.9	1.6		6.6
5	38.0	39.3	16.8					7.3
	46.0	50.5	29.9		7.5	4.1		6.8
	36.9	42.6	26.4					6.1
6	43.3	45.6	21.0		6.7	1.0		7.1
	50.6	50.8	31.5	31.5	8.4	2.9	6.6	7.8
	42.0	45.4	33.2					6.4
9	40.6	41.0	36.9					7.0
	37.2	57.2	20.8		7.4	3.3		5.2
	31.6	34.3	24.8	24.6	6.3	1.9	3.7	6.4
	25.0	25.6	21.2					6.9
12	28.8	29.2	21.3	21.1	8.3	1.4	13.7	7.3

TABLE II

Grain Counts, per 40 μ^2, Resulting from P^{32} in Nucleic Acids and Proteins

Average counts per larva, and their standard errors, are shown for samples of the second experiment. Nucleolar and nuclear diameters are shown in the last columns. N indicates the number of larvae.

Time	Nucleolus	N	Cytoplasm	N	Chromatin	N	Diameter	
							Nucleolar	Nuclear
hr.							μ	μ
1	12.5	1	1.4	1			8.8	16.0
2	29.4±3.1	7	4.2±0.8	7	5.5±1.5	4	8.0	14.8
3	33.5±5.0	5	11.6±2.9	5	13.2±3.8	4	8.6	15.4
4	40.8±0.4	2	19.8±1.0	2			9.8	17.2
5	34.1±3.6	5	22.1±2.1	5	17.9±6.5	3	9.5	17.5
7	33.3±1.5	4	26.9±1.1	4	22.6±0.5	3	9.6	18.1
8	35.4±1.7	4	29.2±0.8	4			10.4	19.5
14	27.4±1.9	7	27.1±2.2	7	23.5±1.4	3	10.3	20.6
18	26.2±0.6	2	30.0±0.2	2	23.4±1.6	2	10.5	23.4

studies were restricted to the cell layers that can be accurately located, *i.e.*, those at the very end of the gland. The last section of the distal end of every gland used was located on the slide, and studies were limited to the last thirty to forty sections (fifteen to twenty on each slide) containing the gland.

Isotope concentration was determined by counting all grains (throughout the thickness of film) in a circular area of 40 μ^2 above the parts of the cell. Counts for all structures were restricted to sections in which the structure extended throughout the thickness of the tissue section. Nuclear counts were made above sections that were free of nucleolar material, and at random positions above the nucleus. Eight to ten nucleolar, cytoplasmic, and nuclear areas were counted from each of the two slides for most larvae; at least five such counts were made for all larvae. Background counts were determined and subtracted from the values for each slide.

The mean counts which are shown in the curves and in Tables II and III were calculated by determining the average activities per cell in each larva of a sample, and then averaging these means to obtain the mean count for the sample of larvae.

Nucleoli of some larvae of the first experiment had a smaller area than the 40 μ^2 area of film that was counted "above nucleoli." The count which would be expected if nucleolar material, instead of nucleolar and chromosomal, filled the cylinder of tissue (40 $\mu^2 \times 4\ \mu$) below the film, was calculated as follows. The volume of the cylinder which was not occupied by nucleolar material was multiplied by the chromatin activity to estimate the contribution of the chromatin to the "nucleolar" count. The nucleolar count was corrected for the chromatin contribution, and then used to calculate the count for 160 μ^3 of nucleolar material. Chromatin activity was taken as equal to cytoplasmic for the calculation (see below). No corrections were necessary for the RNAase-resistant fraction.

The relative concentrations of RNA in nucleoli and cytoplasm were estimated by microphotometric determination of dye concentration after staining with the basic dye azure B (6). Sections were stained for 2 hours (1 mg. dye/cc., pH 4.0, at 40°C.) and differentiated in tertiary butyl alcohol overnight. Extinctions were determined on 3 or 4 μ sections at 480 mμ, with an apparatus of the type described by Pollister (7).

For volume determinations, camera lucida drawings of the cells were used. Nuclear and nucleolar volumes were calculated from their diameters, using the formula for a sphere. The drawings of all sections containing each cell were traced on glassine paper, cut out, and weighed; cytoplasmic volumes were calculated from these weights.

About 10 cells, from the very bottom of the gland, were measured from each larva. Data from two different batches of larvae are shown in the next section; these larvae were not used in the incorporation studies reported here, but were prepared the same way and were of comparable stage of development.

TABLE III

Grain Counts, per 40 μ^2, Resulting from RNAase-Resistant P^{32}

Average counts per larva, and their standard errors, are indicated for larvae of the second experiment. N indicates the number of larvae.

Time	Nucleolus	N	Cytoplasm	N	Chromatin	N
hr.						
2	3.4±0.5	7	0.8±0.2	7	3.7±0.9	4
3	3.5±0.6	5	1.0±0.3	5	5.0±2.3	4
5	7.5±1.0	4	1.5±0.2	4	10.9±3.7	2
7	7.0±0.5	4	2.0±0.3	4	9.6±0.7	3
8	6.5±0.7	2	1.4±0.1	2		—
14	6.7±0.8	7	1.4±0.2	7	10.5±2.9	3
18	6.7±2.1	2	1.6±0.7	2	9.7±5.6	2

OBSERVATIONS AND RESULTS

The counts that were made in the two experiments are shown in Fig. 1. Since the various solvents used extract essentially all tissue components except nucleic acids and proteins, these counts represent P^{32} activity in the nucleic acid and protein fraction of the cell.

As reported previously (3), the nucleolus contains P^{32} shortly after the larvae are placed on the radioactive food. The count increases rapidly while they are eating this food, but reaches a maximum value soon after their removal to non-radioactive media. The same count persists from the 3rd hour until the 8th or 9th hour, after which a large drop in activity occurs. Little further change in count follows in later hours. The curves for the two experiments reach different maximum counts, relative to the cytoplasmic count, but are qualitatively alike.

The cytoplasmic and chromosomal fractions show a very different course of incorporation from that of the nucleolus. They show a definite lag before incorporation begins, do not reach their maximum values until several hours after the larvae have been transferred to the non-radioactive media, and then maintain their maximum values in the later part of the experiment. Nuclei in most larvae of the first experiment were too small to yield sections free of nucleoli, but an estimate of chromosomal activity was made with the few suitable larvae. As seen in Table I, the chro-

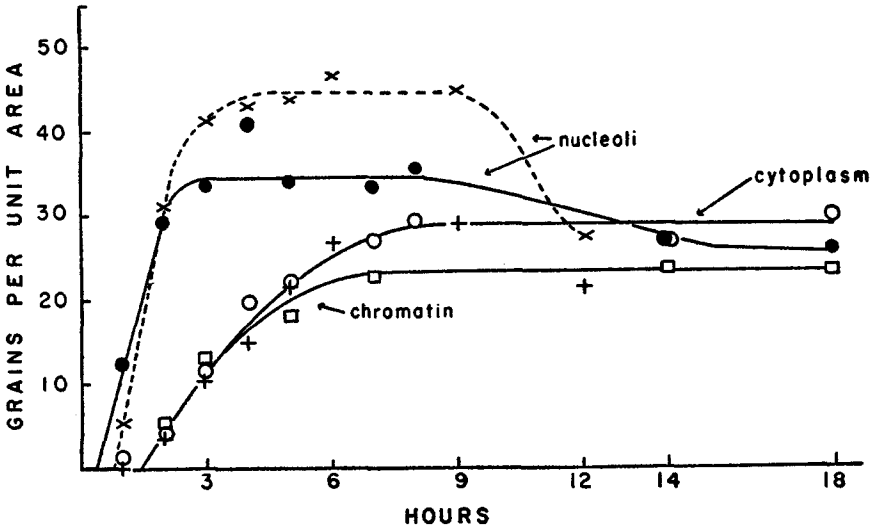

FIG. 1. P^{32} concentration (grain density per unit area) in nucleic acids and proteins of cellular structures. Larvae were fed P^{32} for the first 2 hours of the experiments. Results from two experiments are plotted; crosses refer to the first experiment, and circles and squares refer to the second. (Chromatin counts refer to the second experiment only.)

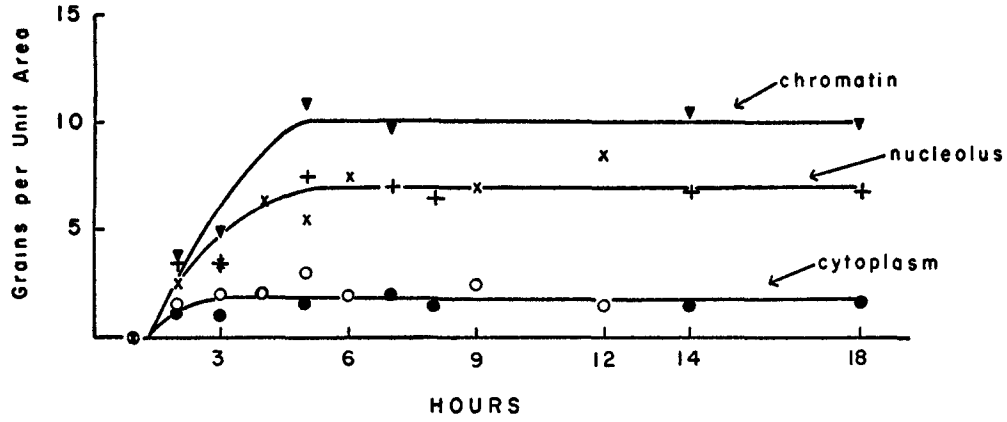

FIG. 2. P^{32} concentration (grain density per unit area) in cellular structures after extraction of RNAase-digestible P^{32}. Same larvae and conditions as those shown in Fig. 1. X and O refer to nucleoli and cytoplasm of the first experiment; other symbols refer to the second experiment.

mosomal count was nearly the same as the cytoplasmic count in each larva studied.

Ribonuclease digestion removes almost all of the cytoplasmic P^{32}, while a fairly large part of the nucleolar and chromosomal P^{32} resists this treatment. The counts which remained after RNAase extraction of the tissue are shown in Fig. 2 and in Tables I and III. Enzyme-resistant P^{32} becomes detectible in all parts of the cell at the same time that P^{32} first appears in the chromosomal and cytoplasmic fractions.

The activities in RNAase-digestible phosphorus fractions, shown in Fig. 3, were obtained by subtracting the curves of Fig. 2 from those of Fig. 1. The resultant curves are similar to those obtained without correction for RNAase-resistant phosphorus.

Half the larvae of the second experiment were left on the radioactive food for the whole period of the experiment. Activities that were found in the RNA fractions (*i.e.* the RNAase-digestible P^{32}) under these conditions indicate a difference between the chromosomal and cytoplasmic fractions. As seen in Fig. 4, the patterns of incorporation into these two RNA fractions are similar to each other

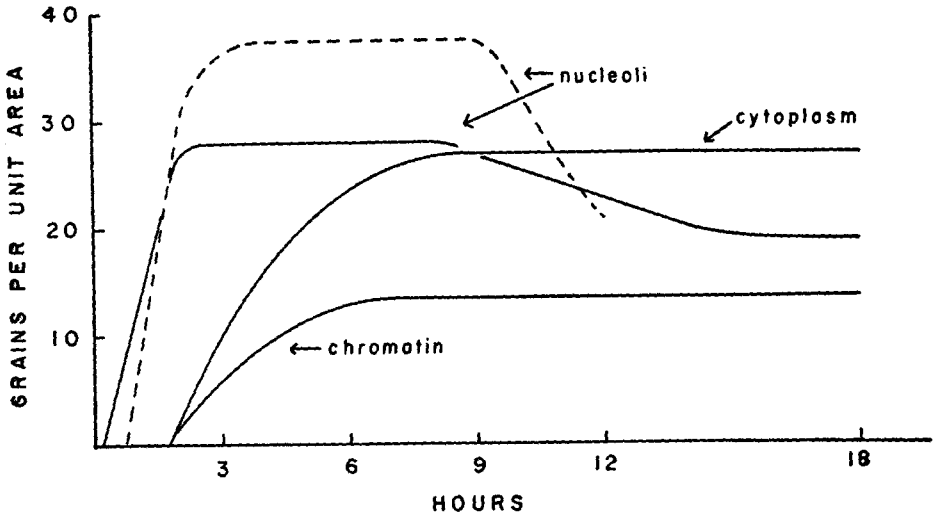

FIG. 3. Concentration of RNAase-digestible P^{32} in cellular structures at various times, calculated as the differences between the curves of Figs. 2 and 1. Nucleoli of the first experiment are represented by a dotted line. The cytoplasmic curves for the two experiments were the same.

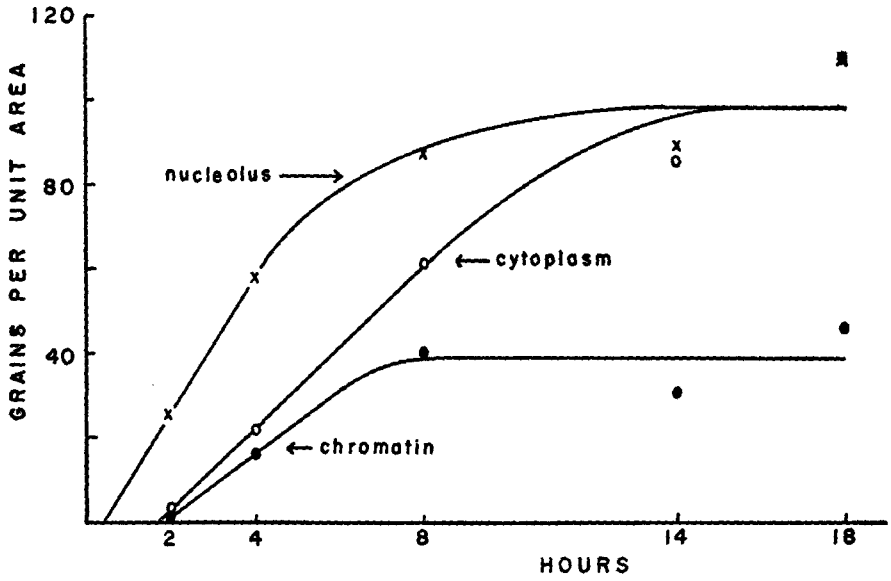

FIG. 4. Concentrations of RNAase-digestible P^{32} at various times in larvae which fed continuously on P^{32}. These larvae, and those of the second experiment shown in previous figures, were from the same culture and were placed on radioactive food at the same time.

n many respects, as with the short feeding on ^{32}, but the chromosomal activity reaches its aximum value when the cytoplasmic activity is ill increasing. The two fractions are therefore etabolically distinct, in spite of their similarities.

Basic staining with the dye Azure B indicates the me concentration of RNA in nucleoli and cytoplasm (Table IV). Included in the table are larvae from the 1st and 2nd day of third instar, and from two different batches of larvae. The cytoplasmic volume of the youngest larva was one-fourth that of the largest; throughout this growth period, extinction is constant, and the same in nucleoli and cytoplasm. Neither RNA concentration, nor

TABLE IV

RNA Concentrations in Nucleoli and Cytoplasm of Salivaries From 9 Different Larvae, as Indicated by Binding of the Basic Dye, Azure B

Cytoplasmic volume, extinctions at 480 mμ, and the ratio of nucleolar E to cytoplasmic E are indicated for each larva.

Cytoplasmic volume $10^3\ \mu^3$	Cytoplasmic E_{480} per μ	Nucleolar E_{480} per μ	Relative E N/C
3.1	0.121	0.126	1.04
3.8	0.120	0.118	0.99
5.2	0.105	0.123	1.17
6.5	0.127	0.128	1.01
7.0	0.121	0.116	0.96
9.1	0.135	0.121	0.90
11.3	0.109	0.115	1.05
11.3	0.111	0.123	1.11
13.3	0.105	0.108	1.03
Average	0.117	0.120	1.02

change in RNA concentration during growth seem to be responsible for the different patterns of P^{32} incorporation into nucleolar and cytoplasmic RNA.

Chromosomal RNA is difficult to reveal or study quantitatively in cytological preparations. In the salivary preparations used in this study, only nucleoli and cytoplasm are stained with the conventional basic staining techniques. Concentrations can be estimated, however, for the RNA fractions that incorporate P^{32}, from the counts found in larvae that had fed continuously on P^{32} (Fig. 4).

In this experiment, the activities of all RNA fractions approached plateaus, which would be expected only when they approached the specific activity of the food the larvae were eating. (Other experiments have shown that incorporation of P^{32} from the food occurs during the periods when activity increases were not apparent in this experiment.) When the RNA fractions of the three structures reach the same specific activity, the RNA-P^{32} concentrations in the structures should reflect their RNA concentrations. The plateau values indicate that nucleoli and cytoplasm have the same concentration, as found with basic staining, while the concentration in the nucleus is less than half that found in the other structures.

The counts per unit volume of nuclear material which were found after a short period of feeding on P^{32} should therefore be multiplied by a factor of about two, if comparable amounts of chromosomal, nucleolar, and cytoplasmic RNA are to be considered. This correction shows that all three RNA fractions (in this second experiment) reach about the same maximum specific activity, after a short period of feeding.

DISCUSSION

The RNA of the chromatin is obviously very different metabolically from the nucleolar RNA, and is surprisingly similar to the cytoplasmic RNA. Incorporation begins at the same time in the cytoplasmic and chromosomal fractions, they both reach their maximum values later than the nucleolar fraction, and maximum activities persist in both fractions.

The chromosomal and cytoplasmic RNA fractions do not have identical patterns of incorporation; with continuous feeding on P^{32} their maximum activities were reached at different times. A similar difference has also been found when adenine-C_{14} is used to label salivary RNA (unpublished observations). In this case, too, the chromosomal RNA reaches its maximum count before the cytoplasmic RNA, even after a short feeding on the label.

The nucleolar RNA of salivaries is unique in several respects; it is labelled before there is any detectible incorporation into the other cellular RNA, its activity reaches a maximum value earlier, and it shows decreases in activity during a period when the other RNA fractions show no loss of isotope.

An early maximum activity and an early decrease in activity, as found for nucleolar RNA, are distinguishing characteristics of the RNA of isolated nuclei. In a comparison of the salivary curves with the curves obtained by studying isolated fractions of cells, nucleolar and cytoplasmic RNA of salivaries are practically indistinguishable, on any qualitative basis, from the "nuclear" and cytoplasmic RNA of various mammalian tissues which have low mitotic activity. (See, for instance, kidney and spleen, studied by Smellie *et al.* (8).) Since chromosomal RNA, on the other hand, shows no resemblance to the RNA of isolated nuclei, it seems certain that the metabolic characteristics which hav been ascribed to "nuclear" RNA apply only to the nucleolar fraction.

The occurrence of two types of RNA in mammalian nuclei has been suggested by Kay *et al.* (9)

from a comparison of nuclei isolated in two kinds of media. They found that liver nuclei isolated in non-aqueous media have a higher RNA content than those isolated in aqueous media, as reported earlier by Dounce *et al.* (10), and that shortly after administration of P^{32}, the specific activity of RNA is lower for non-aqueous nuclei than for aqueous. These data suggest that a nuclear RNA which does not incorporate P^{32} rapidly is extracted from the nuclei in the aqueous isolation procedure. Tyner *et al.* (11) have reported similar data and pointed out that cytoplasmic contamination, rather than a nuclear RNA fraction, may be removed in the aqueous procedure. The salivary data would lead us to expect these results if either cytoplasmic or chromosomal RNA were removed.

The chromosomal RNA of salivaries is an active fraction, when judged by amount, rather than initial rate, of P^{32} incorporation, and is a large fraction relative to the nucleolar RNA. In the experiment shown in Tables II, and III, chromosomal RNA-P^{32} increased slowly until its maximum concentration in the nucleus was about half the maximum in the nucleolus and cytoplasm. When both nucleolus and chromatin have their maximum counts, the nucleus will contain more than three times as much chromosomal RNA-P^{32} as nucleolar RNA-P^{32}, since the nuclei have about eight times the volume of the contained nucleoli. (See diameters in Table II.)

The distribution of RNA-P^{32} in the nucleus is very different shortly after P^{32} is fed to the larvae; at the 1st hour after feeding, only the nucleolus is labelled (see Table I.) The curves indicate that labelled RNA does not begin to appear in the chromatin, or the cytoplasm, until about an hour after the rapid labelling of the nucleolus has begun. Thus, for the first 2 hours essentially all the nuclear RNA-P^{32} is localized in the nucleolus. The total nuclear RNA-P^{32} increases slowly in later hours, and becomes essentially chromosomal, especially after the drop in nucleolar activity.

The time interval between onset of incorporation into nucleoli and other RNA fractions is apparent in salivaries when either P^{32} or adenine-C_{14} (not shown here) is incorporated. The simultaneous occurrence of labelled nucleolar RNA and unlabelled chromosomal RNA demonstrates clearly that the nuclear RNA fractions which occur in two discrete structures do not mix freely, but are separate and distinct in the living cell. It further indicates that nucleolar RNA is synthesized in the nucleolus, rather than being derived from either chromosomal or cytoplasmic RNA; these fractions contain no label when P^{32} has already appeared in the nucleolar RNA.

The possibilities cannot be eliminated that nucleolar RNA serves as the precursor of some of the labelled RNA which appears later elsewhere in the cell, or that there is exchange between the cytoplasmic and chromosomal fractions. It is clear, however, that any transfer would necessarily involve changes in whatever features are responsible for the metabolic characteristics of each type of RNA, in order to maintain the apparent distinctions among the fractions.

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