Effects of *in vitro* Incubation and X-irradiation on the Ribonucleic Acid Fractions of Rabbit Lymphatic Cells

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In previous papers[9, 10] we reported that ribonucleic acid (RNA) of various animal cells could be separated with aqueous phenol into two fractions, i.e., phenol-released RNA (pRNA) and residual RNA (rRNA). They differed in metabolic activity as well as in composition. In pRNA, 2 M NaCl-insoluble fraction (pRNA-B) predominated over 2 M NaCl-soluble fraction (pRNA-A). We later found[4] that the efficiency of the fractionation was affected considerably by the composition of suspending medium.

To explain the results of such a fractionation, we have proposed the following *tentative* hypothesis, which will help to distinguish the actually obtained fractionation products which may vary in properties from what is present in the living cell and can only be approached by reasonable assumptions. If we disregard the soluble RNA which may correspond to our pRNA-A fraction[12], the cellular RNA may be divided into two principal classes: the class I RNA (RNA I) and the class II RNA (RNA II). The former is synthesised rather slowly and may be duplicated only once during the course of mitotic cycle in certain types of cells. This RNA can be recovered as pRNA-B in more or less pure form only under appropriate fractionation conditions. The latter class of RNA (RNA II) has an exceedingly high turnover rate and goes exclusively into the rRNA fraction by the optimal fractionation procedure. The two classes of RNA may differ in the linkage to proteins which is modified by the composition of the suspending medium. Under certain conditions, therefore, RNA I may no longer be separable in pure form as the pRNA-B fraction, which may now be heavily contaminated by RNA II.

In a previous communication[4] it was suggested that such a cross-contamination of pRNA-B fraction may occur also during the course of an *in vitro* incubation of the rabbit lymphatic cells even under the fractionation conditions that may be optimal for the fresh material. The present paper deals with some further experiments about this point and also with the effect of X-irradiation.

MATERIALS AND METHODS

Incubation of lymphatic cells.—Appendices and Peyer's patches of adult albino rabbits weighing about 2 kg were used for the preparation of a lymphatic cell ac-

cording to the procedure of Sibatani[8]. Cells were suspended in Ca-free Tyrode solution to a density of 4×10^8 cells/ml and incubated with 5 μc/ml of ^{32}P-orthophosphate for 1–4 hours. About 1.5×10^9 cells were necessary for each determination.

X-irradiation.—In some experiments, separate tubes containing 3.5–4.0 ml of the cell suspension were irradiated, prior to the addition of ^{32}P, with 800 r of X-rays with 0.5 mm thick copper and aluminium filters at a rate of 31.5 rpm from a Shimadzu Hakuaigo tube (160 KVp, 3 mA) at room temperature. During the irradiation period, control tubes were kept under similar conditions in a separate room.

Phenol fractionation of cellular RNA.—This was made essentially according to a previous report[4]. Upon termination of the desired incubation period, 0.05 volume of 2.5% $Na_2HPO_4 \cdot 12H_2O$ solution was added to the cell suspension. This was then mixed with an equal volume of 90% phenol and shaken for 1 hour at 37°C. pRNA fraction was separated in many experiments into A and B subfractions according to the solubility in 2 M NaCl solution as indicated above. Separated RNA fractions were hydrolysed with 0.5 N KOH at 37°C for 20 hours. DNA was obtained by a hot dodecylsulphate extraction from the residue remaining after the isolation of rRNA. The amount of individual RNA fractions was estimated by the optical density measument at 260 mμ with the alkaline hydrolysate. Details of the procedure were given in a previous report[4].

Radioactivity measurement.—This was also made exactly as in a previous report[4] with the mixture of 2′, 3′-adenylic, cytidylic and uridylic acids. Guanylic acid was discarded because of the possible radioactive contamination of the spot of this nucleotide on the paper chromatogram which separated other three nucleotides as a single spot from guanylic acid[5]. Phosphorus determination for the estimation of the specific activity was conducted according to Gomori[2].

RESULTS

Changes in RNA fractions during the course of in vitro *incubation.* The course of ^{32}P-orthophosphate incorporation into nucleic acids of rabbit lymphatic cells *in vitro* was studied by Sibatani[8]; the incorporation into DNA proceeded, with a short lag, linearly during 4 hours of incubation, while that into total RNA, after an initial linear incorporation for about 2 hours, tended to level off gradually.

In the present experiments, the course of ^{32}P incorporation into individual RNA fractions separated by phenol was followed. It was thus found that the ^{32}P incorporation into rRNA, just like that into DNA, proceeded linearly for 4 hours with a lag of about half an hour (Fig. 1). An enormous difference in the specific activity between rRNA and DNA should be noted. The curve for pRNA showed gradually increasing rate of incorporation during the course of incubation. Thus, the specific

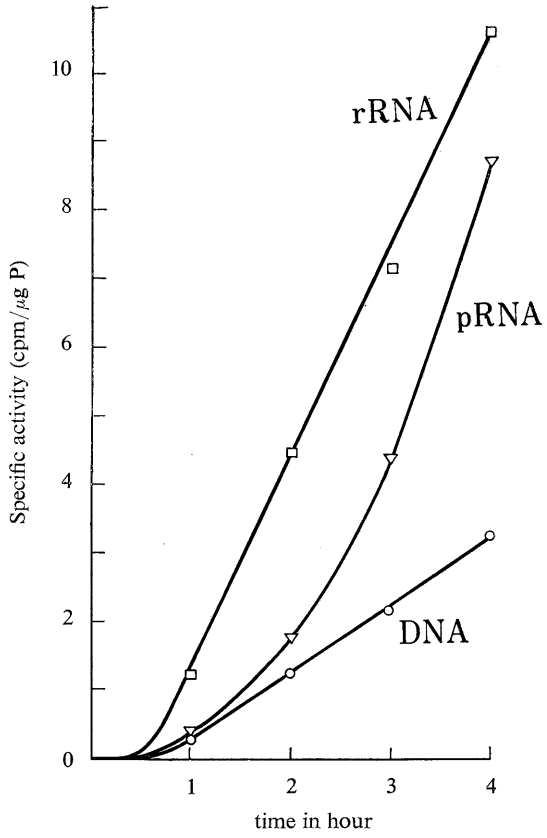

Fig. 1. *In vitro* incorporation of ^{32}P into DNA and RNA fractions of rabbit lymphatic cells. pRNA-A and -B were not separated. The scale of the ordinate is reduced to 1/10 for rRNA.

activity of pRNA which was very close to that of DNA at 1 hour, later became much higher than the latter. In another experiment shown in Fig. 3, A and B fractions of pRNA were separated. Here, the incorporation into pRNA-B proceeded also linearly, so that the progressive rise of the incorporation rate of the whole pRNA fraction as shown in Fig. 1 might be due to an considerable increase of total activity incorporated into pRNA-A fraction. Such a trend of pRNA-A was actually noted in the experiment shown in Fig. 3, but owing to the small amount of material the determination of the specific activity of pRNA-A in this series of experiments was not very reliable and usually gave erratic results, so that exact time curve of incorporation into this fraction could not be shown in Fig. 3.

The change in the specific activity in pRNA fraction in Fig. 1 might be a reflection of the progressive labilization during the incubation of the phenol-resistant linkage between protein and RNA II. If this is the case, the yield of rRNA would

be reduced during the course of incubation.

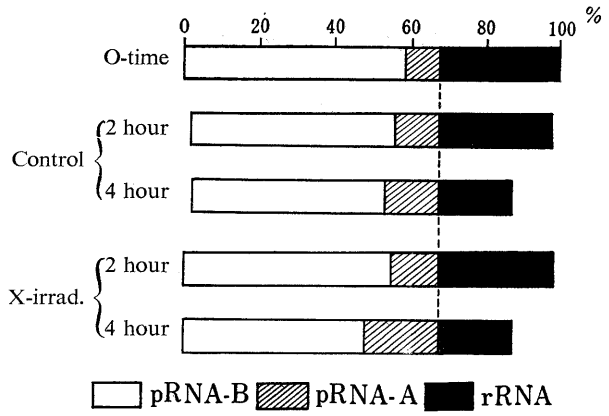

Fig. 2. Changes in amount of individual RNA fractions of rabbit lymphatic cells during the course of an incubation at 37°C. The relative amounts are indicated in % of total RNA at the outset of incubation. Initial amount of total RNA: $E_{260}=130$

Experiments were therefore carried out to clarify whether the yield of rRNA actually decreased during the incubation period. Fig. 2 illustrates results of a typical experiment. The total amount of pRNA did not seem to be much affected by the incubation, but there was relatively more pRNA-A after incubation than at the outset of experiment. As for the yield of rRNA, a remarkable decrease was usually observed after 1–2 hours of incubation. Therefore, the composition of total RNA with regard to the phenol fractionation was progressively changed, and the ratio of the amount pRNA/rRNA rose with time. This change may affect the linearity of the incorporation curve of total RNA, resulting in a progressive decrease in its rate of incorporation, even when the incorporation into both pRNA-B and rRNA proceeds linearly.

Effects of X-irradiation on the synthesis of RNA fractions.

The effect of 800 r X-irradiation *in vitro* on the incorporation of ^{32}P-orthophosphate into individual RNA fractions of rabbit lymphatic cells were examined. Fig. 3 illustrates the results of a typical experiment, from which the data shown in Fig. 2 were also taken. The results clearly indicated that the incorporation into rRNA was definitely depressed by X-irradiation while that into pRNA-B was not affected (Fig. 3). It should also be noted that pRNA-B, when separated from pRNA-A fraction, incorporated the radiophosphate linearly for 4 hours. At the 4th hour of incubation, the ^{32}P incorporation into rRNA and into DNA was inhibited to the same extent (68% of the control). The rate of incorporation into pRNA-A was lowered by X-irradiation down to 82% of the control at 4 hr. of incubation, but this value was not very reliable as stated before.

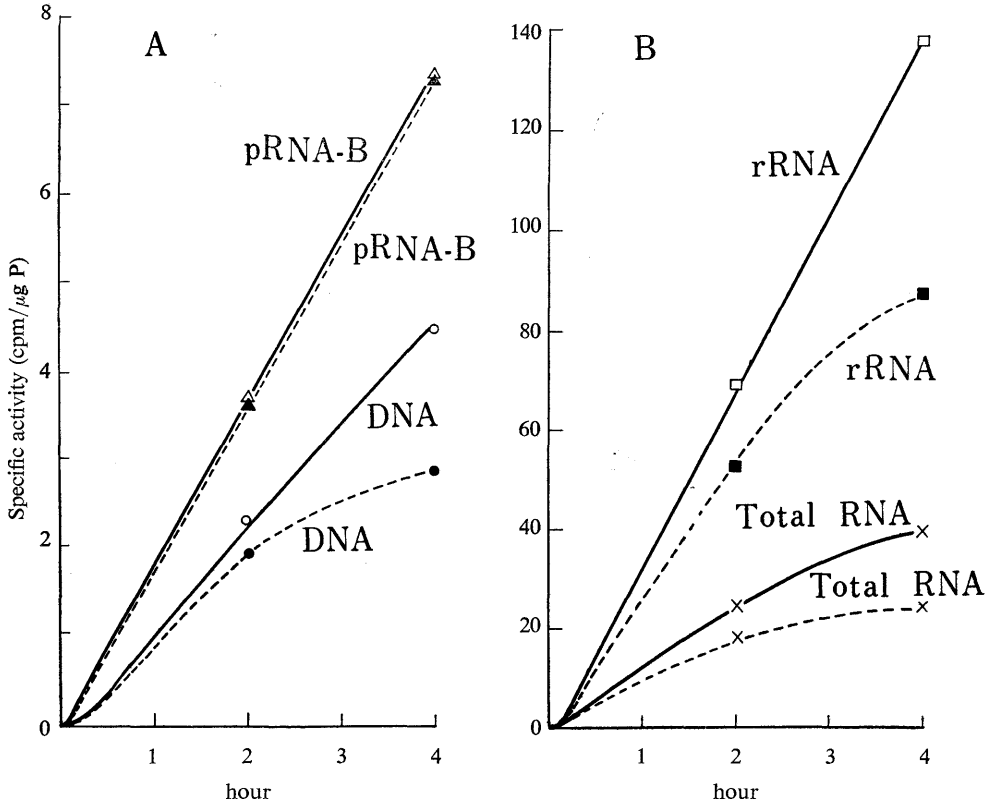

Fig. 3. Effects of 800 r X-irradiation upon the incorporation of ^{32}P into DNA and RNA fractions of rabbit lymphatic cells. Solid lines are controls and broken lines are irradiated cells.

Curves for total RNA was constructed on the basis of the calculation from the specific activities and the relative amounts of three RNA fractions and shown in Fig. 3, B. The feature of the curves is somewhat reminiscent of that reported before[8].

DISCUSSION

It was shown in the present paper that the yield of rRNA markedly decreased during the *in vitro* incubation. This would mean either that under *in vitro* conditions the linkage between protein and RNA II became more susceptible to the action of phenol or that RNA II was more labile than RNA I and preferentially degraded *in vitro*. The progressive rise in specific activity of pRNA fraction suggests that pRNA after *in vitro* incubation may have received some RNA II. But the fact that no significant rise in the yield of pRNA during the incubation was observed

indicates that an appreciable portion of RNA II was degraded. In fact, the calculation indicated the decrease in the yield of rRNA during the incubation is more than sufficient to account for the increment of the specific activity of pRNA over that of DNA.

Effects of X-irradiation on the ^{32}P incorporation into nucleic acids of lymphatic cells have been investigated by several workers[6, 8, 11]. In most cases, ^{32}P uptake of DNA was significantly inhibited by 600–1000 r of X-irradiation, while that of RNA was less sensitive or, in some cases, even not affected at all by the same doses of X-rays. However, there are a few instances including lymphatic cells in which it was indicated that the irradiation affected the synthesis of nuclear RNA but not of cytoplasmic RNA[3]. Comparable finding about the differential effects of X-irradiation on the synthesis of nuclear and cytoplasmic proteins are also reported[7]. Creasey and Stocken[1] suggested that the specific effect of irradiation on the synthesis of nuclear nucleic acids and proteins was due to the nuclear damage which also resulted in an inhibition of nuclear ATP formation. Our observations suggest that the incorporation of ^{32}P into RNA II and DNA was affected by 800 r X-irradiation to the same extent while that into RNA I remained intact. This is apparently consistent with the idea of specific nuclear damage by X-irradiation of the dose employed. However, since either class of RNA separable with phenol occurs in both nuclei and cytoplasm[4, 10], it remains to be seen whether the effect of X-irradiation on the synthesis of RNA confines itself in nuclear RNA or in class II RNA; that is, whether it is related to intracellular localization of RNA or to its linkage with protein. It is true that nuclear RNA and DNA occur in nuclei. But both DNA and class II RNA can not be released with phenol by standard fractionation procedure, and they can both be released from the protein by phenol treatment in the presence of *p*-aminosalicylate (unpublished experiment), suggesting some common features of their linkage to proteins.

SUMMARY

The yield of metabolically distinct fractions of RNA separated from rabbit lymphatic cells by phenol treatment changed during the *in vitro* incubation. The amount of the major component of the phenol-released fraction, pRNA-B, was kept rather constant, while the yield of the residual fraction with a very high turnover rate (rRNA) decreased during the course of the incubation. The *in vitro* incorporation of ^{32}P-orthophosphate into pRNA-B was not affected by a 800 r X-irradiation *in vitro* of lymphatic cells but that into rRNA was depressed to the same extent as that into DNA.

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