may be an important natural substrate for granulocyte alkaline phosphatase.

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In vitro Uptake of Isotopically Labeled RNA by Mammalian Spleen Cells.* (30542)

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The incorporation of nucleic acid or nucleoprotein into mammalian cells has been reported by several investigators(1-3). Bensch and King(4) noted the uptake of particles containing a coacervate of high molecular weight mouse Sarcoma DNA and protein, by a strain of Earle's L cells *in vitro*. Higginbotham(5) reported that fibroblasts incorporate DNA by phagocytosis *in vivo*. Kay (3) presented evidence for incorporation of 2 to 4% of the administered DNA into Ehrlich-Lettre ascites cells *in vitro*. It was found that the DNA retained its macromolecular structure in the host cell. Borenfreund *et al* (6) reported that tritium labeled RNA from pneumococci and human leucocytes was incorporated by growing Hela cells. The amount of DNA incorporated was equivalent to 10% of the normal Hela cell complement. Gartler(7) described uptake of DNA by Earle's L cells of 0.5 to 3% of the DNA administered. A smaller amount was reportedly degraded upon uptake, and the fragments incorporated into host DNA. Schwarz and Rieke(8) demonstrated *in vivo* and *in vitro* incorporation of tritium labeled RNA from normal mouse liver into isologous mouse ascites tumor cells by the method of autoradiography. The cells reported as incorporating RNA were large and medium lymphocytes. Amos and Moore(9) have shown that RNA from various sources stimulates the synthesis of protein in chick embryo fibroblasts. However, the magnitude of the response was not correlated with the concentration of exogenous RNA. This disproportion was apparently due to the rapid depolymerization of the RNA to acid soluble products by ribonucleases of the media. Amos and Kearns (10) have demonstrated significant uptake of P^{32} labeled RNA by chick embryo fibroblasts, and have suggested that the degradation RNA can be prevented by various additives.

In view of the reported ability of RNA extracted from the spleens of immune animals, to transfer the capacity for antibody synthesis to nonimmune spleen cells(11,12), it seemed desirable to examine the conditions necessary to obtain measurable cellular uptake of rat spleen RNA by isologous spleen

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cells *in vitro*. It might then be possible to define the maximal amount of RNA that may be incorporated into a fixed population of cells, and the factors necessary for this occurrence.

Materials and methods. Isotopic labeling of RNA. To determine the time which would give maximum incorporation of isotope into spleen RNA, 12 Sprague-Dawley male rats varying in weight from 250 to 280 g were used for *in vivo* labeling. An incision was made through the skin and peritoneum, the spleen exposed, and 200 or 300 μc of P^{32} labeled disodium phosphate in a volume of 0.2 ml, was injected intrasplenically. The animals were sacrificed in groups of 3 at 1, 2 and 3.5 hours after isotope injection. The spleens were removed and RNA extracted by a modification of the cold phenol technique of Kirby(13). The specific radioactivity of RNA obtained from the above 3 groups of animals was compared to determine the time required for peak labeling. The 3.5-hour labeling period was found to provide RNA of the highest specific activity, and was employed in subsequent experiments.

Extracted RNA was precipitated in 95% ethyl alcohol for 12 hours, and resuspended in 5 ml of TRIS buffer, pH 7.4. The extracted RNA was characterized by the following procedures: The Orcinol test(14) for ribose containing compounds, the Diphenylamine test(15,16) for presence of trace amounts of DNA, and a modification of Lowry's test(17) for protein.

Single cell cultures. A single cell suspension of 4×10^7 viable spleen cells was obtained from untreated rats of the same strain and of similar weight to those from which spleen RNA was extracted. Excised spleens were diced with iris scissors and the cells harvested and pooled. Two-tenths ml cell aliquots were diluted into 0.18 ml of a 0.25% trypan blue solution, and total viable cells estimated using the standard dye exclusion test(18).

Cell cultures were obtained by diluting a suspension of 4×10^7 cells to 40 ml (final conc. 10^6 cells/ml) in medium 199(19), supplemented by 1 μg/ml hydrocortisone hemisuccinate, and 1 unit/ml insulin, after the method of Ambrose(20). The cell suspensions were placed in 100 ml spinner flasks, and agitated mildly at 37°C for 60- and 90-minute periods. To the above cell suspensions were added varying amounts of P^{32} labeled RNA.

Recovery of P^{32} labeled RNA from cells. Following exposure in culture for appropriate intervals, cells were centrifuged from the media at $130 \times g$. Media was decanted, and cells washed twice in RNA-free Sorensen's phosphate buffer, pH 7.3, were then resuspended to a concentration of 5×10^6 cells/ml. Two one-ml aliquots of cells were removed and each was precipitated with 10% trichloroacetic acid (TCA) at 4°C for 2 hours.

The TCA insoluble material obtained from each aliquot was collected on 3 mm Whatman filter paper discs by suction in a millipore filter holder attached to a 500 ml vacuum flask. Discs were removed, allowed to dry, and placed in cold 10% TCA at 4°C for one hour to assure the precipitation of acid insoluble material into the matrix of the filter paper. Discs were air dried, and placed in ethyl ether for 15 minutes for removal of lipids, after the method of Mans and Novelli (21).

The filter discs were then placed in standard 20 ml low potassium glass counting vials, and 5 ml of scintillation mixture added (5 grams/liter 2,5 Diphenyloxazole, 0.3 g/liter 1,4 bis-2 (5 phenyloxazole) benzene in toluene). Samples were counted with a Packard Tri-Carb Liquid Scintillation Spectrometer. Uptake of P^{32} labeled RNA was expressed as acid-precipitable radioactivity, and as a per cent of input radioactivity, recoverable from 5×10^6 cells in culture.

Results. In vivo labeling of RNA. Preliminary experiments were performed to determine the time interval most suitable for maximal incorporation of isotope into RNA. Animals were injected intrasplenically in groups of 3, each animal receiving 200 or 300 μc of P^{32} labeled disodium phosphate (8.5×10^5 cpm/μc). Animals were sacrificed at 1, 2, and 3.5 hours after isotope injection. Counts of RNA obtained from the spleens of each group were: 2300, 4100 and

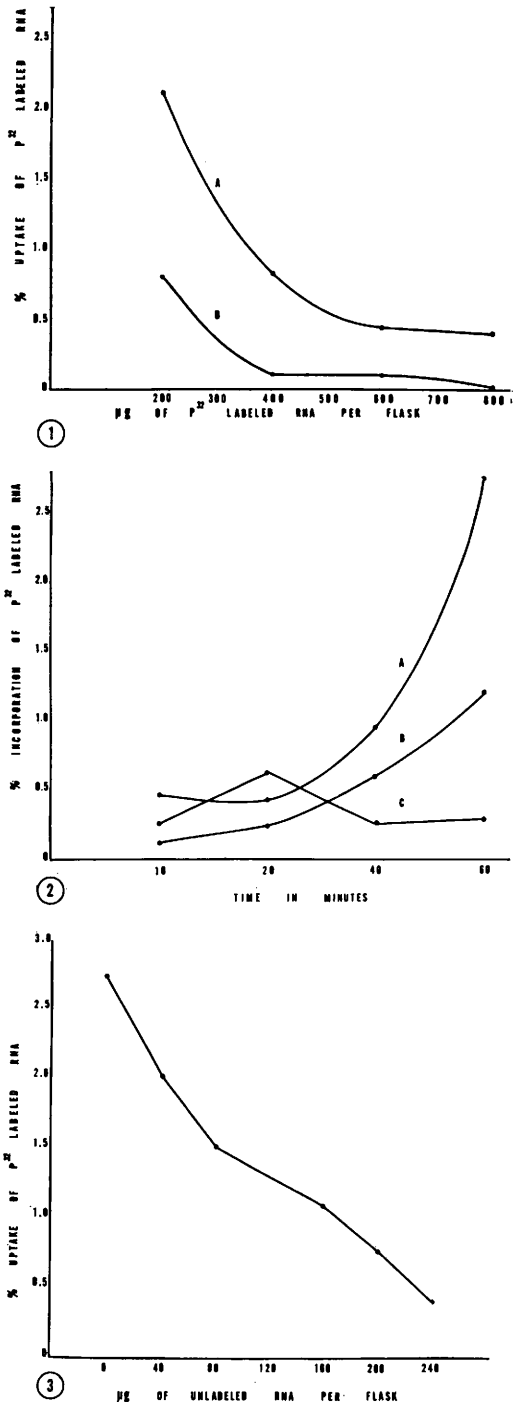

FIG. 1. Uptake of P^{32} labeled RNA by cells exposed to increasing amounts of RNA *in vitro, versus* cells exposed to labeled RNA plus 0.3 mg/ml bovine pancreatic ribonuclease. A. Uptake of RNA. B. Uptake of RNA with ribonuclease present.

9250 cpm/100 μg of RNA, respectively, indicating a rapid increase in the rate of isotope incorporation with time. The RNA from animals in the latter groups was employed in the experiments to be discussed.

Characterization tests of the labeled RNA indicated a protein content of 8 μg, and a DNA content of 4.2 μg/100 μg of RNA.

Excised spleens were found to have an average wet weight of 1 g and an average RNA content of 6190 μg.

Uptake of labeled RNA. The initial experiment was conducted to determine the amount of labeled RNA sufficient to allow measurable uptake when exposed to a fixed population of cells. Cell cultures were exposed for 60-minute periods to increasing amounts of P^{32} labeled RNA. Groups of 4 flask cultures were exposed to 200, 400, 600, and 800 μg of labeled RNA, with input radioactivity as shown in Table I.

The data shown in Fig. 1 indicate that uptake is greatest when cells are exposed to 200 μg of P^{32} labeled RNA. When the amount of RNA is increased in multiples of 200 μg per flask, that per cent of input radioactivity that is recoverable decreases and reaches a plateau.

Cell cultures exposed at zero time to 0.03 mg/ml of bovine pancreatic ribonuclease (RNAse) with the labeled RNA, showed a significant decline in uptake of RNA (Fig. 1). Uptake by cells exposed to 200 μg of RNA in the presence of RNAse is decreased from 2.1% of input radioactivity of 0.8%. A similar decline is seen in uptake by cells exposed to 400 μg of P^{32} labeled RNA in the presence of RNAse. Cells exposed to 600 and 800 μg of RNA in the presence of RNAse show acid precipitable radioactivity which is not significantly above background. The presence of RNAse in the 200 and 400 μg cultures depresses RNA uptake to 38% and

FIG. 2. Rate of labeled RNA uptake as a function of time. A. Cells exposed to 200 μg of labeled RNA per culture. B. Cells exposed to 150 μg of labeled RNA per culture. C. Cells exposed to 100 μg of labeled RNA per culture.

FIG. 3. Competitive inhibition of uptake of P^{32} labeled RNA by unlabeled RNA. Each condition exposed to 200 μg of labeled RNA plus the stated amount of unlabeled RNA.

TABLE I. Uptake of P^{32} Labeled RNA with Increasing Concentrations of RNA.

Labeled RNA per flask (μg)	Input radioactivity (cpm)	TCA precipitable activity recovered per 5×10^6 cells (cpm)
200	18550	391.0
200	18550	149.3 (RNAse)
400	37090	136.3
400	37090	45.8 (RNAse)
600	55640	252.0
600	55640	26.0 (RNAse)
800	74190	304.3
800	74190	15.2 (RNAse)

15%, respectively, of the non-RNAse treated cultures.

The finding that uptake of P^{32} was greatest when cells were exposed to 200 μg of RNA for 60 minutes suggested that an amount less than 200 μg may be optimal. Furthermore, the optimal exposure time may be less than 60 minutes. To test these possibilities cell cultures were maintained for periods of 10, 20, 40, and 60 minutes. Cells of each culture were exposed to 100, 150, and 200 μg of labeled RNA, having an input radioactivity as shown in Table II.

The data in Fig. 2 indicate an increase in rate of labeled RNA uptake with time when cells were exposed to 150 and 200 μg of RNA, while cells exposed to 100 μg showed no net increase over that seen in 10-minute cultures. The per cent of input radioactivity recoverable in cells exposed to 200 μg of RNA was 2.7%, and that of cells exposed to 150 μg was 1.2%, when cultures were terminated at 60 minutes.

TABLE II. Uptake of P^{32} Labeled RNA as a Function of Time.

Labeled RNA per flask (μg)	Time of incubation (min)	Input radioactivity (cpm)	TCA precipitable activity recovered per 5×10^6 cells (cpm)
100	10	5580	14.7
100	20	5580	35.4
100	40	5580	14.9
100	60	5580	16.7
150	10	8390	10.6
150	20	8390	20.4
150	40	8390	—
150	60	8390	101.3
200	10	11190	66.8
200	20	11190	48.1
200	40	11190	107.0
200	60	11190	305.7

The total amount of RNA incorporated into cells under optimal conditions, (200 μg per culture at input) estimated as the RNA-equivalent of the acid-precipitable radioactivity obtained, was 0.137 μg of RNA incorporated per 10^6 cells in culture.

Competitive inhibition by unlabeled RNA. Doubt existed as to whether the acid-precipitable radioactivity obtained may be attributed entirely to the uptake of labeled RNA. The cold TCA technique employed precipitates DNA and protein as RNA contaminants, all of which will contain label when the isotope employed is inorganic P^{32}. An experiment was conducted to determine whether unlabeled RNA placed in culture with a constant amount of P^{32} labeled RNA, will competitively inhibit the uptake of labeled RNA. Cells of each culture were exposed to 200 μg of labeled RNA (4110 cpm), plus an amount of unlabeled RNA as shown in Table III.

TABLE III. Competitive Inhibition of Uptake of P^{32} Labeled RNA by Unlabeled RNA.

Labeled RNA per flask (μg)	Unlabeled RNA per flask (μg)	TCA precipitable activity recovered per 5×10^6 cells (cpm)
200	0	113.9
200	40	83.5
200	80	62.3
200	120	51.8
200	160	44.6
200	200	31.7
200	240	16.4

The data in Fig. 3 indicate that uptake of labeled RNA was inhibited by unlabeled RNA, and that inhibition became progressively greater with larger amounts of unlabeled RNA. This study shows that inhibition of uptake approaches linearity with increasing concentrations of unlabeled RNA and suggests that a majority of the acid-precipitable radioactivity recovered from cultured cells may be attributed to the uptake of labeled RNA.

Discussion. Recent studies indicate that the extent of nucleic acid uptake by mammalian cells differs only slightly with the cell type and the system employed. Gartler(7) demonstrated uptake of DNA (0.1 μg/ml at

input) by Earle's L cells of approximately 0.04 μg per culture when maintained for 1 to 2 hours at 37°C. An average uptake of 2 to 4% of added DNA by Ehrlich-Lettre ascites cells in one hour cultures was reported by Kay(3).

The present data confirm the above findings by showing that rat spleen cells incorporate an average of 2.4% of added RNA, or 0.137 μg per 5×10^6 cells, in 1-hour cultures under optimal conditions. The data indicate that cell cultures exposed to P^{32} labeled RNA show maximal uptake when 200 μg per flask is employed. However, greater values of RNA become increasingly inhibitory, rather than simply ineffective. The cause of inhibition of uptake may be attributed to an increasing degradation of RNA to acid-soluble products as noted by Amos and Kearns(10). Cells may then incorporate labeled acid-soluble nucleotides, which would not appear in the acid precipitable cell extract (unless immediately reutilized within the cell) and would not contribute to the radioactivity obtained.

Addition to cultures of bovine pancreatic RNAse also results in a significant depression of RNA uptake. This effect varies directly with the amount of RNA employed. Cultures containing 200, 400, 600, and 800 μg of RNA in the presence of RNAse have uptake decreased to 38, 15, 11 and 5%, respectively, of non-RNAse treated cultures. The hydrolysis of RNA effected by RNAse resulted in the accumulation of acid soluble nucleotides at a greater rate than that obtained by simple degradation. Inhibition of RNA uptake in the presence of RNAse is correspondingly greater, supporting the belief that macromolecular RNA incorporation is significantly altered by fragments arising from its own breakdown.

The impetus for the present investigation is traceable to recent reports of the ability of RNA from immune animals to induce the synthesis of specific antibody by lymphoid cells from nonimmune animals. It was reported(12) that T^2 phage-specific antibody is formed by lymph node cells *in vitro,* when incubated with RNA extracted from macrophage cells that had been allowed to phagocytize T^2 bacteriophage *in vivo*. A further report(11) indicates that RNA from the spleens of mice immunized with sheep red blood cells successfully induced isologous nonimmune cells to synthesize antibody *in vitro*.

The results of the present study suggest that RNA uptake does occur in an amount sufficient to account for the above reports (11,12). However, no effort was made to determine the molecular size of the spleen RNA, or to measure its biological activity. Also, the possibility is not excluded that uptake represents adsorption sufficiently bound to cells to remain after repeated washing. Definite proof of incorporation will require the reisolation, after uptake, of RNA in macromolecular form, combined with evidence that the isolated molecule is structurally the same as the molecule supplied.

Summary. Suspensions of rat spleen cells were found to incorporate P^{32} labeled rat spleen RNA. A maximal incorporation of 2.4% of the input RNA was obtained when 4×10^7 viable cells were cultured in the presence of 200 μg of P^{32} labeled RNA. Larger amounts of RNA appear to inhibit uptake. It was shown also that the uptake of P^{32} labeled RNA is competitively inhibited by unlabeled RNA, and that inhibition of uptake is proportional to the competitive RNA concentration. It was concluded that the majority of the radioactivity obtained from treated cells may be attributed to the uptake of RNA.

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Observations on Calcareous Corpuscles of Larval *Echinococcus granulosus* of Various Geographic Origins. (30543)

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It has been reported(1) that calcareous corpuscles isolated from different species of tapeworms differ considerably in Ca:Mg:P:CO_2 ratios. As a probable consequence the crystallization patterns which were induced in the amorphous corpuscles by heat showed rather remarkable differences especially with respect to the nature of the P-containing minerals. Evidently the question can be raised whether the observed variations really are species-specific, or a fortuitous consequence of the fact that the worms in all likelihood had different food materials available and thus lived in environments differing in ionic composition. In this respect it should be mentioned that the worms studied were derived from various hosts, or, as was the case with the human parasites *Taenia saginata* and *Diphyllobothrium latum* from people living geographically far apart (Lebanon, Finland) and thus presumably differing in their food habits. It was therefore deemed of interest to study the calcareous corpuscles of a tapeworm that is less host-specific than those used before and that could be secured from different geographic localities. Larval *Echinococcus granulosus* was selected as one of the most suitable species.

Materials and methods. Lyophilized scolices of *Echinococcus granulosus* isolated from sheep livers in Chile and from cattle livers in Lebanon were obtained respectively through the courtesy of Dr. M. Agosin and Dr. R. Thorson. Mixed *Echinococcus* material isolated from liver and lung cysts of New Zealand sheep, first stored in alcohol and then dried, was contributed by Dr. D. J. Morseth.

The calcareous corpuscles were isolated by the ethylenediamine procedure described previously(2) and the methods of analysis coincided with those detailed earlier(2,3).

Results and discussion. Despite their varied origins the parasites contained essentially identical amounts of calcareous corpuscles (Table I) and there was little variation in Ca and CO_2 content of the latter. The Chilean material, however, contained somewhat more Mg and less P than the corpuscles isolated from either the New Zealand or the Lebanon samples. All corpuscles contained appreciable amounts of hydrogen which, when calculated as water, corresponded rather closely to the weight losses observed upon heating to 300°C (Table II), indicating probably that during heating tightly bound water was lost. It should be noted that the corpuscles of another larval cestode, *Cysticercus fasciolaris*, showed a much less pronounced weight loss even after heating to 400°C(2). Our material contained in all cases some nitrogen, undoubtedly referable to organic material that has not been destroyed by the ethylenedi-