Precursor-Product Relationships between Nucleotides and RNA during Differentiation in *Dictyostelium discoideum*

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SUMMARY

Fourteen of the sixteen nucleotides present in extracts of *Dictyostelium discoideum* were identified by thin-layer chromatography. Data are presented describing the incorporation of radioactive bases into the guanine nucleotide pools and into RNA. Studies with labelled guanine suggested that a small fraction of the RNA undergoes rapid turnover at the pre-culmination stage of differentiation. Analytical methods are described for determining flux rates *in vivo* and the percentage turnover of endogenous pools.

INTRODUCTION

We have described previously some aspects of nucleotide metabolism in *Dictyostelium discoideum* (Rutherford & Wright, 1971). This report examines the role of nucleotides as precursors for RNA synthesis during differentiation, and demonstrates that an analysis of RNA turnover requires, in addition to a determination of RNA and nucleotide specific radioactivity, kinetic information on the nucleotide precursor pool. Determination of the fluxes and turnover rates *in vivo* can be complex. Too often these parameters have been calculated from simplified models which represent the flow of radioactivity from precursor to product as would occur in an *in vitro* system, rather than fully considering the complexities *in vivo*. In some cases, it is assumed that the rate of incorporation of exogenous radioactive precursors into a macromolecule truly reflects the absolute rate of synthesis (Hirschberg, Ceccarini, Osnos & Carchman, 1968), but this rate of incorporation rarely reflects synthesis alone and degradation must be considered. Likewise, the macromolecule may be synthesized from only a portion of the soluble precursor pool (Cowie & Bolton, 1957; Plagemann, 1971, 1972). The addition of labelled precursors may cause changes of the internal pool size or of the intracellular flux rates. The rates of cell growth and division, if they occur, must also be taken into account.

The present investigation was undertaken to consider a number of these possibilities during differentiation in *D. discoideum* and to obtain information about the turnover *in vivo* of nucleotides and RNA during this process. From the results and those reported earlier (Rutherford & Wright, 1971), it is apparent that the interpretation of incorporation experiments *in vivo* may be somewhat simplified in this case because of the unique properties of this organism. During differentiation, *Dictyostelium* can best be represented as a closed system. There is no requirement for external nutrients, nor is there any cell growth, and the

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calculation of intracellular flux rates is therefore not complicated by these variables. The primary internal metabolism of carbohydrates during differentiation is geared toward the conversion of soluble glycogen to new carbohydrates, primarily trehalose and cellulose (Ceccarini & Filosa, 1965; P. A. Rosness and B. E. Wright, unpublished results).

The apparent lack of significant compartmentalization of low molecular weight precursors in *D. discoideum* further simplifies the interpretation of *in vivo* incorporation kinetics. For example, the lack of significant compartmentalization of the nucleotides UTP, GTP and ATP has previously been shown (Rutherford & Wright, 1971) and the present analyses provide further evidence. Wright (1968) has developed a kinetic model of carbohydrate metabolism during differentiation based in part upon flux rates *in vivo*. Flux rates are known for the synthesis and degradation of glycogen (Marshall, Sargent & Wright, 1970) the synthesis of trehalose (Sargent & Wright, 1971) and uridine-5'-diphosphoglucose (UDPG) (Pannbacker, 1967), and for the turnover of protein (Wright & Anderson, 1960). In these cases, no significant compartmentalization of the precursors was apparent, and the resulting flux values were consistent with the observed accumulation of the end products of differentiation (Gustafson & Wright, 1972).

METHODS

The harvesting and the conditions of incubation of organisms in the presence of label, and the nucleotide isolation procedures are as described by Rutherford & Wright, 1971. Compounds isolated by thin-layer chromatography (Randerath & Randerath, 1964*a*; Rutherford & Wright, 1971) were identified as follows: (i) Guanine spots were located by exposing the thin-layer sheets to HCl vapours. This treatment causes guanine nucleotides to exhibit a characteristic blue fluorescence when viewed under u.v. light. Other compounds were tentatively identified by reference to nucleotide maps (Randerath & Randerath, 1964*b*). (ii) Ultraviolet absorption spectra of the unknown and authentic compounds. (iii) Incubating organisms in the presence of a labelled base. After chromatography and autoradiography, the compounds containing the radioactive base were detected as dark spots on the X-ray film. (iv) The migration of the unknown compound and an authentic nucleotide during thin-layer chromatography. The unknown spot was cut out and placed in anhydrous methanol for 10 min to remove salt remaining from the first chromatographic solvent. The authentic nucleotide was added and the dry cutout was attached to a fresh thin-layer sheet so that the cellulose layers were in contact. Chromatography was then carried out in LiCl and formate buffer (Randerath & Randerath, 1964*b*). The identity of the radioactive unknown and authentic compound were determined by comparison of their R_F values or by utilizing the finger-print technique (Roberts *et al.* 1963). Chromatographic separation of the nucleotides was greatly facilitated by cutting the sheet in half after development in the first dimension. Thus, in the second dimension the more polar compounds could be chromatographed in a higher-concentration formate buffer (4 M, pH 3.4) and the less polar compounds in a lower-concentration formate buffer (0.5 M, pH 3.4).

Specific radioactivities and pool sizes of the nucleotides were determined from their absorbance maxima and radioactivity (LS-200 liquid scintillation counter, Beckman Instruments). Nucleotide concentrations are expressed as mol/l packed cells (Barravecchio, Baumann & Wright, 1969).

Extraction of RNA and determination of the specific radioactivity of 2'3'-GMP resulting from RNA hydrolysis were accomplished by either of two procedures. Organisms (4 ml; 0.4 ml packed volume) were incubated with a labelled precursor for various times. An equal

volume of cold 7 % (w/v) perchloric acid (PCA) was then added. After 30 min in an ice bath, the PCA extract was centrifuged and the supernatant removed for the isolation of acid-soluble nucleotides. The acid-insoluble precipitate was washed with 8 ml of 95 % (v/v) ethanol to remove the lipids. RNA from the lipid-free residue was hydrolysed with 3.5 % (w/v) PCA at 4 °C for at least 18 h and then neutralized with 1 N-KOH. Alternatively, the lipid-free residue was suspended in 1.5 ml of 0.3 N-KOH and hydrolysed for 18 h at 37 °C. The solution was acidified by adding 0.15 ml of 35 % (w/v) HClO₄, centrifuged, and the supernatant neutralized with 1 N-KOH. The 2'3'-GMP from the RNA hydrolysates was then isolated by the procedure of Randerath & Randerath (1965).

The distribution of radioactivity between the organisms and that free in the medium during incubation with labelled bases (New England Nuclear, Boston, Massachusetts, U.S.A.) was determined in samples (0.2 ml) of suspension added to 10 ml aquasol (New England Nuclear). The remainder of a 0.5 ml sample was centrifuged (1000 g, 1 min) and the radioactivity determined in a sample of the supernatant. The radioactivity associated with the organisms was determined by subtraction.

Labelling experiments were routinely done in liquid suspension. However, a comparative study was made under conditions in which differentiation proceeded normally (Newell, Telser & Sussman, 1969). Millipore absorbant (nutrient) pads (142 mm diam) were saturated with 0.1 M-phosphate buffer (pH 6.5) containing 1.0 mM-EDTA and placed in 150 × 25 mm plastic Petri dishes (Lab-Tek, Westmont, Illinois, U.S.A.). Washed amoebae (1.5 ml) were spread onto Whatman No. 50 filter paper (9 cm) and placed on the pads. At the desired stage of differentiation the filter papers were transferred to Petri dishes containing absorbant pads saturated with Bonner's (1947) 'standard solution', buffered at pH 6.5 with 1.0 mM-2(*N*-morpholino) ethane sulphonic acid and containing a labelled precursor. In other experiments labelled compounds were mixed with 1 ml of Bonner's solution, pipetted directly into the Petri dishes, and the filter papers placed in contact with the radioactive solution. At various times filter papers were removed and the organisms washed into test tubes with 8 ml of 3.5 % (w/v) perchloric acid.

RESULTS

Identification of nucleotides and nucleotide sugars after two-dimensional thin-layer chromatography

Nucleotides and nucleotide sugars were separated by thin-layer chromatography, to determine pool sizes, the amount of radioactive incorporation and their specific radioactivity. Their positions after two-dimensional thin-layer chromatography are shown in Fig. 1 and by using the criteria described in Methods, 14 of the 16 spots were identified. The pattern shown in Fig. 1 was found at all stages of differentiation. Spots corresponding to cytosine nucleotides were not observed. The spot just below NAD⁺ was UMP, resulting from the breakdown of UDPG during chromatography in the acidic formate buffer, and its specific radioactivity was always the same as that of UDPG. Furthermore, in chromatograms in which the UDPG spot was relatively dark, little UMP was present; conversely, when the UDPG spot was faint, a dark UMP spot appeared. The spot just below ADP in Fig. 1 exhibited a brilliant pale-blue fluorescence under u.v. light. Its adsorption spectrum in 0.02 M-tris-HCl pH 7.4 containing 0.7 M-MgCl₂ had two major peaks, one at 260 nm, the other, a broad peak, at 365 nm. The fluorescence emission spectrum showed a peak at 460 nm which was completely destroyed by HCl at a final concentration of 1.2 N. The compound did not co-chromatograph with either NADPH or NADH, nor was it radioactive

Fig. 1. Identification of nucleotides and nucleotide sugars after two-dimensional chromatography. The chromatogram was developed in the first dimension with 0.2 M-LiCl for 2 min, with 1.0 M-LiCl for 6 min, and finally with 1.6 M-LiCl to the top of the sheet. After drying and soaking in absolute methanol for 10 min the thin-layer plate was cut into two sections as shown in the Figure. The section shown on the left was developed in 0.5 M-formate buffer, pH 3.4, the section on the right in 4 M-formate buffer, pH 3.4. Compounds were located by their fluorescence under u.v. light. The Figure is a tracing of a representative experiment. Abbreviations: GTP, guanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; IMP, inosine monophosphate; UDPG, uridine-5'-diphosphoglucose; NAD⁺, nicotinamide-adenine dinucleotide; UNK, unknown.

when labelled adenine, guanine, or uracil was added to a cell suspension (all the other compounds which were identified were labelled by at least one of the radioactive bases). On the basis of these characteristics the compound was judged to be a folic acid derivative although exact identification was not attempted.

When labelled adenine was incubated with the cells, radioactivity was found in the adenine nucleotides (ATP, ADP, AMP, NADP⁺) in inosine monophosphate (IMP) and in the guanine nucleotides (GTP, GDP, GMP). Labelled guanine and uracil were incorporated only into their respective nucleotides or nucleotide sugars during the period of incubation.

Incorporation patterns of ATP, GTP and UTP in the presence of labelled bases

Figure 2 shows the incorporation patterns of ATP, GTP and UTP at the pseudoplasmodium stage of development. Cell suspension (4 ml; 0.4 ml packed organisms) was incubated in the presence of 0.04 μ mol (10 μ M) of [¹⁴C]adenine (52 mCi/mmol), [¹⁴C]guanine (52 Ci/mmol) or [¹⁴C]uracil (56.3 mCi/mmol). Cytosine nucleotides were not present in high enough concentrations for accurate measurement. The initial rate of labelled uracil incorporation into UTP was only about 5 % of that of adenine and guanine into ATP and GTP. The

Fig. 2. Incorporation patterns of nucleoside triphosphates. We added $0.04 \mu\text{mol}$ each of [^{14}C]adenine (52 mCi/mmol), [^{14}C]guanine (52 mCi/mmol) and [^{14}C]uracil (56.3 mCi/mmol), in separate incubation flasks, to 4 ml of organism suspension (0.4 ml packed cell volume) taken at the pseudoplasmodium stage of development. Nucleotides were isolated from acid-soluble extracts of cells, as described in Methods. ○, Specific radioactivity of ATP; □, specific radioactivity of GTP; △, specific radioactivity of UTP.

differences in the initial rates of incorporation probably reflect the permeability of the cell membrane to the three nucleotide bases, rather than actual differences in the rate of turnover of the nucleoside triphosphate. We have shown previously (Rutherford & Wright, 1971) that for both [^{14}C]adenine and [^{32}P]Pi the incorporation of isotope into nucleotides is limited by its transport across the cell membrane.

The plateau curve obtained for GTP incorporation was not related to its cellular concentration, as the pool sizes of UTP and GTP are similar. Furthermore, the organism density and the concentration of the three bases in the incubation medium were the same for the three nucleotides.

Comparison of the radioactivity in the incubation medium and that associated with the organisms during incubation in the presence of free bases

The labelling patterns of nucleoside triphosphates observed in Fig. 2 also occurred at all later stages of differentiation. The relationship between the amount of free base in the medium and that associated with the organisms was therefore measured. When organisms at the culmination stage of development were incubated with labelled adenine there was a slow loss of radioactivity from the medium (Fig. 3) and a nearly linear rate of uptake by

Fig. 3. Comparison of the radioactivity of medium and organisms during incubation in the presence of [^{14}C]adenine or [^{14}C]guanine. We added $0.04 \mu\text{mol}$ of the labelled base to 3 ml of suspension (0.33 ml packed organisms) at the culmination stage of development. $\circ$, [^{14}C]adenine; $\square$, [^{14}C]guanine.

Table 1. *Distribution of radioactivity during incubation of cells in the presence of [^{14}C]guanine*

Time of incubation (min)	$10^{-5} \times \text{PCA soluble (c.p.m.)}$	$10^{-5} \times \text{PCA insoluble (c.p.m.)}$	$10^{-3} \times \text{GDP (c.p.m.)}$	$10^{-3} \times \text{GTP (c.p.m.)}$	$10^{-5} \times 2',3'\text{-GMP (RNA) (c.p.m.)}$
5	1.60	0.46	1.08	2.46	0.17
10	1.26	0.95	0.85	2.00	0.46
15	1.05	1.35	0.70	1.58	0.90
20	0.87	1.62	0.49	0.92	1.04
40	0.71	1.38	0.15	0.27	1.08

[^{14}C]guanine ($0.004 \mu\text{mol}$) was added to 4 ml of suspension (0.28 ml packed cells) at the early sorocarp stage of development. The PCA-insoluble counts refer to the radioactivity released from the acid-insoluble material after hydrolysis in 0.3 N-KOH for 18 h at 37°C . Heating the remaining pellet at 100°C for 18 min did not release additional radioactivity. $2',3'\text{-GMP}$ was released from the PCA-insoluble material after hydrolysis. GTP and GDP were isolated from the acid-soluble fraction.

the organisms. Conversely, for organisms incubated with labelled guanine the radioactivity in the medium and that associated with the organisms was constant after five minutes (Fig. 3), at about tenfold higher than the extracellular level. This difference in adenine and guanine uptake undoubtedly contributes to the differences observed between the incorporation of their respective nucleoside triphosphates.

Distribution of radioactivity after incubation of organisms in the presence of [^{14}C]guanine

Table 1 shows the distribution of radioactivity in each component of the incubation mixture, a slow loss of radioactivity from the PCA-soluble fraction being concomitant with

Fig. 4. Incorporation patterns of GTP and GDP. We added 0.004 μmol [^{14}C]guanine to 4 ml of suspension (0.62 ml packed organisms) at the late culmination stage of development. $\circ$, Specific radioactivity of GDP; $\triangle$, specific radioactivity of GTP.

a corresponding uptake of radioactivity into the PCA-insoluble fraction. The sum of the two fractions was essentially constant with time. Of the radioactivity in the acid-insoluble fractions, about 60% was accounted for in the 2',3'-GMP resulting from RNA hydrolysis. The only nucleotide labelled in the RNA hydrolysate was 2',3'-GMP.

The percentage contribution of the guanine nucleotides to the total soluble radioactivity was also investigated. The combined GTP and GDP radioactivity was about 1% of the total radioactivity in the acid-soluble fraction. The remainder of the radioactivity originally associated with the acid-soluble fraction was found in the methanol wash of the thin-layer chromatography sheets used before the first-dimensional chromatography. In order to identify this radioactive component, the methanol wash was concentrated to 1 ml by evaporation at room temperature under a stream of nitrogen. A sample (20 ml) of this solution was then applied to Whatman No. 1 filter paper and chromatographed in the following solvents: isopropanol:HCl:water (65:16.6:18.4, by vol.); isopropanol:2N-HCl (65:35, v/v); butanol:acetic acid:water (25:4:10, by vol.). The one radioactive spot found was identified as guanine on the basis of the similarity of R_F values between the unknown and authentic guanine, and of the shape of the fingerprint (Roberts *et al.* 1963) after autoradiography.

Incorporation patterns in the presence of low concentrations of labelled guanine

Figure 4 shows the incorporation of labelled guanine into GTP and GDP. By using low levels of exogenous base, the amount of label available to the internal nucleotide pools and to RNA is limited. Therefore, cycling of radioactivity between precursor and product, if it occurs, can be readily studied without the complexity of additional uptake from the medium. Figure 4 shows that after an initial period of uptake, the radioactivity associated with the nucleotides fell to an intermediate plateau value. The incorporation of GMP is not shown because the level was too low to measure accurately. The incorporation curves for GTP and GDP are identical, indicating a very rapid equilibration between these two

Fig. 5. Incorporation of [^{14}C]guanine into GTP and RNA (2',3'-GMP). We added $0.004\ \mu\text{mol}$ of [^{14}C]guanine to 4 ml of suspension ($0.58\ \text{ml}$ packed organisms) at the culmination stage of development. $\circ$, Specific radioactivity of GTP; $\triangle$, specific radioactivity of 2',3'-GMP resulting from RNA hydrolysis. Note that the values for GTP are 100 times those for RNA.

pools. Similar incorporation curves (not shown) were found for the incorporation of labelled uracil into UTP and for labelled adenine into ATP.

In the analysis of the precursor-product relationship between nucleotides and RNA, guanine was used as the precursor because it is incorporated only into guanine nucleotides. Labelled adenine, on the other hand, is incorporated into both adenine and guanine nucleotides, as well as into inosine monophosphate, while uracil labelling is complicated by incorporation into a large pool of UDPG, as well as the uridine nucleotides. In addition both GTP and GDP were widely separated from other nucleotides during chromatography. Thus, errors in determining pool sizes or specific radioactivity was minimized and mathematical analysis greatly simplified in the case of guanine labelling.

Figure 5 shows the pattern of incorporation of low levels of [^{14}C]guanine into GTP and into 2',3'-GMP after RNA hydrolysis. The shapes of the curves indicate a typical product-precursor relationship between GTP and RNA respectively. Label was rapidly incorporated into GTP until the supply of guanine from the medium was exhausted. Labelled material was then lost from the precursor GTP pool to the product RNA, until a constant value was reached at a specific radioactivity of about $3.3 \times 10^4\ \text{c.p.m./}\mu\text{mol}$. After a short lag, RNA increased in specific radioactivity, then reached a plateau value of about $5.7 \times 10^2\ \text{c.p.m./}\mu\text{mol}$. These incorporation curves were obtained in eight separate experiments; in each case a constant plateau value for GTP and RNA was found, although the peak specific radioactivity value for GTP varied from experiment to experiment. Similar results were obtained when using [^{14}C]guanosine as the precursor.

To determine whether the incubation conditions produced a progressive impairment of RNA synthesis, a 10 min pulse of [^{14}C]guanine was added, successively, to each of 10 flasks shaken from 10 to 100 min. The resulting equilibrium specific radioactivities between GTP and RNA did not change significantly. Cells incubated under these conditions differentiate normally when removed to a solid surface (Marshall *et al.* 1970).

Weber & Rubin (1970) have shown that the rate of incorporation of [^3H]uridine into RNA by chick embryo fibroblasts and mouse 3T3 cells is reduced, as a function of increased cell density. We therefore tested whether the amount of cells per incubation solution

could affect the percentage of RNA turnover. The cells were harvested from agar sheets in the usual manner and the packed cell volume determined. Bonner's (1947) 'standard solution' was then added to give packed cell volumes (ml packed cells/ml cell suspension) of 0.045, 0.090 and 0.150. Portions (4 ml) of each suspension were then added to rotating flasks and incubated with labelled guanine as described previously. Although the specific radioactivity of both the precursor and the product were higher for the samples with low cell density than for samples with high cell density, there was no difference in the shape of the incorporation curves. Thus, the density of cells in the incubation flasks did not affect the conclusions to be derived from the incorporation curves.

Long-term incubation studies during differentiation of D. discoideum on filter paper discs

The incorporation kinetics between GTP and RNA were also studied under conditions in which normal differentiation occurred. Organisms were incubated with [^{14}C]guanine in separate Petri dishes as described in Methods, and the nucleotides were isolated at appropriate times. The resulting incorporation curves for the incubations in shaking flasks (Fig. 5) and those on filter paper were similar. In both cases a peak in the specific radioactivity of GTP occurred as precursor was exhausted from the medium, followed by a loss of radioactivity to RNA. Likewise, in both incubation procedures GTP and RNA reached constant specific radioactivities. Incubation under liquid conditions was used routinely, as this method is technically easier.

Test of the significance of bacterial contamination on the in vivo incorporation patterns

Experiments were designed to test whether bacteria, associated with washed slime mould organisms, could contribute to the incorporation of label into either GTP or RNA. Organisms were harvested from agar sheets and washed three times in cold sterile distilled water by centrifuging at 1000 *g*. After the final wash, half the organisms were plated onto Petri dishes containing non-nutrient agar, and the other half onto Petri dishes containing non-nutrient agar plus streptomycin sulphate (0.5 mg/ml). The organisms were allowed to differentiate to the culmination stage, then were washed from the agar with 4 ml of sterile Bonner's solution buffered with 2-(*N*-morpholino)-ethanesulphonic acid. A sample of the suspension was removed for bacterial counting and the remainder incubated for 40 min with radioactive guanosine. Although the streptomycin treatment reduced the level of remaining bacteria from 5×10^4 cells/ml to less than 1×10^2 cells/ml, there was no change in the specific radioactivities of either GTP or RNA. In fact, bacterial contamination significantly contributed to the results of incorporation studies only when the level of bacteria reached 1×10^7 /ml in the incubation suspension. This is the same level of bacteria for measurable incorporation of uridine into RNA as that found by Hirschberg *et al.* (1968).

We also tested whether the numbers of bacteria present at the culmination stage had any effect on the overall incorporation curves. Organisms were allowed to differentiate in the presence and absence of streptomycin as described above, then harvested and incubated in the presence of label. Similar incorporation curves were obtained in both streptomycin-treated organisms and in controls.

Development of a computer model for analysis of nucleotide and RNA turnover

A computer model of the observed precursor-product relationship between nucleotides and RNA was developed in order to determine the flux rate *in vivo* between nucleotides and

Fig. 6. Model of precursor-product relationships between nucleotides and RNA.

Fig. 7. Best fit to the guanine nucleotide-RNA radioactive tracer experiment using the model in Fig. 6. The specific radioactivities of RNA have been multiplied by 43.0 to account for the compartmentalization of the RNA pool. $\square$, Measured nucleotide specific radioactivities; $\circ$, measured RNA specific radioactivities multiplied by the compartment factor.

RNA, the percentage of RNA which was undergoing rapid turnover, and the rate of turnover of this RNA. The calculations were performed on an IBM-360, Model 65, computer using a program, TRACER, developed by Park. TRACER is written in the programming language PL/I, requires 150 bytes of core storage, and is a general purpose program for the analysis of tracer flow in steady state systems.

The model which was used to represent the experimental data (Fig. 6) assumes that the metabolite pools remain constant in size during the experiment; this has been demonstrated experimentally for RNA and the nucleotide pools (Pannbacker, 1966, 1967; Rutherford & Wright, 1971). In accord with this assumption the chemical fluxes, expressed as mm/min

Table 2. Calculated parameters for guanine-RNA experiment using the model

Rapid turnover RNA concn (C_3)	0.12 mM
RNA in rapid turnover pool	2.3 %
Extracellular guanine concn referred to cell volume	4.54×10^{-5} mM
Chemical flux loop 1 (I_1)	1.472×10^{-5} mM/min
Chemical flux loop 2 (I_2)	3.91×10^{-3} mM/min
Turnover time of nucleotide pool	18 min
Turnover time of rapid RNA pool	30 min

(mmol/l packed cells/min) always appear as flux loops (I_1 and I_2). The intracellular guanine nucleotide pool is actually composed of three pools (GMP + GDP + GTP) which turn over among themselves so rapidly that they reach radioactive equilibrium within seconds (Fig. 4), a short time compared with any significant measurement interval in the experiment. In addition, the exogenous or extracellular guanine pool is initially radioactive and all other pools are initially unlabelled.

The guanine RNA pool in the model is composed of two fractions. One fraction, called the rapid turnover RNA, exchanges with the nucleotide pool via the flux loop I_2 , the other fraction comprises the remainder of the RNA pool. The latter fraction has no exchange with the nucleotide pool and hence remains non-radioactive. In the model, C_3 is the concentration of the rapid turnover RNA, C_4 is the total RNA concentration.

Figure 7 shows the best fit of computer-generated curves to the experimental data. The curves were obtained after optimizing the flux loops and adjusting the size of the RNA pool undergoing turnover to the correct RNA specific radioactivity at equilibrium. The parameters resulting from this analysis are shown in Table 2. The concentration of RNA undergoing rapid turnover was about 0.12 mM, or 2 % of the total RNA. The time required for this pool of RNA to turn over was about 30 min.

DISCUSSION

The rate of RNA turnover in *Dictyostelium discoideum* has been estimated previously by several different methods (Hirschberg *et al.* 1968; Inselburg & Sussman, 1967; Pannbacker, 1967). However, the *percentage* of the RNA pool which is undergoing turnover at a particular stage of differentiation has not been accurately determined, primarily because of the lack of an appropriate method for determining this parameter. We have found that a small, rapidly turning over pool of RNA can be detected by using low concentrations of radioactive precursor and following the incorporation pattern of precursor nucleotide and product RNA as radioactive equilibrium occurs. By using low concentrations of radioactive precursors it is possible to attain isotopic equilibrium values for precursor and product. The cell acts as a closed system, since no net radioactivity is lost from the precursor or from the product during the rather short incubation period (Table 1). Thus, the distribution of radioactivity between precursor and product and the resulting incorporation curves are direct reflections of the interconversion of internal pools.

The method for determining the percentage of the RNA which is turning over can best be seen by comparing the incorporation curves for material in which the total RNA pool is turning over and for that in which only a small fraction of the RNA pool is turning over. Figure 8(a) shows the expected incorporation curves when the entire product pool is turning over. The precursor released by degradation of the product would be re-utilized in the synthesis of the product. In this case of total product turnover, the precursor and product

Fig. 8. Schematic incorporation curves of radioactive precursor into GTP and 2',3'-GMP (RNA). (a) Incorporation resulting from complete turnover of the RNA pool. (b) Incorporation resulting from partial turnover of RNA pool. In both (a) and (b) the precursor released by the degradation of the product would be utilized in the resynthesis of the product. —, Specific radioactivity of GTP; ----, specific radioactivity of 2',3'-GMP (RNA).

come to the same equilibrium specific radioactivity. Figure 8(b) shows the incorporation curve when only a small percentage of the total RNA pool is turning over. The portion of the RNA pool undergoing turnover also comes into equilibrium with the precursor. However, when the organism is ruptured before isolation of the RNA, both the radioactive pool of RNA (turnover RNA) and the non-radioactive pool are mixed. The resulting specific radioactivity of the total pool is therefore lower than that of the 'turnover RNA' owing to the large pool of unlabelled RNA. Thus, the equilibrium specific radioactivity as shown in the examples in Figs. 8 and 5 indicates the percentage of the RNA pool which is turning over.

These methods do not distinguish the type of RNA undergoing turnover. For example, we cannot conclude that this 'turnover RNA' is DNA-like heterogeneous RNA. Nevertheless, the value of 2 %, for the fraction of RNA undergoing turnover in 30 min, is well within the corresponding literature values (Kijima & Wilt, 1969; Leighton & Doi, 1971; Nierlich & Vielmetter, 1968; Salser, Janin & Levinthal, 1968; Watts, 1964). This turnover time is also similar to that required for the synthesis and transport of nuclear RNA to the cytoplasm of *D. discoideum*. Kessin (1973) has shown that ribosomal RNA species appear in the cytoplasm 6 to 7 min after the onset of labelling of vegetative cells of *D. discoideum*, and after 30 min only 10 % of the label remains associated with the nucleus. In developing organisms, RNA appears in the cytoplasm at a reduced rate. Similar results were obtained by Firtel & Lodish (1973). Under conditions in which the synthesis of rRNA was inhibited, cells were pulse labelled for 1 min with [^3H]uracil. After a subsequent 20 min chase period, 8 % of the radioactivity remained in the nucleus, while the majority of label appeared in the cytoplasm.

To develop a valid model of precursor-product interactions, the distribution of radioactivity associated with all components of the incubation system must be determined. We found, for example, that within 5 min of exposure to labelled guanine, at least 90 % of the label was associated with the organisms (Fig. 3). During further incubation, radioactivity was lost from the acid-soluble fraction into RNA (Table 1). Although 99 % of the radioactivity remaining in the acid-soluble fraction during the incubation period is apparently free guanine, the shape of the incorporation curve for GTP and RNA is typical of a system in which the radioactive precursor has been exhausted from the medium. Therefore, a large portion of the guanine added to the medium must be metabolically inaccessible to the

GTP and RNA pools during the incubation period. This inaccessibility could result from the ionic properties of the cell membrane, resulting in binding of extracellular guanine. This is suggested from the rapid association of radioactivity with the cells as shown in Fig. 3. Alternatively, the radioactive guanine may permeate the cell, but most of it may be 'trapped' in a compartment inaccessible to the GTP-RNA system.

The experimental data are consistent with models in which the degradation products of RNA re-enter the nucleotide pool. This interpretation is supported by the recent work of Hurlbert & Hardy (1972), who showed that in rat liver nuclei most of the degradation of heterogeneous RNA is via 5'-nucleotides which may be reutilized for RNA synthesis. Since the rapid turnover fraction of RNA is probably heterogeneous, perhaps what is measured is an average of many smaller pools turning over at different rates. However, in fitting a kinetic model to the experimental data it is appropriate to use as simple model as possible, consistent with the data. A number of more complicated models were seriously considered, but did not improve the fit to the data. This does not mean that nucleotide and RNA metabolism is not more complex than is shown by the model used. Nevertheless, by using the model and optimizing the flux loops and adjusting the percentage of RNA turnover to give the correct RNA specific radioactivity when radioactive equilibrium has been achieved, a fit to the data shown in Fig. 7 was obtained.

The modelling of the extracellular guanine pool requires special discussion. This extracellular precursor is distributed in the volume of the suspension, rather than within the organism volume, so its concentration must be referred to the cell volume. (The extracellular volume was approximately 10 times the cell volume.) In addition, only part of the extracellular guanine is converted to the intracellular guanine-nucleotide pool (Table 1). Lastly, the extracellular guanine pool may not remain constant in size during the time of incubation, but may be completely taken up by the organism. This situation has been approximated in the model (Fig. 6) by using an extracellular pool whose radioactivity decreases during the period of incubation. Hence, there is a flux loop, I_1 , which is completed through a residuum pool. The residuum pool is of infinite size, thus accepting radioactive molecules but returning only non-radioactive molecules. The extracellular guanine pool is 1000 times smaller than the intracellular nucleotide pool, so that even if the extracellular guanine pool were taken up by the intracellular nucleotide pool, its effect on pool size would be negligible.

To interpret radioactive tracer experiments it is important to make a kinetic model of the experiment which is as explicit and complete as possible. Such models also help to avoid errors in interpreting experimental data. For example, it is not correct simply to use the specific radioactivity of the extracellular base or of the acid-soluble fraction as a precursor function for the RNA. In the case of the experiment shown in Table 1, much of the extracellular base did not participate in the experiment. The acid-soluble fraction contains these 'inactive' bases as well as those participating as RNA precursors. The extracellular precursor specific radioactivity is not equal to the intracellular product specific radioactivity in the case of GTP in the present analysis, UTP and UDPG (Pannbacker, 1967), or methionine (Wright & Anderson, 1960). Thus, rates of synthesis calculated on the basis of the specific radioactivity of the added extracellular precursor are not valid.

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