Chain Extension of Ribonucleic Acid by Enzymes from Rat Liver Cytoplasm

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1. The 105 000g supernatant fraction of rat liver catalyses the incorporation of ribonucleotides from ribonucleoside triphosphates into polyribonucleotide material. The reaction requires Mg^{2+} ions and is enhanced by the addition of an ATP-generating system and RNA, ATP, UTP and CTP but not GTP are utilized in this reaction. In the case of UTP, the product is predominantly a homopolymer containing 2–3 uridine residues, and there is evidence that these may be added to the 3'-hydroxyl ends of RNA or oligoribonucleotide primers. 2. The microsome fraction of rat liver incorporates ribonucleotides from ATP, GTP, CTP and UTP into polyribonucleotide material. This reaction requires Mg^{2+} ions and is enhanced slightly by the addition of an ATP-generating system, and by RNA but not DNA. Supplementation of the reaction mixture with the three complementary ribonucleoside 5'-triphosphates greatly increases the utilization of a single labelled ribonucleoside 5'-triphosphate. The optimum pH is in the range 7.0–8.5, and the reaction is strongly inhibited by inorganic pyrophosphate and to a much smaller degree by inorganic orthophosphate. It is not inhibited by actinomycin D or by deoxyribonuclease. In experiments with [^{32}P]UTP in the absence of ATP, GTP and CTP, 80–90% of ^{32}P was recovered in UMP-2' or -3' after alkaline hydrolysis of the reaction product. When the reaction mixture was supplemented with ATP, GTP and CTP, however, about 40% of the ^{32}P was recovered in nucleotides other than UMP-2' or -3'. Although the reactions seem to lead predominantly to the synthesis of homopolymers, the possibility of some formation of some heteropolymer is not completely excluded.

Studies on the kinetics of incorporation of labelled nucleotides into RNA, on the effect of actinomycin D on RNA synthesis both *in vivo* and *in vitro*, and on the ability of all known classes of RNA to form specific hybrids with homologous DNA, indicate that all cellular RNA is ultimately derived from DNA templates. In addition, a DNA-dependent RNA polymerase has been observed in bacteria (Chamberlin & Berg, 1962; Krakow & Ochoa, 1963; Nakamoto, Fox & Weiss, 1964), and in nuclear preparations of plant (Rho & Bonner, 1961; Mans & Novelli, 1964; Semal, Spencer, Kun & Wildman, 1964) and animal cells (Furth & Loh, 1965; Eason & Smellie, 1965; Ballard & Williams-Ashman, 1966). In cells infected with RNA viruses (Weissmann, Simon, Borst & Ochoa, 1963; Eason, Cline & Smellie, 1963; Haruna & Spiegelman, 1965; Horton, Liu, Martin & Work, 1966) a new RNA-dependent RNA polymerase is elaborated in response to infection.

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The RNA-dependent incorporation of ribonucleotides into RNA has also been described in fractions from uninfected cells. Such reactions include the synthesis of the terminal pCpCpA sequence of transfer RNA by cytoplasmic enzymes, which is well documented (Heidelberger, Harbers, Leibman, Takagi & Potter, 1956; Daniel & Littauer, 1963), but the significance of other soluble fractions that catalyse the incorporation of ribonucleotides into oligo- and poly-ribonucleotides remains obscure (Chung, Mahler & Enrione, 1960; Burdon & Smellie, 1962; Edmonds & Abrams, 1962; Klemperer, 1963a,b, 1964, 1965; Ventkataraman & Mahler, 1963). There are also reports of the RNA-dependent uptake of ribonucleotides catalysed by particulate fractions of both bacterial (August, Ortiz & Hurwitz, 1962; Smith & August, 1966) and animal cells (Baltimore & Franklin, 1963; Eason & Smellie, 1965; Dalgarno, Liu, Martin & Work, 1966; Naora, 1966; Wykes & Smellie, 1966).

The present work is concerned with the incorporation of ribonucleotides into polyribonucleotides by

the soluble fraction and by the microsomal fraction of rat liver.

MATERIALS AND METHODS

Materials. Ribonucleoside 5'-mono-, -di- and -tri-phosphates and deoxyribonucleotides were purchased from Pabst Laboratories (Milwaukee, Wis., U.S.A.), Sigma Chemical Co. (St Louis, Mo., U.S.A.) and British Drug Houses Ltd. (Poole, Dorset). Phosphocreatine and crystalline bovine serum albumin were obtained from Calbiochem (Los Angeles, Calif., U.S.A.). Highly polymerized yeast RNA and yeast RNA for use as carrier were purchased from British Drug Houses Ltd. Bovine serum albumin was also purchased from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex); phosphocreatine kinase, deoxyribonuclease and ribonuclease were purchased from Sigma Chemical Co., and alkaline phosphatase from *Escherichia coli* was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Actinomycin D was a gift from Merck, Sharp and Dohme Inc. (Rahway, N.J., U.S.A.), and DNA from Landschütz ascites-tumour cells was prepared in this Department by Dr J. B. Shepherd.

[³H]-Ribonucleoside and -deoxyribonucleoside 5'-tri-phosphates were obtained from Schwarz BioResearch Inc. (Mount Vernon, N.Y., U.S.A.). Carrier-free [³²P]orthophosphate and cyanoethyl [³²P]phosphate were purchased from The Radiochemical Centre (Amersham, Bucks.). [α -³²P]UTP was prepared by the method described by Wykes & Smellie (1966).

Iso-octane (2,2,4-trimethylpentane) was obtained from British Drug Houses Ltd. and Tween 80 from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). A.R. dioxan, for use in liquid scintillators, was purchased from British Drug Houses Ltd. and purified by passage through a column of activated alumina, obtained from Peter Spence and Sons (Widnes, Lancs.).

1,4-Bis-(5-phenyloxazol-2-yl)benzene and scintillation-grade naphthalene were purchased from Nuclear Enterprises (G.B.) Ltd. (Edinburgh), 2,5-diphenyloxazole was from Koch-Light Laboratories Ltd. and A.R. toluene was from British Drug Houses Ltd.

Preparation of enzyme fractions. Mature unmated female rats, 10–12 weeks old, of the Wistar strain were used. Each liver was perfused with ice-cold 0.9% NaCl and quickly excised, placed in ice-cold vessels and weighed.

The method of cell fractionation was derived from that of Fisher & Harris (1962). The chilled livers were cut up finely with scissors and homogenized with 5 vol. of ice-cold 10 mM-tris-HCl buffer, pH 8.0, 3 mM with respect to MgCl₂ in 0.1% (v/v) Tween 80. Seven passes sufficed to disrupt the cells, leaving most of the nuclei intact.

The nuclei were obtained as a pellet on centrifugation at 600g for 5.0 min. at 0°. The supernatant fluid was removed with a Pasteur pipette and centrifuged at 10000g for 15 min. at 0° to obtain a large-particle fraction, and the supernatant liquid from this was centrifuged at 105000g or 150000g for 120 min. at 0° to obtain the microsome fraction. Particulate fractions were suspended in 10 mM-tris-HCl buffer, pH 8.0, for enzyme assays.

Nucleotidyltransferase assays. Nucleotidyltransferase activity was determined by measuring the incorporation of radioactive nucleotides into acid-insoluble products. All assays were performed in duplicate. Where possible a

special mixture was made up to include all reagents common to a given assay. This was pipetted into ice-cold reaction tubes followed by any variable reagents. The enzyme fraction was added last. The total volume of the reaction mixtures was 0.5 ml., and details of their composition are given in the legends to Tables and Figures. After incubation the reaction tubes were either frozen rapidly and stored at –15° until required, or processed immediately as follows.

Ice-cold saturated Na₄P₂O₇ solution (0.5 ml.) and 1.0 mg. of carrier yeast RNA or bovine serum albumin were added to the reaction mixtures and these were dispersed by shaking on a Vortex mixer and allowed to equilibrate for 10 sec. Ice-cold 5% (w/v) trichloroacetic acid (5.0 ml.) was added and the tubes were shaken once more. After equilibration for 10 min. the precipitate was collected by centrifugation at 600g for 10 min. and the supernatant fluid discarded. This process was repeated four times.

In experiments with [¹⁴C]- or [³H]-ribonucleotides the precipitate of RNA and protein was drained and taken up in 0.1 ml. of 98–100% (w/w) formic acid. The RNA was hydrolysed at 100° for 15 min. The solutions were then diluted with 1.0 ml. of deionized water and samples were removed for the measurement of radioactivity. Samples of the diluted hydrolysate in formic acid were dissolved in 8.0 ml. of scintillation fluid [0.5% (w/v) 2,5-diphenyloxazole, 0.03% (w/v) 2,4-bis-(5-phenyloxazol-2-yl)benzene and 10% (w/v) naphthalene in scintillation-grade dioxan]. The samples were counted in either a Nuclear-Chicago model 725 liquid-scintillation spectrometer or a Packard series 4000 liquid-scintillation spectrometer. Efficiency of counting was determined from the channel ratios, and was in the range 10–18% for ³H and 70–80% for ¹⁴C.

In experiments with [³²P]ribonucleotides, the precipitates of RNA and protein were washed with 5.0 ml. of 1% (w/v) potassium acetate in 95% ethanol and the tubes drained; they were then dissolved in 1.0 ml. of 98–100% (w/w) formic acid, and samples were removed for counting.

³²P radioactivity was measured in a Nuclear-Chicago model D47 gas-flow counter fitted with a Micromil window, at an efficiency of 40–50% and with a background of 14–17 counts/min. ³²P on chromatograms was measured by using a Nuclear-Chicago Actigraph fitted with a model D47 gas-flow counter and a Micromil window.

Assay and inactivation of alkaline phosphatase. Alkaline phosphatase, supplied commercially as a saturated-(NH₄)₂SO₄ precipitate, was diluted 1:100 for assay purposes. Diluted enzyme (0.1 ml.), 2 μ moles of *p*-nitrophenyl phosphate and 20 μ moles of tris-HCl buffer, pH 8.0, in a total volume of 0.5 ml., were incubated at 37°. At various times assay tubes were cooled in crushed ice, 2.5 ml. of ice-cold 0.1 N-NaOH was added and the extinction at 400 m μ was measured. The amount of phosphate released was calculated by assuming an ϵ at 400 m μ of 12000 (Razzell & Khorana, 1961) for *p*-nitrophenol. The activity of the undiluted enzyme was 120 μ moles of phosphate released/min./ml.

The enzyme was inactivated as follows. The solution was refluxed with an equal volume of redistilled ethanol for 1 hr. and the ethanol was then allowed to evaporate. An equal volume of chloroform was added and the mixture was shaken vigorously for 2 hr., after which the two layers were separated by centrifugation at 1000g for 10 min. The upper aqueous layer was removed, the chloroform was washed twice with small volumes of water, and the washings were

Table 1. Incorporation of uridine nucleotides from [α - 32 P]UTP into the 10000g sediment, the 105000g sediment and the 105000g supernatant fraction of rat liver cytoplasm

The reaction mixture contained 50 μ moles of tris-HCl buffer, pH 8.0, 5 μ moles of MgCl₂, 0.1 μ mole of [α - 32 P]UTP (1.0×10^6 counts/min./ μ mole), 1.8 μ moles of phosphocreatine, 45 μ g. of phosphocreatine kinase, and up to 1 mg. of enzyme protein in a total volume of 0.5 ml. Where indicated 50 μ g. of highly polymerized yeast RNA and 0.1 μ mole each of ATP, GTP and CTP were included. Incubation was for 10 min. at 37°.

Cell fraction	[32 P]UMP incorporated (m μ mole/mg. of enzyme protein)			
	No additions	Plus RNA	Plus ATP, GTP, CTP	Plus RNA, ATP, GTP, CTP
10000g sediment	0.005	0.004	0.020	0.041
105000g supernatant	0.0	0.049	0.006	0.176
105000g sediment	0.023	0.025	0.225	0.253

combined with the aqueous phase. No alkaline phosphatase was detected after the alkaline phosphatase had been subjected to this procedure.

Electrophoretic separation of ribonucleoside monophosphates. The acid-washed insoluble material from reaction mixtures containing [α - 32 P]UTP was hydrolysed for 10 hr. in 0.3N-KOH and neutralized to pH 3.5 with 12.4N-HClO₄, and insoluble KClO₄ was removed by centrifugation in the cold. Samples were applied as narrow bands to 8 cm. $\times$ 70 cm. strips of Whatman 3MM chromatography paper. The strips were moistened with 20 mM-citrate buffer, pH 3.4 (20 mM-citric acid adjusted to pH 3.4 with 20 mM-ammonium citrate). A voltage of 4 kv was applied across the paper for 1.25 hr. in a Shandon high-voltage electrophoresis apparatus. Channels containing the ultraviolet-absorbing spots were cut out and counted directly in a Nuclear-Chicago Actigraph, and the radioactivity was estimated by cutting out and weighing each peak from the recorder trace.

Determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), RNA by the orcinol method as modified by Slater (1956), and DNA by the diphenylamine method of Burton (1956).

RESULTS AND DISCUSSION

In preliminary experiments, the ability of the large-particle, the microsomal and the supernatant fractions of rat liver to promote the uptake of [α - 32 P]UTP into acid-insoluble products was measured (Table 1). All three fractions showed some activity, but the microsome fraction and the supernatant fraction were much more active than the large-particle fraction, and it seemed probable that the activity observed in that fraction could be accounted for by contamination.

The supernatant fraction. Experiments with this fraction were all carried out at pH 8.0 and showed that utilization of labelled UTP increased linearly with protein concentration up to 2.0 mg. of protein/ml. The reaction showed a marked dependence on Mg²⁺ ions (Fig. 1); the finding that the reaction curve did not intersect the ordinate at zero was probably due to the presence of 0.28 μ mole of

Fig. 1. Effect of increasing concentrations of MgCl₂ on the utilization of [3 H]UTP by the 150000g supernatant fraction of rat liver cells. Reaction mixtures contained 50 μ moles of tris-HCl, pH 8.0, 0.1 μ mole of [3 H]UTP (20 μ C/ μ mole), 0.1 μ mole each of ATP, GTP and CTP, 50 μ g. of highly polymerized yeast RNA and 0.424 mg. of enzyme protein in 0.5 ml. Where indicated various amounts of MgCl₂ were included in the reaction mixture. Incubations were at 37° for 10 min.

magnesium chloride/reaction tube derived from the homogenizing medium. In subsequent experiments 3–4 μ moles of magnesium chloride were included in each reaction mixture.

The response of the supernatant enzyme to added RNA is demonstrated in Fig. 2. The reaction was carried out at two different Mg²⁺ concentrations because it is known that RNA is capable of binding bivalent cations (Bannerjee & Perkins, 1962). The reason for the biphasic response, which was observed repeatedly, is not known, but it may reflect the presence of different enzymes in the system.

The supernatant fraction catalysed the incorporation of ATP, UTP and CTP into polynucleotides, but no incorporation of GTP was ever detected. Experiments on the effect of increasing concentrations of [3 H]UTP and [3 H]ATP on the incorporation of these nucleotides by this fraction gave similar

Fig. 2. Effect of increasing concentrations of RNA on the utilization of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ by the 150000g supernatant of rat liver cells. Reaction mixtures contained 50 μmoles of tris-HCl buffer, pH 8.0, either 3.5 or 4.0 μmoles of MgCl_2 , 0.1 μmole of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (0.5×10^6 counts/min./ μmole), 0.1 μmole each of ATP, GTP and CTP, 2 μmoles of phosphocreatine, 20 μg . of phosphocreatine kinase and 0.86 mg. of enzyme protein in a total volume of 0.5 ml. $\square$, 3.5 μmoles of MgCl_2 /tube; $\triangle$, 4.0 μmoles of MgCl_2 /tube.

Fig. 3. Incorporation of $[\text{H}^3]\text{UTP}$, $[\text{H}^3]\text{ATP}$ and $[\text{H}^3]\text{CTP}$ into the 150000g supernatant of rat liver cells. Reaction mixtures contained 50 μmoles of tris-HCl buffer, pH 8.0, 4.0 μmoles of MgCl_2 , 10 μmoles of EDTA, 0.1 μmole of the appropriate ribonucleoside triphosphate, 200 μg . of highly polymerized yeast RNA, 2 μmoles of phosphocreatine, 20 μg . of phosphocreatine kinase and 0.9 mg. of enzyme protein. Where indicated 0.1 μmole each of the other three unlabelled ribonucleoside triphosphates were included. Incubations were at 37°. $\circ$, $[\text{H}^3]\text{UTP}$ only; $\bullet$, $[\text{H}^3]\text{UTP}$ + ATP, GTP, CTP; $\triangle$, $[\text{H}^3]\text{ATP}$ only; $\blacktriangle$, $[\text{H}^3]\text{ATP}$ + UTP, GTP, CTP; $\square$, $[\text{H}^3]\text{CTP}$ only; $\blacksquare$, $[\text{H}^3]\text{CTP}$ + UTP, ATP, GTP.

curves for each nucleotide. Incorporation increased rapidly with up to about 0.1 μmole /tube and more slowly with 0.1–0.4 μmole /tube.

In studies on the time-course of the reaction (Fig. 3), the incorporation of $[\text{H}^3]\text{uridine}$ and $[\text{H}^3]\text{adenosine}$ nucleotides reached a maximum in 20 min. and thereafter declined slightly. $[\text{H}^3]\text{CTP}$ was utilized much more slowly, but incorporation

increased steadily up to 40 min. Little difference was observed between experiments in which $[\text{H}^3]\text{ATP}$, $[\text{H}^3]\text{UTP}$ and $[\text{H}^3]\text{CTP}$ were present singly or supplemented by a mixture of the three complementary unlabelled ribonucleoside triphosphates. The presence of an ATP-generating system stimulated the reaction by about 40%, and 2 μmoles of phosphocreatine kinase and 20 μg . of phosphocreatine were included in subsequent experiments.

Experiments were carried out to study the nature of the product formed in this reaction. Reaction tubes containing $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ of high specific radioactivity were incubated under normal conditions. The ribonucleoside 3'(2')-monophosphates from the polyribonucleotide product were separated by electrophoresis of acidified alkaline hydrolysates as described above, and the distribution of radioactivity in the different nucleotides was measured. The percentage of the total radioactivity recovered in each of the four ribonucleotides is given in Table 2. In experiments with $[\text{H}^3]\text{UTP}$ alone, approximately 80% of the radioactivity was recovered in UMP, and the addition of ATP, GTP and CTP to the reaction mixture caused an increase rather than a decrease in this value. These results suggest that this fraction catalyses the formation of homopolymer sequences, but they do not completely eliminate the possibility of some heteropolymer synthesis, since approximately 20% of the radioactivity was recovered in AMP, GMP and CMP.

In order to clarify this point, the average length of the newly synthesized chains was determined as follows. Reactions were set up with $[\text{H}^3]\text{UTP}$ of high specific radioactivity. After the usual washing procedure, the polynucleotide product was hydrolysed with alkali as described above and the resulting ribonucleosides and ribonucleoside 3'(2')-monophosphates were separated by paper chromatography in propan-2-ol–water–aq. ammonia (sp.gr. 0.88) (35:15:1, by vol.). Areas corresponding to the ribonucleosides and ribonucleotides were eluted and the distribution of radioactivity among them was determined. The ratio of radioactivity in the free nucleosides to that in the ribonucleoside monophosphates therefore provided a convenient method for estimating the average length of the newly synthesized chains of homopolymers.

The results of such an experiment are shown in Table 3. The average length of the newly synthesized chains was very small, only 2–3 units, and the presence of ATP, GTP and CTP in the reaction mixture did not lead to much increase. These experiments, taken in conjunction with those described above, suggest that, in this system, existing polyribonucleotide chains are extended by the addition of 2 or 3 ribonucleotide residues of the same type.

To determine whether 3'-hydroxyl ends on the

Table 2. *Distribution of the ^{32}P in the ribonucleoside 2'(3')-monophosphates obtained on alkaline hydrolysis of the product formed after incubation with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ by the 150 000g supernatant fraction of rat liver*

Reaction mixtures contained 0.5 m-mole of tris-HCl buffer, pH 8.0, 40 μmoles of MgCl_2 , 0.1 μmole of EDTA, 1 μmole of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (20×10^6 counts/min./ μmole), 2 mg. of RNA, 20 μmoles of phosphocreatine, 200 $\mu\text{g.}$ of phosphocreatine kinase and 4 mg. of enzyme protein in 5.0 ml. total volume; 1 μmole each of ATP, GTP and CTP were included where indicated. The tubes were incubated at 37° and the reaction was stopped by rapid cooling.

Time of incubation (min.)	Additions	Radioactivity in nucleotide (% of total recovered)			
		UMP	CMP	AMP	GMP
20	Nil	79.9	5.0	6.1	9.0
20	ATP, GTP, CTP	84.7	4.8	0.4	10.1
40	ATP, GTP, CTP	81.1	4.3	5.1	9.5

Table 3. *Chain lengths of polyribonucleotides formed by the supernatant fraction from rat liver*

Reaction tubes contained 50 μmoles of tris-HCl buffer, pH 8.0, 3.0 μmoles of MgCl_2 , 0.1 μmole of $[\text{H}^3]\text{UTP}$ (200 $\mu\text{C}/\mu\text{mole}$), 200 $\mu\text{g.}$ of highly polymerized yeast RNA, 2 μmoles of phosphocreatine, 20 $\mu\text{g.}$ of phosphocreatine kinase, 10 m μmoles of EDTA and 0.46 mg. of enzyme protein in a total volume of 0.5 ml. In Expt. 2 the protein concentration was 0.85 mg./tube; 0.1 μmole each of ATP, GTP and CTP were added where indicated. Incubation was at 37° for the indicated time.

Time of incubation (min.)	Additions	Ratio $\frac{\text{ribonucleotide}}{\text{ribonucleoside}}$	Average chain length of product (residues)
Expt. 1			
20	Nil	0.95	2
20	ATP, GTP, CTP	1.20	2-3
40	Nil	1.23	2-3
Expt. 2			
20	Nil	0.83	2
20	ATP, GTP, CTP	0.95	2
40	Nil	0.93	2
40	ATP, GTP, CTP	1.76	3

primer RNA chains were important, experiments were performed with oligonucleotides bearing phosphate groups on the 2'(3')-positions and with dephosphorylated oligonucleotides. Yeast RNA (50 mg.) with a sedimentation coefficient of 3.3s was hydrolysed in 0.1 M-potassium hydroxide at 37° for 8.0 min., the hydrolysate was neutralized with perchloric acid, and insoluble salt was removed as before. The new ends produced by alkaline hydrolysis were in the form of 2'(3')-phosphate esters. The hydrolysate was passed through a column of Sephadex G-75 (35 cm. $\times$ 7.5 cm.) equilibrated with 10 mM-tris-hydrochloric acid, pH 7.0 and RNA, with a sedimentation coefficient of 1.8s, was precipitated from the effluent by the addition of 2 vol. of 95% (w/v) ethanol, 1% (w/v) with respect to potassium acetate. This RNA was collected and dissolved in 10 mM-tris-hydrochloric acid, pH 8.0, and a sample was treated with an excess of alkaline phosphatase for 4.0 min. at 37°. The phosphatase was then inactivated as described above. This treatment

with phosphatase did not alter the chromatographic behaviour of the RNA on Sephadex G-75.

The effect of adding increasing amounts of phosphorylated or dephosphorylated, partially hydrolysed, RNA on the uptake of uridine residues by the 150 000g supernatant fraction is shown in Fig. 4. At low primer concentrations the dephosphorylated RNA is a much more effective primer than the RNA with phosphate groups at the 3'-hydroxyl end.

These findings are consistent with the view that, in the reaction with UTP, short stretches of polyuridylic acid are added to the 3'-hydroxyl ends of RNA or oligoribonucleotide primers. It seems likely that the small amount of randomization of ^{32}P observed in the nearest-neighbour analyses (Table 2) can be accounted for by the addition of uridine residues to primer molecules with a more or less random distribution of nucleotides at the 3'-hydroxyl end.

It should be noted that the uptake of uracil and

Fig. 4. Effect of increasing amounts of polyribonucleotide primer, containing predominantly 3'-hydroxyl or 2'(3')-phosphate ends, on the utilization of [³H]UTP by the 150000g supernatant fraction of rat liver. Reaction mixtures contained 50 μmoles of tris-HCl buffer, pH 8.0, 4.0 μmoles of MgCl₂, 10 mμmoles of EDTA, 0.1 μmole of [³H]UTP (20 μC/μmole), 0.1 μmole each of ATP, GTP and CTP, 2 μmoles of phosphocreatine, 20 μg. of phosphocreatine kinase, 0.702 mg. of enzyme protein/0.5 ml. total volume. The incubations were at 37° for 5.0 min. Δ, Polynucleotides with 3'-hydroxyl ends; ●, polynucleotides with 2'(3')-phosphate ends.

Fig. 5. Effect of pH on the utilization of [³H]UTP by rat liver microsomes. Reaction mixtures contained 4 μmoles of MgCl₂, 10 mμmoles of EDTA, 0.1 μmole of [³H]UTP (20 μC/μmole), 0.1 μmole each of ATP, GTP and CTP, 100 μg. of RNA, 2 μmoles of phosphocreatine, 20 μg. of phosphocreatine kinase and 0.769 mg. of microsomal protein in a total volume of 0.5 ml. Where indicated 50 μmoles of tris-HCl buffer of the appropriate pH was included. Incubations were at 37° for 6.5 min.

adenine nucleotides was four to five times greater than that of cytosine nucleotides. Much of the incorporation of the adenine and cytosine nucleotides observed can probably be attributed to turnover of the terminal pCpCpA sequence of transfer RNA (Heidelberger *et al.* 1956; Preiss, Dieckmann & Berg, 1961). The observation that the uptake of adenine nucleotides was much greater than that of cytosine nucleotides, however, suggests either that there is a much higher turnover of the adenine than of the cytosine part of the pCpCpA triplet or that adenine nucleotides are involved additionally in some other reaction.

Daniel & Littauer (1963) reported the incorporation of uracil nucleotides from UTP into transfer RNA by the supernatant fraction of rat liver. The main product was transfer RNA-pXpCpU, and neither transfer RNA-pXpCpC nor transfer RNA-pXpCpCpA could accept the uridylate residues. It seems unlikely that the reaction described in the present paper corresponds to that described by Daniel & Littauer (1963) since 80% of the uridine residues in our system were found to be adjacent to UMP residues and the proportion incorporated adjacent to cytidine residues was at the most 10%.

Klemperer (1963a,b) described an enzyme in the pH 5 fraction of rat liver cytoplasm that incorporates uridine residues into the 3'-terminal positions of certain natural and synthetic ribonucleotides, and this enzyme is similar to that

described in this paper and to an enzyme in the soluble fraction from Ehrlich ascites-carcinoma cells described by Burdon & Smellie (1962).

The present experiments provide further information about the requirements of this reaction and about the nature of the product, but the biological significance of this system remains obscure.

The microsome fraction. In experiments with the microsome fraction, the incorporation of uridine nucleotides increased linearly with protein concentration up to 2 mg. of protein/ml. The general requirements of the system are shown in Table 1, from which it is evident that there was a tenfold increase in activity when ATP, GTP and CTP were added to the reaction mixture. The effect of adding RNA, however, was quite small, but a small degree of stimulation was observed repeatedly. The relationship between activity and pH is shown in Fig. 5; there was a rather broad peak over the pH range 7.0–8.5. The reaction was stimulated by the addition of Mg²⁺ ions (Fig. 6) but the addition of Mn²⁺ ions had no effect. The activity observed in the absence of added Mg²⁺ was probably due to the presence of 0.51 μmole of magnesium chloride/tube derived from the homogenizing medium. Supplementation of the reaction mixture with an ATP-generating system, in the form of phosphocreatine and phosphocreatine kinase, stimulated the uptake of UTP slightly, even in the presence of ATP, GTP and CTP, and this was therefore included regularly in reaction mixtures. The presence of small amounts of EDTA also favoured the reaction,

Fig. 6. Effect of increasing concentrations of Mg^{2+} and Mn^{2+} on the utilization of $[^3\text{H}]\text{UTP}$ by rat liver microsomes. Reaction mixtures contained 50 μmoles of tris-HCl buffer, pH 8.0, 0.1 μmole of $[^3\text{H}]\text{UTP}$ (20 $\mu\text{Ci}/\mu\text{mole}$), 0.1 μmole each of ATP, GTP and CTP, 50 $\mu\text{g.}$ of highly polymerized yeast RNA, 2 μmoles of phosphocreatine, 20 $\mu\text{g.}$ of phosphocreatine kinase and 0.43 mg. of protein in a total volume of 0.5 ml. Various amounts of either MgCl_2 or MnCl_2 were included as indicated. Incubations were at 37° for 10 min. $\circ$, MgCl_2 ; $\triangle$, MnCl_2 .

Fig. 7. Time-course of utilization of $[^3\text{H}]\text{UTP}$ by rat liver microsomes. Reaction mixtures contained 50 μmoles of tris-HCl buffer, pH 8.0, 4 μmoles of MgCl_2 , 0.1 μmole of $[^3\text{H}]\text{UTP}$ (30 $\mu\text{Ci}/\mu\text{mole}$), 0.1 μmole each of ATP, GTP and CTP, 50 $\mu\text{g.}$ of highly polymerized yeast RNA, 2 μmoles of phosphocreatine, 50 $\mu\text{g.}$ of phosphocreatine kinase and 0.87 mg. of microsomal protein in a total volume of 0.5 ml. Incubations were at 37° for the times indicated.

probably owing to the removal of trace amounts of inhibitory heavy-metal ions.

The time-course of the incorporation of labelled uridine nucleotides into polynucleotide material was examined in a reaction mixture containing optimum amounts of Mg^{2+} , ATP, GTP and CTP, yeast RNA primer and phosphocreatine and phosphocreatine kinase. Rapid incorporation was observed during the first 10 min. (Fig. 7) of incubation, but at longer time-intervals the extent of labelling in the polynucleotide product declined sharply. This seemed to be caused by nuclease degradation of the reaction product, but it could not be prevented by the addition of bentonite to inhibit ribonuclease.

The reaction was relatively insensitive to inhibition by inorganic orthophosphate, which caused 50% inhibition at a concentration of 10 $\mu\text{moles/ml.}$; 5 μmoles of inorganic pyrophosphate/ml. abolish incorporation completely. This suggests that pyrophosphate may be released during the reaction, and supports the view that UTP rather than UDP is required in this reaction.

Experiments were carried out to study the effects of RNA, DNA, ribonuclease, deoxyribonuclease and actinomycin D on the uptake of UTP by the microsome fraction. Table 4 shows that the addition of 50 $\mu\text{g.}$ of yeast RNA stimulated the reaction, and that 50 $\mu\text{g.}$ of DNA inhibited incorporation by about 20%. Deoxyribonuclease had

Table 4. *Effect of RNA, DNA, ribonuclease, deoxyribonuclease and actinomycin D on the utilization of $[^3\text{H}]\text{UTP}$ by rat liver microsomes*

Reaction mixtures contained 50 μmoles of tris-HCl buffer, pH 8.0, 4.0 μmoles of MgCl_2 , 20 m μmoles of EDTA, 0.1 μmole of $[^3\text{H}]\text{UTP}$ (30 $\mu\text{Ci}/\mu\text{mole}$), 0.1 μmole each of ATP, GTP and CTP, 2 μmoles of phosphocreatine, 50 $\mu\text{g.}$ of phosphocreatine kinase and 1.10 mg. of microsomal enzyme/tube. Where indicated 50 $\mu\text{g.}$ of highly polymerized yeast RNA, 50 $\mu\text{g.}$ of DNA from Krebs II ascites-tumour cells, 10 $\mu\text{g.}$ of ribonuclease, 10 $\mu\text{g.}$ of deoxyribonuclease or the stated various amounts of actinomycin D was included. Incubations were at 37° for 5 min.

Additions	UMP incorporated ($\mu\text{mole/mg. of protein}$)
Nil	0.368
RNA	0.407
DNA	0.286
Ribonuclease	0
Deoxyribonuclease	0.376
Actinomycin D (1 $\mu\text{g.}$)	0.361
Actinomycin D (10 $\mu\text{g.}$)	0.368
Actinomycin D (50 $\mu\text{g.}$)	0.342
Actinomycin D (100 $\mu\text{g.}$)	0.382

little effect on the system, but the presence of 10 $\mu\text{g.}$ of ribonuclease abolished incorporation completely. Actinomycin D over a wide range of

Table 5. *Distribution of radioactivity in UMP, CMP, AMP and GMP obtained after alkaline hydrolysis of the product of [α - 32 P]UTP uptake by rat liver microsomes*

Reaction mixtures contained 50 μ moles of tris-HCl buffer, pH 8.0, 4 μ moles of $MgCl_2$, 0.1 μ mole of [α - 32 P]UTP ($20\text{--}50 \times 10^6$ counts/min./ μ mole), 2 μ moles of phosphocreatine, 50 μ g. of phosphocreatine kinase, and up to 1 mg. of enzyme protein/total volume of 0.5 ml. Where indicated 0.1 μ mole each of ATP, CTP and GTP and 50 μ g. of highly polymerized yeast RNA were included. Incubations were at 37° for 10 min.

Additions	Nucleotide Expt. no. ...	Radioactivity recovered in the various nucleotides in different experiments (% of total)				
		1	2	3	4	5
Nil	UMP		87.6	89.6	77.2	71.6
	CMP		8.7	3.9	22.8	28.4
	AMP		0	1.0	0	0
	GMP		3.7	5.5	0	0
RNA	UMP		81.1	100	100	100
	CMP		9.3	0	0	0
	AMP		4.5	0	0	0
	GMP		5.1	0	0	0
ATP + GTP + CTP	UMP	56.1	58.0	84.8	61.4	62.4
	CMP	24.0	23.6	10.4	27.3	17.7
	AMP	7.4	7.3	0.2	3.8	12.7
	GMP	12.5	11.2	4.6	7.5	7.2
RNA + ATP + GTP + CTP	UMP	57.6	64.3	83.4	66.6	59.3
	CMP	16.1	15.6	8.2	17.7	24.1
	AMP	16.8	10.4	3.3	4.4	11.1
	GMP	9.5	9.7	5.1	11.3	5.5

concentrations had no effect on the reaction. These results provide convincing evidence that DNA is not required for the uptake of uridine nucleotides but that RNA may be necessary.

In experiments with [α - 32 P]UTP, the distribution of radioactivity was studied in the ribonucleoside 3'(2')-monophosphates obtained by electrophoresis of alkaline hydrolysates of the reaction product. Results from several different experiments are shown in Table 5.

In each experiment, the major portion of radioactivity was recovered in UMP. However, a significant percentage of the 32 P was recovered in the other ribonucleotides, especially when ATP, GTP and CTP were included in the reaction. The results suggested extensive incorporation of UMP adjacent to UMP in the product, but it was not possible to exclude some synthesis of heteropolymer chains, especially in view of the enhancement of the reaction in the presence of all four ribonucleotides, and of the increased degree of randomization of radioactivity in the polynucleotide product under these conditions.

The characteristics of incorporation of labelled ATP, GTP and CTP were also investigated. Substrate-concentration curves for ATP and UTP gave similar results, broad maxima being observed over the range 200–400 $m\mu$ moles/ml. Table 6 shows the uptake of the four ribonucleotides under various

conditions. Similar patterns were observed for UTP and CTP. In each case the addition of RNA or the three complementary nucleotides stimulated the reaction. With UTP and CTP the addition of RNA and the three complementary ribonucleoside 5'-triphosphates produced an effect that was nearly additive. In the case of ATP, addition of UTP, GTP and CTP decreased the amount of adenine nucleotide incorporated and, although supplementation with RNA alone enhanced the reaction, addition of RNA together with UTP, CTP and GTP gave only slightly higher incorporation than was found for the addition of the ribonucleoside 5'-triphosphates alone. No incorporation of GTP was found in the absence of the added ribonucleoside 5'-triphosphate or RNA; very low uptakes were observed in the presence of either RNA or the ribonucleotides but the addition of both gave an additive effect.

The effects on the uptake of any one labelled triphosphate of each of the other triphosphates individually were also examined. The only clear conclusion from these experiments was that in no case did maximum incorporation occur when all four ribonucleoside triphosphates were added to the reaction mixture.

It is evident from the complex pattern of results obtained with the microsome fraction that several reactions occur simultaneously in this system. Thus formation of homopolymers containing uridylylate,

Table 6. *Characteristics of utilization of labelled ATP, GTP, CTP and UTP by rat liver microsomes*

Reaction mixtures contained 50 μ moles of tris-HCl buffer, pH 8.0, 3 μ moles of $MgCl_2$, 100 m μ moles of EDTA, 0.1 μ mole of [3H]ribonucleoside 5'-triphosphate (20 μ C/ μ mole), 2 μ moles of phosphocreatine, 50 μ g. of phosphocreatine kinase and 0.475 mg. of protein in a total volume of 0.5 ml.; 50 μ g. of highly polymerized RNA and 0.1 μ mole each of the three complementary ribonucleoside triphosphates were added where indicated. Incubations were at 37° for 10 min.

[3H]Ribonucleoside 5'-triphosphate	Additions	Nucleotide incorporated (m μ mole/mg. of protein)
UTP	Nil	0.059
	ATP + GTP + CTP	0.576
	RNA	0.199
	RNA + ATP + GTP + CTP	0.803
CTP	Nil	0.201
	UTP + ATP + GTP	0.723
	RNA	0.409
	RNA + ATP + GTP + UTP	0.953
ATP	Nil	0.211
	UTP + GTP + CTP	0.147
	RNA	0.361
	RNA + UTP + GTP + CTP	0.188
GTP	Nil	0.000
	UTP + ATP + CTP	0.022
	RNA	0.028
	RNA + ATP + UTP + CTP	0.057

adenylate and cytidylate but not guanylate residues can occur. It is also possible that some genuine heteropolymer synthesis takes place. The extent of this, however, can be gauged from the extent of incorporation of guanosine residues. This is quite small, and it is at present difficult to reconcile the considerable stimulation of uptake of uridylate, adenylate and cytidylate residues caused by the addition of the corresponding complementary ribonucleotides with the small amount of genuine heteropolymer formation that seems to occur. To resolve these problems, further studies were carried out to fractionate microsomes and determine the characteristics of the subfractions obtained. Some of these results are presented by Wilkie & Smellie (1968).

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