Decreased Ribonucleic Acid Synthesis in Isolated Rat Liver Nuclei during Starvation

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1. Isolated nuclei from starved rats showed a lowered incorporation of [^{14}C]UMP into RNA. 2. The Mg^{2+} -dependent incorporation was decreased by 30% after 1 day of starvation, but incorporation in the presence of Mn^{2+} and ammonium sulphate decreased only after longer periods of starvation. 3. RNA synthesis by nuclei in the presence of excess of added RNA polymerase was unchanged after 1 day of starvation and was inhibited by 20% after 4 days. 4. The capacity of nuclei to bind actinomycin D was unchanged after 1 day and was decreased by 20% after 4 days of starvation.

An increased rate of RNA synthesis in liver has been observed *in vivo* when rats are provided with a protein-deficient diet (Munro, 1964), and nuclei from such livers show a faster rate of RNA synthesis *in vitro* (Quirin-Stricker & Mandel, 1968). In contrast, the present paper studies the decrease in RNA synthesis that was observed with liver nuclei from starved rats. While this work was in progress Henderson (1969) reported that the Mg^{2+} -dependent RNA polymerase activity of isolated nuclei was lowered after overnight starvation of rats and was rapidly restored by feeding with a complete amino acid mixture.

MATERIALS AND METHODS

Animals. Male Wistar-derived albino rats of a caesarian-derived 'specific pathogen-free' stock, each weighing approx. 180g, were maintained under barrier precautions (Bleby, 1967) and under a strict lighting schedule (light from 06.00 h to 20.00 h, dark from 20.00 h to 06.00 h). The diet was Oxoid Pasteurised Breeding Diet (Oxo Ltd., London S.E.1, U.K.). The animals were starved, with free access to water, by withdrawal of food at 09.00 h, and were kept in wire-bottomed cages to prevent coprophagy.

Materials. [$4\text{-}^{14}\text{C}$]UTP was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

RNA polymerase (DNA-dependent nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) was prepared from the ribonuclease-free strain of *Escherichia coli* M.R.E. 600 by the method of Chamberlin & Berg (1962) and had an activity of 2000 units/mg of protein when measured as described by Chamberlin & Berg (1962).

Preparation of nuclei. Two rats subjected to the same starvation regimen were killed at the same time and the livers pooled for homogenization. Nuclei were prepared by the method of Chauveau, Moulé & Rouiller (1956) as modified by Widnell & Tata (1964) except that the pellet of crude nuclei obtained by centrifuging the homogenate

from two livers at 600g was resuspended in 60 ml of 2.2 M-sucrose–1 mM-MgCl₂–10 mM-2-mercaptoethanol–10 mM-tris-HCl buffer, pH 7.4, and 20 ml of this suspension was layered over 10 ml of the same medium in each of three centrifuge tubes. The tubes were centrifuged at 40000g for 60 min in a 3×40 ml swing-out rotor in the MSE Super-Speed centrifuge. After removal of the supernatant the nuclear pellet was resuspended in 0.25 M-sucrose–3 mM-MgCl₂–10 mM-2-mercaptoethanol–20 mM-tris-HCl buffer, pH 8.0. Routine microscopic examination showed the final suspension of nuclei to be free of cytoplasmic particles normally visible with the high-power objective. The yield of nuclei represented approx. 60% of the initial DNA per liver.

Measurement of incorporation of [^{14}C]UMP into RNA. The standard reaction mixture based on that of Widnell & Tata (1966), contained (in 0.5 ml) nuclei (0.3–0.4 mg of DNA), 100 μmol of sucrose, 25 μmol of tris-HCl buffer, pH 8.0, 2.5 μmol of MgCl₂, 5 μmol of 2-mercaptoethanol and 0.1 μmol each of ATP, CTP, GTP and [$4\text{-}^{14}\text{C}$]UTP (2.7×10^6 c.p.m./ μmol). Under these conditions the incorporation of radioactivity into RNA at 37°C was linear for approx. 8 min and was proportional to the amount of nuclei added up to approx. 0.7 mg of DNA. These conditions were modified as follows. When the Mn^{2+} + (NH₄)₂SO₄-dependent polymerase was measured 175 μmol of (NH₄)₂SO₄ and 1.0 μmol of MnCl₂/0.5 ml were added. When RNA polymerase was added the amount of nuclei was decreased to approx. 0.03 mg of DNA/0.5 ml and the complete reaction mixture was preincubated for 10 min at 0°C before incubation for 5 min at 37°C (Hurlbert, Miller & Vaughan, 1969). Under the latter conditions no significant incorporation of radioactivity occurred at 0°C.

The incubations were terminated by cooling the tubes in ice and adding, in turn, 0.3 ml of 0.2 M-sodium pyrophosphate, pH 7.5 [to discharge adsorbed nucleotides (Kammen, Klemperer & Canellakis, 1961)], 0.1 ml of RNA (yeast, high-molecular-weight, 1 mg/ml; Calbiochem, Los Angeles, Calif., U.S.A.), 0.1 ml of bovine plasma albumin (5 mg/ml) and 8.0 ml of 0.4 M-HClO₄. The precipitate was collected

by centrifugation at 1000g for 10 min, and was washed twice by resuspension in 8.0 ml of 0.4M-HClO₄ and centrifugation. The pellet was resuspended in 1 ml of 6% (w/v) trichloroacetic acid and collected quantitatively by filtration on 2.5 cm-diam. glass-paper discs (Whatman GF/C) and washed on the filter with 4 ml of 6% (w/v) trichloroacetic acid. The discs were dried in an oven at 37°C and then placed in vials together with 10 ml of scintillation fluid [toluene containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l] for measurement of radioactivity in a Nuclear-Chicago 725 system liquid-scintillation counter (background 35 c.p.m.; counting efficiency approx. 75%).

Determination of ribonuclease activity. Nuclei were incubated under the same conditions as for incorporation of [¹⁴C]UMP, except that the nucleoside triphosphates were omitted and 0.5 mg of RNA (yeast, high-molecular-weight; Calbiochem) was added to each 0.5 ml of reaction mixture. The incubation was terminated by cooling the tube in ice and adding 2 ml of 0.4M-HClO₄. After centrifugation the extinction of the supernatant was measured at 260 nm, the supernatant from an unincubated tube being taken as the blank. The rate of increase in the extinction at 260 nm remained constant for at least 20 min of incubation, and the increase after a 5 min incubation period was taken as a measure of ribonuclease activity.

Measurement of the binding of actinomycin by nuclei. Nuclei were suspended in 0.7 mM-sodium phosphate buffer, pH 6.8. The suspension was dialysed for 2 h against the same buffer. To 3 ml of suspension (containing 150 µg of DNA) was added actinomycin D in successive 10 µl quantities (each containing 2 µg of actinomycin). After each addition the extinction of the suspension was mea-

sured at 425 nm and at 470 nm to determine the maximum amount of actinomycin that could be bound (Ringertz & Bolund, 1969). Progressive additions of actinomycin cause proportionate (and approximately similar) increases in the extinction at 425 nm and 470 nm due to actinomycin, which binds to DNA with high affinity. At the point where the binding capacity of the DNA is exceeded there is an accumulation of free actinomycin, which has a higher extinction coefficient at 425 nm than bound actinomycin, and the increments in the extinction at this wavelength are correspondingly increased (Ringertz & Bolund, 1969). For comparison, similar experiments were carried out with purified rat liver DNA (30 µg/3 ml of 0.7 mM-sodium phosphate buffer, pH 6.8).

RESULTS

RNA synthesis by isolated nuclei. Nuclei incubated with [¹⁴C]UTP under the conditions shown in Table 1 incorporated radioactivity into acid-insoluble material. In other experiments (results not shown) this incorporation showed the characteristics of RNA synthesis in that it was dependent on the presence of all four ribonucleoside triphosphates and was inhibited by actinomycin D. Nuclei from fed animals incorporated labelled nucleotide at rates similar to those reported previously by other workers (see e.g. Widnell & Tata, 1966). Table 1 shows that with increasing duration of starvation there was a progressive decrease in the rate of incorporation in the presence of Mg²⁺

Table 1. *Incorporation of UMP into RNA and ribonuclease activity in nuclei*

The standard system contained (in 0.5 ml): nuclei (0.3–0.4 mg of DNA), 100 µmol of sucrose, 25 µmol of tris-HCl buffer, pH 8.0, 2.5 µmol of MgCl₂, 5 µmol of 2-mercaptoethanol and 0.1 µmol each of ATP, CTP, GTP and [4-¹⁴C]UTP. Where indicated, 175 µmol of (NH₄)₂SO₄ and 1 µmol of MnCl₂ were added. After incubation at 37°C for 5 min the acid-precipitable radioactivity was collected and counted. The values are corrected for the amount (4 pmol of UMP) found with unincubated controls. Ribonuclease activity was determined under the standard conditions in the presence of 0.5 mg of RNA. All values are for pooled nuclei prepared from two animals.

Starvation (days)	¹⁴ CUMP incorporated (pmol/ 5 min per mg of DNA)		Ribonuclease activity (E ₂₆₀ /5 min per mg of DNA)
	Standard	With (NH ₄) ₂ SO ₄ +MnCl ₂	
0	359	527	0.35
	367	630	0.39
	370		
1	223	505	0.35
	239	594	0.36
	259		
2	184	422	0.39
	186	447	0.40
	202		
4	152	294	0.37
	154	309	0.38
	159

Fig. 1. UMP incorporated by nuclei in the presence of added RNA polymerase. Standard reaction mixtures (Table 1) contained added RNA polymerase as shown, and the tubes were preincubated for 10 min at 0°C. Nuclei were from: ●, fed animals; ▲, 1-day-starved animals; ■, 4-day-starved animals.

Table 2. *Binding of actinomycin D to DNA and to isolated nuclei*

The binding capacities of purified rat liver DNA (30 μ g/3ml) and of rat liver nuclei (150 μ g of DNA/3ml) in 0.7M-sodium phosphate buffer, pH 6.8, were titrated by successive additions of 2 μ g quantities of actinomycin as described by Ringertz & Bolund (1969). Results are given of duplicate experiments performed on different occasions with different preparations of nuclei.

Materials used for binding	Actinomycin D bound (μ g/mg of DNA)	DNA available for binding (% of purified DNA)
Rat liver DNA	325	100
	325	100
Liver nuclei		
From fed animals	65	20
	64	20
From 1-day-starved animals	66	20
	64	20
From 4-day-starved animals	52	16
	53	16

(approx. 30% decrease after 1 day, over 50% decrease after 4 days). The net rate of incorporation could have been affected by the ribonuclease activity of the nuclei. However, there was no change in the ribonuclease activity of the nuclei under the same conditions, suggesting that the decreased rate of incorporation reflected a diminished rate of RNA synthesis in the nuclei from starved animals. In the presence of Mn^{2+} and ammonium sulphate the decrease of [^{14}C]UMP incorporation during starvation was less marked, especially in the early stages (little decrease after 1 day, approx. 50% decrease after 4 days).

Template activity. In the presence of excess of RNA polymerase the rate of RNA synthesis will reflect the amount of DNA that is acting as a template for RNA synthesis. Fig. 1 shows the effect of adding saturating amounts of RNA polymerase to nuclei in order to measure the amount of

template available for RNA synthesis. The maximum rate of RNA synthesis was the same for nuclei from fed and from 1-day-starved rats, but was decreased by about 20% after 4 days of starvation, suggesting that the amount of available template was decreased at this time. These results were confirmed by estimation of the relative amounts of DNA available for binding actinomycin D (Table 2). The binding capacity was unchanged after 1 day of starvation but was lowered by about 20% after 4 days.

DISCUSSION

In the presence of Mg^{2+} nuclei make predominantly ribosomal-like RNA, whereas with Mn^{2+} and ammonium sulphate they synthesize DNA-like RNA (Widnell & Tata, 1966; Pogo, Littau, Allfrey & Mirsky, 1967; Chambon, Ramuz, Mandel & Doly,

1968). RNA synthesis under these two sets of conditions is apparently a measure of the activity of two distinct RNA polymerases (Widnell & Tata, 1966; Stirpe & Fiume, 1967; Kedinger, Gniazdowski, Mandel, Gissinger & Chambon, 1970; Roeder & Rutter, 1970).

The early decrease in the Mg^{2+} -dependent RNA synthesis, which is also evident from the results of Henderson (1969), therefore probably reflects an inhibition of rRNA synthesis, which is also observed *in vivo* in the livers of starved rats (Hirsch & Hiatt, 1966). This fall was not associated with a decrease in the amount of template available in the nuclei and therefore probably represents a decrease in the amount of Mg^{2+} -dependent RNA polymerase. This in turn may be due to a decreased rate of synthesis of this enzyme, since Henderson (1969) showed that restoration of this activity by re-feeding was inhibited by puromycin or cycloheximide. In contrast with the early inhibition of Mg^{2+} -dependent RNA synthesis, the template activity of the nuclei and the Mn^{2+} -dependent RNA synthesis were inhibited only after long periods of starvation. Thus the inhibition of the capacity to make DNA-like RNA was a delayed response. These changes in starvation recall the effects often observed after hormone administration in that the first effect on RNA synthesis involves mainly rRNA (Tata, 1966; Drews & Wagner, 1970).

An inhibition of RNA synthesis has also been found in tissue-culture cells during amino acid deprivation (e.g. Maden, Vaughan, Warner & Darnell, 1969; Smulson & Thomas, 1969). In contrast, RNA synthesis is stimulated in the livers of rats on protein-deficient diets (see the introduction), a difference in response that remains unexplained. Possibly there are important differences in the individual concentrations of certain free amino acids. Thus the restoration of RNA synthesis by feeding after starvation is less effective when an amino acid mixture deficient in tryptophan is used (Henderson, 1969), and the concentration of aminoacyl-transfer RNA (which may be important in regulating RNA synthesis) is most depressed by feeding with incomplete amino acid mixtures (Allen, Raines & Regen, 1969).

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REFERENCES

- Allen, R. E., Raines, P. L. & Regen, O. M. (1969). *Biochim. biophys. Acta*, **190**, 323.
- Bleby, J. (1967). In *Handbook on the Care and Management of Laboratory Animals*, p. 201. Ed. by UFAW. Edinburgh and London: E. and S. Livingstone Ltd.
- Chamberlin, M. & Berg, P. (1962). *Proc. natn. Acad. Sci. U.S.A.* **48**, 81.
- Chambon, P., Ramuz, M., Mandel, P. & Doly, J. (1968). *Biochim. biophys. Acta*, **157**, 504.
- Chauveau, J., Moulé, Y. & Rouiller, C. (1956). *Expl Cell Res.* **11**, 317.
- Drews, J. & Wagner, L. (1970). *Eur. J. Biochem.* **13**, 231.
- Henderson, A. R. (1969). *Biochem. J.* **114**, 41 p.
- Hirsch, C. A. & Hiatt, H. H. (1966). *J. biol. Chem.* **241**, 5936.
- Hurlbert, R. B., Miller, E. G. & Vaughan, C. L. (1969). In *Advances in Enzyme Regulation*, vol. 7, p. 219. Ed. by Weber, G. Oxford: Pergamon Press Ltd.
- Kammen, H. O., Klemperer, H. G. & Canellakis, E. S. (1961). *Biochim. biophys. Acta*, **51**, 175.
- Kedinger, C., Gniazdowski, M., Mandel, J. L., Gissinger, F. & Chambon, P. (1970). *Biochem. biophys. Res. Commun.* **38**, 165.
- Maden, B. E. H., Vaughan, M. H., Warner, J. R. & Darnell, J. E. (1969). *J. molec. Biol.* **45**, 265.
- Munro, H. N. (1964). In *Mammalian Protein Metabolism*, vol. 1, p. 382. Ed. by Allison, J. B. & Munro, H. N. New York: Academic Press Inc.
- Pogo, A. O., Littau, V. C., Allfrey, V. G. & Mirsky, A. E. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 743.
- Quirin-Stricker, C. & Mandel, P. (1968). *Bull. Soc. Chim. biol.* **50**, 31.
- Ringertz, N. R. & Bolund, L. (1969). *Biochim. biophys. Acta*, **174**, 147.
- Roeder, R. G. & Rutter, W. J. (1970). *Proc. natn. Acad. Sci. U.S.A.* **65**, 675.
- Smulson, M. E. & Thomas, S. (1969). *J. biol. Chem.* **244**, 5309.
- Stirpe, F. & Fiume, L. (1967). *Biochem. J.* **105**, 779.
- Tata, J. R. (1966). *Prog. nucleic Acid Res. molec. Biol.* **5**, 191.
- Widnell, C. C. & Tata, J. R. (1964). *Biochem. J.* **92**, 313.
- Widnell, C. C. & Tata, J. R. (1966). *Biochim. biophys. Acta*, **123**, 478.