Comparison of the Effects of Zinc Deprivation and Actinomycin D on Ribonucleic Acid Synthesis by Stimulated Lymphocytes

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1. EDTA inhibited incorporation of [^3H]uridine into RNA of lymphocytes, but did not decrease uptake into the cold-acid-soluble fraction of the cells. The inhibition by EDTA was largely reversible by simultaneous addition of Zn^{2+} . 2. Low concentrations of actinomycin D (3 ng/ml) added at the time of stimulation of the cells inhibited [^3H]uridine incorporation into RNA, but concentrations of 50–100 ng/ml were required to produce the same degree of inhibition if addition of actinomycin D was delayed until just before the incorporation was measured. This difference in sensitivity did not relate to the period of time available for concentration of the drug within the cells. 3. When added immediately before phytohaemagglutinin, actinomycin D (3 ng/ml) and EDTA produced similar time-courses of inhibition of uridine incorporation. 4. Uridine incorporation at 32 h was inhibited when actinomycin D (3 ng/ml) or EDTA was added just before stimulation of the cells, but was only slightly affected when they were added at 32 h. At intermediate times the incorporation of uridine remained sensitive to addition of EDTA for longer than it was sensitive to actinomycin D. 5. Polyacrylamide-gel separation of RNA synthesized in EDTA-treated cultures in the presence or absence of added Zn^{2+} showed that lower availability of Zn^{2+} resulted in a decreased rate of transfer of radioactivity from 32S to 28S rRNA and decreased survival of 28S rRNA relative to 18S rRNA. 6. Close similarities have been shown to exist between the effects of EDTA and low concentrations of actinomycin D. Not all the effects of EDTA could be explained by postulating that Zn^{2+} was a constituent of RNA polymerase I, nor were the effects of actinomycin D readily explained by previously suggested mechanisms of action of this antibiotic.

Previous studies (Chesters, 1972) showed similarities between the effects of EDTA and actinomycin D on DNA and protein synthesis by lymphocytes stimulated with phytohaemagglutinin. The inhibition of DNA synthesis by EDTA was specifically reversible by addition of Zn^{2+} . The known effects of actinomycin D on RNA metabolism prompted the present investigation of the effects of EDTA and Zn^{2+} on RNA synthesis by lymphocytes. A preliminary report of some of these findings has already been presented (Chesters, 1974).

Methods

The preparation of lymphocytes from pig blood and the culturing of them after stimulation with phytohaemagglutinin were performed as previously described (Chesters, 1972). At least four replicate cultures were prepared for each experimental condition. When present in the culture EDTA was always at a final concentration of $600\text{ }\mu\text{M}$ and was added in combination with Fe^{3+} at a final concentration of $100\text{ }\mu\text{M}$ to satisfy the Fe^{3+} requirements of the lymphocytes (Chesters, 1972).

Incorporation of [^3H]uridine and [^3H]thymidine

After the specified period of preincubation of the stimulated cultures, $5\text{ }\mu\text{Ci}$ of [$5\text{-}^3\text{H}$]uridine ($27\text{ mCi}/\mu\text{mol}$) or $2\text{ }\mu\text{Ci}$ of [$\text{Me-}^3\text{H}$]thymidine ($18.9\text{ mCi}/\mu\text{mol}$) was added to each culture and incubation was continued for 2 h unless otherwise stated. The cultures were then diluted with 2 ml of ice-cold 0.9% NaCl and centrifuged at 0°C . The cell pellet was resuspended in 0.2 M-HClO_4 and left at 0°C for 30 min. The precipitate was collected by centrifugation at 0°C , washed once with 0.2 M-HClO_4 , resuspended in 0.5 M-HClO_4 and extracted at 70°C for 30 min. The samples were centrifuged, and the pellet was re-extracted once for 20 min. The supernatant fractions from the two extractions were combined and 1 ml samples were counted for radioactivity in a liquid-scintillation counter (Packard Instrument Co., Reading, Berks., U.K.) after addition of 10 ml of scintillation fluid. The scintillation fluid contained 1 vol. of Triton X-100 (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) and 3 vol. of toluene scintillator [4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene].

Under the conditions used the incorporation of radioactivity was linear with time up to 4h.

Polyacrylamide-gel separation of RNA

RNA was extracted from the cells by the method of Loening (1969), modified by extraction at 55°C. The ethanol-precipitated nucleic acid was dissolved in Tris-magnesium acetate buffer and treated with deoxyribonuclease [EC 3.1.4.5.; electrophoretically pure, Sigma (London) Chemical Co., London S.W.6, U.K.] according to the method of Loening *et al.* (1969). After reprecipitation with ethanol the RNA was dissolved in buffer 1 (Loening, 1969) and subjected to gel electrophoresis in the same buffer.

RNA labelled during a 45min exposure of the cultures to [³H]uridine was subjected to electrophoresis on a 9cm gel at 5mA/tube for 2.5h. These gels contained acrylamide and bisacrylamide [total concn. (T)=2.1% (w/v), ratio bisacrylamide: total (C)=8%] (Eastman-Kodak, Kirby, Liverpool, U.K.) re-purified by the method of Loening (1967). RNA from cultures labelled for 20h was separated on gels containing acrylamide with bisacrylamide cross-linking (T=2.5%, C=8%) run for 3.5h at 5mA/gel. Both gels terminated in a 1cm segment of more concentrated polyacrylamide gel (T=10%, C=3%). After electrophoresis the gels were stored in 0.2M-HClO₄ at 0°C for 1h and then their absorbance at 260nm was scanned with the linear-transport accessory of a Gilford model 240 spectrophotometer (Gilford Instruments Ltd., Morden, Surrey, U.K.). The gels were re-equilibrated to buffer 1 and minced in 2mm segments with a Gilson Gel Slicer (Gilson Medical Electronics Inc., Villiers-Le-Bel, France). The fractions of minced gel were oxidized with H₂O₂ in counting vials at 60°C overnight and counted for radioactivity in a liquid-scintillation counter after addition of 10ml of Triton X-100-toluene scintillator (1:3, v/v).

The molecular weights of the RNA species associated with peaks of radioactivity in the polyacrylamide gels were estimated on the basis of their migration rates relative to those of the mature rRNA species (Loening, 1969). Values for the molecular weights of 28S and 18S rRNA were taken from Loening *et al.* (1969) and the calculated molecular weights of 45S and 32S RNA agreed closely with those given by Loening *et al.* (1969). The identity of 4S-5S RNA species was based on their co-electrophoresis with calf liver soluble RNA (type IV, Sigma Chemical Co.).

Radioactivity in cold-acid-soluble pool

When the radioactivity associated with the cold-acid-soluble fraction of the cells was to be determined, the cultures were centrifuged without prior dilution

with 0.9% NaCl, the medium was decanted and the cells were resuspended in 0.2M-HClO₄ at 0°C. The decanted medium was made 0.2M with respect to HClO₄, stored at room temperature for 30min and centrifuged. The supernatant fractions from the acidified medium and from the first cold 0.2M-HClO₄ extract of the cells were decolorized before being used for radioactivity determinations. The 0.2M-HClO₄ precipitate of the cells was used to determine the radioactivity in hot HClO₄-soluble material as described above.

The acidified medium and cold-acid-soluble fractions were decolorized as follows. One drop of conc. HCl and approx. 50mg of MnO₂ were added to each fraction and the mixture was heated at 70°C for 30min. Excess of MnO₂ was removed by centrifugation and samples of the resulting colourless fluid were counted for radioactivity in Triton X-100-toluene scintillator (1:3, v/v) in a liquid-scintillation counter.

It was considered inadvisable to wash the cells before determination of the radioactivity in the cold-acid-soluble fraction because of the possibility of loss of intracellular radioactivity during the washing procedure. In the absence of washing, however, there was inevitably some contamination of the fraction with radioactivity derived from the medium. In order to correct for this, use was made of the relatively high Na⁺ concentration of the medium compared with that of the cells. The Na⁺ concentrations of the cold-acid-soluble fraction and of the acidified medium were determined by flame-emission spectrophotometry in a Techtron AA-5 spectrophotometer (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.). Na⁺ in excess of the normal intracellular concentration of 20mM (McCance & Widdowson, 1956) was considered to have come from contaminating medium, the volume of which was calculated and used to correct the radioactivity in the cold-acid-soluble fraction for that derived from the medium.

Actinomycin D uptake by lymphocytes

Cultures were incubated in the presence of [³H]-actinomycin D (5.4mCi/μmol) at 3.5ng/ml from the initiation of the cultures or at 92ng/ml from 39.5h. Incorporation was stopped at 42h by transfer of the cultures to ice, and subsequent treatment of the cultures was similar to that described for determination of radioactivity in the cold-acid-soluble fraction. The cells were not, however, treated with HClO₄ but were digested at 55°C with 1ml of Hyamine hydroxide. Water (2ml) was then added and the Hyamine hydroxide was neutralized with conc. HCl. Samples were taken for determination of radioactivity in the Triton X-100-toluene scintillation fluid and for estimation of Na⁺ concentration. The quantity of actinomycin D in the cells was corrected for medium contamination by the Na⁺ method described above.

Results and Discussion

Effects of EDTA and Zn²⁺ on [³H]uridine incorporation and RNA synthesis

Addition of EDTA (600 μ M)–Fe³⁺ (100 μ M) to lymphocyte cultures immediately before stimulation with phytohaemagglutinin inhibited [³H]uridine incorporation into RNA, but this inhibition was largely reversed by simultaneous addition of Zn²⁺ (Table 1). Kay & Handmaker (1970) have suggested that many factors may affect the radioactivity associated with the precursors for RNA synthesis and therefore that uridine incorporation may be an unreliable measure of RNA synthesis. Table 2 shows the relative rates of incorporation of [³H]uridine into RNA in EDTA-treated cultures either with or without additional Zn²⁺ and the corresponding incorporation into the cold-acid-soluble fraction of the cells. The inhibition of uptake of uridine into the cold-acid-soluble fraction of cultures treated with EDTA alone was slight compared with the effect on incorporation into RNA. Hausen & Stein (1968) have shown that most of the radioactivity in the cold-acid-soluble fraction of lymphocytes labelled with [³H]uridine was present in UTP, the immediate precursor of RNA. Although it is possible that the distribution of radioactivity from uridine among the components of the cold-acid-soluble fraction was altered by deprivation of Zn²⁺, it seems more likely that availability of Zn²⁺ influenced uridine incorporation into RNA by an effect on the rate of synthesis of RNA.

Inhibition of [³H]thymidine incorporation into DNA of lymphocytes by addition of EDTA has been shown to be reversed specifically by Zn²⁺ (Chesters, 1972). However, EDTA is a relatively non-specific chelator of bivalent cations and Mg²⁺ or Mn²⁺ are required for RNA synthesis *in vitro* and therefore might have been implicated in the effects of EDTA in the cultures. The culture medium contained Ca²⁺ at

three times and Mg²⁺ at twice the final molar concentration of EDTA in the cultures. Since the affinity of EDTA for Ca²⁺ is approximately a hundredfold greater than for Mg²⁺ it is extremely unlikely that addition of the chelator would have appreciably affected the availability of Mg²⁺ in the culture. On the other hand, Mn²⁺ was not specifically added to the cultures and has a much higher affinity for EDTA than that of Ca²⁺. Attempts were therefore made to replace Zn²⁺ with Mn²⁺ in reactivation of EDTA-inhibited cultures. At concentrations up to 100 μ M, Mn²⁺ addition did not significantly increase uridine incorporation, but at 200 μ M Mn²⁺ resulted in a 40% reversal of the inhibition of incorporation caused by EDTA. A similar effect was obtained by addition of less than 5 μ M–Zn²⁺ and it is reasonable to suggest that the effect of 200 μ M–Mn²⁺ was mediated through displacement from the EDTA of Zn²⁺ derived from the medium.

Table 1. *Effect of concentration of added Zn²⁺ on [³H]uridine incorporation into RNA by EDTA-treated lymphocytes between 20 and 22 h after stimulation with phytohaemagglutinin*

EDTA (600 μ M)–Fe³⁺ (100 μ M) with or without Zn²⁺ was added just before phytohaemagglutinin and the Zn²⁺ content of the basal medium was 5 μ M. Incorporations are expressed as a percentage of that obtained with addition of phytohaemagglutinin alone and were based on values from three experiments with four replicates per treatment. Values from the separate experiments were pooled within groups for calculation of means and S.E.M.

Added Zn ²⁺ concentration (μ M) ...	Incorporation (%)						
	0	5	10	20	50	100	200
Mean	40	72	83	86	88	84	87
S.E.M.	6	6	4	2	2	2	3

Table 2. *Effects of addition of EDTA (600 μ M)–Fe³⁺ (100 μ M) or EDTA–Fe³⁺ + Zn²⁺ (50 μ M) at the time of stimulation with phytohaemagglutinin on the incorporation of [³H]uridine and [³H]thymidine into RNA or DNA and into the cold-acid-soluble pool of lymphocytes*

Uridine incorporation was measured between 20 and 22 h and thymidine between 40 and 42 h after phytohaemagglutinin stimulation. Results are expressed as percentages of the incorporation obtained with phytohaemagglutinin alone and represent means $\pm$ S.E.M. of three experiments with four replicates each. Values from separate experiments were pooled within groups for calculation of means and S.E.M.

Fraction		Incorporation (%)			
		+ EDTA–Fe ³⁺		+ EDTA–Fe ³⁺ + Zn ²⁺	
		Mean	S.E.M.	Mean	S.E.M.
[³ H]Uridine	Pool	88	2	96	2
	RNA	40	3	89	2
[³ H]Thymidine	Pool	50	6	79	5
	DNA	51	4	100	4

Sensitivity of [³H]uridine incorporation in EDTA-treated cultures to actinomycin D

The effects of actinomycin D have been shown to vary qualitatively as well as quantitatively with concentration (Reich & Goldberg, 1964). For comparison with the effects of EDTA it appeared advisable to use actinomycin D at a concentration which produced a similar degree of inhibition of [³H]uridine incorporation when the compounds were added at the time of stimulation with phytohaemagglutinin. The sensitivity of uridine incorporation to actinomycin D was determined and on the basis of the results shown in Table 3, actinomycin D was used at a concentration of 3 ng/ml in the following investigations. This experiment also showed that the residual incorporation occurring at concentrations of actinomycin D of 4 ng/ml and greater was insensitive to Zn²⁺ deprivation and was enhanced by the presence of EDTA.

In general, when actinomycin D has been used in incorporation studies as an inhibitor of rRNA synthesis, it has been added just before administration of the radioactive isotope. When added at this time in the present study, a concentration of 50–100 ng of actinomycin D/ml was required to inhibit uridine

incorporation by lymphocytes to the same extent as that obtained when it was added at only 3 ng/ml at the initiation of the cultures. Further, the percentage distribution of actinomycin D between medium and cells was the same after 2.5 h as after 42 h (Table 4), suggesting that equilibrium was rapidly established and that the difference in sensitivity with time of administration did not reflect a continuing slow concentration of actinomycin within the cells. The long-term effects of very low concentrations of actinomycin D were obtained without the intracellular concentration of the drug reaching anything like the concentration associated with a direct effect on rRNA synthesis (Table 4).

Time-course of the effects of EDTA and actinomycin D on [³H]uridine incorporation

The effects of adding EDTA-Fe³⁺, EDTA-Fe³⁺ + Zn²⁺ or actinomycin D to cultures immediately before phytohaemagglutinin stimulation on the time-course of the rate of [³H]uridine incorporation are shown in Fig. 1.

In stimulated lymphocytes, increase in the rate of RNA synthesis has been shown to be proportional to

Table 3. *Effect of concentration of actinomycin D on the incorporation of [³H]uridine by lymphocytes stimulated with phytohaemagglutinin*

Actinomycin, EDTA-Fe³⁺ and Zn²⁺ (50 μM) were added to the cultures, as indicated, just before stimulation with phytohaemagglutinin. The period of incorporation was for 2 h from 40 h after phytohaemagglutinin addition. Values are based on four replicates per treatment.

		10 ⁻³ × Incorporation (c.p.m.)					
Actinomycin D (ng/ml)	...	0	1	2	4	7	10
Culture conditions							
Phytohaemagglutinin alone	Mean	46.1	42.9	39.3	2.0	1.5	0.8
	S.E.M.	1.3	2.5	1.9	0.3	0.2	0.2
EDTA-Fe ³⁺	Mean	14.6	9.8	9.1	5.3	2.1	1.5
	S.E.M.	1.5	0.6	1.1	0.5	0.3	0.1
EDTA-Fe ³⁺ + Zn ²⁺	Mean	50.6	42.4	37.0	5.8	2.1	1.5
	S.E.M.	3.9	6.4	1.3	0.7	0.2	0.1

Table 4. *Effect of concentration and length of exposure on the uptake of [³H]actinomycin D by stimulated lymphocytes*

All cultures received EDTA-Fe³⁺ and Zn²⁺ before stimulation with phytohaemagglutinin. Half of the cultures from each treatment received [³H]uridine after 40 h incubation and incorporations were determined 2 h later. These cultures were treated with [³H]actinomycin D as indicated for the individual treatments and the other half of the cultures in each treatment received corresponding amounts of [³H]actinomycin D. Results are means ± S.E.M. for groups of four replicate cultures.

Concentration of actinomycin D (ng/ml)	Time of addition of actinomycin D	Intracellular actinomycin D after 42 h incubation (ng/culture)	Proportion of actinomycin D within cells (%)	10 ⁻³ × Uridine incorporation (c.p.m.)
0	—	—	—	17.1 ± 0.9
3.5	At initiation of culture	0.43 ± 0.02	4.9 ± 0.3	11.7 ± 0.4
92	After 39.5 h incubation	12.3 ± 1.5	5.3 ± 0.6	9.8 ± 0.2

Fig. 1. Time-course of the effects of actinomycin D, EDTA-Fe³⁺ and EDTA-Fe³⁺+Zn²⁺ on the rate of incorporation of [³H]uridine into RNA in phytohaemagglutinin-stimulated lymphocyte cultures

All additions were made immediately before phytohaemagglutinin stimulation and incorporation periods were for 2h from the times indicated. Unless otherwise indicated the S.E.M. fell within the area of the symbol. ○, +EDTA (600 μM)-Fe³⁺ (100 μM); ●, +EDTA-Fe³⁺+Zn²⁺ (50 μM); △, +actinomycin D (3 ng/ml).

increase in RNA polymerase activity (Cooke *et al.*, 1973). In the present experiment the period of incorporation of [³H]uridine (2h) was such that most of the cold-acid-insoluble radioactivity would have been present in rRNA synthesized by RNA polymerase I (EC 2.7.7.6) (Cooper, 1968). The above effects of EDTA on uridine incorporation might be explained if Zn²⁺ was an essential component of RNA polymerase I. RNA polymerase from *Escherichia coli* has been shown to contain Zn²⁺ (Scrutton *et al.*, 1971), and Valenzuela *et al.* (1973) have speculated that all nucleotidyltransferases are Zn²⁺ enzymes.

If it is accepted that Zn²⁺ plays a role in RNA polymerase I activity, two mechanisms of inhibition of uridine incorporation by EDTA can be envisaged. It may inactivate pre-existing enzyme by removal of Zn²⁺ or it may decrease Zn²⁺ availability to an extent that prevents entry of Zn²⁺ into newly synthesized apo-enzyme. Valenzuela *et al.* (1973) have shown that inactivation of RNA polymerases *in vitro* by *o*-phenanthroline took 2–4h and that EDTA was less effective than *o*-phenanthroline. Therefore regardless of the mechanism of inhibition of RNA polymerase I by EDTA, a delay would be expected between addition of the chelator and its effect being manifest. The experimental observations in Table 5 show this to have occurred.

Table 5. Effects of the time of addition of actinomycin D (3 ng/ml) or EDTA-Fe³⁺ to stimulated lymphocytes on the incorporation of [³H]uridine into RNA

In all cases [³H]uridine was added to the culture 32h after stimulation and the incorporation was determined 2h later. The results are expressed as percentages of the incorporation obtained with phytohaemagglutinin alone and the values represent means and S.E.M. for four replicates per treatment.

Time of EDTA-Fe ³⁺ addition (h after stimulation) ...	Incorporation (%)					
	0	16	20	24	32	
Mean	51	43	59	65	92	
S.E.M.	4	3	2	4	2	
Time of actinomycin D addition (h after stimulation) ...	Incorporation (%)					
	0	16	20	24	32	
Mean	55	69	72	78	92	
S.E.M.	1	2	3	2	1	

Actinomycin D is thought to inhibit RNA synthesis and therefore uridine incorporation by binding to DNA, thus preventing its transcription by the appropriate polymerase (Reich & Goldberg, 1964). Since equilibrium of actinomycin D between medium and cells was achieved rapidly (Table 4), inhibition of uridine incorporation by the drug would be expected to occur soon after its addition to the medium. In fact, uridine incorporation 32h after stimulation of the cells was significantly less inhibited by actinomycin D added 16h after the start of the incubation than when it was added initially (Table 5). It appears therefore that very low concentrations of actinomycin D inhibited a process necessary for uridine incorporation at 32h but completed at least partially during the first 16h of lymphocyte transformation. Further, since inhibition by EDTA of uridine incorporation 32h after the start of the cultures was not significantly different whether the chelator was added initially or 20h later, the process sensitive to very low concentrations of actinomycin D must have occurred before that which required Zn²⁺.

Types of RNA synthesized in cultures treated with EDTA-Fe³⁺ or EDTA-Fe³⁺+Zn²⁺

If Zn²⁺ was required solely for the synthesis or maintenance of RNA polymerase I, treatment with EDTA would be expected to result in a decreased rate of synthesis of 45S rRNA precursor with corresponding decreases in the rates of production of 28S and 18S mature rRNA. Figs. 2(a) and 2(b) show the effects of adding EDTA-Fe³⁺ or EDTA-Fe³⁺+Zn²⁺ to lymphocyte cultures at the time of stimulation with phytohaemagglutinin on the patterns of uridine incorporation into RNA after short- and long-term labelling periods commencing 20h after stimulation. The addition of Zn²⁺ to EDTA-treated cultures in-

Fig. 2. Representative polyacrylamide-gel separations of RNA extracted from cultures labelled with [^3H]uridine

(a) Radioactivity was incorporated between 20 and 20.75 h after stimulation, and RNA separated on 2.1% (w/v) polyacrylamide gels. (b) Incorporation period was 20–40 h after stimulation and the gels contained 2.5% (w/v) polyacrylamide. Cultures received either EDTA-Fe^{3+} or $\text{EDTA-Fe}^{3+} + \text{Zn}^{2+}$ immediately before stimulation. —, + EDTA-Fe^{3+} ; ----, + $\text{EDTA-Fe}^{3+} + \text{Zn}^{2+}$.

Table 6. Effects of EDTA-Fe^{3+} and $\text{EDTA-Fe}^{3+} + \text{Zn}^{2+}$ on the incorporation of radioactivity into RNA species after 45 min labelling with [^3H]uridine from 20 h after phytohaemagglutinin stimulation

Group means were derived from values for three replicate gels from each of two experiments.

RNA species	Distribution of radioactivity between RNA species (%)				Significance of difference between groups
	EDTA-Fe ³⁺		EDTA-Fe ³⁺ +Zn ²⁺		
	Mean	S.E.M.	Mean	S.E.M.	
45S	21.8	2.0	19.9	3.3	N.S.
32S	30.5	1.0	24.2	1.5	<i>P</i> <0.01
28S	18.2	0.4	24.7	1.4	<i>P</i> <0.01
18S	12.9	0.5	14.1	0.3	N.S.
4S-5S	16.5	1.9	17.1	0.9	N.S.
	Ratios				
32S/28S	1.67	0.10	0.98	0.06	<i>P</i> <0.001
(32S+28S)/18S	3.81	0.24	3.47	0.15	N.S.
28S/18S	1.42	0.06	1.75	0.10	<i>P</i> <0.05

creased the overall incorporation of radioactivity and influenced the distribution of that radioactivity within the various RNA species investigated. After a short period of incorporation, Zn^{2+} -deprived cultures had a significantly higher proportion of radioactivity present in 32S RNA and a smaller proportion present in 28S

RNA than did EDTA-treated cultures supplemented with Zn^{2+} (Table 6). The 45S precursor molecule of rRNA is successively cleaved to yield one molecule of 18S rRNA and one molecule of 28S rRNA, the immediate precursor of the latter being 32S RNA (Weinberg & Penman, 1970). The sum of the radio-

activity present in both 32S and 28S RNA as a ratio of that present in 18S RNA was not significantly different in the EDTA-treated cultures compared with that found in similar cultures supplemented with Zn^{2+} . It appears therefore that the relative rates of synthesis of precursors of 18S and 28S rRNA were not altered by Zn^{2+} deprivation, although the rate of transfer of radioactivity from 32S to 28S RNA was decreased.

Over a longer period of incorporation, the ratio of radioactivity in 32S and 28S to 18S RNA was not significantly different in cultures supplemented with EDTA- Fe^{3+} and Zn^{2+} from that in cultures stimulated with phytohaemagglutinin alone. EDTA- Fe^{3+} in the absence of additional Zn^{2+} resulted, however, in a significantly lower ratio. The means ($\pm$ S.E.M.) of the ratios obtained with duplicate gels in each of two experiments were: phytohaemagglutinin alone, 2.89 ± 0.29 ; +EDTA- Fe^{3+} , 1.56 ± 0.02 ; and +EDTA- Fe^{3+} + Zn^{2+} , 2.60 ± 0.11 . At this time the radioactivity in 32S RNA was only a small proportion of that in 28S RNA and even with the better resolution of the two species achieved with more concentrated gels it was impossible to quantify the radioactivity in the two fractions separately.

The radioactivity incorporations suggest that deprivation of Zn^{2+} resulted in diminished survival of 28S rRNA. An alteration in the concentration of 28S rRNA was also seen in the pattern of u.v.-absorbing material in polyacrylamide-gel separations of RNA extracted from cultures 40h after phytohaemagglutinin stimulation. RNA from EDTA-treated cultures supplemented with Zn^{2+} showed more than twice as much u.v.-absorbing material in 28S rRNA as in the 18S rRNA. This would be expected from the ratio of the molecular weights of the two species and the equimolar proportion in which they are normally present (Cooper, 1972). The RNA from cultures treated with EDTA alone showed, however, approximately equal quantities of u.v.-absorbing material in these two peaks.

It does not seem possible to explain the difference in survival of 28S rRNA in Zn^{2+} -deprived cultures on the basis of a role for Zn^{2+} in RNA polymerase I. Kay & Cooper (1969) found a similar differential decrease in survival of 28S rRNA in lymphocyte cultures treated for 24h with low concentrations of actinomycin D. The lowered stability of 28S rRNA might be associated with previously reported increases in activity of ribonuclease (EC 3.1.4.22) found in zinc-deficient animals (Prasad & Oberleas, 1973) and after actinomycin D treatment (von Tigerstrom, 1972). The effects of EDTA and actinomycin D contrast with the balanced decrease in survival of both 18S and 28S rRNA found in unstimulated lymphocytes and in stimulated cultures treated with protein-synthesis inhibitors (Cooper, 1972).

Relationship of the effects of EDTA and low concentrations of actinomycin D

A number of other similarities have been demonstrated between the effects of low concentrations of actinomycin D and those caused by Zn^{2+} deprivation. In lymphocyte cultures both EDTA and low concentrations of actinomycin D have been found to inhibit increase in the rate of protein synthesis from approx. 8–10h after phytohaemagglutinin addition (Kay *et al.*, 1969; Chesters, 1972). Neither inhibitor, however, affected the increase in the rate of protein synthesis that occurred before 8h, nor did they prevent the continuation of synthesis for several hours at the rate achieved at that time. It appears that EDTA and actinomycin D prevented assembly of the components for translation rather than inhibited polypeptide synthesis.

In both primary cultures of rabbit kidney cells and stimulated lymphocytes addition of actinomycin D at the time of initiation of the culture inhibited the incorporation of [3H]thymidine into DNA at concentrations which did not inhibit incorporation when added immediately before the onset of DNA synthesis (Liebermann *et al.*, 1963a; Agarwal & Loeb, 1972). A similar pattern of sensitivity of thymidine incorporation was found when EDTA was added to lymphocyte cultures (Chesters, 1972), and in all three experimental situations the inhibition of incorporation was proportional to the decrease in the number of cells synthesizing DNA. The inhibitors appear to have acted by decreasing the number of cells capable of incorporating thymidine rather than by decreasing the rate of incorporation per cell. Thymidine incorporation involves uptake of the radioactive deoxynucleoside into the cell, phosphorylation and incorporation into DNA. In the present experiments EDTA inhibited thymidine uptake and incorporation to similar extents (Table 2), and inhibition of thymidine incorporation in rabbit kidney cells by either EDTA or actinomycin D was associated with similar decreases in the activity of both DNA polymerase and thymidine kinase (Liebermann *et al.*, 1963b). It seems likely that inhibition of DNA synthesis by EDTA or actinomycin D resulted from a failure of individual cells to acquire the group of proteins required for thymidine uptake and incorporation.

Many of the actions of actinomycin D and EDTA listed above suggest an effect on the control of synthesis of new proteins and enzymes. Actinomycin D is known to inhibit mRNA synthesis, but only at concentrations significantly greater than those required for inhibition of rRNA synthesis (Prescott, 1964). In the experiments quoted the concentrations of actinomycin D used were, however, often below those required to cause major inhibition of rRNA synthesis and therefore a direct effect of actinomycin D on the synthesis of mRNA does not appear to be an

acceptable explanation of its effects. Further, in the present experiments, prolonged exposure to very low concentrations of actinomycin D inhibited rRNA synthesis by an effect distinct from direct inhibition of transcription of rRNA genes. This suggests that low concentrations of actinomycin D inhibited an uncharacterized process with consequences which resemble those resulting from Zn^{2+} deprivation.

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