Biochem. J. (1972) **130**, 133–139
Printed in Great Britain

The Role of Zinc Ions in the Transformation of Lymphocytes by Phytohaemagglutinin

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(*Received* 15 *May* 1972)

1. Incorporation of [^{3}H]thymidine into DNA was inhibited by EDTA and diethylenetriamine-*NNN'N''N''*-penta-acetate but not by nitrilotriacetate even when Ca^{2+} was present at more than twice the concentration of the chelators. 2. The inhibition caused by EDTA was most effectively reversed by Zn^{2+} but also to a lesser extent by Cd^{2+}. Very little if any activation of the EDTA-inhibited system was obtained with Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+} or Ni^{2+} added alone. 3. Fe^{3+} added to the Zn^{2+}-activated lymphocytes in the presence of EDTA markedly increased thymidine incorporation. Addition of Cd^{2+} prevented the inhibition of incorporation which occurred at high Zn^{2+} concentrations. 4. If EDTA was added more than 15h after phytohaemagglutinin, the inhibition of incorporation was less than that obtained by its addition at zero time. If Zn^{2+} was added later than 12h after EDTA and phytohaemagglutinin, the inhibition of incorporation by EDTA was not fully reversed. A study of the time-course of the effects of delayed additions of EDTA and Zn^{2+} suggested that, on average, the cells required Zn^{2+} between 20 and 30h after phytohaemagglutinin addition to allow the full rate of thymidine incorporation at 37h. 5. The increase in the rate of protein synthesis caused by phytohaemagglutinin was not inhibited by EDTA until about 8h. The further increase after this was totally inhibited by EDTA but this inhibition was fully reversible with Zn^{2+}. The rate of protein synthesis in EDTA-inhibited cultures at 40h was the same as that at 10h. 6. There was a close similarity between the effects of EDTA on lymphocyte stimulation and those reported by Kay *et al.* (1969) with low doses of actinomycin D.

In previous investigations at this Institute, rats which were fed with a zinc-deficient diet showed a decline in the rate of [^{3}H]thymidine incorporation into DNA of several tissues. This decline was progressive and statistically significant before growth of the rats was adversely affected by the deficiency (Williams & Chesters, 1970). Further progress in defining the role of Zn^{2+} in DNA synthesis in partially hepatectomized rats was hampered by an inability to control the redistribution of Zn^{2+} between the tissues of the rats even when they were zinc-deficient. It seemed likely that a tissue-culture system where Zn^{2+} availability could be controlled by addition of chelators would yield further information on the role of Zn^{2+} in DNA synthesis. Working with human lymphocytes transformed by phytohaemagglutinin, Alford (1970) reported that addition of EDTA to the culture medium decreased the incorporation of [^{3}H]thymidine into DNA even in the presence of twice the molecular concentration of Ca^{2+}. The inhibition caused by EDTA was most effectively reversed by the addition of Zn^{2+}, and further supplementation with Fe^{3+} resulted in a complete recovery of activity. This paper reports investigations into the specificity and nature of the bivalent metal-ion requirements for DNA synthesis by lymphocytes and the relationship of these requirements to lymphocyte transformation by phytohaemagglutinin. A preliminary report of some of this work has already been published (Chesters, 1971).

Methods

Lymphocyte preparation

Pig blood (800ml) taken at slaughter was mixed immediately with heparin (50000i.u.) and the mixture was allowed to stand at 37°C for 1h in a pear-shaped separating funnel. The lower layer, containing most of the erythrocytes, was run off and the upper layer of plasma and leucocytes was centrifuged at $500g_{av.}$ for 15min in glass centrifuge bottles in an MSE Super-magnum centrifuge. Approx. 85% of the plasma was aspirated from the cell pellet, centrifuged again at $1000g_{av.}$ for 10min and used in the culture medium as described below. The cells were resuspended in the residual 15% of plasma and diluted to approx. 70ml with phosphate-buffered saline (Dulbecco & Vogt, 1954). The cells in plasma–saline were poured on to a 40cm×3.2cm glass column packed with dry Ballotini beads (approx. 0.3mm

diam.). The cells were incubated in the column at 37°C for 25 min and then eluted from the column at a rate of 3 ml/min with phosphate-buffered saline. Lymphocytes generally accounted for 95–99% of the leucocytes in the column eluate, in which there were two to six erythrocytes per leucocyte. Lymphocyte counts were made with a Fuchs–Rosenthal haemocytometer by using as a diluent 1% acetic acid with a trace of Brilliant Green dye. The cell preparations were diluted with culture medium to give a final cell concentration in culture of 3.2×10^6 lymphocytes/ml.

Cultures

Lymphocytes were cultured in bijou bottles sealed with aluminium caps and silicone rubber liners, and with 2.5 ml of Eagle's minimal essential medium (Flow Laboratories, Irvine, U.K.) containing 20% autologous plasma. The medium was supplemented with both penicillin and streptomycin (each 150 i.u./ml) and 5 μg of amphotericin B/ml. The medium and cultures were gassed with air + CO_2 (95:5) and incubated at 37°C in stationary culture. The lymphocytes were stimulated with phytohaemagglutinin (reagent grade; Wellcome Reagents Ltd., Beckenham, Kent, U.K.) diluted 1:60 in the culture medium (final concn. approx. 160 μg/ml), which was shown to produce maximal stimulation of [^{3}H]-thymidine incorporation. The cultures were prepared by adding the components in the following order: EDTA, 2 ml of medium, metal ions as sulphates or chlorides, cells in 0.5 ml of medium and, finally, phytohaemagglutinin. At least three replicate cultures were used for each determination.

Incorporation of [^{3}H]thymidine

After 37–40 h at 37°C, 2 μCi of [6-^{3}H]thymidine (2.7 mCi/μmol) was added to each culture and incubation continued for a further 2 h. The cells were then diluted with 2 ml of 0.9% NaCl and chilled to 0°C before being centrifuged at 500 $g_{av.}$ for 5 min. The cell pellet was resuspended in 1% (w/v) acetic acid, centrifuged and further prepared for counting as described below.

In early experiments, the cells after acetic acid washing were suspended in 5% (w/v) trichloroacetic acid, left at 0°C for 30 min and then filtered on 2 cm membrane filters (Oxoid; Oxo Ltd., London, U.K.). The filters were washed with 5% (w/v) trichloroacetic acid followed by 5% (w/v) acetic acid and dried. Radioactivity on the filters was estimated by suspension of the filter in toluene scintillator [4 g of 2,5-diphenyloxazole, 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene] and counting in a liquid-scintillation counter (Packard Instrument Co., Reading, Berks., U.K.). Results were expressed as c.p.m. incorporated/culture, the counting efficiency being approx. 4%.

In later experiments it was found that this method tended to give variable counting efficiencies if the cultures to be compared had been incubated for different times and had reached different stages of transformation. To overcome this, subsequent experiments used an extraction procedure which permitted evaluation of the counting efficiency of each sample. After acetic acid washing, the cells were suspended in 0.2 M-perchloric acid at 0°C for 30 min, centrifuged and the pellet was washed once by centrifugation in cold 0.2 M-perchloric acid. The residue was extracted at 70°C with 0.5 M-perchloric acid for 30 min, centrifuged and re-extracted for 20 min. The two extracts were combined, neutralized with 10 M-KOH, chilled to 0°C and centrifuged. Samples (1 ml) of the supernatant fraction were counted with 14 ml of Bray's (1960) scintillator containing 5% (w/v) Cab-o-Sil (Packard Instrument Co.) in a liquid-scintillation counter with automatic external standardization of the counts. The results were expressed as d.p.m./culture.

Incorporation of [^{14}C]valine

After incubation of the cultures for the required period, L-[U-^{14}C]valine (2 μCi at 5 μCi/μmol in the first experiment; 1 μCi at 10 μCi/μmol in the second experiment) was added to the cultures and incubation was continued for a further 2 h. The cultures were then diluted with 2 ml of 0.9% NaCl, chilled to 0°C and centrifuged at 500 $g_{av.}$ for 5 min. The cell pellet was resuspended in 0.5 M-perchloric acid, extracted at 70°C for 30 min, centrifuged and re-extracted with 0.5 M-perchloric acid for 20 min. The residue was washed twice with acetone, dried at 55°C and digested with 1 ml of Hyamine hydroxide (Nuclear Enterprises, Edinburgh, U.K.) at 55°C for 1 h. The Hyamine hydroxide solution was transferred to 9 ml of toluene scintillator (composition given above) and the mixture acidified by addition of 0.1 ml of conc. HCl before counting by liquid scintillation. Automatic external standardization allowed correction for variations in colour quenching between samples and results were expressed in d.p.m./culture.

Cell division

In an experiment to investigate the effects of EDTA on cell division, cultures were incubated at 37°C for 65 h in the presence or the absence of EDTA and Zn^{2+}. Colcemid (8 μg; CIBA, Duxford, Cambs., U.K.) was added to each culture and incubation continued for 5 h. The cells were washed with 0.9% NaCl and transferred in saline to glass slides. After staining with Leishman's stain, the cells were examined microscopically and the mitotic index was determined.

Estimation of Zn^{2+} and Cu^{2+} in the culture medium

Addition of trichloroacetic acid to a final concentration of 5% (w/v) at 0°C. was to precipitate the proteins of the culture medium and to disrupt the binding of Zn^{2+} and Cu^{2+} to them. After 30min the precipitated proteins were removed by centrifugation and the concentrations of Zn^{2+} and Cu^{2+} in the supernatant fluid were estimated by atomic absorption with a Techtron AA-5 atomic absorption spectrometer (Varian Associates Ltd. Walton-on-Thames, Surrey, U.K.). Solutions of Zn^{2+} and Cu^{2+} prepared in 5% (w/v) trichloroacetic acid were used to standardize the estimation.

Results

Table 1 shows the inhibition of [^{3}H]thymidine incorporation caused by adding EDTA (600μM) to cultures of pig lymphocytes stimulated with phytohaemagglutinin and the reversal of this inhibition by added Zn^{2+}. Although there were considerable differences in the degree of incorporation between experiments, the incorporation in the presence of EDTA and Zn^{2+} was generally as great as, if not greater than, that obtained with phytohaemagglutinin alone. In this and all subsequent experiments, the addition of 20% plasma to the medium provided endogenous Zn^{2+} and Cu^{2+} concentrations of approx. 5μM and 8μM respectively, and the medium was approx. 1.8mM with respect to Ca^{2+}.

Effects of different chelators

Fig. 1 illustrates the effects of adding chelators with differing affinities for Zn^{2+} on the activity of the system and its reactivation by added Zn^{2+}. Both EDTA and diethylenetriamine-*NNN′N″N″*-penta-acetate produced a marked inhibition of incorporation, which was reversible with added Zn^{2+}, the concentrations required being related to the chelators' affinity for this ion (Figs. 1*a* and 1*b*). The strength of binding of Zn^{2+} by nitrilotriacetate was insufficient to inhibit incorporation but did prevent the inhibition of the system found when similar concentrations of Zn^{2+} were added to the system in the absence of an added chelator (Figs. 1*c* and 1*d*).

Table 1. *Incorporation of [^{3}H]thymidine into DNA of pig lymphocytes stimulated with phytohaemagglutinin*

The conditions of culture and counting were as described in the Methods section. Values represent means of triplicates±S.E.M.

Culture additions	Incorporation (c.p.m./culture)
Cells alone	280± 30
Cells+phytohaemagglutinin	63600±2700
Cells+phytohaemagglutinin +EDTA (600μM)	8300±1300
Cells+phytohaemagglutinin +EDTA+Zn^{2+} (50μM)	82700±3100

Effects of different bivalent metal ions

The activities obtained by adding a range of different metal ions to the EDTA-inhibited lympocyte cultures are illustrated in Table 2. For comparative purposes the results have been expressed relative to the activity of the system at optimum Zn^{2+} concentration (55μM). Clearly Zn^{2+} was much more effective at reactivating the lymphocytes than any of

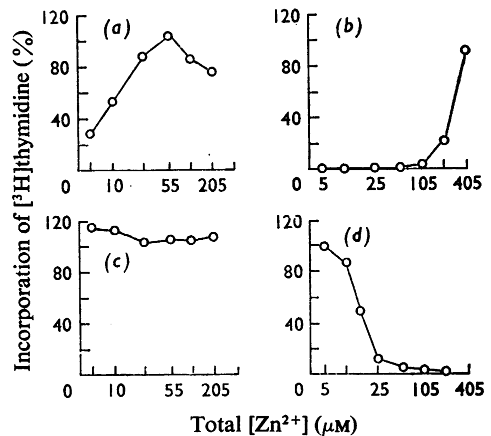

Fig. 1. *Effects of Zn^{2+} and different chelators on [^{3}H]-thymidine incorporation by lymphocytes stimulated with phytohaemagglutinin*

(*a*) EDTA (logK_s for Zn 16.5), 600μM; (*b*) diethylenetriamine-*NNN′N″N″*-penta-acetate (logK_s 18.6), 600μM; (*c*) nitrilotriacetate (logK_s 10.7), 600μM; (*d*) Zn^{2+} alone. The first point in graphs (*a*)–(*c*) represents the incorporation in the presence of chelator but without added Zn^{2+}; in this instance the Zn^{2+} present (5μM) was derived from the plasma added to the medium. The incorporations were expressed as percentages of that obtained by phytohaemagglutinin stimulation in the absence of added chelators or Zn^{2+}. Incorporation at 100% was equivalent to approx. 60000c.p.m./culture. The stability constant, K_s, for a metal–chelator complex is defined as follows:

$$\text{if}\quad M+L \rightleftharpoons ML$$

where M is the metal ion and L the chelator, then:

$$K_s = [ML]/[M][L]$$

Table 2. *Bivalent metal-ion requirements for [^{3}H]thymidine incorporation by lymphocytes in the presence of EDTA* (600 μM)

The activities were expressed relative to that at optimum Zn^{2+} concentration (55 μM). The medium always contained endogenous Zn^{2+} derived from the plasma (5 μM) as well as the added metal-ion concentration indicated. The values represent means of three replicates and, overall, the S.E.M. was approx. 3%. Full incorporation at 55 μM-Zn^{2+} varied between experiments within the range 0.5–2 × 10^6 d.p.m./culture.

		Incorporation (%)						
Metal	Added concn. of activating ion (μM) ...	10	20	50	100	200	Ion	$\log K_s$ for EDTA
Zn		50	81	100	85	75	Zn^{2+}	16.5
Cd		8	12	26	19	4	Cd^{2+}	16.5
Co		2	0	3	4	5	Co^{2+}	16.5
Cu		0	2	2	3	3	Cu^{2+}	18.8
Ni		0	0	2	3	16	Ni^{2+}	18.3
Mn		0	0	2	6	9	Mn^{2+} Mn^{3+}	13.8 24.8
Fe		0	0	0	7	12	Fe^{2+} Fe^{3+}	14.3 25.1

Table 3. *Effect of Cd^{2+} and Zn^{2+} on the reactivation of phytohaemagglutinin-stimulated lymphocytes in the presence of EDTA* (600 μM)

Incorporations were expressed as a percentage of the incorporation at optimum Zn^{2+} concentration. This incorporation was equivalent to approx. 3 × 10^5 d.p.m./culture. The concentrations listed were the added concentrations of the metal ions. Figures are means of triplicates; the S.E.M. was approx. 11% as determined by analysis of variance.

Zn^{2+} (μM)	50	50	50	50	200	50	100	50	20	0
Cd^{2+} (μM)	0	10	20	50	200	100	200	200	200	200
Cd^{2+}/Zn^{2+}	—	0.2	0.4	1	1	2	2	4	10	—
Incorporation of [^{3}H]thymidine (%)	100	98	115	143	146	172	146	156	140	4

the other metal ions tested. Certain metals with higher affinities for EDTA than that of Zn^{2+} produced slight activation at high concentration, which may have been caused by displacement of endogenous Zn^{2+} from the EDTA. In contrast, Cd^{2+} activated the lymphocytes at the same ionic concentration as Zn^{2+} but gave a lower maximum activity.

In view of the similarities between the activations by Zn^{2+} and Cd^{2+} and their known antagonism in animals (Underwood, 1971), the effects of combined additions of these ions were investigated (Table 3). At 200 μM, Cd^{2+} produced a stimulation of incorporation at all Zn^{2+} concentrations. At optimum Zn^{2+} concentration, the degree of stimulation increased from 20 μM-Cd^{2+} up to a maximum at 100 μM.

Alford (1970) found that the activation of incorporation by Zn^{2+} was enhanced by additional Fe^{3+}, and this effect was reinvestigated (Table 4). Fe^{3+} at as low a concentration as 10 μM stimulated incorporation in the presence of Zn^{2+} and was not inhibitory even at high concentrations.

Effect of Zn^{2+} on mitosis

The effects of EDTA and Zn^{2+} on the mitotic index were examined 72 h after adding phytohaemagglutinin, with colcemid present for the final 5 h. EDTA (600 μM) added alone prevented the appearance of mitotic figures but added with 55 μM-Zn^{2+} gave a mitotic index (150/1000 cells) higher than that with phytohaemagglutinin alone (44/1000 cells). The enlargement of both nucleus and cytoplasm characteristic of lymphocyte transformation did not appear to be affected by EDTA.

Timing of the Zn^{2+} requirement

The time-course of the increase in the rate of [^{3}H]thymidine incorporation into DNA is shown in Fig. 2. The major increase in the rate of incorporation

Table 4. *Effect of combinations of Fe^{3+}, Zn^{2+} and Cd^{2+} on the incorporation of [^3H]thymidine by lymphocytes in the presence of EDTA* (600 μM)

Incorporation at 100% was equivalent to approx. 5×10^5 d.p.m./culture. Values without a common superscript were significantly different at the 5% level as assessed by an analysis of variance with eight replicates per treatment.

Metal ions (and added concn.) (μM)	Incorporation of thymidine (%)
Zn^{2+} (50)	100[a]
Cd^{2+} (100)	24[b]
Fe^{3+} (200)	37[b]
Zn^{2+} (50) + Fe^{3+} (10)	193[c]
Zn^{2+} (50) + Fe^{3+} (20)	192[c]
Zn^{2+} (50) + Fe^{3+} (50)	167[c,d]
Zn^{2+} (50) + Fe^{3+} (100)	204[c]
Zn^{2+} (50) + Fe^{3+} (200)	179[c]
Zn^{2+} (50) + Fe^{3+} (200) + Cd^{2+} (100)	202[c]
Zn^{2+} (50) + Cd^{2+} (100)	149[d]

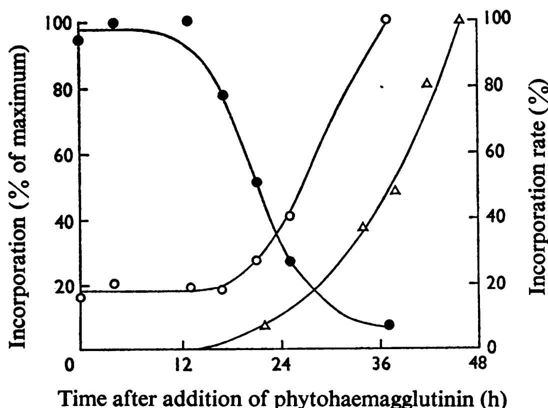

Fig. 2. *Effects of delayed addition of EDTA and Zn^{2+} on the incorporation of [^3H]thymidine into DNA*

△, Incorporation of thymidine in a 2h period starting at the time indicated; ○, effect of delaying the addition of EDTA (600 μM) for the time indicated; ●, EDTA (600 μM) added at zero time, Zn^{2+} (50 μM) added at the time indicated. For ○ and ●, thymidine incorporation was measured during the period 37–39h. In each investigation the incorporation was measured by perchloric acid extraction of the DNA and the results were corrected for variations in counting efficiency. The incorporations were expressed as percentages of the maximum incorporation obtained in each case.

began about 20h after addition of phytohaemagglutinin.

When EDTA (600 μM) was added just before phytohaemagglutinin, thymidine incorporation was inhibited to the full extent but, when EDTA addition was delayed until just before the incorporation period, incorporation was maximal. The incorporations obtained when EDTA was added at intermediate times are shown in Fig. 2. The inhibition of incorporation caused by adding EDTA at time zero was totally reversed by 55 μM-Zn^{2+} added just before the phytohaemagglutinin. Zn^{2+} added to the EDTA-inhibited system just before the incorporation period failed to cause any reactivation. Incorporations obtained by adding Zn^{2+} at intermediate times are shown. The greater inhibition shown for zero time addition of EDTA when Zn^{2+} was added just before the incorporation period than when Zn^{2+} was never added resulted from a difference in the degree of inhibition caused by EDTA in the two experiments and not from the late addition of Zn^{2+}.

The results suggest that removal of available Zn^{2+} by adding EDTA still permitted at least 50% of the maximal incorporation if the EDTA was added after about 30h incubation with phytohaemagglutinin. On the other hand, at least 50% incorporation was obtained when EDTA had been added at zero time provided that Zn^{2+} was added before about 20h. It would appear therefore that, on average, the cells required Zn^{2+} during the period of 20–30h after phytohaemagglutinin addition to permit full incorporation of thymidine at 37h.

Effect of EDTA and Zn^{2+} on amino acid incorporation

Table 5 shows the effect of adding EDTA just before phytohaemagglutinin on the incorporation of [^{14}C]-valine into lymphocyte protein. The partial inhibition caused by EDTA was reversible by Zn^{2+}. Fig. 3 illustrates the time-course of the effect of EDTA in a second experiment. In the presence of EDTA alone, the rate of protein synthesis did not alter markedly between 10 and 40h. Furthermore, the initial rise in the rate of protein synthesis after addition of phytohaemagglutinin occurred in the presence of EDTA even without added Zn^{2+}. It seems that protein synthesis *per se* was not inhibited by EDTA. However, the later increase in the rate of protein synthesis from about 8h onwards was inhibited by EDTA but this inhibition was reversed when Zn^{2+} was also added before the phytohaemagglutinin.

An obvious possibility arising out of the results shown in Fig. 3 was that the EDTA took approx. 8h to equilibrate with Zn^{2+} within the cells. However, adding EDTA 10h before phytohaemagglutinin resulted in the same incorporation of valine as occurred when the EDTA was added immediately before phytohaemagglutinin. Furthermore, addition

Table 5. *Effect of EDTA and Zn^{2+} on [^{14}C]valine incorporation by phytohaemagglutinin-stimulated lymphocytes*

Times represent the period of incorporation (h) after the addition of phytohaemagglutinin. Values are the means of three replicates ± S.E.M.

Conditions	10^{-3} × Incorporation (d.p.m./culture)	
Incorporation period (h) ...	16–18	40–42
No phytohaemagglutinin	1.53 ± .03	0.67 ± .01
Phytohaemagglutinin alone	8.99 ± .27	12.98 ± .55
Phytohaemagglutinin + EDTA (600 μM)	6.80 ± .19	6.28 ± .14
Phytohaemagglutinin + EDTA + Zn^{2+} (55 μM)	9.56 ± .24	13.39 ± .36

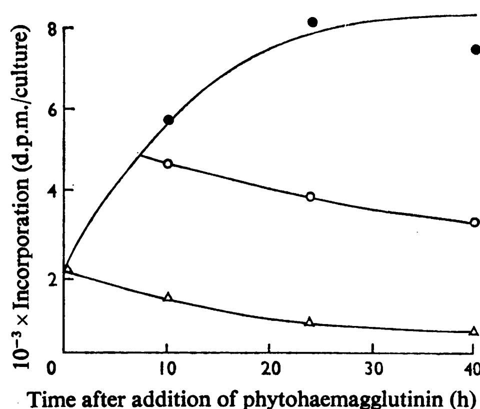

Fig. 3. *Time-course of incorporation of* [^{14}C]*valine by lymphocytes in the presence or absence of EDTA and* Zn^{2+}

The incorporations were measured in a 2h period starting at the time indicated. △, No phytohaemagglutinin or other additions to the cultures; ○, EDTA (600 μM) added to stimulated cultures at zero time; ●, EDTA (600 μM) and Zn^{2+} (50 μM) added to the stimulated cultures at zero time.

of EDTA 10h previously still allowed full recovery of activity if Zn^{2+} was added just before phytohaemagglutinin.

It appears that whereas the initial rise in protein synthesis after phytohaemagglutinin stimulation was insensitive to EDTA and therefore did not require Zn^{2+}, the later increase in the rate of incorporation was dependent on the presence of Zn^{2+}.

Discussion

Lymphocyte transformation is accompanied by an increase in genetic expression (Darzynkiewicz *et al.*, 1969), which results in a prolonged increase in the rate of protein synthesis (Neiman & MacDonnell, 1970) and later in the synthesis of enzymes required for DNA replication (Loeb *et al.*, 1970). The results described above have confirmed the findings of Alford (1970) that lymphocyte transformation by phytohaemagglutinin was inhibited by EDTA even in the presence of 2 Ca^{2+} ions/molecule of EDTA This contrasts with the results of Kay (1971), who concluded that the effect of EDTA on lymphocytes was to inhibit phytohaemagglutinin binding but that the effect was reversible with a twofold concentration of Ca^{2+}. The concentrations of EDTA used by Kay (1971) were, however, approx. 16 times those used in the present experiments where Ca^{2+} was always present at more than twice the concentration of EDTA. Furthermore, EDTA in the present experiments, although inhibiting thymidine incorporation, still permitted morphological transformation of the lymphocytes and the initial rise in protein synthesis. It seems clear that the phytohaemagglutinin was not prevented from binding to the lymphocytes under the present conditions.

It is of interest that the effects of EDTA on lymphocyte DNA and protein synthesis described above showed close similarities to those reported by Kay *et al.* (1969) in studies of the action of actinomycin D on human lymphocytes. A concentration of actinomycin D just sufficient to inhibit ribosomal RNA synthesis (0.05 μg/ml) totally inhibited [^{3}H]thymidine incorporation into DNA. The time-course of the sensitivity of thymidine incorporation to actinomycin D found by Kay *et al.* (1969) was very similar to that shown in Fig. 2 for EDTA. Kay *et al.* (1969) also investigated the inhibition of amino acid incorporation by actinomycin D (0.05 μg/ml). If allowance is made for the different degrees of incorporation in the control cultures, then the results they obtained with actinomycin D are virtually superimposable on those shown in Fig. 3 for the effect of EDTA on amino acid incorporation.

Lieberman *et al.* (1963), studying rabbit kidney cells in primary culture, showed that thymidine incorporation could be inhibited by the addition of EDTA or actinomycin D but, in each case, only if the inhibitor was added several hours before the onset of DNA synthesis. In this type of culture

(Lieberman & Ove, 1962) EDTA also produced an inhibition of protein synthesis similar to that shown in Table 5. When the effects of EDTA on thymidine incorporation were investigated further (Lieberman *et al.*, 1963), the synthesis of both ATP–thymidine 5′-phosphotransferase (EC 2.7.1.21) and deoxynucleoside triphosphate–DNA deoxynucleotidyltransferase (EC 2.7.7.7) was found to be impaired. This suggests an effect on the induction of a metabolic pathway rather than on a single zinc-containing enzyme. Fujioka & Lieberman (1964) found that EDTA infused into partially hepatectomized rats for different periods resulted in different degrees of inhibition of thymidine incorporation but that in all cases the incorporation obtained was proportional to the number of cells labelled with tritium. The effect of EDTA would appear to have been to control whether in any particular cell the induction of the enzymes responsible for thymidine incorporation occurred, rather than to modify the rates of synthesis of these enzymes. In addition Lieberman & Ove (1962) showed that, although thymidine incorporation by kidney cells in primary culture was sensitive to EDTA, this chelator did not affect DNA synthesis by permanently cultured HeLa and L cells. The combined evidence suggests that, in each of the systems discussed, EDTA inhibited the expression of the cells' genetic potential to synthesize the enzymes required for DNA synthesis and cell division. This process of 'gene activation' (Baserga & Stein, 1971) is essential for cell replication in primary cultures but unnecessary in continuously cultured cell lines where the enzymes required for growth are permanently present. The present series of experiments has confirmed the conclusions of Alford (1970) that Zn^{2+} is of prime importance in reversing the inhibition caused by EDTA and it seems likely therefore that Zn^{2+} is required for 'gene activation'.

Of the metal ions tested, only Zn^{2+} and Cd^{2+} produced marked reactivation of incorporation of thymidine when added alone and, of these, Cd^{2+} reactivation gave only a quarter of the activity obtained with Zn^{2+}. However, Cd^{2+} did produce a consistent stimulation of incorporation when added in conjunction with optimum Zn^{2+} concentration (Table 3). Since Cd^{2+} is generally considered to be toxic to animals and antagonistic to Zn^{2+} (Underwood, 1971), the reason for the activation of lymphocytes by Cd^{2+} is not obvious. The most likely explanation would appear to be that Zn^{2+} activated two processes, one of which stimulated thymidine incorporation whereas the other, active at high Zn^{2+} concentrations, tended to inhibit it. This would explain the observed response of thymidine incorporation to increasing concentrations of Zn^{2+} (Fig. 1 and Table 2). If only the latter activity were antagonized by Cd^{2+}, the presence of Cd^{2+} would tend to stimulate incorporation by preventing the inactivation normally associated with high concentrations of Zn^{2+}. Reference to Table 3 shows that such an explanation does fit with the observed effects of Cd^{2+} on the system.

Fe^{3+} added alone produced only a slight increase in the rate of incorporation in the presence of EDTA, but added with optimum Zn^{2+} concentration it caused almost a doubling of incorporation. One explanation for this effect could be that Zn^{2+} was required for the initial processes of gene activation as suggested above and Fe^{3+} was required for a subsequent step in the events leading to DNA synthesis. In the absence of Zn^{2+}, gene activation would be minimal and the presence of Fe^{3+} would do little to enhance the activity of the system. However, with Zn^{2+} added to the medium, Fe^{3+} would be required for optimum expression of the gene activation caused by Zn^{2+}.

I am very grateful to Miss M. Nicol for excellent technical assistance with this work and to my colleagues for many helpful discussions.

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