oxidation and produces a semiquinol or aryl radical (27). The short-lived radical can spontaneously participate in a variety of intermolecular and intramolecular reactions such as coupling, disproportionation, hydrogen abstraction, peroxide and epoxide formation (28). These reactions are governed only by the electronic properties of the specific radical and the nature of the immediate environment.

In vivo studies have found radioactivity from 2,4-D-2^{14}C bound to proteins including the lysine-rich histones in cultured Jerusalem artichoke tuber tissue (29). The authors report that they recovered intact 2,4-D upon hydrolysis and chromatography (30). If this is the case, then a different mechanism is responsible for the 2,4-D protein binding than that binding the 2,4-D derivative to sRNA observed in our experiments.

Present knowledge about the molecular mechanisms involved in auxin action is limited, however, the positive correlations between growth and peroxidase activity and the data presented here that a peroxidase can produce reactive auxin derivatives able to bind to a cellular nucleophile such as sRNA suggests that this oxidation may be responsible for generating the ultimate molecular regulators of growth and differentiation *in vivo*. The biological effects would most likely be governed by the proximate molecular environment in which the reactive species are generated.

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tumor cells under certain conditions. Secondly, recent work has shown that the association of initiator Met-tRNA$_f$ with native 40S ribosomal subunits in Ehrlich ascites tumor cells is regulated by factors, such as amino acid supply, which influence the rate of polypeptide chain initiation *in vivo* (16). It was therefore of interest to determine whether non-erythroid cells possess an inhibitor of initiation analogous to that identified in reticulocytes. The existence of a simple partial purification procedure for the reticulocyte inhibitor (7, 12) has allowed us to apply the method to non-hemoglobin synthesizing cell types. We report here the preliminary characterization of a soluble macromolecular inhibitor of protein synthesis from Ehrlich cells which resembles the reticulocyte inhibitor in a number of its properties. An abstract of this work has already been published (17).

MATERIALS AND METHODS

Growth of Ehrlich ascites tumor cells. Ehrlich cells were grown in suspension culture in Eagle's medium containing 5% calf serum as described previously (18). They were maintained at a concentration of 3 x 10^5 cells per ml by dilution with fresh medium every 24 hr.

Preparation of translational inhibitors from Ehrlich ascites cells and rabbit reticulocytes. Ehrlich cells were harvested and a post-mitochondrial supernatant was prepared exactly as described by Smith and Henshaw (19) except that the cell lysis buffer was slightly different (10mM Tris. HCl pH 7.6, 10mM KCl, 1.5mM Mg acetate). This supernatant was then centrifuged at 164,000 x g for 150 min in a Beckman 50Ti rotor to prepare the post-ribosomal supernatant. A typical preparation from 12 litres of cell suspension gave approximately 35 ml of post-ribosomal supernatant.

For preparation of the translational inhibitor from the post-ribosomal supernatant of Ehrlich cells essentially the same procedure was followed as previously described for preparation of the reticulocyte inhibitor.(7, 12). Briefly, a pH 5.0 precipitate was obtained from the post-ribosomal supernatant and redissolved in half the original volume of 36 mM N-2-hydroxyethyl-piperazine N'-2-ethanesulfonic acid (Hepes), pH 7.2. This solution was clarified and then subjected to precipitation by $(NH_4)_2SO_4$ at 30% saturation. The precipitate from this step was again dissolved in Hepes buffer (one twelfth the original volume of post-ribosomal supernatant) and dialysed for 16 hr against this buffer. Aliquots were then frozen and stored under liquid nitrogen. Twelve litres of cell suspension yielded approximately 3 ml of an inhibitor solution containing 10-13 mg of protein per ml.

Preparation of the translational inhibitor from rabbit reticulocytes was exactly as previously described (12) with the same pH 5.0 and $(NH_4)_2SO_4$ precipitation steps as above.

Preparation and incubation of rabbit reticulocyte lysates. The procedures used were exactly as described before (3, 12). The radioactive amino acids used, either ^{14}C leucine (273 Ci/mole) or ^{14}C valine (273 Ci/mole), were supplied by New England Nuclear and were added at a final concentration of 4μCi/ml.

Preparation of the Met-tRNA$_f$ binding factor (IF-MP). The highly purified initiation factor, IF-MP, was generously supplied by Drs. W.C. Merrick and W.F. Anderson. It was prepared from reticulocyte ribosomal salt wash as described

by Safer et al., (20) and is functionally equivalent to the initiation factors IF-E2 (21), IF-1 (22, 23) and IF-L3 (24).

RESULTS AND DISCUSSION

To determine whether Ehrlich cells possess an inhibitor of protein synthesis similar to that present in rabbit reticulocytes, we carried out a protein fractionation procedure on Ehrlich cells identical to that previously used for the partial purification of the "irreversible" inhibitor formed in reticulocyte lysates on incubation in the absence of hemin (12). The effects of such a preparation from Ehrlich cells on the kinetics of protein synthesis in a hemin-supplemented rabbit reticulocyte lysate system are shown in Figure 1a. The Ehrlich cell preparation had no effect on the initial rate of amino acid

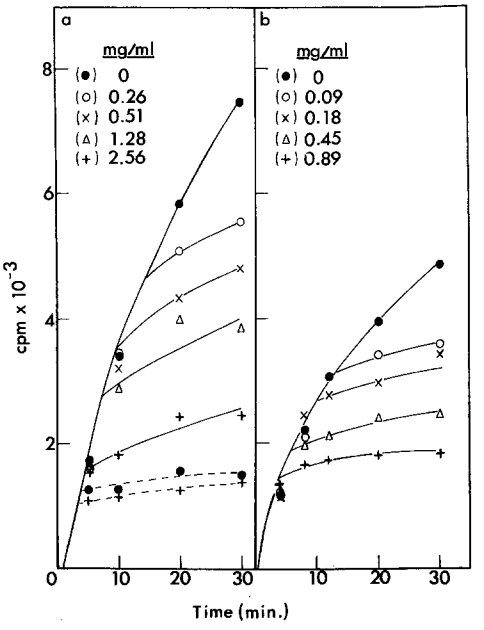

Figure 1. Protein Synthesis in Reticulocyte Lysates in the Presence of Various Concentrations of Partially Purified Inhibitor Preparations.

Reaction mixtures (50 or 60 µl) were incubated at 30°C in the presence of various amounts of inhibitor preparations from (a) EAT cells and (b) rabbit reticulocytes. Aliquots (10 µl) were removed at the times indicated for assay of (a) valine or (b) leucine incorporation into protein. (————), plus 40 µM hemin; (- - - -), minus hemin. The inhibitor concentrations shown refer to mg protein added per ml of reaction mixture.

incorporation but caused a rather abrupt decrease in the rate of protein synthesis at later times of incubation. The time at which this decrease occurred was dependent on the concentration of inhibitor. If hemin was not added to the incubation, protein synthesis shut off after a lag period of several minutes, and in this case the presence of a high concentration of the inhibitor had little additional effect on the kinetics of amino acid incorporation (Figure 1a). Figure 1b shows the results of a similar assay of various dilutions of rabbit reticulocyte inhibitor. The kinetics of protein synthesis in the presence of this inhibitor are identical to those shown in Figure 1a, but less reticulocyte protein than Ehrlich cell protein was needed to produce the same degree of inhibition.

The results illustrated in Figure 2b demonstrate that the inhibitor preparation does not act by virtue of endonuclease action on mRNA *in vitro*. Reticulocyte polysomes were incubated for 30 minutes at 30°C in the presence and absence of the inhibitor under conditions not permitting possible loss of polysomes due to protein synthesis. They were then analysed for changes in size distribution by sucrose density gradient centrifugation. This highly sensitive test revealed very little loss of polysomes in the incubation containing added inhibitor. In contrast, when protein synthesis could take place, the inhibitor caused an almost complete loss of polysomes within 30 minutes (Figure 2d). The latter effect indicates that polypeptide chain initiation is the major site of action, as is the case for the hemin-controlled reticulocyte inhibitor also (8, 13).

We have previously reported that the effects on the reticulocyte lysate system of hemin deficiency and of the inhibitory fraction obtained from reticulocyte post-ribosomal supernatant can be overcome by addition of highly purified preparations of a reticulocyte initiation factor (12, 13, 25). This factor, called IF-MP by Safer *et al.*, (20), forms a ternary complex with Met-$tRNA_f$ and GTP and binds Met-$tRNA_f$ to 40S ribosomal subunits. To test for a similar mode of action of the Ehrlich cell inhibitor to that of the reticulocyte

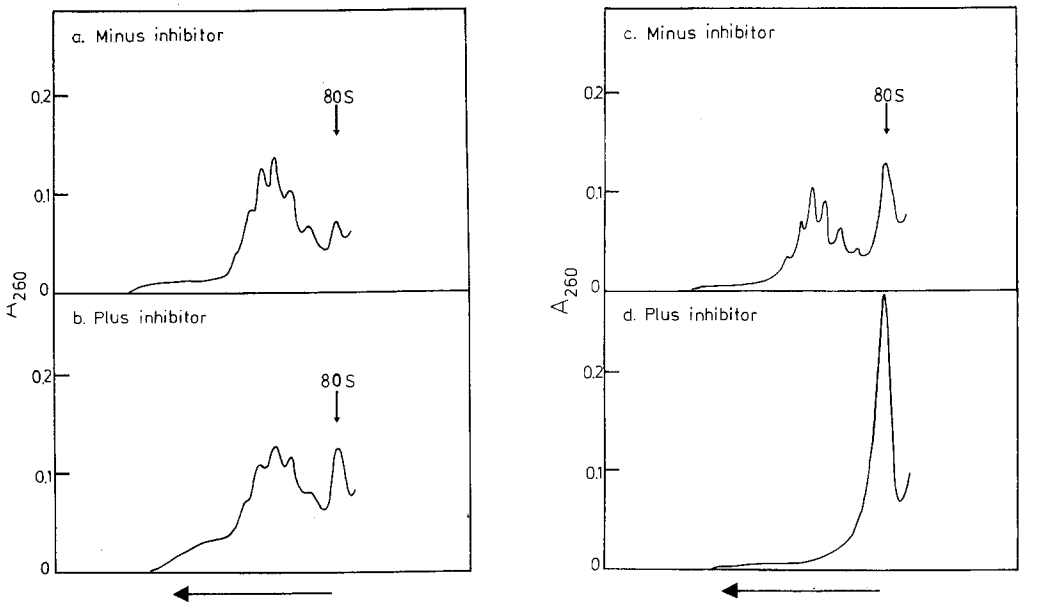

Figure 2. Size Distribution of Reticulocyte Polysomes after Incubation in the Presence and Absence of EAT Cell Inhibitor.

50 µl of reticulocyte lysate was incubated at 30°C in a final volume of 100 µl containing either (a and b), 6 mM Tris (pH 7.8), 40 mM KCl, 1.5 mM Mg acetate and 10 µM hemin but lacking added ATP, GTP, energy generating system or amino acids, or (c and d), 10 mM Tris (pH 7.8), 80 mM KCl, 2 mM Mg acetate, 20 µM hemin and optimal concentrations of ATP, GTP, energy generating system and amino acids for protein synthesis. Inhibitor was present where indicated at a final protein concentration of 2 mg per ml. After 30 minutes an equal volume of cold gradient buffer (20 mM Tris, pH 7.8, 100 mM KCl, 5 mM Mg acetate) was added to each incubation and the samples were layered on to 20-50% sucrose gradients containing the above buffer. The gradients were centrifuged at 4°C for 100 min at 39,000 rpm in a Beckman SW40 rotor. Fractionation was performed by pumping the gradients from the bottom through a flow-cell of a Unicam SP-1800 spectrophotometer recording optical density at 260 nm. (a and c) no inhibitor; (b and d) inhibitor present. The 80S monomeric ribosome peak is indicated by the vertical arrow; the direction of sedimentation was from right to left.

inhibitor, we determined whether the effect of the Ehrlich cell inhibitor in the reticulocyte lysate assay could also be counteracted by the Met-$tRNA_f$ binding factor. Figure 3a shows that leucine incorporation in the presence of the ascites cell inhibitor was restored to 84% of the control value by the initiation factor. In the absence of the Ehrlich cell inhibitor the Met-$tRNA_f$ binding factor had no stimulatory effect, but was capable of restoring protein

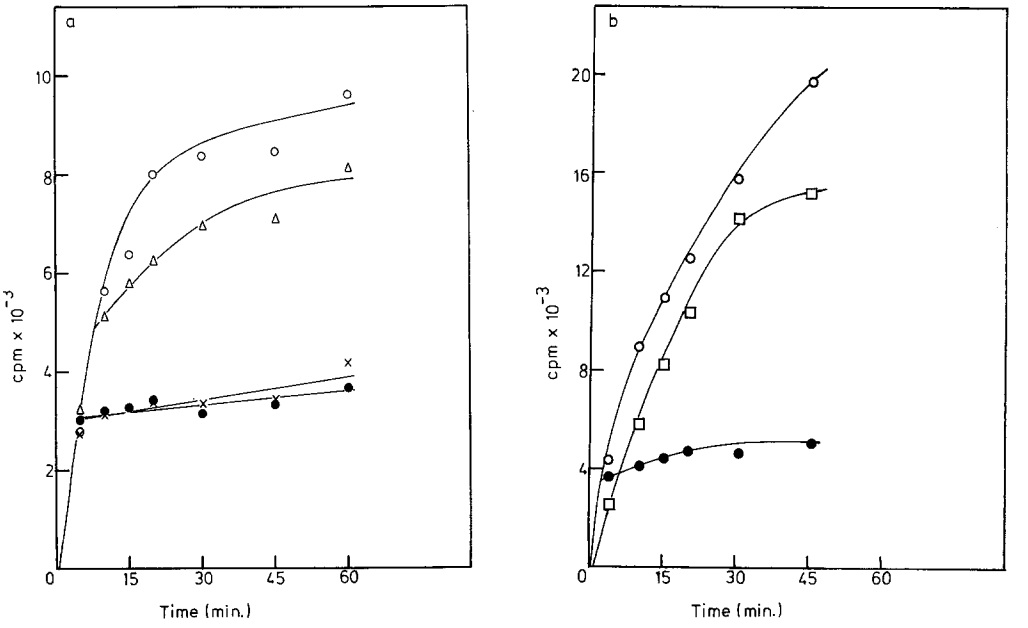

Figure 3. Effects of the Met-tRNA$_f$ Binding Factor on the Inhibition of Reticulocyte Protein Synthesis by the EAT Cell Inhibitor (panel a) and by Heme-deficiency (panel b).

Reaction mixtures were incubated at 30°C and aliquots were removed at intervals for assay of leucine incorporation into protein. Panels (a) and (b) illustrate results from separate experiments. (a) (o——o), plus hemin (38 μM); (●——●), minus hemin; (✕——✕), plus hemin and EAT cell inhibitor (2.32 mg/ml); (△——△), plus hemin, EAT cell inhibitor and Met-tRNA$_f$ binding factor (0.16 mg/ml); (b) (o——o), plus hemin; (●——●), minus hemin; (□——□), minus hemin, plus Met-tRNA$_f$ binding factor (0.16 mg/ml).

synthesis in the absence of hemin to 85% of the control (plus hemin) value as shown in Figure 3b.

The experimental results described in this paper indicate that Ehrlich ascites tumor cells possess a soluble, non-dialyzable inhibitor of protein synthesis which has properties in common with the "irreversible form" of the hemin controlled translational inhibitor present in reticulocytes. It is not known at present how widespread the occurrence of such translational inhibitors in different cell types may be. There is evidence that the hemin-controlled reticulocyte inhibitor persists into the mature erythrocyte stage (26) and a translational repressor with similar properties in Friend virus transformed

murine proerythroblasts has been described (27). In addition to the inhibitor from the ascites tumor cells used in the present study we have also obtained evidence for the presence of similar inhibitors in rat liver and muscle tissue (M.J.C. and V.M.P. unpublished studies; J. Delaunay and I.M. London, manuscript in preparation). Since the work described here was completed, Weber et al., (15) reported stimulatory effects of hemin addition to cell-free extracts of HeLa cells on the subsequent protein synthetic activity of these extracts. These effects were attributed to the existence of a hemin-suppressible inhibitor of polypeptide chain initiation in HeLa cells.

The possible existence of inhibitors of polypeptide chain initiation in eukaryotic cells raises the question of how their activities could be controlled. Evidence from several laboratories shows that, in the reticulocyte, a "pro-inhibitor" exists which gives rise, in the absence of hemin, to a hemin-reversible inhibitor (5-9). The latter then becomes transformed to the irreversible inhibitor of initiation. It is not known to what extent hemin might control translation in non-erythroid cells, but it is well established that other environmental factors including hormones and nutritional conditions can influence rates of polypeptide chain initiation. For example, deficiency of a single essential amino acid or of glucose from the medium in which Ehrlich ascites cells are grown results in a substantial inhibition of polypeptide chain initiation and a loss of 40S ribosomal subunit-Met-$tRNA_f$ complexes in the intact cells (16). It is possible that such an effect in these cells occurs through operation of a mechanism analogous to that established for control of protein synthesis by hemin. Knowledge of whether environmental factors can control initiation by reversible activation of pre-existing "proinhibitor" molecules or whether translational inhibitors are rapidly synthesized and degraded in response to changing conditions must await further purification and characterization.

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