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EFFECT OF THE TOXIC CASTOR BEAN PROTEIN, RICIN, ON THE PHOSPHORYLATION PATTERN OF [^{32}P]-LABELLED RIBOSOMES FROM MOUSE L CELLS

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Mouse L cells were grown in the presence of [^{32}P] phosphate and two dimensional gel electrophoresis and radioautography was used to examine the labelling pattern of ribosomal proteins in the presence and absence of ricin. The major proteins that were phosphorylated were L40/L41, although others were phosphorylated to a smaller extent. There was an apparent decrease in the extent of L1/L2 phosphorylation brought about by ricin; however, the extent of L40/L41 phosphorylation was unchanged.

The toxic action of ricin is generally considered to be enzymatic (1) although Hedblom et al. (2) have shown that the binding of ricin and its constituent polypeptide chains is stoichiometric. Ricin specifically inactivates the 60S ribosomal subunit of susceptible ribosomes (3,4,5) and is thought to affect EF-1 and EF-2 associated functions (6,7), but not peptidyl transferase activity or initiation (8). Ricin requires no addition of cofactors to result in inactivation (1) and does not appear to be a endonuclease (9). Since no change was observed in the two dimensional pattern after treatment of 80S rabbit reticulocyte ribosomes with ricin, it was concluded that ricin has no apparent proteolytic action, although the possibility was not ruled out that ricin could have a slight and very specific proteolytic activity (1). Based on the 2 to 1 stoichiometry of binding of ricin A chain to the 60S subunit of rat liver, the possibility has been put forward that L40 and L41 might serve as the site of binding (2). Because phosphorylated ribosome proteins, such as S6, migrate on the edge of the Coomassie Blue stained spot (11,12) and L40/L41 stain so weakly, studies such as that of Lugnier et al. (1) may not adequately detect ricin induced changes. Therefore, we wanted to determine if L40/L41 was phosphorylated in mouse L cells and if ricin was able to dephosphorylate the two acidic proteins, an action requiring no cofactor.

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MATERIALS AND METHODS

Ricin was purified by Bio-Gel and CM-Bio-Gel A chromatography (13). The preparation of ricin$_1$ shows only a single band upon pH 8.3 gel electrophoresis, has a molecular weight of 64,000 and inhibits the translation of polyuridylic acid by rat liver ribosomes at levels of 1-10 ng/ml.

NCTC clone 929 mouse cells, strain L, were grown without serum on the surfaces of 2 liter roller bottles containing 100 ml of culture medium (14). The medium was replaced on the fourth day to insure maximal growth. On the sixth day, the bottles were rolled at 5 RPM for 60 min. at 2 hour intervals over an 8 hour period to dislodge rounded, mitotic cells. The remaining cells were labelled with 20 to 60 µl of [^{32}P] (25 mCi/ml, Amersham Searle) in medium containing one tenth as much phosphate as the complete medium. The cells were harvested after 24 hours of labelling and the ribosomes isolated (15). The polysomes from the Palmiter method were suspended in 0.6 M NH_4Cl in buffer A of Staehelin and Falvey (16), collected by centrifugation through 0.5 M sucrose in buffer A, treated with 20 µg/ml of ribonuclease, collected by centrifugation through 0.7 M sucrose in buffer A, treated with 0.5% sodium deoxycholate and centrifuged through 0.7 M sucrose in buffer A. Ribonuclease treated monosomes were sensitive to ricin and were more active than untreated preparations in polyuridylic acid translation. Samples of ribosomes were treated with ricin at final concentrations up to 0.59 mg/ml for 30 minutes at 37°. The concentrations of ricin used were sufficient to produce maximal inactivation within 10 minutes (2). The ribosomal proteins were extracted in 3 M LiCl-4 M urea (17) and precipitated with trichloroacetic acid (18). Equal amounts of control or ricin-treated ribosomes, between 30 and 50 A_{260} units, were used for gel electrophoresis (19). Rat liver protein synthesis was assayed using polyuridylic acid as described previously (2,3).

RESULTS

Labelling pattern of 80S ribosomes: Radioautograms of labelled 80S ribosomal proteins are compared in Fig. 1 to the pattern of the same gel obtained by staining with Coomassie Blue. The origin was intensely labelled and up to twelve [^{32}P] positive spots have been observed with 80S ribosomes. The most intensely labelled proteins were those corresponding to L40/L41, using the Wool and Stöffler numbering system (20). The other proteins which contain [^{32}P] are L1, L2, S12 and S6. In other experiments we found that S17, S20, S31 or L20, S21, L14 and L21 contained small amounts of label. All of these proteins have been weakly labelled compared to the extent of phosphorylation of L40/L41.

Alkaline phosphatase treatment (0.7 mg/ml) of the labelled ribosomes for 30 min. at 37° resulted in the disappearance of all the [^{32}P] spots except for the origin, which is probably RNA. Treatment of the extracted proteins to remove phospholipids (21) from possible membrane contamination resulted in no change in the radioautograms.

Fig. 2 shows the effect of ricin treatment on both the [^{32}P] labelling and Coomassie Blue staining pattern of identical quantities of 80S ribosomal proteins. The

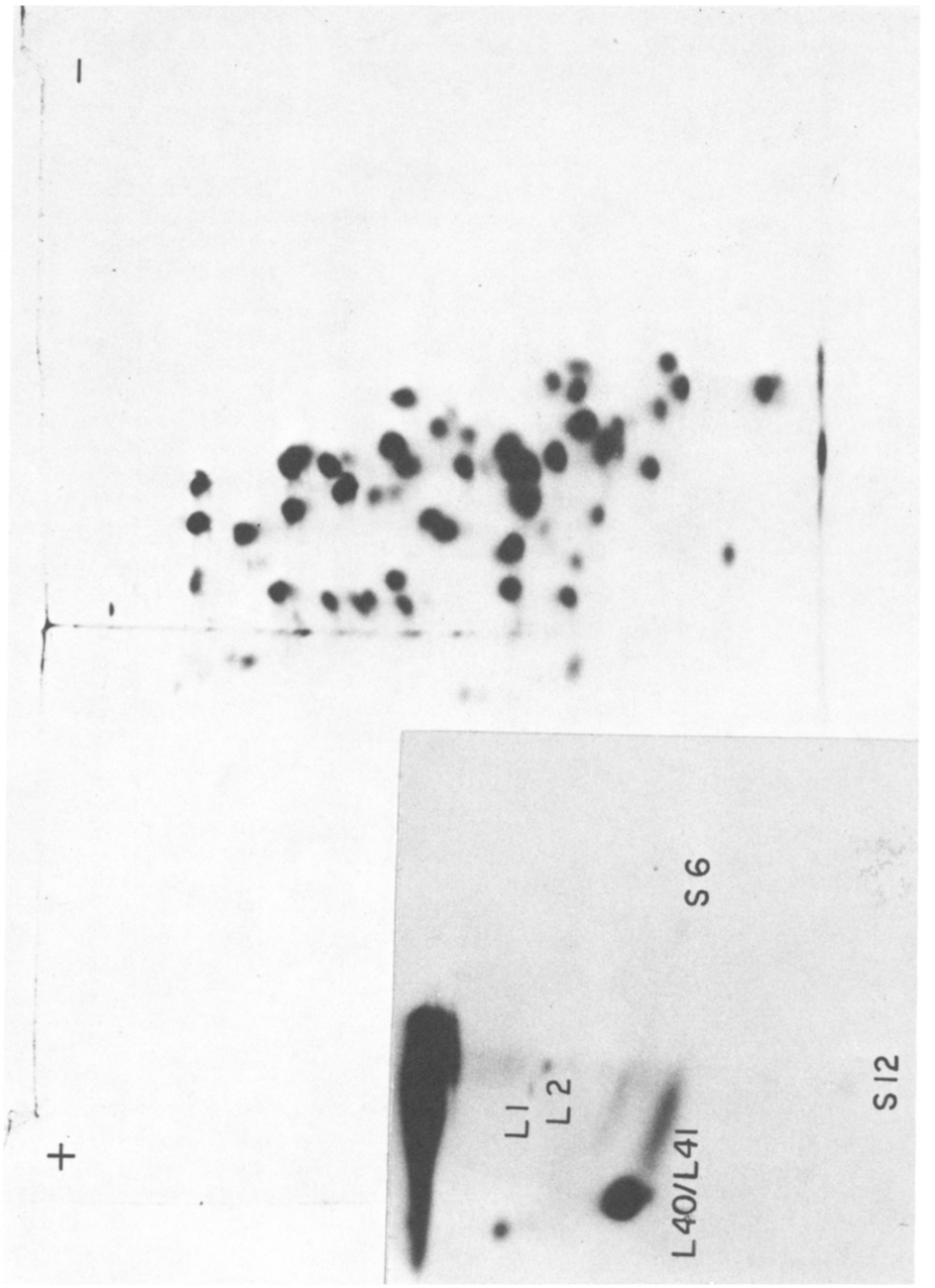


FIG. 1. Phosphorylation pattern of untreated 80S ribosomes from mouse L cells: A sample of 80S [^{32}P]labelled ribosomes was extracted in LiCl/urea and 200 µg of the proteins were applied to each anionic and cationic gel in the first dimension. The gel was first stained with Coomassie Blue and then radioautographed at -80° C (inset) for one day.

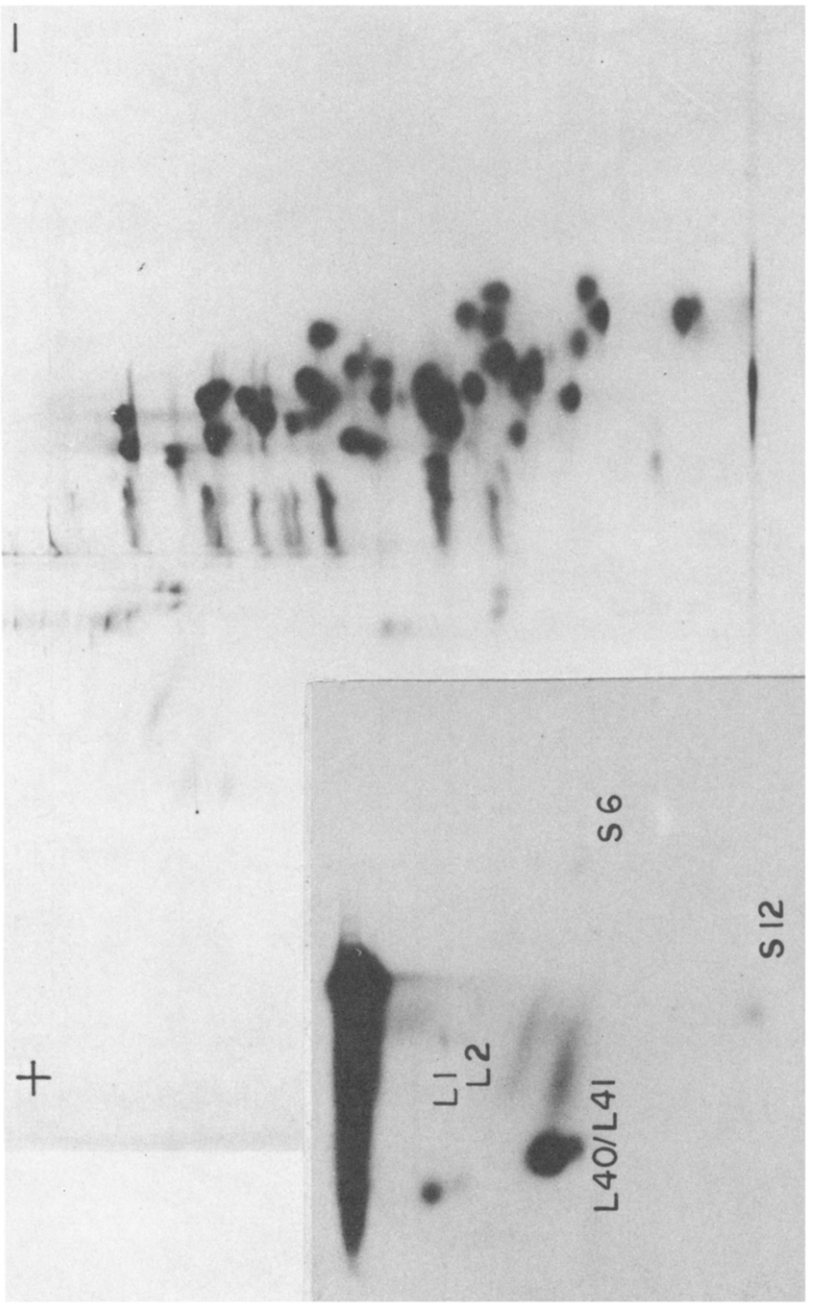
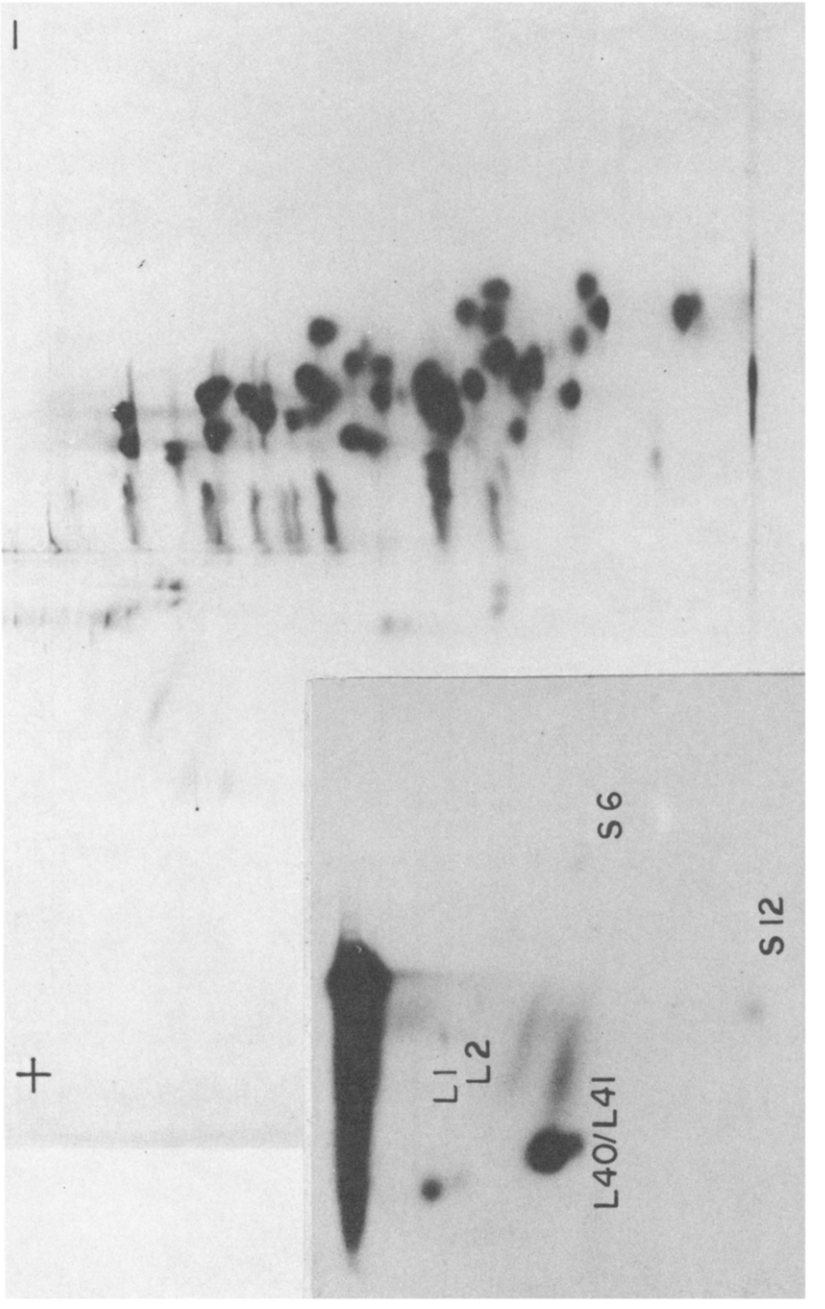


FIG. 2. Phosphorylation pattern of 80S mouse L cell ribosomes treated with ricin: In parallel with the experiment in Figure 1, an identical sample was treated with a 10-fold molar excess of ricin for 30 minutes at 37°C.

relative amounts of [^{32}P] under each spot were estimated using a Densichron densitometer after correcting for the background density. L40/L41 were only weakly stained by Coomassie Blue; however, no significant change in the intensity of the [^{32}P] labelling could be detected in L40/L41 between ricin-treated (Fig. 2) and control ribosomes (Fig. 1). No changes in intensity of L40/L41 were observed with either short or long periods of film exposure. As an example, the L40/L41 spot in one experiment could be divided into two regions of intensity which were reasonably well resolved on the film, the upper spot having an absorbance of 1.59 in the ricin-treated gel and 1.62 in the control gel. The lower region had an absorbance of 1.78 in the ricin-treated gel and 1.80 in the control gel. Only one change in the pattern of phosphorylation resulted from ricin treatment. The intensity of the [^{32}P] spots we identify as L1 and L2 was reduced by ricin treatment; however, the intensity (which was weak) of Coomassie Blue staining of L1 and L2 between ricin-treated and untreated controls appeared to be unchanged. Because ribosomes were not reisolated to remove ricin, any differences in quantity of L1 and L2 would have to be due to a change in solubility in LiCl/urea or TCA or to a changed mobility upon ricin treatment. The reduction in [^{32}P]was easily observed in three of four experiments; one experiment did not show this difference.

Effect of ricin on phosphorylation pattern of 60S subunits: The two dimensional electrophoretograms of the 60S proteins after ricin treatment are shown in Fig. 3. The spots above L1 and L2 in Fig. 3B are due to added ricin. The [^{32}P]spots identified as 40S proteins in Fig. 1 and 2 are not present in the 60S radioautograms (Fig. 3C and 3D). A weak spot is present which is marked with a question mark since proteins from both subunits, S31 and L20, comigrate at this position. The poorly labelled proteins were not observed in 80S ribosomes when the short exposure time was employed. The elongated streaks to the right of L40/L41 seen with 80S monosomes are absent. As expected, the L40/L41 proteins were intensely labelled compared to the L1 and L2 proteins. In agreement with the results using undissociated ribosomes, the intensity of the [^{32}P] spots of L40/L41 (2.67 and 2.81 in ricin-treated and control samples, respectively) did not appear to change significantly when identical quantities of control and ricin-treated 60S subunits were compared. Furthermore, treatment with


-
+
L1
L2
L40/L41
S6
S12
L40/L41
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L40/L41
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ricin appeared to decrease the amount of [^{32}P]label found associated with L1 and L2 and in one case the L1 and L2 [^{32}P]spots were completely absent from the ricin-treated sample. The intensity of the L1/L2 [^{32}P]spots in Fig. 3 are 0.05 and 0.24 for ricin-treated and control samples, respectively. The reduction in the intensity of L1/L2[^{32}P] spots by ricin treatment, although somewhat variable, was reproduced on three separate occasions using three different preparations of 60S ribosomal subunits.

Effect of alkaline phosphatase on protein synthesis: Rat liver ribosomes (2) were treated with E. coli alkaline phosphatase. No significant difference in the ability of the treated ribosomes to synthesize polyphenylalanine as compared to controls was observed except at very high concentrations of phosphatase (Fig. 4). Identical results were obtained when phosphatase was added to polyuridylic acid for 10 minutes before ribosomes were added suggesting that the message was being degraded at high alkaline phosphatase concentrations.

DISCUSSION

The major proteins that are phosphorylated are L40/L41 in mouse L cells. Acidic proteins are known to be phosphorylated in other cells (22,23) and although no quantitative data are available on the extent of labelling, it is likely that L40/L41 contain several phosphate groups in view of the intensity of the [^{32}P] spots compared to the other phosphorylated proteins. The elongated L40/L41 spots seen especially with proteins extracted from polysomes may be indicative of some heterogeneity in the extent of phosphorylation. No significant change could be demonstrated in the state of phosphorylation of L40/L41 brought about by ricin treatment and no differences could be detected in the position of the phosphorylated ribosomal proteins after two dimensional gel electrophoresis. The only changes in phosphorylation pattern that were observed are with the L1 and L2 proteins; however, L1 and L2 are so poorly labelled that the conclusion that ricin is a specific phosphatase is premature.

FIG. 3. Comparison of the pattern of phosphorylation between ricin treated and untreated purified 60S ribosomal subunits: The 40S and 60S subunits from [^{32}P] labelled mouse L cells were prepared by the procedure of Blobel and Sabatini (27). A sample (55.3A_{260} units) of 60S ribosomal subunits was extracted with LiCl/urea and prepared for gel electrophoresis (A) and radioautography (C). A sample of identical size was treated with a 10-fold molar excess of ricin and prepared in parallel for gel electrophoresis (B) and radioautography (D).

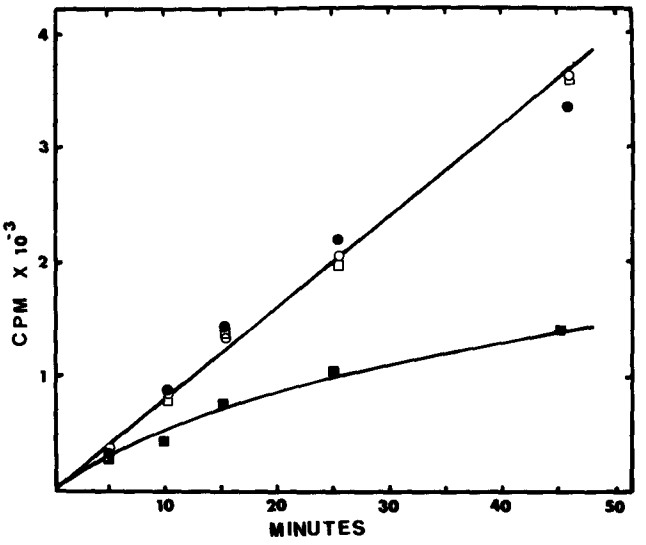

FIG. 4. Effect of alkaline phosphatase on synthesis of polyphenylalanine: Dialyzed alkaline phosphatase was added to a concentration of 2.34 (■), 0.234 (●) and 0.023 (□) mg/ml to about 3 A_{260} units of rat liver ribosomes in 100 µl of buffer. After a 10 minute incubation at 37°C, 200 µl of a mixture containing the necessary components for the translation of polyuridylic acid was added. Samples were quenched in TCA, filtered for liquid scintillation counting, and compared to controls (○) without ricin treatment.

Nevertheless, the apparent dephosphorylation of L1 and L2 is consistent with the known location of ricin action on the 60S subunit.

There are several arguments against a hypothesis that this apparent action of ricin is directly involved in inhibiting ribosome activity. No data are available which correlate the state of ribosomal phosphorylation with any change in the pattern or specificity of translation (24). Furthermore, E. coli alkaline phosphatase at low to moderate concentrations had no effect of polyuridylic acid translation activity. If the action of ricin were to dephosphorylate L1 and L2, resulting in inactivation of the ribosome, this would imply a role for L1 and L2 in which they were necessarily present in a phosphorylated state in order for the ribosome to be active. This would appear not to be the case since L1 and L2 are not universally phosphorylated (11,12,25). Detection of L1 and L2 on gels is complicated by the fact that they stain poorly and are not consistently seen by all laboratories (26).

From this work, we conclude that ricin does not act as a phosphatase removing phosphoryl groups from L40/L41, but we cannot definitely rule out the possibility that ricin is a highly specific phosphatase acting on L1 and L2. However, if this is a true action of ricin, it is not at all clear how this effect would be directly associated with the inhibitory effect of ricin on protein synthesis.

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