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TOSYL LYSINE CHLOROMETHYL KETONE INHIBITION OF THE INITIATION OF HEMOGLOBIN SYNTHESIS*

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Abstract—Tosyl lysine chloromethyl ketone (TLCK) a potent inhibitor of cellular enzymes, rapidly and irreversibly inhibited protein synthesis in the anucleate rabbit reticulocyte. This inhibition was accompanied by a conversion of the polyribosomes to single ribosomes not attached to mRNA, without an increase in the amount of ribosomal subunits. The polyribosomal conversion to single ribosomes required ribosomal movement along mRNA, suggesting that the major effect was on polyribosome formation (initiation of globin chain synthesis). This was confirmed by demonstrating that single ribosomes do not attach to mRNA in the presence of TLCK. The level of reduced glutathione was also decreased upon treatment with TLCK. It remains to be seen if this is the cause of the inhibition of translation.

TOSYL LYSINE chloromethyl ketone (TLCK) is a specific and irreversible inhibitor of both serine and sulfhydryl cellular enzymes. It has been shown to react with histidine in the prosthetic site of serine proteases[1,2] and to alkylate the sulfhydryl group of sulfhydryl proteases.[3] Previously it has been shown in a variety of systems that, when cellular enzymes are inhibited by this agent, protein synthesis is also retarded.[4–7] TLCK has been used to prevent tumor promotion by phorbol ester,[8,9] phytohemagglutinin activation of lymphocytes,[7] the embryological development of sea urchins, and the growth of bacteria.[6] It has been suggested that in lymphocytes the predominant effect of TLCK on cell proliferation may be due to inhibition of transcription.[7] There are, however, no data on the effect of TLCK on translation. The rabbit reticulocyte hemoglobin-synthesizing system is an ideal means of studying translation, as no transcription occurs in these anucleate cells. Using this system, we were able to demonstrate that TLCK irreversibly inhibits translation, with its major effect at the site of attachment of ribosomes to messenger RNA (initiation).

MATERIALS AND METHODS

Incubation of intact cells. Reticulocyte-rich blood was collected in heparin by cardiac puncture of phenylhydrazine-treated rabbits.[10] The cells were centrifuged free of plasma and washed twice with low magnesium saline.[11] Incubations were carried out n a "metabolite water bath shaker" at 37° with air as the gas phase. The washed

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reticulocytes were incubated in the low-magnesium saline containing Tris (hydroxymethyl) amino methane hydrochloride buffer, 5 mM, pH 7·4, at 37°. The density of cells in the incubation medium was 1 vol. of packed cells/7 vol. of medium. The concentrations of amino acids, glucose, iron-transferrin were as previously described.[12] In some experiments, hemin was substituted for iron-transferrin.[10] TLCK was placed into solution immediately before each experiment and the pH adjusted to 7·4.

Measurement of protein synthesis in intact cells. In experiments measuring the incorporation of radioactive amino acids into the nascent chains on polyribosomes, the total volume of the incubation mixture was 2 ml. The cells were incubated for 10 mins in the presence of 5×10^{-5} μM L-leucine to satisfy the leucine requirement. L-(U-^{14}C)-leucine (16·5 nmoles, 5 μCi) was added for the last 2 min of incubation. The cells were washed, lysed, centrifuged in sucrose density gradients, and 0·75-ml fractions of the sucrose density gradients were collected and counted in a Beckman liquid scintillation spectrometer (efficiency 92 per cent) as previously described.[13]

When the incorporation into hemoglobin was measured, the total volume of the incubation was 0·2 ml. L-(U-^{14}C)-leucine (3·3 nmoles, 1 μCi) was present throughout the incubation as the only source of leucine. Metabolism was stopped by removing a 50-μl sample into cold 5% TCA. The protein was then washed three times with cold 5% TCA, once at 90° with 5% TCA for 20 min, three times with an ethanol–ether (3:1) wash at 62°, and twice with ether. The protein was suspended in ether and plated on 0·45 μm millipore filters. Radioactivity was determined in a Nuclear of Chicago gas-flow counter with micromil window (efficiency 15 per cent).

Analysis of the ribosome–polyribosome component. Cells were washed twice with low magnesium saline, lysed and the stroma removed as previously described.[10] One ml of the 1:7 stroma-free lysate was layered on 36 ml of a 15–30% (w/w) linear sucrose gradient in standard buffer (0·01 M Tris, pH 7·4 at 4°, 0·01 M KCl and 0·0015 M $MgCl_2$). After centrifugation in a Spinco SW 27 swinging bucket rotor at 4° for speeds and times shown with individual experiments, the gradient was pumped through the flow-through cell of a Beckman Kintrac VII spectrophotometer and absorbance at 260 nm measured.

Determination of ATP concentrations. ATP concentrations were determined on perchloric acid extracts of reticulocytes by the method of Bucher.[14] Reagents were obtained from the Boehringer Mannheim Company.

Proteolytic dissociation of single ribosomes not attached to mRNA. Pronase selectively dissociates only 80 S ribosomes not attached to mRNA into 60 S and 40 S subunits.[15,16] Selective proteolysis was used, therefore, to ascertain whether or not ribosomes were attached to mRNA. In these experiments the total volume of the incubation was 8 ml. The ribosome–polyribosome component was isolated from the cell lysate by centrifugation through a cushion of 30% sucrose at 100,000 *g* for 3 hr. The resultant adherent pellet was gently rinsed and resuspended in 1 ml standard buffer. The resuspended ribosomes (2·5 mg) were mixed with 0·5 mg pronase at a final volume of 1 ml at 4° for 1 hr. The concentration of ribosomes was determined at 260 nm using an extinction coefficient of 11·2 absorbance units/mg/ml.[17] After proteolysis, the solution was centrifuged at 600 *g* for 10 min and any pellet was discarded. The ribosomal suspension was then layered on a 36-ml, 15–30% sucrose gradient and analysed as described above.

Assay of polyribosome formation. Reticulocytes were incubated in the presence of

a known reversible inhibitor of initiation of hemoglobin synthesis, either 0·1 M *n*-butanol[18] or 0·1 mM 2,2′-bipyridine,[19] for 20 min at 37°. At the end of these incubations, polyribosomes have been converted to single ribosomes not attached to mRNA.[16] The cells were then washed twice with buffered saline to remove the inhibitor, and then re-incubated for 1 hr at 37° in the complete medium. This results in the single ribosomes again attaching to mRNA to form polyribosomes identical to control patterns. Since initiation of protein synthesis occurs when ribosomes attach to mRNA, this regeneration of the polyribosome profile was used as an assay of initiation. Comparisons were made between TLCK and inhibitors of elongation (puromycin and cycloheximide).

Determination of reticulocyte reduced glutathione (GSH). Incubation of reticulocytes, with and without TLCK, was performed as described above. Aliquots (0·2 ml) of the incubation mixture were assayed colorimetrically at 410 nm after reaction with 5,5′-dithio,bis(2-nitrobenzoic acid) according to the method of Beutler *et al.*[20] GSH for standard curves was obtained from the Aldrich Company.

RESULTS

TLCK inhibition of hemoglobin synthesis in intact reticulocytes. When rabbit reticulocytes, incubated in a medium which fully supports hemoglobin synthesis, were treated with TLCK at a final concentration of 1 mM at 37°, there was a prompt inhibition of protein synthesis (Fig. 1). This effect is very concentration-dependent (Fig. 2). In subsequent experiments the concentration used was 1 mM.

The inhibition of protein synthesis by TLCK is accompanied by an irreversible conversion of polyribosomes to single ribosomes and a lack of incorporation of radioactive leucine into nascent chains (Fig. 3). When the ribosomal subunits were resolved on sucrose density gradients, no change was observed in the amount of 60 S

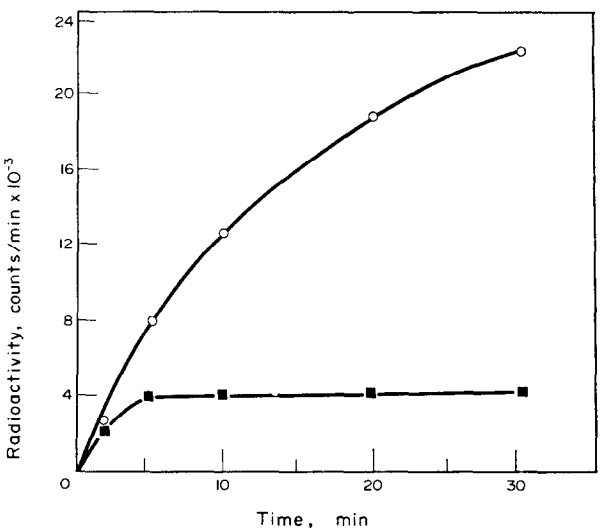

FIG. 1. Effect of TLCK on the rate of incorporation of L-(U-^{14}C)-leucine into protein of intact rabbit reticulocytes. Each point represents a separate incubation tube from which a 50-μl sample was removed into cold 5% TCA. The details of the incubation and radioactivity determinations are given in Materials and Methods. (○), Control cells, (■) TLCK, 1 mM, added at time zero.

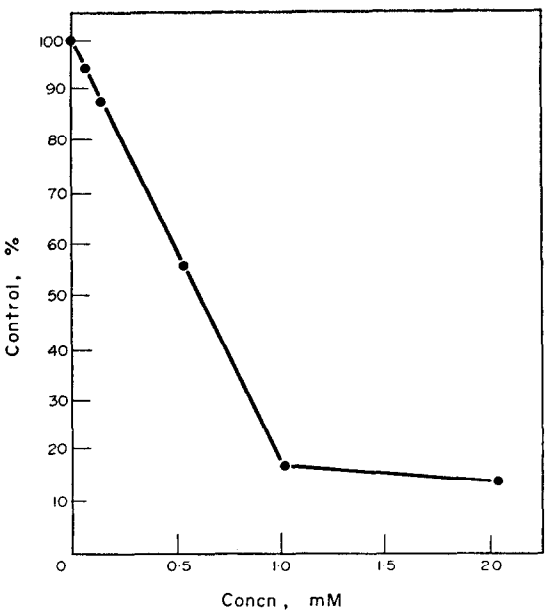

FIG. 2. Effect of TLCK concentration in the incorporation of L-(U-^{14}C)-leucine into protein of intact rabbit reticulocytes. The radioactive leucine was present throughout the 20-min incubation. Inhibition is expressed as per cent of control.

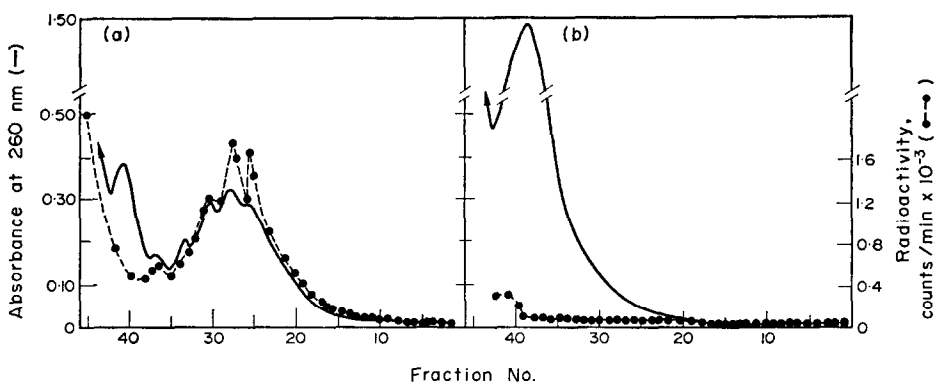

FIG. 3. Sucrose density gradient analysis of the ribosome–polyribosome component of intact rabbit reticulocytes incubated with 1 mM TLCK. Incubation was for 10 min in the presence of 5×10^{-5} M L-(^{12}C)-leucine. L-(U-^{14}C)-leucine was added for the last 2 min of incubation. Centrifugation of 1 ml of the stroma-free lysate was for 3 hr at 25,000 rev/min in a Spinco SW 27 rotor. (a) control; (b) TLCK added at time zero.

and 40 S subunits following TLCK treatment (Fig. 4), even though the increase in single ribosomes was once again seen. Identical results were obtained in all of these experiments using either iron-transferrin or hemin in concentrations ranging from 0·1 to 1 mM.

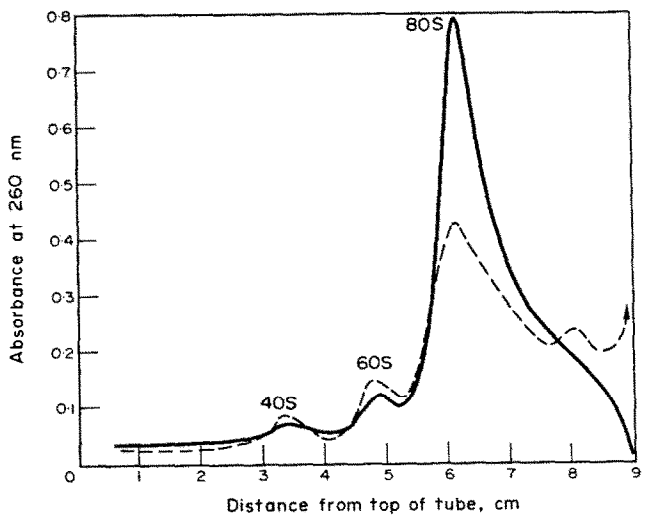

FIG. 4. Sucrose density gradient analysis of the isolated single ribosomes and ribosomal subunits from cells incubated in the presence of 1 mM TLCK. The total volume of incubation was 8 ml. The ribosome component was isolated from the cell lysate by centrifugation through a cushion of 30% sucrose at 100,000 *g* for 3 hr. The pellet was resuspended in 1 ml standard buffer, layered on a 15–30% sucrose density gradient and centrifuged for 16 hr at 22,500 rev/min. (———), Ribosomes from cells incubated with TLCK for 10 min; (- - - - -), ribosomes from control cells.

In order to confirm that all of the polyribosomes had been converted to single ribosomes, a sample of the 1:7 stroma-free lysate from cells treated with TLCK was exposed to 0·25 μg/ml of bovine pancreas ribonuclease (five times crystallized) (Fig. 5). The addition of ribonuclease did not further alter the ribosome profile from TLCK-treated cells, demonstrating that indeed there were no polyribosomes remaining. A simultaneous control lysate from cells incubated in the complete medium was likewise exposed to ribonuclease. This resulted in conversion of the polyribosomes to single ribosomes, with a ribosome profile identical to those shown.

The TLCK-induced conversion of the polyribosomes to single ribosomes did not occur when the cells were incubated simultaneously with TLCK and cycloheximide (Fig. 6). Cycloheximide inhibits both protein synthesis and the incorporation of amino acids into nascent chains while it stops ribosomal movement.[18,21] Likewise, no conversion occurred at 4° with TLCK alone. It appears, therefore, that continued ribosomal movement is necessary for TLCK to cause polyribosomal conversion to single ribosomes. This was confirmed by incubating cells together with TLCK and puromycin ($2{\cdot}5 \times 10^{-5}$ M). Puromycin also inhibits protein synthesis and the incorporation of amino acids into nascent chains. It differs from cycloheximide in that ribosomal movement is not retarded.[22] When cells were incubated together with puromycin and TLCK, conversion to single ribosomes occurred to the same extent as shown in Fig. 3.

No hemolysis occurred during incubation of the cells with TLCK. Addition of 1 mM methionine or increasing the concentration of trypophan 100-fold in the medium did not protect against TLCK inhibition of hemoglobin synthesis or the

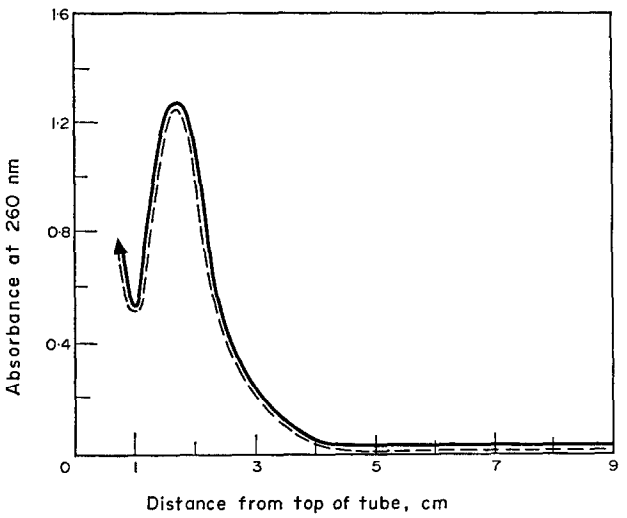

FIG. 5. Effect of ribonuclease on the ribosome component of reticulocytes incubated with 1 mM TLCK for 10 min. The stroma-free lysate was exposed to 0·25 μg/ml of ribonuclease (bovine pancreas) for 1 hr at 4°. Centrifugation of 1 ml lysate in a sucrose density gradient was for 3 hr at 25,000 rev/min. (———) Without ribonuclease; (- - - - -) with ribonuclease.

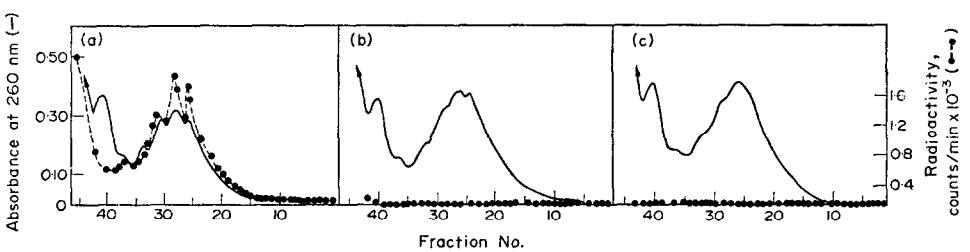

FIG. 6. Effect of cycloheximide on preventing ribosomal disaggregation by TLCK. (a) control cells; (b) with 10 mM cycloheximide; (c) with 10 mM cycloheximide and 1 mM TLCK. Incubation was for 10 min with cycloheximide and TLCK present throughout. L-^{12}C-leucine, 5×10^{-5} M, was present to satisfy the leucine requirement and L-(U-^{14}C)-leucine was added for the last 2 min of incubation.

conversion of polyribosomes to single ribosomes. ATP concentrations were determined on cells from five different rabbits. Reticulocytes were incubated with and without 1 mM TLCK for 10 min. The mean value of ATP concentration, either with or without TLCK, was 0·32 μmole/0·25 ml of cells.

Evidence that TLCK-induced single ribosomes are not attached to mRNA. It has been previously shown that pronase selectively dissociates only single ribosomes not attached to mRNA into ribosomal subunits.[15,16] Ribosomes attached to MRNA, even fragments of mRNA, completely resist this dissociation.[23] Single ribosomes from TLCK-treated cells were completely dissociated into ribosomal subunits by pronase (Fig. 7). This indicates that TLCK converts polyribosomes to single ribosomes not attached to mRNA.

TLCK inhibition of polyribosome formation. Initiation of protein synthesis occurs

when a ribosome attaches to mRNA. The ability of single ribosomes not attached to mRNA to form polyribosomes is, therefore, one method of studying the initiation process. Reticulocytes were incubated with an inhibitor of initiation, 0·1 M *n*-butanol,[18] which results in a conversion of the polyribosomes to single ribosomes (Fig. 8, A). It has been previously shown that these ribosomes are not attached to mRNA.[16]

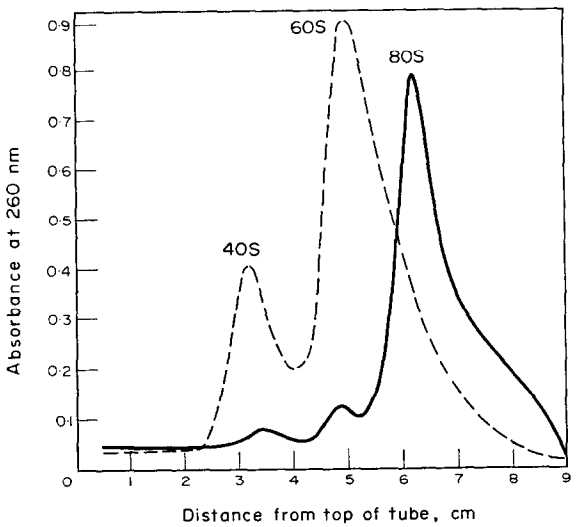

FIG. 7. Effect of pronase on the single ribosomes isolated from reticulocytes incubated in the presence of 1 mM TLCK for 10 min. Incubation and isolation of single ribosomes were as described in Fig. 4. After isolation, the ribosomes (2·5 mg) were mixed with 0·5 mg pronase in standard buffer at a final volume of 1 ml at 4° for 1 hr. The ribosomal suspension was then analyzed as described in Fig. 4. (———) Without pronase; (- - - - -) with pronase.

After removing the *n*-butanol and reincubating the cells in the complete medium for 1 hr at 37°, a total regeneration of the polyribosome profile occurred (Fig. 8, b). This profile is identical to that derived from control cells. In the presence of 1 mM TLCK in the complete medium, no polyribosomes were re-formed (Fig. 8, c). When the ability of these single ribosomes to form polyribosomes in the complete medium was assayed in the presence of inhibitors of elongation, puromycin and cycloheximide, partial regeneration occurred. The results with 2 mM puromycin are shown in Fig. 8, d. The polyribosome formation seen with puromycin is particularly significant, as this concentration of drug itself induces an alteration in the polyribosome profile to smaller polyribosomes and single ribosomes not attached to mRNA.[16] Similar results were obtained with 0·01 M cycloheximide and have been reported previously.[18] Identical results were obtained with cells where the single ribosome not attached to mRNA was obtained by exposing cells to 0·1 mM bipyridine.

Effect of TLCK on reduced glutathione. TLCK was added to rabbit reticulocytes to a final concentration of 1 mM in the complete incubation medium. Aliquots of the incubation mixture were taken at 5 and 20 min, and the intracellular GSH levels measured (Table 1). The molar ratio of TLCK to normal intracellular GSH in these experiments was 2·42:1. TLCK produced a rapid decrease in the GSH concentration of the intact cell.

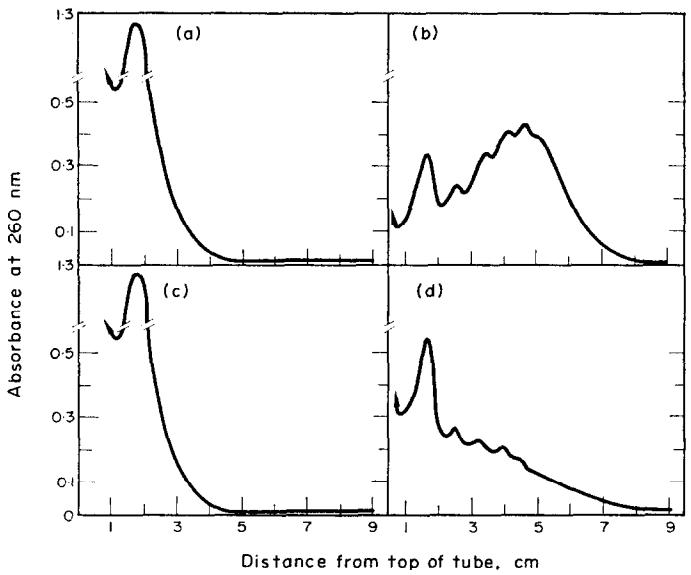

FIG. 8. TLCK inhibition of polyribosome formation. Cells were incubated with 0·1 M *n*-butanol to form single ribosomes not attached to MRNA. After washing out the inhibitor, the cells were re-incubated in the complete medium for 1 hr at 37°. Centrifugation of 1 ml lysate in a sucrose density gradient was for 3 hr at 25,000 rev/min. (a) single ribosomes from cells incubated with 0·1 M *n*-butanol; (b) polyribosome formation after *n*-butanol was removed; (c) inhibition of polyribosome formation in the presence of 1 mM TLCK; (d) polyribosome formation in the presence of 2 mM puromycin.

In order to see if this reaction was due to direct reaction of TLCK and GSH, the reaction between pure GSH and TLCK was studied in aqueous solution. When TLCK was added to a solution of GSH (25 μg/ml) so that the final concentration of TLCK

TABLE 1. EFFECT OF TLCK ON RETICULOCYTE REDUCED GLUTATHIONE (GSH)

Time (min)	Treatment	GSH (μg/0·2 ml)	Control (%)
5	None	63·0	100
	TLCK	8·5	13·5
20	None	68·0	100
	TLCK	3·0	4·4

was $3{\cdot}82 \times 10^{-4}$ M, no decrease in GSH concentration was observed during 20 min of incubation. The ratio of TLCK to GSH in this experiment was approximately five times that required to produce polyribosome disaggregation in intact reticulocytes.

DISCUSSION

The demonstration that TLCK irreversibly inhibits hemoglobin synthesis in intact rabbit reticulocytes strongly indicates that TLCK inhibits translation directly. It should be emphasized, however, that the concentration of TLCK required to inhibit protein synthesis in this system was greater than what has been employed to inhibit transcrip-

tion in nucleated mammalian cells.[7] It is possible that this agent may inhibit transcription by a mechanism completely independent of the one shown here. Any conclusions regarding its biological effects, however, must take into account this inhibition of translation.

In the presence of 1 mM TLCK, the polyribosomes were all converted to single ribosomes not attached to mRNA. The number of ribosomes on mRNA during hemoglobin synthesis is directly proportional to the rate of initiation of chain synthesis and inversely proportional to the rate of release of ribosome from mRNA.[19] When initiation is selectively inhibited so that elongation and release of globin chain occur normally, the polyribosomes will be converted to single ribosomes not attached to mRNA. When elongation is selectively inhibited, as with specific amino acid deficiencies, the polyribosome profile depends upon the site of inhibition. Elongation proceeds from the amino terminal end of the protein to the carboxyl terminal end (or from the 5′-nucleotide to the 3′-nucleotide end of mRNA).[24,25] When ribosomal movement is delayed at a specific codon, ribosomes pile up proximal to the delay.[26] Consequently, a rate-limiting step near the 5′-nucleotide end of mRNA results in smaller polyribosomes and an increase in single ribosomes not attached to mRNA.[27] A rate-limiting step near the 3′-nucleotide end of mRNA results in larger than normal polyribosomes.[19,26] If elongation is inhibited at uniformly distributed sites along the mRNA, the polyribosomal profile appears normal but no protein is synthesized.[27] The polyribosomal pattern in intact cells, therefore, reflects the major site of translational inhibition of a drug.

We have shown that the TLCK inhibition of hemoglobin synthesis and the conversion of polyribosomes to single ribosomes did not result from any change in ATP concentration in these cells. It seemed unlikely that TLCK would chelate any ions (iron or magnesium) in the incubation medium. However, to further exclude this possibility it was shown that hemin did not protect or reverse the inhibition, which it would do if iron was chelated.[19] The observation that there was no dissociation of single ribosomes into subunits, as occurs in the absence of magnesium,[17] excludes this as a possibility.

On the basis of these observations, the following explanations for the TLCK-polyribosomal disaggregation were considered: (1) ribonuclease degradation of polyribosomes; (2) premature release of ribosomes from mRNA; (3) delay in elongation towards the 5′-nucleotide end of mRNA; and (4) an inhibition of polyribosome formation (initiation).

Polyribosomes are disaggregated by ribonuclease to single ribosomes attached to fragments of mRNA. It seemed unlikely that TLCK activated ribonuclease, since in the presence of 1 mM hemin, which previously has been shown to inhibit reticulocyte ribonuclease,[28,29] the TLCK-induced disaggregation still occurred. To exclude further a ribonuclease effect, single ribosomes obtained by TLCK treatment of intact cells were exposed to pronase. Ribosomes attached to fragments of mRNA, derived from ribonuclease degradation of polyribosomes, completely resist dissociation by pronase.[23] The single ribosomes from the TLCK-treated cells were completely dissociated into ribosomal subunits by pronase, which indirectly demonstrates that they are not attached to either intact or fragmented mRNA. Furthermore, if a ribonuclease were activated by TLCK, cycloheximide would not be expected to stabilize the polyribosome pattern as its does.

The polyribosomal structure is quite stable, and premature release of ribosomes from mRNA does not occur either when ribosomal movement is inhibited,[18] or when nascent chains are removed.[22] It therefore seemed unlikely that TLCK was causing premature release of ribosomes from mRNA. However, to exclude this possibility, it was shown that continued protein synthesis and ribosomal movement are necessary for the polyribosomal conversion to single ribosomes seen with TLCK to occur. The cells were incubated together with TLCK and either cycloheximide (10 mM) or puromycin ($2{\cdot}5 \times 10^{-5}$ M). Cycloheximide inhibits both protein synthesis and the incorporation of amino acids into nascent chains while it stops ribosomal movement.[18,21] Puromycin likewise inhibits protein synthesis and the incorporation of amino acids into nascent chains, but ribosomal movement along mRNA is not inhibited.[22] When cells were incubated with cycloheximide and TLCK, no conversion to single ribosomes occurred. Likewise, no conversion occurred at 4° with TLCK alone. However, when cells were incubated with puromycin and TLCK, conversion occurred to the same extent as with TLCK alone. It is clear, therefore, that continued ribosomal movement is necessary for TLCK to cause conversion of polyribosomes to single ribosomes not attached to mRNA.

An inhibition of elongation toward the 5′-nucleotide end of mRNA also seemed unlikely. The only unique requirements at this end of both globin chains are trytophan and methionine.[30,31] Increasing the concentration of tryptophan 100-fold or adding 1 mM methionine to the medium did not protect against TLCK inhibition of hemoglobin synthesis. In addition, with an early elongation inhibition some ribosomes should be attached to mRNA.[16] In the case of TLCK inhibition there were no detectable ribosomes attached to mRNA. It has previously been demonstrated that an inhibition of chain elongation toward the 5′-nucleotide end of mRNA will result in an increase in ribosomal subunits.[32] This was not found in the case of TLCK-treated cells. Further evidence against a deficiency of an amino acid was the failure of $2{\cdot}5 \times 10^{-5}$ M puromycin to protect or reverse the conversion of polyribosomes to single ribosomes. This concentration of puromycin has been shown to both protect and reverse the conversion to single ribosomes due to tryptophan deficiency.[22]

We have, therefore, presented evidence that the TLCK-induced conversion to single ribosomes did not result from ribonuclease degradation, premature release, or an early delay of elongation. This suggests that TLCK inhibits polyribosome formation (initiation) directly. This conclusion is supported by the observation that ribosomal movement is necessary for the conversion of polyribosomes to single ribosomes to occur, which is evidence that the ribosomes complete translation ("run-off"), but are unable to initiate new globin chain synthesis. Furthermore, there were no detectable ribosomes attached to mRNA. More direct evidence of inhibition of initiation was shown in the experiments measuring polyribosome formation from single ribosomes not attached to mRNA. No polyribosome formation was detectable in the presence of TLCK. Conversely, some polyribosome formation could be detected in the presence of inhibitors of elongation (cycloheximide and puromycin). It appears, therefore, that this assay indeed differentiates between inhibitors of initiation and elongation.

The studies are consistent with the idea that the major effect of TLCK in intact reticulocytes is on initiation. However, it has not been excluded that there is a concomitant but lesser inhibition of elongation or release. Indeed, a similar agent, tosyl phenylalanyl chloromethane (TPCK), has been shown to inhibit *Escherichia coli*

elongation factor T in a cell-free system.[33] Studies, in progress, with the fractionated cell-free system should definitively elucidate the site or sites of translation inhibited by TLCK.

Both initiation and elongation of globin chain synthesis in intact reticulocytes have been shown to require GSH.[34] The effects of TLCK that we have observed in the reticulocyte might be, therefore, at least partially explained by inhibition of the cellular enzymes required to maintain GSH. GSH might be necessary by itself for initiation or by its ability to maintain sulfydryl groups in other proteins. In addition, TLCK might directly react with any one or more of the factors required for initiation.

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