Biochem. J. (1966) **101**, 627

Effect of Cycloheximide on Protein Biosynthesis in Rat Liver

By A. KORNER
Department of Biochemistry, University of Cambridge

(*Received* 5 *May* 1966)

1. The liver ribosomes of rats given cycloheximide by intraperitoneal injection incorporate less amino acid into protein than ribosomes from control rat liver when they are incubated *in vitro* with excess of Sephadex-treated cell sap. The effect is rapid, marked and persistent. 2. Cell sap from liver of cycloheximide-treated animals is inhibitory but the inhibition can be relieved almost entirely by treating the cell sap with Sephadex. No damage has been done to the cell-sap factors: it is suggested that the dissolved cycloheximide in the cell sap causes the inhibition. 3. Cycloheximide added *in vitro* inhibits amino acid incorporation into protein in the presence or absence of polyuridylic acid. The inhibition is lessened by addition of excess of cell sap but is not abolished. 4. The differences between these results and those obtained with mouse liver (Trakatellis, Montjar & Axelrod, 1965) might arise because of species differences in sensitivity to the drug.

Cycloheximide (Actidione) is an antibiotic, isolated from *Streptomyces griseus*, that was shown to inhibit protein biosynthesis in *Saccharomyces carlsbergensis* (Kerridge, 1958). Subsequent work showed it to have similar effects in a number of mammalian tissues including rabbit liver (Young, Robinson & Sacktor, 1963), rabbit reticulocytes (Colombo, Felicetti & Baglioni, 1965), rat liver (Wettstein, Noll & Penman, 1964), mouse liver (Trakatellis, Montjar & Axelrod, 1965) and human lymphocytes (Kay & Korner, 1966). There is evidence from a number of sources (Ennis & Lubin, 1964; Siegel & Sisler, 1963, 1964; Wettstein *et al.* 1964) that cycloheximide does not inhibit the charging of s-RNA* with amino acids but that it affects some stage of protein synthesis subsequent to charging of s-RNA.

Trakatellis *et al.* (1965) reported that injection of cycloheximide into the mouse had no effect on the ability of liver ribosomes to incorporate amino acids into protein *in vitro* in the presence of liver cell sap from uninjected mice. The inhibitory action of cycloheximide on protein synthesis was found to reside in the cell-sap fraction. My experiments with rat liver, which are reported below, showed different results. Possible reasons for these differences are discussed.

MATERIALS AND METHODS

Materials. DL-[1-^{14}C]Phenylalanine (38mc/m-mole) and DL-[1-^{14}C]leucine (36·6mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Cycloheximide was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and polyU from Miles Chemical Co., Elkart, Ind., U.S.A. Phosphocreatine was obtained from British Drug Houses Ltd., Poole, Dorset, and phosphocreatine kinase was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Animals. Female albino rats, bred in this Laboratory and weighing 150–200g., were used. In some experiments they were starved overnight to deplete the liver of glycogen. Cycloheximide (10mg. in 0·9% NaCl) was administered by intraperitoneal injection and control rats were given 0·9% NaCl. This amount of cycloheximide was found to inhibit the incorporation of injected leucine into liver protein almost completely (A. E. Pegg, unpublished work).

Preparation of liver cell fractions. Microsomes, polysomes and cell sap were separated from a liver homogenate in 0·25M-sucrose in medium M [20mM-tris buffer (pH7·6)–40mM-NaCl–100mM-KCl–10mM-magnesium acetate–6mM-mercaptoethanol] by the methods described by Munro, Jackson & Korner (1964). The 10000*g* supernatant material of the rat-liver homogenate was treated at 0° with freshly prepared sodium deoxycholate (final concn. 1%) in 20mM-tris buffer, pH8·2, and the ribosomes were isolated by centrifugation at 100000*g* for 4hr. through M-sucrose in medium M. This procedure (see Munro *et al.* 1964) produces a preparation of particles less contaminated by deoxycholate and ferritin than that obtained by an earlier method (Korner, 1961). The pellet is almost free of deoxycholate and is fairly easily suspended.

The polysomes were suspended in medium M by gentle agitation and pipetting, and the ribosomes and microsomes in medium M by gentle homogenization. The suspensions were kept for about 10 min. and a portion of the material that did not sediment was removed and diluted for use.

The cell sap was passed through a column of Sephadex G-25, equilibrated with medium M and of volume at least 30 times that of the cell sap, to remove inhibitors of protein synthesis present in untreated cell sap (Mansbridge &

* Abbreviations: s-RNA, 'soluble' or transfer RNA; polyU, polyuridylic acid.

Korner, 1963; Munro *et al.* 1964). Unless this is done it is not possible to add saturating concentrations of cell sap to the incubation mixture for the amount required contains sufficient inhibitors to cause serious depression of amino acid incorporation.

Incubation conditions. The standard incubation mixture for measuring the incorporation of amino acids into protein contained about 25 μg. of ribonucleoprotein-particle RNA and a minimum of 2mg. of Sephadex-treated cell-sap protein. It is necessary to have at least 25 times as much cell-sap protein as ribosomal protein to ensure that cell-sap factors are not limiting (Munro *et al.* 1964). The incubation medium was medium M and it was supplemented with GTP (0·1mM) and ATP (5mM). An ATP-generating system is not necessary for deoxycholate-treated particles (Korner, 1961; Munro *et al.* 1964), but it was added, as phosphocreatine (5mM) and phosphocreatine kinase (0·1mg./ml.), when microsomes were used. Radioactive amino acid (0·5 μC) was added to each incubation tube. The incubation volume was 1ml. In some experiments an amino acid mixture, lacking the radioactive one used, was added. All additions to the incubation medium were in 20mM-tris buffer, pH7·6, and compensating amounts of tenfold-concentrated medium M were added to keep the final salt concentrations those of medium M. The tubes were shaken in a water bath at 37° and the reaction was stopped by addition of 5ml. of 0·5N-$HClO_4$ containing unlabelled amino acid corresponding to the labelled one used.

The proteins were washed and prepared for radioactivity measurement in a Nuclear–Chicago gas-flow counter as described by Munro *et al.* (1964). Efficiency of counting was about 20%. RNA and protein were determined as described by Munro *et al.* (1964). The results are all calculated as counts/min. incorporated into protein/mg. of particle RNA incubated, so that different preparations of particles could be compared.

RESULTS

Effect of injected cycloheximide on ribosomes. Table 1 shows the results of a typical experiment in which groups of rats were given 10mg. of cycloheximide in 0·9% sodium chloride by intraperitoneal injection and killed at various times afterwards. The ribosomes were prepared as described and assayed with saturating amounts of cell sap from control rats. The cycloheximide treatment of rats had a rapid, marked and persistent effect on the ability of the ribosomes to incorporate amino acids into protein *in vitro*. This finding, which differs from that of Trakatellis *et al.* (1965), who found no cycloheximide inhibition effect on ribosomes of mouse liver, has been obtained repeatedly.

The inhibition by cycloheximide is observed within 15min. of injection of the material and is not greatly augmented by waiting for 2hr. before assaying the ribosomes. By 4hr. further inhibition is evident.

The results in Table 1 were obtained with a group of unstarved rats. Similar results were obtained with animals starved overnight before cycloheximide treatment.

It seemed possible that the difference between these results and those of Trakatellis *et al.* (1965) might be explained if washing the ribosomes could remove the cycloheximide. The ribosomes in their experiments were prepared as described by Korner (1961). According to this method the pellet of ribosomes is resuspended and recentrifuged to remove deoxycholate. The ribosomes used in the present work were not washed by recentrifuging because they had been prepared by spinning through a M-sucrose step, which washed out much of the deoxycholate (see the Materials and Methods section). Portions of the ribosomes were therefore washed by resuspension and recentrifugation or were passed through a Sephadex G-25 column, equilibrated with medium M, and reassayed. The results (Table 1) show that either treatment enhanced incorporation somewhat in all the ribo-

Table 1. *Effect of injected cycloheximide on the amino acid-incorporating activity of rat-liver ribosomes*

Rats (two per group) were given 10mg. of cycloheximide by intraperitoneal injection and killed at the times indicated. Ribosomes were prepared as described in the Materials and Methods section and were used as such or after Sephadex treatment or after recentrifuging. Incubation was with excess of cell sap from control rats. An amino acid mixture was added in this experiment.

Time after injection of cycloheximide (hr.)	[^{14}C]Leucine incorporated into protein (counts/min./mg. of ribosomal RNA incubated)		
	Ribosomes used as prepared	After treatment of ribosomes with Sephadex	After resuspension and recentrifuging of ribosomes
0	12560	14800	14100
0·25	6100	6300	8270
1·0	5960	6150	8600
2·0	5600	—	—
4·0	4400	4170	—

some preparations assayed but did not remove all the inhibition from ribosomes of cycloheximide-treated rats.

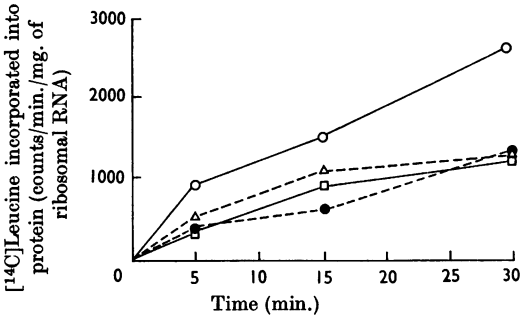

Fig. 1. Time-course of incorporation of [14C]leucine into protein in a cell-free system with excess of cell sap from normal rat liver and liver ribosomes of normal (○) and cycloheximide-treated rats killed 15min. (△), 60min. (□) and 150min. (●) after injection of 10mg. of the drug. Incubation conditions were as described in the Materials and Methods section. An amino acid mixture was added in this experiment.

Fig. 1 shows that the inhibition by injected cycloheximide is observable at all times during incubation of ribosomes from treated rats. After 5min. incubation, for example, a clear difference exists between treated and control preparations of ribosomes. Cycloheximide treatment has inhibited rate, as well as extent, of amino acid incorporation.

Effect of injected cycloheximide on cell sap. Table 2 shows the effect of injected cycloheximide on the ability of cell sap of liver to stimulate the incorporation of [14C]leucine into protein of normal rat-liver ribosomes. The cell sap was markedly inhibitory, as reported for mouse-liver cell sap by Trakatellis *et al.* (1965), but no change in degree of inhibition was noted with change in time of treatment of rats with cycloheximide. When the cell sap was passed through a Sephadex G-25 column (see the Materials and Methods section), the inhibitory effect almost entirely disappeared. Indeed, cell sap from rats that had been treated with cycloheximide for 4hr. seemed slightly more active than normal cell sap.

Effect of cycloheximide in vitro. Table 3 shows the effect of various amounts of cycloheximide

Table 2. *Effect of injected cycloheximide on the activity of cell sap*

Incorporation of [14C]leucine into protein was measured with ribosomes from control rat liver and cell sap from rats given 10mg. of cycloheximide by intraperitoneal injection. No amino acid mixture was added in this experiment.

Time after injection of cycloheximide (hr.)	[14C]Leucine incorporated into protein (counts/min./mg. of ribosomal RNA of control rats incubated with cell sap from rats given cycloheximide)	
	Not treated with Sephadex	Treated with Sephadex
0	7450	11250
0·25	3410	10900
1·0	3490	10520
2·0	3360	11600
4·0	3300	12400

Table 3. *Effect of cycloheximide added* in vitro *on the incorporation of* [14C]*leucine into protein*

Incubation conditions were as described in the Materials and Methods section. No amino acid mixture was added in this experiment. Values in parentheses are percentages of control values.

Amount of cycloheximide added (μg./ml.)	[14C]Leucine incorporated into protein (counts/min./mg. of ribosomal RNA incubated)	
	Incubated with adequate cell sap (25 times ribosomal protein)	Incubated with excess of cell sap (200 times ribosomal protein)
0	6760	8560
10	3140 (46·5)	5390 (62·2)
25	2600 (38·4)	4900 (56·7)
50	1820 (26·9)	4050 (46·7)
100	1470 (21·7)	3480 (40·2)
200	520 (7·7)	2630 (30·3)

Table 4. *Effect of cycloheximide and polyU added* in vitro *on the incorporation of* [^{14}C]*phenylalanine into protein*

Incubation conditions were as described in the Materials and Methods section. The Mg^{2+} concentration was 10mM in all cases. No amino acid mixture was added in this experiment.

Type of particle used	[^{14}C]Phenylalanine incorporated into protein (counts/min./mg. of particle RNA incubated)			
	No additions	PolyU (100μg.) added	Cycloheximide (100μg.) added	PolyU (100μg.) and cycloheximide (100μg.) added
Ribosomes	3150	13150	1000	4130
Polysomes	4200	12900	1420	2550
Microsomes	3270	13600	1000	3660

added to the amino acid-incorporating system *in vitro*. Marked inhibition was noted with 10μg./ml. and the inhibition became greater, but at a lower rate, as the concentration of cycloheximide was increased.

The percentage inhibition by a given amount of cycloheximide was larger if smaller amounts of cell sap were present (Table 3), but, even when the cell-sap protein concentration was 200 times that of the ribosomal protein concentration, marked inhibition of incorporation was obtained. This result is different to the observation of Trakatellis *et al.* (1965), who noted almost complete suppression of the inhibition caused by 100μg. of cycloheximide when extra cell sap of mouse liver was added.

Inhibition of amino acid incorporation by cycloheximide, added *in vitro*, was observed with microsomes and with polysomes as well as with ribosomes (Table 4). The inhibition by cycloheximide was also observed in the presence of polyU (Table 4), although polyU still stimulated phenylalanine incorporation in the presence of cycloheximide.

DISCUSSION

These results show that cycloheximide injection into rats results in a rapid, persistent and marked inhibition of the ability of liver ribosomes to incorporate amino acids into protein when they are incubated with cell sap from control rat livers. This result is in direct contradiction to that obtained by Trakatellis *et al.* (1965) with mouse-liver ribosomes: these workers reported that cycloheximide had no effect on the ribosomes.

At least three possible explanations exist for the difference between the results of Trakatellis *et al.* (1965) and those reported in the present paper. The methods used to prepare ribosomes were not the same. It is possible that washing or other manipulations of the ribosomes could decrease the inhibition observed (Table 1) so that, if the particles used by Trakatellis *et al.* (1965) were washed more than those used in the present work, some of the differences between our results would disappear. But my results show that washing or treatment with Sephadex did not remove all the inhibitory material from the ribosomes.

A second possibility is that the assay of ribosome activity in the experiments of Trakatellis *et al.* (1965) was not carried out in the presence of saturating amounts of cell sap. Munro *et al.* (1964) have found that a plateau of incorporation is only reached when more than 25 times as much cell-sap protein as ribosome protein is added during incubation. Without this ratio, differences between different ribosome preparations may be obscured because of relative lack of cell-sap factors.

A third possibility arises from the results of Fiala & Fiala (1965). These workers reported that the action of injected cycloheximide is modified by the increased adrenal function brought about by the drug. They observed differences between the response of mice and rats to cycloheximide injection: rats lost liver glycogen and accumulated RNA in the microsome fraction of liver but neither of these changes occurs in mice. It seems possible, in view of these findings, that mice are more resistant than rats to the effects of cycloheximide, possibly because of differences in adrenal response to the drug. This difference in sensitivity may, possibly, together with the differences in technique used by Trakatellis *et al.* (1965) and in the present experiments, allow reconciliation of our differing conclusions on the influence on ribosome activity of cycloheximide treatment.

Several observers, using a variety of systems, conclude that species differences in sensitivity to cycloheximide reside in the differences in ribosomes of the different species (Siegel & Sisler, 1965; Clark & Chang, 1965).

Jondorf, Simon & Avnimelech (1966) reported that injection of cycloheximide at a dose of 1 mg./kg. body wt. stimulated amino acid incorporation into protein with isolated rat-liver microsomes. No effect was observed if the rats were adrenalectomized. It seems likely that this dose of cycloheximide is large enough to cause secretion of corticosteroids, which stimulate amino acid incorporation in the liver microsome system (Korner, 1960), but the dose is too small to inhibit protein synthesis very much.

The cell sap from liver of cycloheximide-treated rats is inhibitory, in agreement with the observation of Trakatellis *et al.* (1965) for mouse liver. However, almost all the inhibitory activity can be removed by treatment of the cell sap with Sephadex (Table 2). Since cycloheximide is active when added *in vitro*, this result suggests that the decreased activity of cell sap from cycloheximide-treated rats does not result from drug-induced damage or alteration of cell-sap factors, but merely because the cycloheximide dissolved in the cell sap acts on the amino acid-incorporating system in the same way as the drug added *in vitro*.

Cycloheximide, added *in vitro*, caused a marked inhibition of amino acid incorporation (Table 3) at 10 μg./ml. Inhibition increased somewhat with increased amounts of cycloheximide. The presence of an excess of cell sap diminished the percentage inhibition found but inhibition was by no means abolished. Abolition of inhibition was found by Trakatellis *et al.* (1965) on addition of extra cell sap in their experiments with mouse liver. Perhaps species differences in sensitivity to cycloheximide of the cell-free system could explain this difference.

I am grateful to Miss C. Manning for highly skilled assistance and to Professor F. G. Young for his encouragement. This work was supported by grants from the British Empire Cancer Campaign and the Medical Research Council.

REFERENCES

Clark, J. M. & Chang, A. Y. (1965). *J. biol. Chem.* **240**, 4734.
Colombo, B., Felicetti, L. & Baglioni, C. (1965). *Biochem. biophys. Res. Commun.* **18**, 389.
Ennis, H. L. & Lubin, M. (1964). *Fed. Proc.* **23**, 269.
Fiala, E. & Fiala, S. (1965). *Biochim. biophys. Acta*, **103**, 699.
Jondorf, W. R., Simon, D. C. & Avnimelech, M. (1966). *Biochem. biophys. Res. Commun.* **22**, 644.
Kay, J. E. & Korner, A. (1966). *Biochem. J.* **100**, 815.
Kerridge, D. (1958). *J. gen. Microbiol.* **19**, 497.
Korner, A. (1960). *J. Endocrin.* **21**, 177.
Korner, A. (1961). *Biochem. J.* **81**, 168.
Mansbridge, J. & Korner, A. (1963). *Biochem. J.* **89**, 15P.
Munro, A. J., Jackson, R. J. & Korner, A. (1964). *Biochem. J.* **92**, 289.
Siegel, M. R. & Sisler, H. D. (1963). *Nature, Lond.*, **200**, 675.
Siegel, M. R. & Sisler, H. D. (1964). *Biochim. biophys. Acta*, **87**, 83.
Siegel, M. R. & Sisler, H. D. (1965). *Biochim. biophys. Acta*, **103**, 558.
Trakatellis, A. C., Montjar, M. & Axelrod, A. E. (1965). *Biochemistry*, **4**, 2065.
Wettstein, F. O., Noll, H. & Penman, S. (1964). *Biochim. biophys. Acta*, **87**, 525.
Young, C. W., Robinson, P. F. & Sacktor, B. (1963). *Biochem. Pharmacol.* **12**, 855.