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Protein Synthesis in the Liver of Rats Injected with Cholesteryl 14-Methylhexadecanoate

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1. Rats were injected intraperitoneally with cholesteryl 14-methylhexadecanoate and killed after various intervals of time up to 3 days; ribosomes and cell sap were isolated from their liver tissue. These fractions were tested for their ability to participate in protein synthesis. 2. Protein synthesis in complete systems containing ribosomes, cell sap and all necessary cofactors was significantly enhanced at 12 and 72h after the injection and significantly inhibited at 24h. At early times after injection isolated ribosomes had a slightly enhanced ability to bind nRNA. Peptide-elongation processes (i.e. binding of aminoacyl-tRNA to ribosomes, peptidyl transfer and polyphenylalanine synthesis) showed significant stimulation or inhibition depending on the time after injection of the ester. 3. A correlation was found between the ability of cell sap to stimulate polyphenylalanine synthesis and the relative cholesteryl 14-methylhexadecanoate content in the postmicrosomal supernatant at different time-intervals after administration of the ester. No significant changes were found in its content in the whole liver tissue. 4. Since the injected ester has previously been shown to accumulate in some enzymic fractions, the changes in its relative content may represent a regulatory mechanism modulating the rate of protein synthesis.

Cholesteryl 14-methylhexadecanoate affects the activity of some enzymes required for protein synthesis in rat liver and evidence has been obtained that this ester is present in molecules of aminoacyl-tRNA synthetases (Hradec & Dušek, 1969), and of peptide-elongation factors (Hradec *et al.*, 1971), as a normal constituent essential for the function of these enzymes. Significant changes of RNA polymerase activity in the nuclei of liver cells (Komárková & Hradec, 1971*b*), as well as of L-leucyl-tRNA synthetase activity (Komárková & Hradec, 1971*a*) follow the injection of this compound into rats. For a better understanding of the mode of action of this ester, which seems to be an important cofactor in protein synthesis, further studies are required on its possible effect on different enzymic systems, in particular at the level of the expression of the genetic message on the ribosome.

The synthesis of polypeptide chains occurring on mammalian ribosomes may be divided into three principal steps: initiation, elongation and termination (Matthaei *et al.*, 1968). The initiation of peptide synthesis on prokaryotic ribosomes requires several protein factors, some of them binding mRNA to the smaller ribosomal subunit (Dubnoff & Maitra, 1971), the others catalysing the formation of the initiating aminoacyl-tRNA and its binding to ribosomes (Dubnoff & Maitra, 1969). Although the initiation processes on eukaryotic ribosomes have not been investigated in great detail, evidence has been presented indicating that similar mechanisms operate in eukaryotic cells and bacterial ribosomes (Kerwar *et al.*, 1970; Heywood & Thompson, 1971). Peptide elongation is started by the binding of aminoacyl-tRNA to the acceptor site on the ribosome, catalysed by the binding enzyme (McKeenan & Hardesty, 1969) or transferase I (Schneir & Moldave, 1968). The next step of peptide elongation is represented by peptidyl transfer from the donor to the acceptor site on the ribosome, where the formation of the peptide bond takes place. The peptidyl transferase, an integral constituent of the larger ribosomal subunit, apparently catalyses this reaction step in mammalian cells in a way similar to that in bacterial ribosomes (Vasquez *et al.*, 1969). The peptide-elongation step is completed by the translocation of peptidyl-tRNA from the acceptor to the donor site of the ribosome. For this reaction the presence of transferase II (Skogerson & Moldave, 1968) or peptidyl-tRNA translocase (Siler & Moldave, 1969), a normal constituent of the cell sap, is required. Insufficient details are as yet available for the peptide-termination step.

In our present experiments several steps involved in the translation of the genetic message on the liver ribosome were studied in rats injected with cholesteryl 14-methylhexadecanoate. Evidence is presented indicating that the ester affects the activity of some of the components required for these processes *in vitro*.

Materials and Methods

Animals

Random-bred Wistar rats of both sexes, weighing 150–180g, were kept on a standard diet. Animals were

injected intraperitoneally with 0.1 ml of olive oil containing, unless otherwise indicated, 1 mg of cholesteryl 14-methylhexadecanoate. Control rats were injected with the same volume of olive oil. Animals were killed 12, 24, 36, 48 or 72h after injection. For each time-interval after injection a group of at least three control and five experimental rats was used. Animals were starved for 24h before death to secure standard experimental conditions and to obtain comparable results. The statistical analysis was carried out by using all control animals as the control group (i.e. at least 15 rats). Values obtained with experimental rats for each time-interval were then compared with the results of the control group by using Student's t test.

Chemicals

ATP (sodium salt) was a product of C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. GTP (sodium salt), phosphoenolpyruvate (Ag/Ba salt) and pyruvate kinase were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. PolyU was obtained from Calbiochem, Los Angeles, Calif., U.S.A., and puromycin from Serva Feinbiochemica, Heidelberg, Germany. Cholesteryl (+)-14-methylhexadecanoate was synthesized as described by Hradec & Dolejš (1968).

Radioactive compounds. Algal protein hydrolysate (100mCi/g), L-[U-^{14}C]phenylalanine (118mCi/mmol) and [5-^{3}H]orotic acid (15.6Ci/nmol) were purchased from the Institute for Research, Production and Utilization of Radioisotopes, Prague, Czechoslovakia. [4-^{3}H]Phenylalanine (18Ci/mmol) was a product of The Radiochemical Centre, Amersham, Bucks., U.K. Aminoacyl-[^{32}P]tRNA was purified by the method of Hradec (1972) from ^{32}P-labelled soluble RNA obtained from the same source. [^{14}C]Phenylalanyl-tRNA was prepared as described by Hradec *et al.* (1971) and contained 5.95μmol of phenylalanine/nmol of tRNA. [^{3}H]Phenylalanyl-tRNA was prepared from this product by acetylation, the procedure of Haenni & Chapeville (1966) being used. The final preparation contained 6.20μmol of acetylphenylalanine/mmol of tRNA. For the preparation of ^{3}H-labelled nRNA, rats weighing 300–350g were injected intraperitoneally with 32nmol of [^{3}H]orotic acid and killed after 60min. Before that they were injected with actinomycin D as described by Naora & Kodaira (1969). Nuclei of liver cells were isolated by the method of Mertelsmann (1969) and nRNA was extracted and purified according to Naora & Kodaira (1969). The final preparations of nRNA had a specific radioactivity of 3735c.p.m./E_{260} unit.

Subcellular fractions

Isolation. Subcellular fractions were isolated as described by Hradec *et al.* (1971).

Sucrose-gradient analysis of polyribosomes. Samples (0.2ml) of polyribosomes isolated as described by Hradec *et al.* (1971) were layered on to 4.5ml linear sucrose gradients ranging from 10 to 30% sucrose and containing 20mM-tris–HCl buffer, pH7.5, 100mM-KCl, 5mM-$MgCl_2$ and 7mM-2-mercaptoethanol. The gradients were centrifuged at 30000rev./min for 90min in a 3×5ml swing-out rotor of a VAC 60 ultracentrifuge (Janetzki, Leipzig, E. Germany). The E_{260} profile of the gradients was recorded continuously by passing the fluid through a flow cell mounted in a recording spectrophotometer (C. Zeiss, Jena, E. Germany).

Incubation procedures

Enzyme preparations from one experimental series were incubated simultaneously. Mixtures for the assay of protein synthesis in the complete ribosomal system contained, in a final volume of 0.1ml: 50mM-tris–HCl buffer, pH7.5, 5mM-$MgCl_2$, 50mM-KCl, 1mM-ATP, 10mM-phosphoenolpyruvate, 5μg of pyruvate kinase, 0.2mM-GTP, 300–350μg of ribosomes, 450–500μg of cell-sap protein and 5.0ng of ^{14}C-labelled algal protein hydrolysate. They were incubated at 37° for 60min. For the assay of nRNA binding to ribosomes the reaction mixtures contained, in a final volume of 0.2ml: 10mM-tris–HCl buffer, pH7.5, 3mM-$MgCl_2$, 5mM-KCl, 1.3mg of ribosomes and 3.67E_{260} units of nRNA. They were incubated at 0°C for 20min. The composition of incubation mixtures for the assay of aminoacyl-[^{32}P]tRNA binding to ribosomes was described by Hradec (1971) and of those mixtures used for the assay of polyU-directed polyphenylalanine synthesis were described by Hradec *et al.* (1971). Mixtures used for the assay of peptidyl transfer were composed essentially according to I. Rychlík (personal communication). Mixtures with mRNA contained, in a final volume of 0.1ml: 50mM-tris–HCl buffer, pH7.5, 10mM-$MgCl_2$, 100mM-NH_4Cl, 10μg of polyU, 31μg of acetyl[^{3}H]-phenylalanyl-tRNA and 200–250μg of ribosomes. They were preincubated at 37°C for 30min and then puromycin was added to a final concentration of 0.1mM and the incubation was continued for another 60min. Mixtures without mRNA contained, in a final volume of 0.1ml: 30μl of the T_1 salt mixture (2.4ml of 1M-tris–HCl buffer, pH7.4, 8.0ml of 2M-KCl, 0.8ml of 1M-$MgCl_2$ and 0.8ml of water), 30μl of acetone, 10mM-puromycin, 200–250μg of ribosomes and 31μg of acetyl[^{3}H]phenylalanyl-tRNA; incubation was at 37°C for 70min.

Assay of radioactivity

Mixtures for the assay of nRNA binding to ribosomes were diluted after the incubation with ice-cold buffer of the same composition as that in the incuba-

tion mixture, and were filtered through cellulose nitrate filters. Mixtures for the binding of aminoacyl-tRNA to ribosomes were filtered as described by Hradec (1972). From the reaction mixtures for the assay with the complete ribosomal system, and for the phenylalanine polymerization, 75μl samples were plated on Whatman GF-83 filters. The filters, placed in slots of a Teflon container, were washed as described by Hradec *et al.* (1971). After the incubation of mixtures for peptidyl transfer, 0.1 ml of a saturated solution of $MgSO_4$ in 0.1 M-potassium acetate, pH 5.2, was added (Monro & Marcker, 1967), followed by 1.3 ml of ethyl acetate (Leder & Bursztyn, 1966). The mixture was then briefly centrifuged at low speed and 1.0 ml of the upper phase was carefully aspirated and mixed with 10 ml of a SLE-31 scintillation mixture (Tesla, Přemyšlení, Czechoslovakia). Cellulose nitrate and GF-83 filters were placed in scintillation vials and 5 ml of a scintillation mixture (Mans & Novelli, 1961) was added. The radioactivity was counted in the NZB315 liquid-scintillation counter (Tesla, Přemyšlení, Czechoslovakia) or with the Ansitron II liquid-scintillation spectrometer (Picker Nuclear, White Plains, N.Y., U.S.A.).

Chemical determinations

Protein and RNA were determined by the method of Warburg & Christian (1942). Cholesteryl 14-methylhexadecanoate content in the cell sap was determined by the method of Hradec (1968).

Results

Protein synthesis in the complete ribosomal system

Values obtained in this assay for control rats were comparable with those found earlier in similar experiments (Hradec & Dušek, 1968). At early times after injection of rats with cholesteryl 14-methylhexadecanoate a stimulation of protein synthesis was found in the experimental animals. At 24h after the injection, however, a significant inhibition of these processes resulted from the injection of the ester. Protein synthesis was again stimulated 72h after the injection, this time to a high level (Table 1).

Binding of nRNA to ribosomes

This reaction was stimulated soon after injection of the ester. However, the degree of this stimulation was very low, although significant, at 24h after the injection. At 36 and 48h a significant inhibition of the nRNA binding was demonstrated. Three days after the injection the binding of nRNA to ribosomes of experimental animals did not differ from that in control rats (Table 2). Sucrose-gradient analysis revealed no differences in the polyribosome pattern between experimental and control animals at any time after injection of the ester.

Table 1. *Protein synthesis in the complete ribosomal system from the liver of rats injected with 1 mg of cholesteryl 14-methylhexadecanoate*

The subcellular fractions were isolated and incubation mixtures were made up as described in the Materials and Methods section. The enzymic activity is expressed as pg of algal protein hydrolysate incorporated into the trichloroacetic acid-insoluble portion of ribosomes under standard assay conditions. Activities are mean values ±S.D. with the number of observations in parentheses.

Time after injection (h)	Enzymic activity	*P*
12	220±11.0 (10)	0.001
24	108±10.4 (10)	0.001
36	170±32.5 (5)	0.02
48	166±26.8 (10)	0.4
72	282±19.9 (10)	0.001
Controls	147± 6.3 (27)	—

Table 2. *Binding of [^{3}H]nRNA to the liver ribosomes of rats injected with cholesteryl 14-methylhexadecanoate*

Animals were injected with 1 mg of the ester, the subcellular fractions were isolated and incubation mixtures were made up as described in the Materials and Methods section. The binding is expressed as c.p.m. of [^{3}H]nRNA bound/mg of ribosomes under standard assay conditions. Values are means ±S.D. with the number of observations in parentheses.

Time after injection (h)	nRNA bound to ribosomes	*P*
12	1110±176 (5)	0.1
24	1295± 63 (5)	0.001
36	785±152 (5)	0.001
48	568±284 (5)	0.001
72	942±187 (5)	0.8
Controls	968± 41 (5)	—

Binding of aminoacyl-tRNA to ribosomes

When both ribosomes and postmicrosomal supernatants from animals injected with the ester were used for this assay a rather small stimulation was found at 24 and 72h after the injection. A slight inhibition of this reaction was demonstrated at 12 and 48h (Table 3).

Different results were found if postmicrosomal supernatants from animals injected with cholesteryl

Table 3. *Binding of aminoacyl-tRNA to the liver ribosomes of rats injected with cholesteryl 14-methylhexadecanoate*

Animals were injected with 1 mg of the ester, subcellular fractions were isolated and incubation mixtures were made up as described in the Materials and Methods section. Incubation mixtures contained in both cases cell sap from rats injected with the ester. They were supplemented by ribosomes isolated either from the same animals or from control rats injected with olive oil only. The enzymic activity is expressed as pmol of aminoacyl-[^{32}P]tRNA bound to ribosomes under standard assay conditions. Activities are mean values ±S.D. with the number of observations in parentheses.

Time after injection (h)	Enzymic activity with ribosomes: Experimental	*P*	Control	*P*
12	0.0213±0.000600 (5)	0.01	0.0140±0.00190 (5)	0.001
24	0.0276±0.002500 (5)	0.01	0.0277±0.00190 (5)	0.8
36	0.0234±0.002000 (5)	0.7	0.0263±0.00110 (5)	0.7
48	0.0191±0.001000 (5)	0.02	0.0109±0.00280 (5)	0.001
72	0.0262±0.001000 (5)	0.01	0.0246±0.00060 (5)	0.05
Controls	0.0236±0.000266 (15)	—	0.0280±0.00017 (15)	—

Table 4. *Peptidyl transfer in the liver ribosomes of rats injected with cholesteryl 14-methylhexadecanoate*

Animals were injected with 1 mg of the ester, ribosomes were isolated and incubation mixtures were made up as described in the Materials and Methods section. The enzymic activity is expressed as pmol of acetyl[^{3}H]phenylalanine transferred to puromycin under standard assay conditions. Activities are mean values ±S.D. with the number of observations in parentheses.

Time after injection (h)	Enzymic activity: With polyU	*P*	Without polyU	*P*
12	4.312±0.18 (5)	0.001	1.478±0.071 (5)	0.8
24	4.655±0.13 (5)	0.001	0.943±0.094 (5)	0.001
36	1.697±0.13 (5)	0.05	0.851±0.057 (5)	0.02
48	2.340±0.12 (10)	0.1	1.909±0.042 (5)	0.001
72	2.770±0.11 (5)	0.4	1.659±0.060 (5)	0.4
Controls	2.059±0.02 (18)	—	1.526±0.007 (15)	—

14-methylhexadecanoate were combined with ribosomes obtained from control animals. In this case no stimulation appeared, whereas a significant inhibition of this reaction was found in the same periods as with systems containing both ribosomes and cell sap from experimental rats.

No significant differences from control values were found in systems containing ribosomes of animals injected with the ester supplemented with cell-sap samples from control rats.

Peptidyl transfer reactions

In incubation mixtures supplemented with polyU, the transfer of acetylphenylalanyl residues to puromycin was greatly stimulated when ribosomes derived from animals 12–24h after injection with cholesteryl 14-methylhexadecanoate were used. On the other hand, 36h after the injection this reaction was significantly inhibited. At later periods a stimulation was again apparent but it was not significant (Table 4).

The peptidyl transfer reaction in the absence of mRNA was significantly inhibited in animals injected with cholesteryl 14-methylhexadecanoate for 36h after the injection. A relatively low, nevertheless significant, stimulation of this reaction appeared in the ribosomes of animals 2 days after the injection of the ester (Table 4).

Phenylalanine polymerization

The polyU-directed polyphenylalanine synthesis was only slightly affected in animals injected with

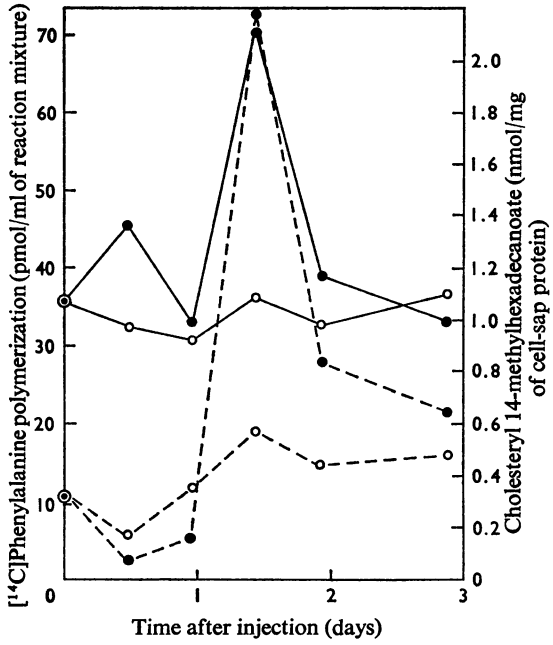

Fig. 1. *PolyU-directed polyphenylalanine synthesis and cholesteryl 14-methylhexadecanoate content in the cell sap of the liver in animals injected with the ester*

Rats were injected with 1 mg (○) or 0.1 mg (●) of this compound and phenylalanine polymerization was assayed by the standard procedure (——). The ester content (----) was determined by the method of Hradec (1968).

1 mg of cholesteryl 14-methylhexadecanoate. However, injection of 0.1 mg of the ester resulted in a significant enhancement of polyphenylalanine synthesis after 12 h ($P<0.02$). Another highly significant stimulation ($P<0.001$) occurred at 36 h after the injection of 0.1 mg of the ester (Fig. 1). No significant changes in polyphenylalanine synthesis resulted from the injection of 0.01 mg of cholesteryl 14-methylhexadecanoate into experimental animals.

When ribosomes isolated from the livers of rats injected with 0.1 mg of the ester were supplemented with the postmicrosomal supernatant from control animals, no significant changes in the polyU-directed polyphenylalanine synthesis were found at any time after injection of cholesteryl 14-methylhexadecanoate. When the cholesteryl 14-methylhexadecanoate content was determined in the cell sap of experimental animals, striking similarities were found between its pattern and that of the activity of the cell sap. Thus 36 h after injection of 0.1 mg of the ester, a significant ($P<0.002$) increase of cholesteryl 14-methylhexadecanoate in the postmicrosomal supernatant coincided with significant enhancement of polyphenylalanine synthesis (Fig. 1). Also at 48 h after the injection, the cholesteryl 14-methylhexadecanoate content in this subcellular fraction was significantly increased ($P<0.01$). On the other hand, no significant changes in the cholesteryl 14-methylhexadecanoate content of the whole liver tissue could be demonstrated during the whole period of the experiment.

Discussion

Results of our experiments indicate that the administration of cholesteryl 14-methylhexadecanoate to rats significantly affects the activity of several components required for the ribosomal peptide synthesis. Evidence has been obtained suggesting that this ester may induce changes in enzymic activity but it does not apparently influence the binding sites on tRNA or ribosomes. This is demonstrated by the fact that no changes in enzymic reactions were found in systems containing ribosomes from experimental animals supplemented with supernatant enzymes from normal rats, in both aminoacyl-tRNA binding to ribosomes and with phenylalanine polymerization. However, significant changes were found in mixtures containing at least supernatant enzymic factors from experimental animals. It seems that cholesteryl 14-methylhexadecanoate interacts primarily with components of the supernatant fraction in a way similar to pH5 enzymes (Komárková & Hradec, 1971*a*).

Since no changes in polyribosome pattern and, in particular, in the proportion of free monomers were found in animals injected with cholesteryl 14-methylhexadecanoate, differences in the binding of nRNA to ribosomes between control and experimental rats are apparently due to changes in activities of enzyme(s) catalysing this binding reaction.

Rats used for the isolation of nRNA in our experiments were given low doses of actinomycin D that are known to block preferentially rRNA synthesis without affecting mRNA formation (Cozzone & Marchis-Mouren, 1967). Such a nRNA preparation apparently thus contains mostly different species of mRNA. This opinion is supported by the results of Naora & Kodaira (1969, 1970), who used a similar preparation of nRNA for their studies on the interaction of informational macromolecules with ribosomes. Thus the binding of nRNA assayed in our experiments may reflect the binding of mRNA to ribosomes, i.e. the first step in peptide-chain initiation (Heywood, 1970).

The assay of peptidyl transferase activity in mixtures without mRNA, similar to the fragment reaction, reveals the activity of the binding site of peptidyl transferase for peptidyl-tRNA, whereas in systems containing artificial mRNA the activity of the

aminoacyl-tRNA binding site is assayed (Monro *et al.*, 1969). Differences in our results obtained with both these systems may be due to different sensitivities of the two binding sites towards cholesteryl 14-methylhexadecanoate.

A relatively low binding of aminoacyl-[^{32}P]tRNA found in the present experiments is apparently due to a rather rapid loss of label from ribosomes (Hradec, 1972). Even highly purified ribosomes may contain traces of translocase, which seems to release deacylated tRNA from ribosomes during the translocation step (Lucas-Lenard & Haenni, 1969). The absolute values obtained with control animals in the present experiments for the binding of aminoacyl-[^{32}P]-tRNA to ribosomes and for the phenylalanine polymerization are in good conformity with the results of our previous experiments (Hradec, 1972; Hradec *et al.*, 1971).

The enhancement of the cell-sap activity occurring early after injection of the ester and accompanied by an increased cholesteryl 14-methylhexadecanoate content in enzyme fractions may be explained as an accumulation of the administered dose in target subcellular fractions.

Since the quantity of the ester injected was relatively high, an inhibition of the normal production of this compound regulated apparently by some feedback mechanisms may take place later on. This may result in a decrease of the ester content in subcellular fractions at later times after its administration and, consequently, to a decreased activity of some enzymic systems found in our experiments. It is not clear, however, why protein synthesis in the complete system is stimulated at this period.

The dose of cholesteryl 14-methylhexadecanoate injected is apparently of great importance for the final effect. Thus with most systems tested 1 mg of the ester was most active. However, in experiments on polyphenylalanine synthesis this dose was obviously too high and may have resulted in an elimination of the excess of this compound or perhaps in an inhibition of its normal biosynthesis. This may help to explain why this dose is far less active than 0.1 mg and why no significant increase in the ester content in the cell sap appears after its administration. No definite conclusions may be made, however, before more knowledge is gained on the metabolism of this cholesteryl ester in the animal organism and on the connexion between protein-synthesizing activity and the relative content of cholesteryl 14-methylhexadecanoate in a given tissue.

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