Biochem. J. (1969) **115**, 873
Printed in Great Britain

Effect of Cholesteryl 14-Methylhexadecanoate on the Activity of Some Amino Acid–Transfer Ribonucleic Acid Ligases from Mammalian Tissues

By J. HRADEC and Z. DUŠEK
Department of Biochemistry, Oncological Institute, Prague 8, Czechoslovakia

(*Received* 2 *July* 1969)

1. L-Tyrosine–, L-alanine–, L-tryptophan– and L-threonine–tRNA ligases (where tRNA is transfer RNA) were purified from mammalian tissues and the relative contents of cholesteryl 14-methylhexadecanoate were determined in fractions obtained during the isolation. Purified enzymes were extracted with various organic solvents. 2. Cholesteryl 14-methylhexadecanoate contents in purified ligases were up to 210-fold that in the starting material. Different enzymes showed different contents of this cholesteryl ester. 3. Extracted enzymes lost in most cases their ability to catalyse formation of the aminoacylhydroxamate and aminoacyl-tRNA complexes. Enzymes extracted with various solvents showed a variable decreased activity. 4. The original activity could be restored to 70–100% by the addition of cholesteryl 14-methylhexadecanoate. Cholesteryl palmitate, cholesteryl margarate and cholesteryl stearate were inactive in this respect. 5. Incubation mixtures of extracted enzymes with cholesteryl 14-methylhexadecanoate added showed an initial delay in the time-course of both reactions assayed. 6. It is concluded that the effect of cholesteryl 14-methylhexadecanoate on the activity of amino acid–tRNA ligases seems to be specific and that this compound may play some role in the function of these enzymes.

Extraction of rat liver pH 5 enzymes with organic solvents results in a decreased combination of labelled amino acids with tRNA* in the presence of such preparations (Hradec & Dušek, 1968). The original activity can be fully restored by the addition of CMH in quantities supplementing those that had been extracted from the pH 5 enzyme. Since the cellular fraction of pH 5 enzymes apparently contains several groups of different enzymes it is difficult to decide if these results represent the effect of CMH on enzymes directly involved in protein synthesis.

Amino acid–tRNA ligases (amino acid-activating enzymes, aminoacyl–tRNA synthetases, EC 6.1.1.–) present in the pH 5 enzyme fraction initiate protein synthesis by catalysing the following reactions (Allende *et al.* 1966):

$$\text{Amino acid} + \text{ATP} + \text{enzyme} \rightleftharpoons \text{aminoacyl-AMP–enzyme} + \text{PP}_i \quad (1)$$

$$\text{Aminoacyl-AMP–enzyme} + \text{tRNA} \rightleftharpoons \text{aminoacyl-tRNA} + \text{AMP} + \text{enzyme} \quad (2)$$

* Abbreviations: tRNA, transfer RNA; CMH, cholesteryl 14-methylhexadecanoate.

The first step of this reaction may be assayed by the amino acid-dependent ATP–pyrophosphate exchange (Hoagland, Keller & Zamecnik, 1956) or by trapping the activated amino acid with hydroxylamine (Hoagland, 1955). As a result of the second step, the labelled amino acid combines with tRNA and the radioactivity of the product may be determined (Webster, 1961). Amino acid–tRNA ligases are strictly specific for individual amino acids. Moreover, more than one ligase was found for those amino acids where more than one codon exists (Allende *et al.* 1966; Vescia, 1967). Isolation of several amino acid–tRNA ligases was reported from mammalian tissues (Allende *et al.* 1966, Davie, Koningsberger & Lipmann, 1956; Fraser, 1963; Schweet, 1962; Webster, 1961), *Escherichia coli* (Calendar & Berg, 1966; George & Meister, 1967; Stern & Mehler, 1965) or baker's yeast (Makman & Cantoni, 1965).

For the study of the effect of CMH on the activity of amino acid–tRNA ligases some of these enzymes were isolated by using procedures that resulted in good purification. Mammalian tissues were chosen as the starting material since mammalian systems were used in all previous experiments with CMH performed in our laboratory (Hradec, 1961; Hradec

& Dolejš, 1968; Hradec & Dušek, 1968). Similar procedures were used in our present study for the demonstration of the effect of CMH on amino acid–tRNA ligases as in our earlier paper on rat liver pH 5 enzymes (Hradec & Dušek, 1968). Preliminary results of these experiments were reported by Hradek & Dušek (1969).

MATERIALS AND METHODS

Animals. Wistar rats of both sexes, weighing 150–200 g., bred in this laboratory and kept on a standard diet, were used for the preparation of the L-threonine tRNA ligase and rat liver tRNA. Hog liver and pancreas as well as ox pancreas were obtained from the slaughterhouse and used within 1 hr. after delivery.

Chemicals. ATP (sodium salt) was a product of Reanal, Budapest, Hungary, and was purified as described by Hradec (1967). All solvents were redistilled before use. Cholesteryl (+)-14-methylhexadecanoate, cholesteryl palmitate, cholesteryl margarate and cholesteryl stearate were kindly prepared by Dr J. Sommerau in this laboratory from corresponding acyl chlorides and purified cholesterol as described by Kritchevsky & Anderson (1952). (+)-14-Methylhexadecanoic acid was isolated from ox liver cholesteryl esters (Hradec & Menšik, 1968); other fatty acids were commercial preparations (Koch–Light Laboratories Ltd., Colnbrook, Bucks.). All fatty acids were purified by preparative g.l.c. to a purity better than 98%. Rat liver tRNA was isolated as described by Moldave (1963). For the purification of tRNA from hog liver and ox pancreas the method of Brunngraber (1962) was followed.

Radioactive compounds. L-[U-^{14}C]Alanine (75 mc/m-mole), L-[U-^{14}C]tyrosine (110 mc/m-mole) and L-[U-^{14}C]-threonine (57 mc/m-mole) were obtained from the Institute for Research, Production and Utilization of Radioisotopes, Prague, Czechoslovakia; DL-[1-^{14}C]tryptophan (33 mc/m-mole) and L-[U-^{3}H]tryptophan (6860 mc/m-mole) were products of The Radiochemical Centre, Amersham, Bucks.

Purification of amino acid–tRNA ligases. L-Alanine–tRNA ligase from hog liver was prepared by the method of Webster (1961). For the purification of L-tyrosine–tRNA ligase from hog pancreas the method of Schweet (1962) was followed. DEAE-cellulose chromatography was used in the final purification step instead of the ethanol fractionation. L-Tryptophan–tRNA ligase from ox pancreas was prepared by the method of Davie (1962) and L-threonine–tRNA ligase from rat liver by the method of Allende *et al.* (1966).

Extraction of amino acid–tRNA ligases. Solutions of purified enzymes in 0·01 M-tris–HCl buffer, pH 7·5, were mixed with DEAE-cellulose (Whatman DE-41) and stirred gently for 30–60 min. The suspension was then filtered by suction through a sintered-glass filter and DEAE-cellulose with adsorbed protein was washed with the same buffer. A thick suspension of DEAE-cellulose in the buffer was then placed into beakers and freeze-dried. For the extraction of threonine–tRNA ligase the solution of the purified enzyme was soaked into fibrous cellulose (Whatman CF-41) in some cases and freeze-dried. DEAE-cellulose powders containing enzyme protein were placed into extraction cartridges and extracted with 200 ml. of the solvent in a Soxhlet apparatus. Extraction with diethyl ether for 4 hr. was chosen as a standard procedure for all enzymes. Other solvents and times were used as indicated. After the extraction, the extracted material was dried *in vacuo* (0·05 mm.Hg) for 2–4 hr. For the extraction of enzyme protein, DEAE-cellulose was suspended in an appropriate volume of cold 1 M-tris–HCl buffer, pH 7·5, and the mixture was stirred for 60 min. The suspension was filtered by suction through a glass filter and the filtrate stored at −50° until used. Control non-extracted enzyme preparations were treated in the same way, omitting only the extraction step. All operations, except the extraction with organic solvents, were performed at 0–5°.

Incubation procedures. The charging of tRNA with labelled amino acids in the presence of amino acid–tRNA ligases was assayed with the following incubation mixture: 70 μmoles of tris–HCl buffer, pH 7·60, 5 μmoles of $MgCl_2$, 10 μmoles of ATP, 5–30 μg. of enzyme protein, 0·8–2·0 mg. of homologous tRNA and 1·5–3·6 nmoles of labelled amino acid in a total volume of 2·0 ml. The optimum ratio of enzyme protein to tRNA was determined for each batch of enzyme. Mixtures were incubated for 20 min. at 37·5°. Mixtures for the hydroxamate assay contained 70 μmoles of tris–HCl buffer, pH 7·60, 5 μmoles of $MgCl_2$, 10 μmoles of ATP, 400–800 μmoles of salt-free hydroxylamine (Beinert *et al.* 1953), 10–25 μg. of enzyme protein and 0·8–3·5 nmoles of labelled amino acid in a total volume of 2·2 ml. The optimum ratio of hydroxylamine to enzyme protein was determined for each batch of enzyme. Mixtures were incubated for 45 min. at 37·5°. These procedures are termed 'standard conditions' in the tables. All samples for both assays were incubated in duplicate together with blank mixtures without ATP. Each experimental series contained extracted and non-extracted preparations of the same enzyme incubated simultaneously.

Assay of radioactivity. For the assay of tRNA radioactivity, mixtures were washed after the incubation and tRNA was extracted and its radioactivity measured as described by Hradec & Dušek (1968). The following procedure resulting from a modification of the method of Loftfield & Eigner (1963) and that of Parin, Kukhanova & Kisselev (1967) was used for the determination of hydroxamate formation. After the incubation mixtures had been heated for 10 min. at 90°, the precipitated protein was centrifuged off and a portion of the supernatant was filtered by suction through discs of Whatman CM-50 paper. Discs were washed with 0·01 M-sodium phosphate buffer, pH 7·0, containing 1 mM unlabelled amino acid and finally with water, and their radioactivity was measured in the NRB 213 low-background windowless gas-flow counter (Tesla, Přemyšlení, Czechoslovakia). The efficiency was about 30%. For calculation of the final results the radioactivity of the blank sample (without ATP) was subtracted from that of the ATP-containing mixture and this value was taken as the energy-dependent labelling of tRNA or hydroxamate formation.

Analytical methods. CMH contents in enzyme solutions were determined as described by Hradec (1968). For the determination of CMH in extracts of enzymes, internal standard was added to the extract, the solvent was evaporated to dryness in a stream of N_2 and the residue was dissolved in chloroform. This solution was then applied to thin layers of silicic acid and samples were processed further by the method of Hradec (1968). Protein and RNA were determined as described by Hradec (1967).

RESULTS

CMH contents in amino acid–tRNA ligases. The relative concentration of CMH increased during the purification of L-tyrosine–tRNA ligase from an average value of 0·863nmole/mg. of protein (range 0·703–0·972, four different preparations) in the starting hog pancreas supernatant to 39·8nmoles/mg. of protein (range 37·5–40·7) in the purified enzyme. Results obtained during the purification of one particular batch of this enzyme are given in Table 1.

The starting material for the purification of L-alanine–tRNA ligase (hog liver postmicrosomal supernatant) showed an average content of 0·091nmole of CMH/mg. of protein (range 0·073–0·101, three different preparations) whereas in the purified enzyme an average content of 19·3nmoles/mg. of protein (range 17·9–20·1) was found. The relative concentration of the cholesteryl ester increased again during the purification of this enzyme (Table 2).

Whereas the ox pancreas postmicrosomal supernatant contained on average 0·105nmole of CMH/mg. of protein (range 0·098–0·202, three different batches of the tissue), the average concentration of CMH in the purified L-tryptophan–tRNA ligase was 14·6nmoles/mg. of protein (range 12·7–16·8). The relative concentration of CMH increased again during enzyme purification (Table 3).

As in our previous experiments (Hradec & Dušek, 1968), an average content of 0·125nmole of CMH/mg. of protein (range 0·116–0·146, six batches of rat liver) was found in the rat liver cell sap. The relative concentration of the cholesteryl ester rose progressively during the purification of L-threonine–tRNA ligase up to an average value of 22·6nmoles/mg. of protein (range 22·2–23·1) in the purified enzyme preparation (Table 4).

Activity of amino acid–tRNA ligases extracted with organic solvents. A good labelling of rat liver tRNA with L-tyrosine was obtained in experiments with L-tyrosine–tRNA ligase, and rat liver tRNA was therefore used throughout. The values obtained with four different batches of non-extracted L-tyrosine–tRNA ligase ranged from 1985 to 3250c.p.m./mg. of RNA. The same enzymes extracted by the standard procedure showed an activity corresponding to 0–5% of that of non-extracted enzymes. Different solvents extracted different quantities of CMH from the same enzyme

Table 1. *Specific activity and CMH contents of fractions obtained during the purification of a preparation of* L-*tyrosine–tRNA ligase*

The specific activity is expressed as c.p.m./mg. of tRNA incorporated in the presence of 1mg. of enzyme protein or c.p.m. in hydroxamate incorporated by 1mg. of enzyme protein under standard conditions. CMH contents are given in nmoles/mg. of protein.

		Specific activity		
Step no.	Fraction	tRNA	Hydroxamate	CMH content
1	Postmicrosomal supernatant	2500	54	0·875
2	Calcium phosphate gel eluate	26000	76	2·17
3	First $(NH_4)_2SO_4$ precipitate	33200	85	3·29
4	Second $(NH_4)_2SO_4$ precipitate	225000	2580	9·13
5	DEAE-cellulose column eluate	465000	5920	40·7

Table 2. *Specific activity and CMH contents of fractions obtained during the purification of a batch of* L-*alanine–tRNA ligase*

The specific activity is expressed as c.p.m./mg. of RNA incorporated in the presence of 1mg. of enzyme protein under standard conditions. CMH contents are given in nmoles of CMH/mg. of protein.

Step no.	Fraction	Specific activity	CMH content
1	Crude supernatant	18	0·090
2	pH5 enzymes	310	0·219
3	Second $(NH_4)_2SO_4$ precipitate	830	0·934
4	Calcium phosphate gel eluate	1472	2·03
5	DEAE-cellulose eluate	35000	19·25

Table 3. *Specific activity and CMH contents of fractions obtained during the purification of a preparation of* L-*tryptophan–tRNA ligase*

The specific activity is expressed in c.p.m./mg. of tRNA incorporated in the presence of 1mg. of enzyme protein or c.p.m. incorporated into hydroxamate in the presence of 1mg. of enzyme protein under standard conditions. CMH contents are given in nmoles of CMH/mg. of protein.

		Specific activity		
Step no.	Fraction	tRNA	Hydroxamate	CMH content
1	Crude supernatant	0·82	29·8	0·097
2	pH6·5 supernatant	5·00	255·0	1·38
3	Combined acid precipitates	99·0	908·0	1·97
4	$(NH_4)_2SO_4$ precipitate	150·2	1960	14·55

Table 4. *Specific activity and CMH contents of fractions obtained during the purification of a batch of* L-*threonine–tRNA ligase*

The specific activity is expressed as c.p.m./mg. of tRNA incorporated in the presence of 1 mg. of enzyme protein under standard conditions. CMH contents are given in nmoles of CMH/mg. of protein.

Step no.	Fraction	Specific activity	CMH content
1	Postmicrosomal supernatant	464	0·125
2	pH 4·5 precipitate	4825	1·520
3	Calcium phosphate gel eluate	5820	3·490
4	$(NH_4)_2SO_4$ precipitate	15620	5·720
5	DEAE-cellulose column eluate	35100	5·850
6	Hydroxyapatite column eluate	185000	22·90

Table 5. *Effect of the extraction with various solvents on the activity of* L-*tyrosine–tRNA ligase*

The enzyme preparation (1·5 mg. of protein) was extracted with 200 ml. of the solvent for 1 hr. Incubation mixtures for tRNA assay contained 25 μg. of enzyme protein and 1·0 mg. of rat liver tRNA, those for hydroxamate assay contained 15 μg. of enzyme protein and 0·6 m-mole of hydroxylamine. The other components added to incubation mixtures are described in the Materials and Methods section. Results are expressed as the energy-dependent combination of L-tyrosine with tRNA (c.p.m./mg. of tRNA) or hydroxylamine (c.p.m.). CMH contents in extracts are given as nmoles of CMH/mg. of protein. CMH was added to extracted enzymes in quantities replacing exactly those extracted.

	Radioactivity				
	In tRNA		In hydroxamate		CMH
Solvent	−CMH	+CMH	−CMH	+CMH	extracted
Ether	400	4050	51	257	26·9
Benzene	2400	4100	82	274	15·8
Cyclohexane	2460	3950	54	260	17·2
Chloroform	210	3880	0	231	30·5
Carbon tetrachloride	100	3940	0	205	33·4
Iso-octane	1000	4150	75	244	21·4
n-Hexane	3900	4200	134	239	3·8
Control values (unextracted enzymes)	4245	—	252	—	—

preparation and different residual activities were found with these enzymes. These results together with recoveries of the enzymic activity after the addition of CMH (see below), are given in Table 5.

Table 6. *Effect of the extraction with various solvents on the activity of* L-*alanine–tRNA ligase*

The enzyme preparation (0·42 mg. of protein) was extracted with 200 ml. of the solvent for 1 hr. Incubation mixtures contained 7·8 μg. of enzyme protein, 0·7 mg. of hog liver tRNA and the other constituents as given in the Materials and Methods section. Results are expressed as the energy-dependent combination of labelled L-alanine with tRNA (c.p.m./mg. of RNA). CMH contents in extracts are given in nmoles of CMH/mg. of enzyme protein. CMH was added to extracted ligases in quantities replacing exactly those extracted.

	Radioactivity in tRNA		
Solvent	−CMH	+CMH	CMH extracted
Ether	480	2070	9·28
Chloroform	80	2120	14·32
Carbon tetrachloride	64	1840	16·74
n-Hexane	1390	2410	6·08
Control values (unextracted enzymes)	2640	—	—

A relatively low radioactivity was found in tRNA if the purified L-alanine–tRNA ligase was incubated with rat liver tRNA. More than 20-fold greater charging of tRNA with labelled L-alanine was obtained with tRNA from hog liver. However, a good agreement was demonstrated between both these tRNA preparations in experiments with extracted enzymes. For example, hog liver tRNA was charged with 1400 c.p.m./mg. of RNA in the presence of an extracted enzyme (this preparation was extracted with *n*-hexane for 2 hr.), the control value with the same non-extracted enzyme being 3180 c.p.m./mg. of RNA. With rat liver tRNA the corresponding values were 72 and 152 c.p.m./mg. of RNA respectively.

When incubated with the non-extracted L-alanine–tRNA ligase the hog liver tRNA was charged with 2640–3180 c.p.m./mg. of RNA (four different preparations). Enzymes extracted by the standard procedure (9·8–11·0 nmoles of CMH/mg. of protein removed) completely lost their ability to catalyse the energy-dependent combination of labelled L-alanine with tRNA. Different solvents again extracted different quantities of CMH from a given enzyme preparation and different residual activities were found with these ligases (Table 6).

When non-extracted L-tryptophan–tRNA ligases (three batches) were incubated with L-[^{3}H]tryptophan, the energy-dependent labelling of ox pancreas tRNA with radioactivity was 172–203 c.p.m./mg. of RNA. The standard extraction procedure removed 5·95–6·24 nmoles of CMH/mg. of protein from this enzyme and no energy-dependent incorporation was found with such extracted ligases.

When different solvents were used for the extraction of a particular enzyme preparation widely differing quantities of CMH were removed. However, most of these extracted ligases showed a very low residual activity or no activity at all (Table 7).

The threonine–tRNA ligase was the only enzyme of all those used in the present study the activity of which was seriously affected by freeze-drying and decreased to 8–15% of the activity of the preparation stored in the frozen state. No differences were found in this respect between preparations adsorbed before being freeze-dried.

Table 7. *Effect of the extraction with various solvents on the activity of* L-*tryptophan–tRNA ligase*

The enzyme preparation (5·2 mg. of protein) was extracted with 200 ml. of the solvent for 1 hr. Incubation mixtures contained 24·8 μg. of enzyme protein and 0·8 m-mole of hydroxylamine together with the other components described in the Materials and Methods section. Results are expressed as the energy-dependent conversion of ^{14}C-labelled DL-tryptophan into its hydroxamate (c.p.m.). CMH contents of extracts are given as nmoles of CMH/mg. of protein. CMH was added to extracted enzymes in quantities replacing exactly those removed by the extraction.

Solvent	Radioactivity in hydroxamate −CMH	Radioactivity in hydroxamate +CMH	CMH extracted
Benzene	0	143	6·20
Ether	0	173	8·92
Chloroform	0	147	10·80
Carbon tetrachloride	0	152	12·95
n-Hexane	54	169	5·64
Control values (unextracted enzymes)	199	—	—

Table 8. *Effect of the extraction with various solvents on the activity of* L-*threonine–tRNA ligase*

The enzyme preparation (0·95 mg. of protein) was extracted with 200 ml. of the solvent for 1 hr. Incubation mixtures contained 7·8 μg. of enzyme protein, 1·1 mg. of rat liver tRNA and the other components as described in the Materials and Methods section. Results are expressed as the energy-dependent combination of L-threonine with tRNA (c.p.m./mg. of RNA). CMH contents in extracts are given in nmoles of CMH/mg. of enzyme protein. CMH was added to extracted enzymes in quantities supplementing exactly those extracted.

Solvent	Radioactivity in tRNA −CMH	Radioactivity in tRNA +CMH	CMH extracted
Ether	137	340	8·93
Chloroform	56	312	18·20
n-Hexane	298	397	6·80
Benzene	164	400	14·90
Cyclohexane	227	423	5·68
Iso-octane	267	416	5·44
Ethylene glycol monomethyl ether	151	398	15·25
Control values (unextracted enzymes)	445	—	—

Rat liver tRNA was charged with 215–445 c.p.m./mg. of RNA in the presence of non-extracted L-threonine–tRNA ligases. No energy-dependent combination of L-threonine with tRNA was found with enzymes extracted by the standard procedure (five different preparations) which removed 17·5–19·8 nmoles of CMH/mg. of protein. However, some residual activity was left in enzymes extracted with other solvents (Table 8).

In the hydroxamate assay three different batches of L-tyrosine–tRNA ligase showed 462–620 c.p.m. incorporated into tyrosylhydroxamate. The activity of enzymes extracted by the standard procedure (25·4–33·7 nmoles of CMH/mg. of protein removed) was 15·3–30·5% of that of the corresponding non-extracted enzyme. Different residual activities were found with enzymes extracted with different solvents (Table 5).

Non-extracted L-tryptophan–tRNA ligases incorporated 240–310 c.p.m. into tryptophanylhydroxamate when incubated with hydroxylamine and [^{14}C]tryptophan. Enzymes extracted by the standard procedure showed a residual activity of 10–15% of the control preparation.

Both L-alanine– and L-threonine–tRNA ligases showed a very low activity in the hydroxamate assay, and no experiments in this respect were therefore performed with extracted enzymes.

Effect of CMH on the activity of extracted amino acid–tRNA ligases. After the addition of CMH to extracted L-tyrosine–tRNA ligases in quantities replacing exactly those removed by the standard procedure of extraction (23·5–33·7 nmoles of CMH/mg. of protein) a charging of tRNA with L-tyrosine corresponding to 83–97% of the control value was found. Similar results were obtained with L-alanine–tRNA ligases extracted by the standard procedure, when a recovery of 76–93% of the original activity resulted from the addition of CMH. For L-tryptophan– and L-threonine–tRNA ligases the corresponding values were 72–85% and 76–89%, respectively.

Addition of greater of smaller quantities of CMH than those that had been removed to extracted L-alanine–tRNA ligases was less effective (Fig. 1). Similar results were obtained with the other enzymes tested.

Different recoveries of the enzymic activity were found with ligases extracted with different solvents when CMH was added in quantities replacing those which had been removed by the extraction (Tables 5–8).

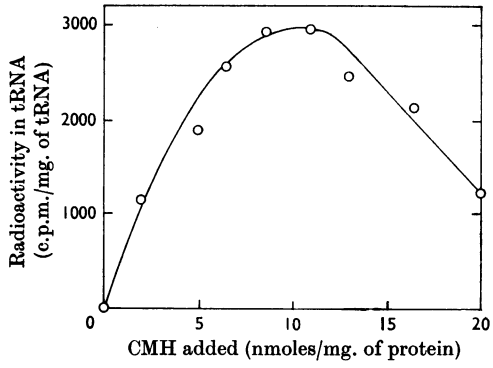

Fig. 1. Effect of different doses of CMH on the charging of tRNA with labelled L-alanine in the presence of an extracted L-alanine–tRNA ligase. The control non-extracted enzyme incorporated 3180 c.p.m./mg. of tRNA; 10·2 nmoles of CMH/mg. of protein had been removed from the extracted enzyme by the standard procedure (see the Materials and Methods section).

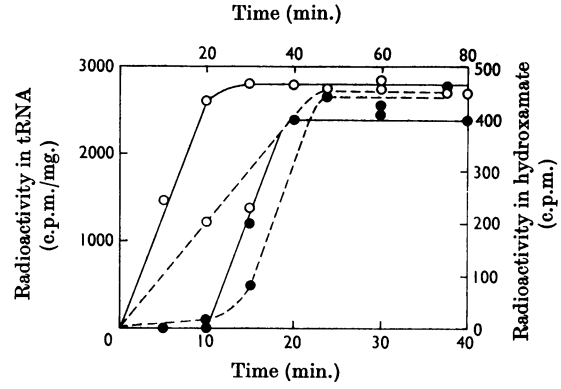

Fig. 2. Time-course of formation of the L-tyrosyl–tRNA complex (——) and of the hydroxamate (– – – –) in the presence of a non-extracted (○) and extracted (●) L-tyrosine–tRNA ligase with CMH added. The enzyme was extracted by the standard procedure (see the Materials and Methods section) and CMH was added to the mixture before incubation in a quantity replacing that extracted (28·7 nmoles/mg. of protein). Mixtures for the hydroxamate assay were incubated for 20–75 min. (upper time scale), those for the tRNA assay for 5–40 min. (lower time scale).

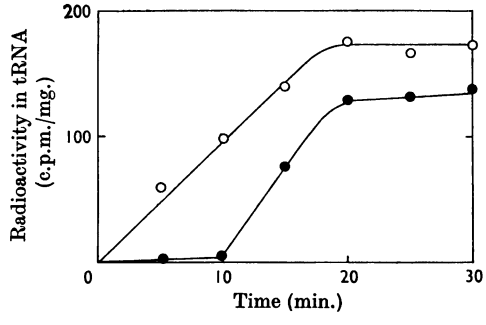

Fig. 3. Combination of L-[U-^{3}H]tryptophan with tRNA in the presence of a non-extracted L-tryptophan–tRNA ligase (○) and the same enzyme extracted by the standard procedure (see the Materials and Methods section) (●) with CMH added (6·12 nmoles of CMH/mg. of enzyme protein were added, replacing the quantity removed by the extraction).

In the hydroxamate assay, a recovery of 94·5–118% of the original activity was obtained with L-tyrosine–tRNA ligases extracted by the standard procedure when the appropriate quantity of CMH was added. Similarly, a complete recovery of the enzymic activity (92–106% of the control value) was found with extracted L-tryptophan–tRNA ligase supplemented with CMH.

Effect of other cholesteryl esters on the activity of extracted ligases. The other cholesteryl esters tested in this study showed no reactivating effect on the activity of extracted ligases as did CMH. Thus, for example, when a preparation of L-alanine–tRNA ligase (extracted with diethyl ether for 1 hr.) was supplemented with the appropriate quantity of CMH (7·24 nmoles of CMH/mg. of enzyme protein), the charging of tRNA with L-alanine increased from 900 to 2450 c.p.m./mg. of RNA (the incorporation with the control non-extracted enzyme was 2875 c.p.m./mg. of RNA). Addition of the same amount of cholesteryl palmitate had no effect on the enzymic activity (900 c.p.m./mg. of RNA), whereas in the presence of the same quantities of either cholesteryl margarate or cholesteryl stearate the combination of L-alanine with tRNA was even less (520 and 500 c.p.m./mg. of RNA, respectively) than with the extracted enzyme alone. Similar results were obtained with all other ligases tested.

Time-course of reactions catalysed by extracted ligases in the presence of CMH. Extracted L-tyrosine–tRNA ligases with CMH added immediately before the incubation showed a delayed time-course in both the tRNA and hydroxamate assays (Fig. 2). Similar results were obtained with extracted L-tryptophan–tRNA ligases (Fig. 3) as well as with the other enzymes used in this study.

DISCUSSION

Some amino acid–tRNA ligases catalyse a high combination of labelled amino acids with tRNA but show a very low activity in the hydroxamate assay. A similar divergence in reactivity with hydroxyl-

amine was reported by Hirsch & Lipmann (1968) for different amino acid–tRNA ligases of *E. coli*. Similarly, in our experiments, very low hydroxamate formation was found with L-alanine– and L-threonine–tRNA aligses. Moreover, the latter enzyme is apparently deactivated not only by a prolonged contact with DEAE-cellulose, as reported by Allende *et al.* (1966), but also by freeze-drying.

No exact agreement was found in our experiments between the results of the hydroxamate assay and of aminoacyl-tRNA complex-formation. Apparently these functions of amino acid–tRNA ligases are largely independent of each other, the activity for the aminoacyl-tRNA formation being more sensitive to different treatments. Papas & Mehler (1968) reported that prolyl-tRNA formation in the presence of proline–tRNA ligase of *E. coli* is more readily affected by the temperature than is the pyrophosphorolysis of prolyl-AMP. Similarly, treatment of the methionine–tRNA ligase of *E. coli* with an organic mercurial resulted in the loss of the transfer activity of the enzyme without affecting the amino acid-activation reaction (Cassio, 1968).

It is not clear why extraction of only a portion of total CMH present in the enzyme preparation is sufficient for complete deactivation of the latter. Contamination of enzyme preparations with free CMH not bound to protein can hardly be suspected in this respect, since procedures securing high purification were used in our experiments for the isolation of individual ligases. Moreover, increasing contents of CMH during enzyme purification as well as relatively drastic conditions necessary for the extraction of larger quantities of the cholesteryl ester from enzyme preparations indicate a rather firm binding of CMH to protein in amino acid–tRNA ligases. It is not possible to decide if CMH present in a chloroform–methanol extract of the enzyme represents the true total quantity of this compound present in the enzyme preparation. However, this solvent mixture is extensively used in lipid research and is supposed to secure an efficient extraction of lipids from biological materials (Folch, Ascoli, Lees, Meath & Le Baron, 1951).

Various amino acid–tRNA ligases show different CMH contents. It is not clear if this is due to structural differences of a particular enzyme from different tissues or to specific differences between individual enzymes. To answer this question it will be necessary to compare amino acid–tRNA ligases from different tissues. The inability to reactivate fully some extracted enzymes with appropriate quantities of CMH may be explained as a result of a different sensitivity of individual ligases towards the extraction. Such results were found mainly with the most effective solvents and it is possible that these solvents altered irreversibly the structure of these enzymes. The possibility cannot be excluded, however, that another factor, beside CMH, necessary for the normal enzymic function is removed by some solvents, although this explanation seems to be less probable.

No exact correspondence was found between the increase of the specific activity of the enzyme and of its CMH content, in particular at early steps of the purification of amino acid–tRNA ligases. This may be due to a contamination of such crude fractions with free CMH not bound to enzyme protein. Alternatively, some protein rich in CMH may be removed during the initial steps of enzyme purification.

Some of the present results seem to support the opinion that CMH may play some role in the function of amino acid–tRNA ligases. This is indicated not only by the presence of relatively large quantities of this compound in purified synthetases but also by its ability to reactivate extracted enzymes. There is apparently a specific requirement for this cholesteryl ester in extracted synthetases. Cholesteryl esters with closely related structures, some of which are also present in enzyme extracts (cholesteryl palmitate and cholesteryl stearate), did not reactivate extracted ligases. Moreover, it was demonstrated that only two other cholesteryl esters could replace CMH in this respect when extracted rat liver pH5 enzymes were used (Sommerau & Hradec, 1968). This ability apparently depends on the configuration and chain length of the fatty acid. Moreover, the delay in the time-course of both hydroxamate formation and aminoacyl-tRNA complex-formation found only in the presence of extracted enzymes with CMH added indicates that some time may be necessary for the recombination of this compound with the enzyme protein before the original activity is regained.

The excellent technical assistance of Miss N. Krejčová, Mrs Z. Tuháčková and Mrs M. Čechová is gratefully acknowledged.

REFERENCES

Allende, C. C., Allende, J. E., Gatica, M., Celis, J., Mora, G. & Matamala, M. (1966). *J. biol. Chem.* **241**, 2245.

Beinert, H., Green, D. E., Hele, P., Hift, H., Von Korff, R. W. & Ramakrishnan, C. V. (1953). *J. biol. Chem.* **203**, 35.

Brunngraber, E. F. (1962). *Biochem. biophys. Res. Commun.* **8**, 1.

Calendar, R. & Berg, P. (1966). *Biochemistry*, **5**, 1681.

Cassio, D. (1968). *Europ. J. Biochem.* **4**, 222.

Davie, E. W. (1962). In *Methods in Enzymology*, vol. 5, p. 718. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Davie, E. W., Koningsberger, V. V. & Lipmann, F. (1956). *Arch. Biochem. Biophys.* **65**, 21.

Folch, J., Ascoli, I., Lees, M., Meath, J. A. & Le Baron, F. N. (1951). *J. biol. Chem.* **191**, 833.

Fraser, M. J. (1963). *Canad. J. Biochem. Physiol.* **41**, 1123.
George, H. & Meister, A. (1967). *Biochim. biophys. Acta*, **132**, 165.
Hirsch, D. I. & Lipmann, F. (1968). *J. biol. Chem.* **243**, 5724.
Hoagland, M. B. (1955). *Biochim. biophys. Acta*, **16**, 288.
Hoagland, M. B., Keller, B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **218**, 345.
Hradec, J. (1961). *Biochim. biophys. Acta*, **47**, 149.
Hradec, J. (1967). *Biochem. J.* **105**, 251.
Hradec, J. (1968). *J. Chromat.* **32**, 511.
Hradec, J. & Dolejš, L. (1968). *Biochem. J.* **107**, 129.
Hradec, J. & Dušek, Z. (1968). *Biochem. J.* **110**, 1.
Hradec, J. & Dušek, Z. (1969). *Abstr. 6th Meet. Fed. Europ. biochem. Soc., Madrid*, p. 251.
Hradec, J. & Menšík, P. (1968). *J. Chromat.* **32**, 502.
Kritchevsky, D. & Anderson, M. E. (1952). *J. Amer. chem. Soc.* **74**, 1857.
Loftfield, R. B. & Eigner, E. A. (1963). *Biochim. biophys. Acta*, **72**, 372.
Makman, M. H. & Cantoni, G. L. (1965). *Biochemistry*, **4**, 1434.
Moldave, K. (1963). In *Methods in Enzymology*, vol. 6, p. 757. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Papas, T. S. & Mehler, A. H. (1968). *J. biol. Chem.* **243**, 3767.
Parin, A. V., Kukhanova, M. K. & Kisselev, L. L. (1967). *Biokhimiya*, **32**, 735.
Schweet, R. S. (1962). In *Methods in Enzymology*, vol. 5, p. 722. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Sommerau, J. & Hradec, J. (1968). *Abstr. 5th Meet. Fed. Europ. biochem. Soc., Prague*, p. 67.
Stern, R. & Mehler, A. H. (1965). *Biochem. Z.* **342**, 400.
Vescia, A. (1967). *Biochem. biophys. Res. Commun.* **29**, 496.
Webster, G. (1961). *Biochim. biophys. Acta*, **49**, 141.