A Translational Control Mechanism in Mammalian Protein Synthesis Modulated by Cyclic Adenosine Monophosphate. Translational Control of Tyrosine Aminotransferase Synthesis in Neonatal Rat Liver†

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ABSTRACT: The release of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) from polysomes isolated from neonatal rat liver specifically requires adenosine 3′,5′-cyclic monophosphate (cAMP) and a release factor which can be extracted from liver microsomes. The system can be demonstrated *in vitro*. The system is exclusively located in the rough-surfaced endoplasmic reticulum. Evidence is also presented for the presence of adenylyl cyclase in this fraction. *In vitro*, the addition of amino acids, pH 5 enzymes, GTP, ATP, and a triphosphate regenerator results in a sixfold increase in the amount of enzyme released from the polysomes. The deletion of pH 5 enzymes or GTP or amino acids or the addition of cycloheximide to the complete system prevents the increased yield of enzyme which therefore appears due to the completion of nascent enzyme chains on the polysome. Pyridoxal phosphate is also required for the effect but it is shown that enzyme polypeptides are completed in its absence, the apoenzyme is formed and the cofactor is subsequently bound to yield activity. Zone centrifugation of the active polysomes indicates a sedimentation coefficient of 270–290 S. This corresponds to six ribosomes on the polysome. The increased yield of enzyme due to chain completion also indicates the same size for the polysome. Thus it is deduced that the enzyme is a tetramer. The phenomenon constitutes evidence for a translational control mechanism in mammalian protein synthesis which is located exclusively in the cell cytoplasm and is under the control of cAMP.

In previous papers from this laboratory (Chuah *et al.*, 1971; Chuah and Oliver, 1971), adenosine 3′,5′-cyclic monophosphate (cAMP[1]) has been implicated as an effector in the postnatal synthesis of tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5) in rat liver. The initial evidence indicated that the effect was restricted to posttranscriptional events (Holt and Oliver, 1969) and further studies indicated that a cAMP-dependent control operates at the termination or release step of tyrosine aminotransferase synthesis in neonatal rat liver (Chuah and Oliver, 1971).

In the latter paper, cAMP was shown to be an obligatory compound for the release of polysome-bound enzyme *in vitro* and a microsomal protein, which strongly and specifically binds the cyclic nucleotide, was demonstrated as a further component of the release system. The exclusive location of the system in the rough endoplasmic reticulum membrane is reported here. In addition, a "cascade"phenomenon is described, in which the release of the C-terminal polypeptide allows partially completed polypeptides on the polysome to complete their synthesis, and in turn be released by the cAMP-activated mechanism. This phenomenon constitutes evidence for a translational control mechanism in mammalian protein biosynthesis located exclusively in the cell cytoplasm.

Methods

Animal Testing. Rats (1–2 day old) of the Wistar albino strain of *Rattus norwegicus* were used. From each litter, one animal was injected (intraperitoneally) with epinephrine in saline (1.4 μg/g of body weight) and another was injected with the same volume of saline. The remaining animals of the litter were killed, the livers pooled, and homogenates were prepared in 0.25 M sucrose and stored in the ice bath. After 4 hr in a humidicrib at 37°, the test rats were killed, liver homogenates were prepared in 0.25 M sucrose, and tyrosine aminotransferase activity was assayed in the supernatant prepared by centrifugation at 15,000*g* for 10 min.

Those litters which showed an increase in enzyme activity in the test animals of at least twofold after epinephrine treatment were defined as inducible.[2] Some animals show no increase in enzyme activity after hormone treatment and their littermates are defined as noninducible. The stored homogenates were used in the *in vitro* experiments described below.

Preparation of Liver Fractions. The pooled liver homogenates were prepared in a coaxial homogenizer in 0.25 M sucrose (4 ml/g of liver) using 15 strokes of the homogenizer at the same rheostat setting of the drive motor each time.

The homogenates were centrifuged at 15,000*g* for 10 min in the PR-2 International centrifuge. The supernatant was removed and centrifuged at 105,000*g* × hr in the type 50 or 65 rotor of the Spinco L2-65 ultracentrifuge at 2–4°. The

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[1] Abbreviations used are: adenosine 3′,5′-cyclic monophosphate (cyclic AMP or cAMP), adenosine 2′,3′-cyclic monophosphate (cyclic 2′,3′-AMP), deoxyadenosine 3′,5′-cyclic monophosphate (cyclic dAMP). Other nucleotides are abbreviated conventionally: TKM buffer, 50 mM Tris-HCl–275 mM KCl–5 mM $MgCl_2$ (pH 8.0); TAM buffer, 50 mM Tris-HCl–275 mM NH_4Cl–5 mM $MgCl_2$ (pH 8.0).

[2] The term inducible is used for convenience and makes no implications concerning mechanisms.

TABLE I: Localization of Nascent Tyrosine Aminotransferase and Release Function in "Rough" Microsomes.[a]

Microsome Fraction	Additions	Tyrosine Aminotransferase Act.
RM (2.6 mg of protein)	Nil	0.00
	cAMP (1 mM)	2.65
SM (1.6 mg of protein)	cAMP (1 mM)	0.00
RM + SM (0.5:0.5)	cAMP (1 mM)	1.40
RM[b] (2.6 mg of protein)	SM (0.43 mg) + cAMP (1 mM)	0.0
	Microsome extract (0.45 mg)	0.0
	Microsome extract + cAMP (1 mM)	2.82

[a] The liver microsome fraction was prepared from inducible 2-day postnatal rats and subfractionated into rough (RM) and smooth (SM) microsomes. Aliquots of 0.5 ml were incubated at 37° for 1 hr in a final volume of 1 ml, containing TKM buffer, 5 mM theophylline, and the additions given above. Enzyme activities were then assayed on 100-μl aliquots of the incubates. [b] The RM fraction was TKM washed while the SM was sucrose washed.

supernatant was removed and the pellet was washed three times by suspension and centrifugation in 0.25 M sucrose or TKM buffer. The final washed pellet was resuspended in sucrose, TKM, or TAM buffer as given in experimental protocols. This fraction is called microsomes.

Subfractionation of Rough and Smooth Microsomes (Dallner et al., 1966). Three milliliters of the 15,000*g* (10 min) supernatant prepared as above was layered with care over 2 ml of 1.3 M sucrose. After centrifugation in the SW 39 rotor for 210 min at 36,000 rpm, a fluffy layer was apparent at the sucrose interface (smooth microsomes) and a brown pellet at the bottom (rough microsomes). The reddish, clear upper phase above the fluffy layer was removed with a pipet provided with a rubber aspirator and was discarded. The fluffy layer was completely collected in about 1 ml and transferred into another 5-ml tube (SW 39 rotor). It was then diluted with 3 ml of distilled water, brought up to tube volume with 0.25 M sucrose, mixed, and centrifuged at 36,000 rpm for 90 min. The pellets from both centrifugations [rough microsomes (RM) and smooth microsomes (SM)] were suspended in TKM buffer.

Preparation of Deoxycholate Polysomes. These were prepared as described previously (Chuah and Oliver, 1971) essentially by the method of Blobel and Potter (1967).

Preparation of Microsomal Protein Fraction. This fraction was prepared by the procedure previously described (Chuah and Oliver, 1971).

Preparation of pH 5 Fraction. The supernatant from neonatal rat liver homogenates (105,000*g* × hr) was diluted with three volumes of buffer (10 mM $MgCl_2$–25 mM KCl–35 mM Tris-HCl (pH 7.8)–0.15 M sucrose) and titrated to pH 5.15 as described by Hoagland *et al.* (1965) with 10% acetic acid.

The precipitate was collected by centrifugation at 15,000*g* for 10 min and suspended in the buffer at pH 5.15 and recentrifuged. Occluded tyrosine aminotransferase activity was completely removed after eight such washes. The tyrosine aminotransferase free precipitate was then suspended in the "cascade" buffer.

Experiments with Liver Fractions. In release experiments, 0.5 ml of the microsome suspension (RM or SM) was incubated in a final volume of 1 ml of TKM buffer containing 5 mM theophylline at 37°. Various additives which were used are given in the results. In the cascade system, aliquots of microsome suspension were incubated at 37° in a final volume of 1 ml containing pH 5 fraction (1.9 mg of protein), 0.85 mM ATP, 0.20 mM GTP, 5 mM P-enolpyruvate, 250 μg of pyruvate kinase, 2.7 mM $MgCl_2$, 35 mM NH_4Cl, 47 mM KCl, 4 mM mercaptoethanol, 44 mM sucrose, 5 mM theophylline, 1 mg/100 ml each of the most common 21 L-amino acids, 34 mM Tris-HCl (pH 7.6), 1 or 5 mM cAMP, and 1 mM pyridoxal 5′-phosphate. This medium is similar to that used by Tryfiates (1969).

The reaction was stopped by dilution of 100 μl of the incubation mixture with 2.0 ml of the assay medium for tyrosine aminotransferase (3 mM L-tyrosine, 2.5 mM diethyl dithiocarbamate, 0.06 mM pyridoxal 5′-phosphate, 0.25 M sucrose, and 0.1 M potassium phosphate buffer (pH 7.4) modified from Sereni *et al.*, 1959).

After preincubation for 10 min at 37°, the enzyme reaction was initiated by addition of 100 μl of 0.3 M 2-oxoglutaric acid. After 20-min incubation, 0.5 ml of 30% trichloroacetic acid was added and the tubes were iced immediately. A reaction blank was prepared by adding 0.5 ml of 30% trichloroacetic acid to the enzyme assay mixture prior to the addition of the microsomal incubate. In the zone centrifugation experiments, enzyme assays were incubated for 60 min at 37°.

The assay tubes were centrifuged in a bench centrifuge at 3000 rpm for 5 min to remove denatured protein, and 1.0-ml aliquots of the clear supernatant were assayed for *p*-hydroxyphenylpyruvate by a modification of the Brigg's reaction (Lin and Knox, 1957).

The tubes were incubated at room temperature for 30 min and centrifuged for a further 15 min when necessary to remove turbidity. Absorbance was determined at 850 mμ against a color reagent blank. *p*-Hydroxyphenylpyruvate was used as a standard. The activity of tyrosine aminotransferase was calculated as micromoles of *p*-hydroxyphenylpyruvate formed per hour.

Zone Centrifugation. This technique was carried out in SW 56 rotor of the Spinco L2-65 ultracentrifuge. Sucrose concentration gradients were prepared essentially according to Britten and Roberts (1960) in a multiple gradient maker (Hoefer Scientific Instruments, San Francisco, Calif.). Tubes were fractionated by bottom puncture and drop counting from the bottom of the tube using a fractionator made by Hoefer Scientific Instruments.

Protein Determination. The method of Lowry *et al.* (1951) was used to determine protein concentrations in suitable diluted aliquots. Lyophilized crystalline bovine serum albumin was used as a standard.

Gel Electrophoresis. Electrophoresis of protein fractions was carried out in 7.5% polyacrylamide gels (6 mm diameter ×10 cm). The gels were made in 0.012 M Tris–0.095 M glycine (pH 8.3) and the electrode buffer was 0.05 M Tris–0.38 M glycine (pH 8.3). No spacer gel was used and samples were layered under 0.25 M sucrose which filled the tubes. "Nonionic" start conditions were thus used. Electrophoresis was carried out at 3 mA/tube for the whole run at 4°. Electrophoresis was continued until a Bromophenol Blue marker

TABLE II: Effect of Pyridoxal Phosphate and Inhibitors on the Tyrosine Aminotransferase Activity Released from Liver Fractions under Conditions Allowing Protein Synthesis.

Liver Fraction	Conditions	Additions (1 mM Except Where Noted)	Transferase Act. Released (μmoles of PHPP/hr)
DOC polysomes[a] (3.6 mg of protein, microsomal release factor 0.47 mg of protein)	Release[b]	cAMP	2.75
	Cascade[c]	Nil	0.00
	Cascade[c]	PALP	0.00
	Cascade[c]	cAMP	2.68
	Cascade[c]	cAMP, PALP	7.77
	Cascade[c]	cAMP, PALP, cycloheximide (3.5 mM)	2.75
	Cascade[c]	cAMP, PALP, puromycin (3.5 mM)	2.75
Microsomes[d] (5.6 mg of protein)	Release[b]	cAMP (5 mM)	2.72
	Cascade[c]	cAMP (5 mM), PALP	16.95
	Cascade[c]	cAMP (5 mM), PALP, cycloheximide (3.5 mM)	2.72
	Cascade[c]	cAMP (5 mM), PALP, puromycin (3.5 mM)	2.72

[a] DOC polysomes were prepared from 2-day-old inducible rats and aliquots were incubated under different conditions with additions noted above for 60 min at 37° in a final volume of 1 ml. Aliquots were then taken for enzyme assay. Abbreviations used are: DOC, sodium deoxycholate; PALP, pyridoxal-5′-P; PHPP, *p*-hydroxyphenylpyruvate. [b] The release conditions were as in footnote *c* except that the ATP regenerator, pH 5 fraction, GTP, and free amino acids were omitted. [c] The cascade conditions included buffers and all factors for protein synthesis as described in Methods. [d] Liver microsomes were prepared from inducible 2-day postnatal rats and incubated under the conditions shown for 3 hr at 37°. P-enolpyruvate concentration was increased to 5 mM. Enzyme activities were subsequently assayed in aliquots from each incubation mixture.

dye just left the bottom of the tube. Proteins were stained with Naphthalene Black.

Chemicals. ATP, GTP, cAMP, P-enolpyruvate, *p*-hydroxyphenylpyruvate, cycloheximide, pyridoxal-5′-P, and sodium deoxycholate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Pyruvate kinase was from Boehringer and Soehne. Theophylline was obtained from Prosana Laboratories, Sydney, Australia. Puromycin was from Nutritional Biochemical Corp. (Cleveland, Ohio). Epinephrine tartrate was from Burroughs Wellcome and Co. Ltd., Sydney, Australia. Most of the L-amino acids were obtained from California Corp. for Biochemical Research, Los Angeles, Calif. L-Glutamate was from L. Light & Co. Ltd., England, L-cysteine hydrochloride was from Ajax Chemicals Ltd., Sydney-Melbourne, and L-asparagine was from the British Drug Houses Ltd., England.

Results

Table I shows that the release of tyrosine aminotransferase activity in response to incubation with cAMP occurs only in the rough endoplasmic reticulum fraction (rough microsomes) and that smooth microsomes are devoid of the effect. It is also shown that the release of tyrosine aminotransferase activity resulting from incubation of TKM-washed rough microsomes with cAMP is dependent on the addition of the microsomal release factor. The failure of smooth microsomes to substitute for the release factor shows that the essential factor is derived from the rough microsomes alone.

In experiments not shown in detail, it was found that puromycin, ATP, and cAMP all promote release of tyrosine aminotransferase from rough microsomes, but there is no enzyme that can be released by these agents in the smooth microsomes.

Evidence for the presence of adenylyl cyclase in the rough microsomes was also found since this fraction released tyrosine aminotransferase after incubation with ATP in the usual system. The addition of smooth microsomes increased the rate of release which indicates the additional presence of adenylyl cyclase in this fraction since smooth microsomes are devoid of the release factor.

The results of adding all the known cofactors and substrates of protein synthesis to the tyrosine aminotransferase system are presented in Table II. In preparations of the deoxycholate polysomes, it is seen that the addition of the complete system for protein synthesis results in an increase in the enzymic activity which is about three times that obtained by incubation in cAMP alone. Pyridoxal phosphate is also required for the cascade effect, and the inhibitors of protein synthesis, cycloheximide and puromycin, reduce the yield of enzyme to the basal "release" level resulting from cAMP alone.

In microsomes, enzyme activity is increased sixfold under cascade conditions and the inhibitors of protein synthesis completely eliminate the increment of enzyme activity. cAMP at 1 mM concentration is just as effective in microsomes as at 5 mM; the effect of increasing the concentration of the cyclic nucleotide was determined to eliminate the possibility of incomplete enzyme release due to destruction of the nucleotide by cyclic phosphodiesterase. The deletion of GTP or the pH 5 fraction from cascade experiments reduced the enzyme yield to the release level. Deletion of either of the two most abundant amino acids in the enzyme from the amino acid mixture (leucine or alanine) had the same effect. Increase in the concentration of all the amino acids to 10 mg/ml did not increase the yield of enzyme.

The procedures for preparation of microsomes were also examined and the results of several experiments demonstrated

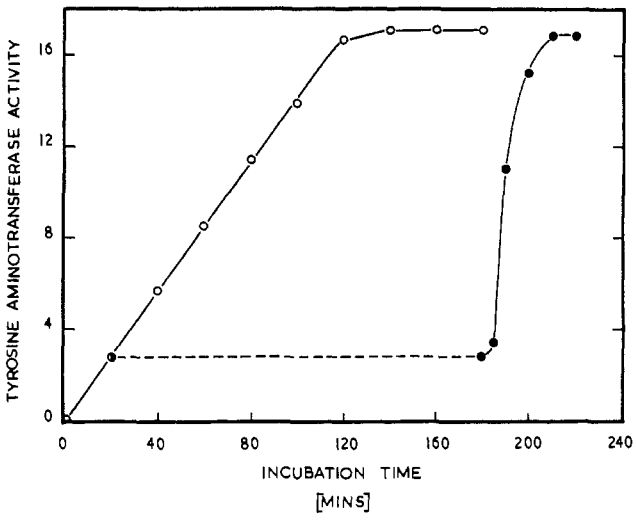

FIGURE 1: The effect of pyridoxal phosphate on cascade synthesis of tyrosine aminotransferase by liver microsomes *in vitro*. Liver microsomes were prepared from 2-day-old inducible rats. Aliquots (0.5 ml, 5.6 mg of protein) were incubated under cascade conditions (final volume of 1.0 ml with P-enolpyruvate and cAMP increased to 5 mM each) for various times at 37° before enzyme assay. In a second run, pyridoxal-5′-P was omitted from the incubation which was for 180 min. The incubate was chilled in ice and centrifuged at 150,000*g* for 42 min. The total microsomal-free supernatant was incubated with 1 mM pyridoxal-5′-P for various times at 37° before enzyme assay. The 180-min point in the second curve thus represents 0-min incubation with pyridoxal-5′-P. (○) Complete cascade system; (●) microsomal-free supernatant incubated with PALP.

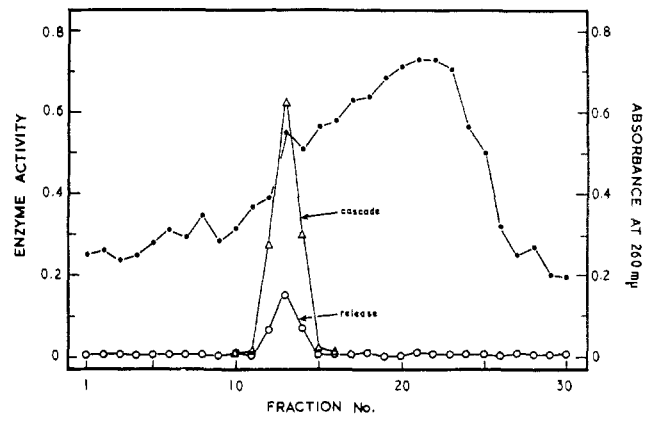

FIGURE 2: Zone centrifugation of the deoxycholate polysomes active in release and cascade. Polysomes were prepared by the deoxycholate procedure from 2-day-old inducible rats. The polysome pellets were gently resuspended in 0.2 M sucrose–2.7 mM $MgCl_2$–34 mM Tris-HCl (pH 7.6) with a stirring rod and 200-μl aliquots layered over 4.0 ml of a linear sucrose gradient (20–30%) prepared in 34 mM Tris-HCl–47 mM KCl–2.7 mM $MgCl_2$ (pH 7.6). Tubes were centrifuged at 2–4° for 45 min at 37,000 rpm in the SW 56 rotor. Fractions were collected by bottom puncture (8 drops each). Fractions from one tube were incubated under release conditions in the presence of the microsomal release factor while a parallel series were incubated under cascade conditions including also the microsomal release factor. Fractions from a triplicate tube were diluted with 0.1 ml of 0.1 M NaOH for determination of absorbance at 260 mμ. (○) Release, (△) cascade, and (●) 260-mμ absorbance.

the complete recovery of all microsomes active in the system under the conditions routinely used for their harvest.

Figure 1 shows an experiment on the kinetics of the system. When microsomes were incubated under cascade conditions in the presence of pyridoxal phosphate, enzyme activity accumulated to a maximum value in about 120 min. Microsomes were also incubated in cascade conditions in the absence of pyridoxal phosphate. After 180 min the incubates were centrifuged at 150,000*g* for 42 min to remove microsomes and the supernatants then reincubated with addition of pyridoxal phosphate. Enzyme activity reached the same level as in the previous run within 30-min incubation and the time course of activity increase was sigmoidal. Centrifugation for 120 min to ensure that all microsomes were removed did not alter this result. Inclusion of 3.5 mM cycloheximide also had no effect on the accumulation of enzyme activity in the microsomal supernatant.

In a further experiment using zone centrifugation it was shown that the molecular weight of the enzyme is the same whether the cofactor is present during or after cascade. Since the yield of enzyme is also the same in these two conditions, the effect of pyridoxal phosphate seems to be confined to activation of the apoenzyme.

Figure 2 presents typical results of a polysome-sizing experiment using zone centrifugation. Polysomes active in release and cascade were located by incubating each fraction under the usual conditions, followed by assay of tyrosine aminotransferase. The profile of polysomes active in the system is remarkably homogeneous and allows the ready calculation of the sedimentation coefficient (see Discussion).

Further experiments similar to those previously reported (Chuah and Oliver, 1971) were conducted with cognate cyclic nucleotides on the release of tyrosine aminotransferase from microsomal polysomes. Cyclic dAMP neither promotes release of enzyme nor inhibits its release at a concentration excess of tenfold over cAMP; and cyclic 2′,3′-AMP which was previously reported to be ineffective in release (Chuah and Oliver, 1971) is also without inhibitory effects.

Microsomal supernatants obtained by centrifugation after release of the enzyme by cAMP were analyzed for protein by gel electrophoresis in polyacrylamide gels. Supernatants obtained after incubation in TKM buffer in presence and absence of 1 mM cAMP were compared. As many as six protein bands appear in the cAMP incubates which are not present in the controls. This situation is similar using either TKM-washed or sucrose-washed microsomes as the starting materials.

Further experiments are necessary to determine whether these proteins are nascent proteins on polysomes or proteins leached from the microsomes. Leaching by cAMP appears unlikely as the ionic strength of the TKM buffer used in release experiments is already high and the incubation of microsomes in unbuffered sucrose yields only a small amount of soluble protein which is unchanged by addition of 1 mM cAMP to the incubation mixture.

Discussion

In previous papers from this laboratory, cAMP was implicated as an effector in the postnatal synthesis of tyrosine aminotransferase in rat liver. The results of experiments *in vivo* (Holt and Oliver, 1969a) and *in vitro* (Chuah *et al.*, 1971; Chuah and Oliver, 1971) were all consistent with this interpretation of the locus of action of cAMP. In the most recent published work (Chuah and Oliver, 1971) the effect was shown to be located at the polypeptide release step in enzyme synthesis. The intracellular location of the system was shown to be in the endoplasmic reticulum of liver cells and evidence was presented that the mechanism operates by release of the C-terminal polypeptide from polysomes attached to mem-

branes (microsomal polysomes). It was suggested that such polysomes are loaded with nascent enzymatic polypeptides at various stages of assembly. In addition, a microsomal factor which strongly binds cAMP was shown to be an essential component of the system.

The effect is confined to microsomes, but since ATP can be substituted for cAMP in the release system, it was suggested that ATP functions in the system through the formation of cAMP by adenylyl cyclase located in the membranous elements of the microsomes. This conclusion was strengthened by the fact that inclusion of cyclic phosphodiesterase (ATPase free) or the removal of membranes by deoxycholate treatment abolished the ATP effect (Chuah and Oliver, 1971). Further studies in the present paper on the subfractionation of microsomes shows that the fraction of microsomes which corresponds to the rough endoplasmic reticulum contains the preloaded nascent enzyme which can be released by cAMP. The results also indicate that the smooth membranous elements of the endoplasmic reticulum contain neither the nascent enzyme polypeptides nor the essential release factor, which can be extracted from liver microsomes (Table I). Experiments with ATP and puromycin confirm that the rough microsomes exclusively contain the pool of nascent enzyme. Experiments with ATP and the microsomal subfractions have also shown that adenylyl cyclase is present in rough microsomes. However, the smooth membrane fraction increases the rate of release of enzyme from the rough microsomes in the presence of ATP, which indicates that adenylyl cyclase is found in both the rough and smooth endoplasmic reticulum. It is quite possible that the smooth elements, as isolated here, are contaminated with plasma membrane fragments, but the data indicate that the ribosome-containing fractions of the endoplasmic reticulum also contain adenylyl cyclase. This fact raises interesting possibilities for the differential control of various cellular activities by cAMP due to regional location of the synthetic enzyme. The potential regulation of adenylyl cyclase activity in different sites by different agents might well elucidate, in a rational way, the bewildering array of biochemical processes which appear to be stimulated by cAMP. A previous report has demonstrated the occurrence of adenylyl cyclase in the endoplasmic reticulum of adrenal cortex cells (Satre *et al.*, 1971).

The recovery of the tyrosine aminotransferase system in liver fractions was also assessed and the method used for preparation of microsomes recovers all the activity, since a further fraction which might be expected to contain large or heavy microsomes has no activity. Previous studies (Chuah and Oliver, 1971) have shown the release system to be absent from the supernatant as usually prepared.

In the previous paper, the phenomenon of cascade was suggested (Chuah and Oliver, 1971). If the microsomes active in the release process contain polysomes preloaded with nascent enzymic peptides, the release of the C-terminal peptide should allow completion and release of the nascent peptides in the presence of the activated release factor, provided that the other necessary factors for protein synthesis are present, as they must be in the intact cell.

The results of Table II demonstrate the occurrence of this process *in vitro*. When the deoxycholate polysomes or microsomes are incubated in the cascade system, *i.e.*, in the presence of all the necessary factors for protein synthesis and the requirements for release of tyrosine aminotransferase, the amount of enzyme is considerably increased over the control. The control is incubated under release conditions in which only the C-terminal polypeptide should be released and previous studies with inhibitors have suggested that no peptide chain elongation occurs under these conditions (Chuah and Oliver, 1971). The cascade effect requires the presence of pyridoxal phosphate but this cofactor appears to be necessary for activation of the apoenzyme. The cascade effect is completely blocked by inclusion of either cycloheximide or puromycin or by the exclusion of GTP, pH 5 enyzmes, leucine, or alanine from the reaction mixture. These results indicate that peptide chain elongation is indeed taking place in the cascade conditions since cycloheximide prevents translocation on the polysome (McKeehan and Hardesty, 1969; Baliga *et al.*, 1970), and puromycin results in the abortive release of incomplete polypeptides (Allen and Zamecnick, 1962) which in the tyrosine aminotransferase system have no enzymatic activity (Chuah *et al.*, 1971; Chuah and Oliver, 1971). The deletion experiments confirm the results with the inhibitors.

The inherent cascade properties predicted for a protein synthetic system controlled at the release step was discussed previously in relation to other workers' data from various systems in which apparent induction of tyrosine aminotransferase synthesis occurs (Chuah and Oliver, 1971). It would seem unnecessary to repeat that discussion in the present paper. However, a recent paper of Butcher *et al.* (1971) in which tyrosine aminotransferase induction has been studied in a tissue culture cell line (H-35) derived from a Reuber hepatoma, deserves comment. The H-35 cell line has different properties to HTC cells as extensively studied in Tomkins' laboratory. One important difference is that the enzyme is inducible by dibutyryl cyclic adenosine monophosphate in H-35 cells (Butcher *et al.*, 1971), while this agent is ineffective in HTC cells (Granner *et al.*, 1968). Both cell lines seem to lack adenylyl cyclase.

In the work of Butcher *et al.* (1971) it is shown that the inductive effect of dibutyryl cyclic adenosine monophosphate is rather insensitive to actinomycin D, a fact which the authors interpret to mean a translational effect of the cyclic nucleotide. More importantly perhaps, cycloheximide appears to block only 85% of the enzyme induction, a figure which is predictable from the effects of cycloheximide on the rat liver *in vitro* system described in this paper (see Table II). Although the authors carried out only two determinations on the effect of cycloheximide, the duplicates were in very close agreement and the difference between the cycloheximide controls and the cycloheximide–dibutyryl cyclic adenosine monophosphate tests would probably be highly significant at the statistical level if sufficient data were available. The results of Wicks (1968, 1969) on cycloheximide effects in fetal liver explants in organ culture have previously been discussed in similar terms to the above (Chuah and Oliver, 1971). These interpretations do not apply to HTC cells, but in H-35 cells cAMP could exert its effect at the release step in enzyme synthesis and the subsequent cascade effect would then result in apparent *de novo* synthesis of enzyme.

The cascade yield of enzyme from the deoxycholate polysomes expressed as a ratio (cascade:release) is variable (2.8–5.9 in different experiments) while that from microsomes is consistently about 6.2. This might indicate that the method of preparation results in some damage to the deoxycholate polysomes while polysomes attached to microsomal membranes are less readily damaged by physical or enzymatic processes. However, in all sizing experiments with the deoxycholate polysomes, the sedimentation profiles were remarkably homogenous which argues against the breakage of polysomes by such factors as shearing forces. If chain initiation does not occur in the *in vitro* system, the maximum cascade yield can be used

as a rough measure of the size of the polysome which is preloaded with nascent enzyme. Thus, the polysomes isolated in the deoxycholate would contain three to six ribosomes, while the corresponding microsomal polysome would contain six ribosomes. Evidence is presented in Figure 1 that chain initiation does not take place in the *in vitro* system. The accumulation of enzyme activity occurs for about 120-min incubation and then ceases. The cascade yield is the same even when the concentration of cAMP, the energy-rich phosphate source (P-enolpyruvate) or the amino acid mixture are increased 5-, 2-, and 10-fold, respectively. Similar kinetic properties are found with the deoxycholate polysome preparations but the reaction is completed in 60–90 min. In the same figure, a further experiment bears on the mechanism of action of pyridoxal phosphate. When microsomes are incubated in cascade conditions but without pyridoxal phosphate, the enzyme yield reaches a maximum equal to that attained in the release condition only. Removal of microsomes by centrifugation of the incubates will remove the essential apparatus of protein synthesis, but the addition of pyridoxal phosphate to the supernatant then results in rapid accumulation of enzyme activity to the final cascade level. Cycloheximide has no effect on the yield; a result which verifies the complete removal of polysomes by the centrifugal procedure. The sigmoidal kinetics of this process suggest the interaction of the cofactor with apoenzyme to form the active enzyme, and further suggests the interaction of subunits to form the holoenzyme in polymeric form. The molecular weight of the enzyme is the same whether pyridoxal phosphate is included in the cascade mixture or added afterward.

The rough estimate of the maximum size of the tyrosine aminotransferase polysome as containing six ribosomes (above) indicates that the enzyme has four subunits since such a polysome could not code for an enzyme with a molecular weight of 110,000 (Valeriote *et al.*, 1969) but for only about one-quarter of this size. A tetrameric structure for the holoenzyme has been suggested previously (Valeriote *et al.*, 1969; Auricchio *et al.*, 1970).

Tryfiates (1969) has also reported a similar quantitative requirement for pyridoxal phosphate in a cell-free system from liver apparently capable of the synthesis of tyrosine aminotransferase which is probably very similar to the system described here (see also Chuah and Oliver, 1971). The question of endogenous cofactor binding to ribosome-bound but completed enzymic peptides cannot be answered at this stage since the assay medium always contains pyridoxal phosphate.

Further experiments to measure the size of the tyrosine aminotransferase polysome have also been performed and are summarized in Figure 2. The deoxycholate polysomes were prepared as gently as possible and zone centrifuged on sucrose concentration gradients. Fractions were incubated in both release and cascade conditions and enzyme activities subsequently determined. The cascade yield as discussed previously and the sedimentation rate give two measures of polysome size.

The sedimentation coefficient of polysomes containing the tyrosine aminotransferase system was calculated by the methods of McEwen (1967) and in two separate experiments the values 270 and 290 S were obtained assuming particle densities of 1.70. In the first experiment the cascade yield was 4.4 and in the second it was 5.9 (not shown).

The size of the polysome in terms of ribosome numbers can then be simply calculated by the equation of Martin and Ames (1961) relating s values to molecular weight and assuming a unit value for 80S monosomes. In such calculations the contribution of mRNA and nascent polypeptide can be ignored as they introduce an error of less than 5%. Such calculations yield a ribosome number of between 6 and 7 for the tyrosine aminotransferase polysome which is in good agreement with the figure derived from the maximum cascade yield.

Analysis of the proteins released by cAMP from liver microsomes in gel electrophoresis indicates that at least six new anionic bands are apparent in gels run from cAMP incubates compared with control gels run from supernatant protein obtained from microsomes incubated with TKM buffer only. Of the total protein released by cAMP, it can be calculated from the tyrosine aminotransferase activity and the turnover number of the enzyme that less than 1% is due to the enzyme. These results suggest that the release mechanism controlled by cAMP is involved in the synthesis of a range of liver proteins and is not restricted to the single enzyme, tyrosine aminotransferase. Since the gel analyses do not constitute definitive evidence for the controlled release of other proteins, further experiments are in progress to test this hypothesis. It should be noted that Khairallah and Pitot (1967) have previously presented evidence for a polypeptide release control in hepatic protein synthesis mediated by cAMP, and nearly 15 years ago Vogel (1957) suggested the operation of controls at the polypeptide release step in adaptive enzyme formation in bacteria.

The data of the present paper clearly show the existence of a specific controlled release step in tyrosine aminotransferase synthesis in higher cells which is cytoplasmic in location and is under the control of an intracellular effector molecule, cAMP. The concentration of the effector in turn is controlled by hormones. The mechanism appears to be of importance in the postnatal production of hepatic tyrosine aminotransferase and may be of more general significance in both cellular differentiation and the modulation of the level of gene products in differentiated cells.

The cytoplasmic mechanism appears to control the release of the ribosome-bound but completed enzyme subunits from the terminus of the synthetic polysome. This property leads to a cascade effect in which all other nascent, incomplete peptides have their synthesis completed and are in turn released at the polysome terminus. Expressed in current terminology, such a system qualifies as a translational control mechanism, since the presence or absence of the release effector molecule (cAMP) has effects on the translation process even though they be indirect.

Cytoplasmic controls over protein synthesis have been deduced to occur in eucaryote cells for a long time and to have an important role in cellular differentiation (see Harris, 1970, for review), but detailed knowledge of mechanisms has previously been lacking.

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Subpopulations of Human Serum Very Low Density Lipoproteins†

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ABSTRACT: Very low density human serum lipoproteins (VLDL, *d* 0.98–1.006 gm/cm³) have been found to be composed of at least four distinct subpopulations of molecules, which differ with respect to protein and polypeptide composition. Differential preparative ultracentrifugation, electrophoretic, and immunological techniques were used to detect and partially characterize the subpopulations. The composition of subpopulations (LDL = low density lipoproteins, polypeptides are designated by their C-terminal amino acids except R-x which has a blocked C-terminal amino acid) is: (1) LDL, R-Ala, R-Thr, R-Gln, R-x; (2) LDL,—, R-Thr, R-Gln, R-x; (3) LDL, R-Ala, —, —, R-x; (4) LDL, R-Ala, R-Thr, —, —. In addition, evidence is presented which indicates the existence of a subpopulation which does not react with anti-LDL antiserum.

Various physical and chemical data indicate that VLDL[1] as a class is heterogeneous. The heterogeneity of VLDL is exemplified by a broad S_f range of 20–5000 (Gofman *et al.*, 1954), particle size variations from 275 to 2592 Å (Gustafson *et al.*, 1965) and widely different ratios of lipid to protein (Rodbell, 1958).

In previous studies, it was shown that HDL-2 and HDL-3 are each composed of at least two subpopulations of molecules, one, approximately 90%, which contains both R-Gln and R-Thr, and the other, approximately 10%, which does not contain R-Gln but does contain R-Thr (Borut and Aladjem, 1971; Albers and Aladjem, 1971).

The present investigation was undertaken to ascertain whether subpopulations which differ with respect to polypeptide composition also occur among VLDL.

It was found that VLDL is composed of at least four subpopulations which differ with respect to protein and polypeptide composition, and of an additional subpopulation, which does not react with anti-LDL antiserum.

Materials and Methods

Isolation and Characterization of VLDL. Fresh pooled human serum was obtained from the blood bank. Each pool consisted of the serums of several hundred donors. VLDL was isolated from a given pool as previously described (Pearlstein *et al.*, 1971) except that the final dialysis against distilled water was omitted.

Hydrated density classes of VLDL were isolated by the method of Gustafson *et al.* (1965). The hydrated density classes were characterized by their S_f ranges as follows: B (S_f 400–5000), C (S_f 100–400), D (S_f 50–100), and E (S_f 20–50).

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[1] Abbreviations used are: VLDL, very low density lipoproteins (*d* 0.98–1.006 g/cm³); LDL, low density lipoproteins (*d* 1.025–1.050 g/cm³); HDL, high density lipoproteins (*d* 1.075–1.195 g/cm³); polypeptides are designated by their C-terminal amino acids as R-Thr, R-Gln, R-Val, R-Glu, and R-Ala except R-x which has a blocked C-terminal amino acid.