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Role of Adenosine Cyclic Monophosphate in the Synthesis of Tyrosine Aminotransferase in Neonatal Rat Liver. Release of Enzyme from Membrane-Bound Polysomes *in Vitro**

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ABSTRACT: Previous results suggested that the synthesis of at least one form of tyrosine aminotransferase was under the control of adenosine 3′,5′-cyclic monophosphate in neonatal rat liver *in vivo*. The locus of action of the cyclic nucleotide appeared to be at a posttranscriptional step in enzyme synthesis. In postnatal Wistar albino rats of 1- to 2-days old, epinephrine and puromycin injection results in elevation of hepatic tyrosine aminotransferase activity. Microsome fractions isolated from liver homogenates prepared from such rats show a threefold elevation of tyrosine aminotransferase activity when incubated *in vitro* with cAMP, puromycin or ATP, in the absence of GTP and a nucleotide triphosphate regenerator. No additives from the supernatant fraction are necessary for the effect. Other 3′,5′-cyclic nucleotides, 5′-AMP, and 2′,3′-cAMP are without effect but cIMP and GMP inhibit the elevation of activity mediated by cAMP. Zone centrifugal analysis of the cAMP-treated microsomes shows that enzyme is released in a form having the usual molecular weight. The effect is localized to the membrane-bound polysomes which can be washed free from all apparent enzyme activity. cAMP is then required to release the bound enzyme in an active form. The ATP effect is due to adenylylcyclase activity in the microsome fraction. A microsomal protein which strongly binds cAMP is essential for enzyme release. Results with cycloheximide indicate that the system releases only finished enzyme molecules (or subunits) and that polypeptide-chain elongation does not occur. The results are discussed in relation to regulatory mechanisms of protein synthesis in higher organisms and a terminator release control mechanism is suggested which may operate in the synthesis of a range of specific proteins.

In recent papers from this and other laboratories, 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 (for summary, see Holt and Oliver, 1971) but the evidence indicates that the effect is restricted to posttranscriptional events (Holt and Oliver, 1969a). Multiple forms of tyrosine aminotransferase in rat liver have been demonstrated by analysis in polyacrylamide gel electrophoresis (Holt and Oliver, 1969b, 1971; Sadleir *et al.*, 1970), and cAMP has its effect on the synthesis of only one of the forms *in vivo* (form C) (Holt and Oliver, 1969b).

Chuah *et al.* (1971) suggested the operation of a cAMP-dependent control at the termination or release step of tyrosine aminotransferase synthesis in neonatal rat liver and presented evidence for such a hypothesis. This evidence was largely based on an anomalous effect of puromycin *in vivo*, in that drug administration to intact animals under certain

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[1] Abbreviations used for nucleoside 3′,5′-cyclic monophosphates have the word cyclic preceding the conventional abbreviation for the 5′-nucleoside monophosphate. Adenosine 2′,3′-cyclic monophosphate (2′,3′-cAMP), N^6-2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dibutyryl-cAMP). Other nucleotides are abbreviated conventionally. Phosphoenolpyruvate (PEP), phosphoenolpyruvate kinase (PK), *p*-hydroxyphenylpyruvate (PHPP), reduced glutathione (GSH), sodium deoxycholate (DOC), rat hepatoma cell (HTC), tyrosine aminotransferase (TAT). 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).

conditions resulted in elevation of enzyme activity. The microsome–polysome fraction of liver homogenates isolated from such animals was shown to release active enzyme upon incubation with RNase *in vitro*. Thus the concept of polysomes preloaded with tyrosine aminotransferase polypeptide (or subunits) at various stages of assembly was postulated. Such polysomes, in the absence of a release signal, would be "frozen" in the preloaded condition. It was suggested that cAMP was involved in some way in the control mechanism of the release step.

This paper presents evidence for the operation of such a control mechanism by experiments *in vitro* and demonstrates an obligatory requirement for the cyclic nucleotide. Evidence is also presented for the involvement of a cAMP binding protein (or proteins) and for the location of the phenomenon in the membrane-bound polysomes of liver.

Methods

Animal Testing. Rats (1- to 2-days old) of the Wistar albino strain of *Rattus norwegicus* were used unless otherwise stated. From each litter, one animal was injected intraperitoneally with epinephrine (1.4 μg/g body weight) and another was injected with the same volume of saline. The remaining animals of the litter were killed, and liver 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 prepared in 0.25 M sucrose, and tyrosine aminotransferase activity assayed in the supernatant prepared by centrifugation at 25,000*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. Some animals show no increase in enzyme activity after hormone treatment and their litter mates are defined as noninducible. The stored homogenates were used in the *in vitro* experiments described below. In some experiments late-fetal animals were delivered by uterine section and maintained in a humidicrib at 37° (see Yeung and Oliver, 1967, for details). Three hours after delivery some animals of the litter received 750 μg of puromycin in saline by intraperitoneal injection. At 6 hr after delivery all animals were killed and the hepatic tyrosine aminotransferase activity measured in the puromycin-treated animals and compared to a control. The results of the enzyme assays allowed the recognition of inducible or noninducible animals by the same criteria as used for epinephrine treatment (see Chuah *et al.*, 1971).

Preparation of Liver Fractions. Livers from several animals from the same litter were pooled and homogenized 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.

Procedure 1. Liver homogenates were centrifuged at 25,000*g* for 10 min at 2° in rotor 295 of the PR-2 International refrigerated centrifuge. The supernatant was removed and centrifuged at 400,000*g* for 1 hr in the SW56 rotor of the Spinco L2-65 ultracentrifuge at 2–4°. The supernatant was removed and the pellet (microsomes and free polysomes) suspended in TKM buffer using a loose-fitting Teflon pestle machined for the centrifuge tubes. The volume of TKM buffer was usually one-half the original volume of supernatant used. This fraction is called the microsome–polysome fraction since it contains both free and membrane-bound polysomes.

For some experiments the pellet was washed by centrifugation and resuspension three times in TKM buffer or in 0.25 M sucrose. The final washed pellet was suspended in TKM buffer as above. When the wash procedure was used it is specified in the experimental protocol.

Procedure 2. Liver homogenates were prepared as described above and centrifuged at 15,000*g* for 10 min in the PR-2 centrifuge. The supernatant was removed and centrifuged at 105,000-*g* (hr) in the type 50 or type 65 rotor of the L2-65 ultracentrifuge at 2–4°. The supernatant was removed and the pellet washed three times with 0.25 M sucrose or TKM buffer or merely resuspended in TKM buffer as in procedure 1. This fraction is called microsomes.

Subfractionation of the Microsome–Polysome Fraction. Bound polysomes (microsomes) were separated from free polysomes using the discontinuous sucrose gradient described by Blobel and Potter (1967) with the following modifications. The cellulose tubes for rotor 65 of the L2-65 Spinco ultracentrifuge received 3 ml of 2 M sucrose and then 7 ml of 0.5 M sucrose. The 15,000*g* (10 min) supernatant (2 ml) prepared as in procedure 2 was layered on top. The tubes were centrifuged for 6 hr at 65,000 rpm in rotor 65 of the Spinco L2-65 ultracentrifuge. The uppermost 2 ml in the tube was carefully removed (supernatant) and most of the 0.5 M sucrose layer then discarded. The microsomes were harvested from the discontinuous region of the gradient, pooled, and homogenized. The suspension was diluted with cold 0.25 M sucrose and centrifuged at 105,000*g* (hr) in rotor 65. The pellet of microsomes was suspended in either TKM or TAM buffer as specified in individual experiments. The free polysome pellet was suspended in TKM or TAM buffer after removal of the 2.0 M sucrose layer.

A total polysome fraction derived from both microsomes and free polysomes was prepared by treatment with deoxycholate. Essentially the same centrifugal procedure was used to prepare this fraction except that the sucrose gradient contained TKM buffer (pH 8.0) and the 15,000*g* (10 min) supernatant was made to 1.2% sodium deoxycholate before layering and centrifugation. After centrifugation all the supernatant solution was discarded and the total polysome pellet resuspended in TKM or TAM buffer.

Preparation of Microsomal Protein Fraction. Microsomes from adult rat liver were prepared in 0.25 M sucrose buffered in 50 mM Tris-HCl–25 mM KCl–5 mM $MgCl_2$ (pH 7.4) by centrifugal procedure 2. They were washed with the same medium and suspended in 50 mM Tris-HCl–6 mM β-mercaptoethanol–0.5 M NH_4Cl (pH 7.4). After centrifugation of the suspension, the supernatant was fractionated with ammonium sulfate (25–55% saturation), and the pellet redissolved and dialyzed for 16 hr on a rotating dialyzer against 200 volumes of 50 mM Tris-HCl–6 mM β-mercaptoethanol (pH 7.4) at 4°.

This preparation will bind [^{3}H]cAMP in equilibrium dialysis with an approximate binding constant of 10^8 M. Further studies will be published elsewhere.

Experiments with Liver Fractions. In most experiments, 0.5 ml of the microsome or polysome suspension was incubated in a final volume of 1 ml of TKM buffer at 37°. Various additives which were used are given in the results. Reaction was always initiated by addition of the microsomal suspension.

The reaction was stopped by diluting 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 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

TABLE I: Effect of cAMP on Tyrosine Aminotransferase Activity of the Rat Liver Microsome–Polysome Fraction *in Vitro*.[a]

Addns to Medium	Increase in Enzyme Act. (Test Control), μmoles of PHPP Formed/hr	
	Inducible	Noninducible
Medium 1		
1 mM cAMP	0.91	−0.01
1 mM cAMP, 5 mM theophylline	1.83	0.00
0.1 mM cAMP, 5 mM theophylline	0.59	−0.01
0.01 mM cAMP, 5 mM theophylline	0.41	0.00
5 mM theophylline	0.00	−0.01
1 mM dibutyryl-cAMP	1.69	0.00
1 mM 5′-AMP	0.00	0.01
1 mM ATP, 0.25 mM GTP	0.91	0.00
1 mM cAMP, 1 mM ATP, 0.25 mM GTP, 5 mM theophylline	1.93	
1 mM ATP	1.87	
0.25 mM GTP	0.03	
1 mM ATP, 5 mM theophylline	1.97	
0.1 mM ATP, 5 mM theophylline	0.68	
0.01 mM ATP, 5 mM theophylline	0.42	
Medium 2		
1 mM cAMP, 5 mM theophylline	2.89	
1 mM cAMP, 5 mM theophylline, 5 mM PEP, 10 IU of PK	2.89	
5 mM PEP, 10 IU of PK	0.00	

[a] The liver microsome–polysome fraction (0.5 ml, 5.6 mg of protein) prepared from 2-day postnatal rats by procedure 1, was incubated at 37° for 60 min in a final volume of 1 ml in either medium 1 (TKM buffer + 5 mM GSH, 5 mM PEP, 10 IU of PK, pH 8.0) or medium 2 (TKM buffer, pH 8.0), and tyrosine aminotransferase activity subsequently determined. Various additions were made at the final concentrations given in the table. The preparation of the microsome–polysome fraction from inducible and noninducible animals and their identification is described under Methods. The activity figures were obtained by subtracting the control value from the value obtained after additions. Controls were incubated in the basic media without additions. Control activities ranged from 0.83 to 1.3 μmoles of PHPP per hr in different experiments and there was no change in activity during 60-min incubation.

TABLE II: Effect of Cyclic Nucleotides on Tyrosine Aminotransferase Activity of the Rat Liver Microsome–Polysome Fraction *in Vitro*.[a]

	Increase in Enzyme Act. (Test Control), μmoles of PHPP Formed/hr
Expt 1	
1 mM cAMP	1.98
1 mM cIMP	0.01
1 mM cUMP	0.01
1 mM cGMP	0.01
1 mM cdTMP	0.01
1 mM cCMP	0.01
1 mM dibutyryl-cAMP	1.98
1 mM 2′,3′-cAMP	0.01
Expt 2	
0.5 mM cAMP	1.76
0.5 mM cAMP + 5 mM cIMP	0.95
0.5 mM cAMP + 5 mM cUMP	1.76
0.5 mM cAMP + 5 mM cGMP	0.99
0.5 mM cAMP + 5 mM cdTMP	1.76
0.5 mM cAMP + 5 mM cCMP	1.76

[a] The liver microsome–polysome fraction (0.5 ml, 5.1 mg of protein), prepared from 2-day inducible postnatal rats by procedure 1, was incubated at 37° for 60 min in a final volume of 1 ml containing TKM buffer, 5 mM theophylline (pH 8.0), and the additives shown. Enzyme activities were subsequently determined. Control values were 1.28 in expt 1 and 0.50 in expt 2. Increase in activity was calculated as in Table I.

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 immediately iced. 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 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 the SW56 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 suitably diluted aliquots. Lyophilized crystalline bovine serum albumin was used as a standard.

Chemicals. ATP, CTP, cAMP, PEP, 5′-AMP, PHPP, cycloheximide, and sodium deoxycholate were obtained from Sigma Chemical Co. (St. Louis, Mo.). cUMP, cIMP, cGMP, cCMP, cdTMP, dibutyryl-cAMP, and 2′,3′-cAMP were obtained from Boehringer und Soehne (Mannheim, GmbH) or from Sigma. p*K* and cAMP phosphodiesterase (from beef heart) were from Boehringer und Soehne. Theophylline was obtained from Prosana Laboratories, Sydney, Australia. Puromycin was from Nutritional Biochemical Corp. (Cleveland, Ohio).

TABLE III: Effect of Temperature and pH on the cAMP-Potentiated Increase of Tyrosine Aminotransferase Activity in the Rat Liver Microsome–Polysome Fraction.[a]

Temp (°C)	Increase in Enzyme Act.	pH	Increase in Enzyme Act.
0	0.09	6.0	0.64
20	0.56	7.0	1.64
37	2.89	8.0	2.89
60	0.64	9.0	1.55
		10.0	0.57

[a] The liver microsome-polysome fraction (0.5 ml, 5.6 mg of protein) prepared from 2-day inducible postnatal rats by procedure 1 was incubated for 60 min in a final volume of 1 ml containing TKM buffer, 5 mM theophylline, 1 mM cAMP, and enzyme activities subsequently determined. In the optimum temperature experiment the pH was brought to 8.0 with HCl. In the pH optimum experiment the pH was adjusted to the final value shown with HCl. Endogenous enzyme activity was determined after 60-min incubation without cAMP at pH 8.0 and 37° and was subtracted from all other values to obtain the increase in enzyme activity (endogenous value 0.83 μmole of PHPP/hr).

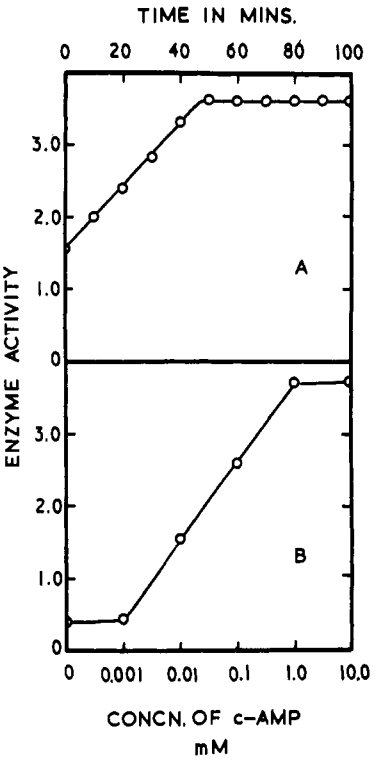

FIGURE 1: Effect of cAMP on tyrosine aminotransferase activity of the rat liver microsome–polysome fraction. The liver microsome–polysome fraction was prepared from 2-day postnatal rats by procedure 1 and incubated (0.5 ml, 5 mg of protein) in TKM buffer, 5 mM theophylline, and 1 mM cAMP for various times at 37° before enzyme assay (A). The liver microsome–polysome fraction was incubated at 37° for 60 min in TKM buffer, 5 mM theophylline, and various concentrations of cAMP (B). Tyrosine aminotransferase activity was assayed on 100-μl aliquots after incubation (micromoles of PHPP per hour).

Epinephrine tartrate was from Burroughs Wellcome and Co., Ltd. Sydney, Australia.

Results

In some experiments microsome–polysome fractions were prepared from 2-day-old rats and used without washing. There is accordingly some enzymatic activity occluded in the pellets but it is always low compared to the enzyme activity found in the 400,000*g* (hr) supernatant.

Table I shows that incubation of such a fraction with cAMP results in an increase in tyrosine aminotransferase activity of two- to threefold. Theophylline addition increases the effect although theophylline alone is ineffective. Dibutyryl-cAMP is more effective than the parent compound and the response to cAMP is concentration dependent. ATP can substitute for cAMP and the response is concentration dependent, but GTP, a triphosphate regenerator and GSH are all unnecessary for the effect. 5′-AMP is not effective.

It should be noted that the effect is obtained only in cell fractions isolated from epinephrine-inducible animals and is not obtained when the liver fractions are prepared from non-inducible animals. At the time of year the experiments were done (June–August), 60–80% of neonatal litters was inducible with epinephrine or puromycin (see also Chuah *et al.*, 1970).

In Figure 1A,B it is shown that the increase in tyrosine aminotransferase activity resulting from incubation with cAMP is complete under the conditions of the experiment in 40–50 min at 37° and that 1 mM cAMP produces a maximal response. As shown in Table II, cyclic nucleotides other than cAMP or its esterified derivative are not active in the system at 1 mM concentration and 2′,3′-cAMP is also without activity. There is no inhibitory effect of the cognate cyclic nucleotides at equimolar concentration with cAMP, but at a concentration ratio of 10:1, cIMP and cGMP inhibit the increase in enzyme activity about 50%. Puromycin is not inhibitory.

The stimulatory effect of cAMP on the enzyme activity is maximal at pH 8.0 and at 37°, and it is perhaps surprising that the loss of activity at 60° is not greater; the system retains 20–25% of its maximal activity at this temperature (Table III).

When the microsome–polysome fraction is washed with TKM buffer, the endogenous enzyme activity disappears and the cyclic AMP effect now fails to occur (Table IV). However, the use of 0.25 M sucrose as a wash medium results in retention of the cAMP effect although the endogenous enzyme activity is removed. This table presents evidence for the occlusion of enzyme in the unwashed pellets and provisional evidence for a release function of cAMP.

The addition of pyridoxal phosphate at a concentration of 1 mM did not alter the increase in tyrosine aminotransferase activity obtained by incubation of liver polysomes in the presence of 1 mM cAMP and 5 mM theophylline under the routine conditions (as in Tables I–V).

Figure 2 shows that the addition of the 400,000*g* (hr) supernatant of 2-day-old rat liver to the microsome–polysome preparation accelerates the cAMP potentiated increase in activity. The absolute increase in activity is always the same, but the time taken for completion is decreased by increases in the amount of supernatant added.

Table V shows that puromycin will in some respect substitute for cAMP in the system. The puromycin effect requires NH_4^+ and is not active in K^+. Washing the preparation in 0.25 M sucrose, TKM, or TAM buffer does not affect the ability of puromycin to bring about the increase in enzyme activity pro-

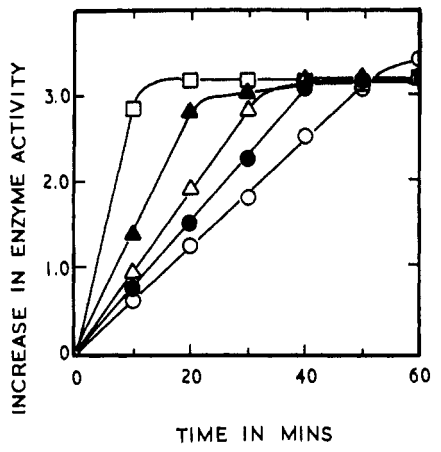

FIGURE 2: Effect of liver supernatant on the cAMP-potentiated tyrosine aminotransferase activity of the microsome–polysome fraction. The liver microsome–polysome fraction was prepared from 2-day inducible postnatal rats by procedure 1 and the postpolysomal supernatant retained. Microsome–polysome fraction (0.5 ml) (approximately 5 mg of protein) was incubated in a final volume of 1.0 ml of TKM buffer, 5 mM theophylline, 1 mM cAMP, and increasing amounts of the postpolysomal supernatant. Aliquots (100 μl) were assayed for tyrosine aminotransferase activity after various times of incubation. The curves are normalized by subtracting the zero time activity from each of the other values since the supernatant contains enzyme activity (micromoles of PHPP per hour). Volume of supernatant added: (○) none, (●) 25 μl, (△) 50 μl, (▲) 100 μl, and (□) 200 μl.

vided that NH_4^+ is supplied. GSH and the triphosphate generating system are not required for the puromycin effect and the quantitative effect is the same as that obtained with cAMP in the absence of GTP or ATP. As previously shown, the use of ionic wash media causes the cAMP effect to disappear but in sucrose-washed preparations, NH_4^+ or K^+ are effective.

TABLE IV: Effect of Washing on the Tyrosine Aminotransferase System of the Rat Liver Microsome–Polysome Fraction.[a]

Expt	Preparation	Additions	Enzyme Act. (μmoles of PHPP/hr)
1	Microsome–polysome fraction, unwashed	None	0.86
		1 mM cAMP	2.67
	Microsome–polysome fraction, washed with TKM buffer	None	0.00
		1 mM cAMP	0.00
2	Microsome–polysome fraction, unwashed	None	0.60
		1 mM cAMP	2.68
	Microsome–polysome fraction, washed with 0.25 M sucrose	None	0.00
		1 mM cAMP	2.14

[a] The liver microsome–polysome fraction was prepared from inducible 2-day postnatal rats by procedure 1 or washed three times with TKM buffer or 0.25 M sucrose. Aliquots (0.5 ml, 5.6 mg of protein) 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.

TABLE V: Effect of Puromycin on Tyrosine Aminotransferase Activity in Rat Liver Microsome–Polysome Fractions.[a]

Additions	Unwashed	Sucrose Washed	TKM Washed
	TKM Incubation Buffer		
None	0.33	0.22	0.00
+ 1 mM cAMP	2.76	2.42	0.00
+ 1 mM puromycin	0.33	0.22	0.00
+ 1 mM puromycin 275 mM NH_4Cl			2.44
+ 1 mM cAMP 275 mM NH_4Cl			0.00
			TAM Washed
	TAM Incubation Buffer		
None	0.33	0.19	0.00
+ 1 mM cAMP	2.81	2.43	0.00
+ 1 mM puromycin	2.79	2.42	2.42
Control (−GSH)	0.33	0.19	0.00
+ 1 mM puromycin	2.78	2.42	2.40
Control − GSH − PEP − PK	0.33	0.19	0.00
+1 mM puromycin	2.76	2.43	2.42

[a] The liver microsome–polysome fraction was prepared from inducible 2-day postnatal rats by procedure 1 and used without further treatment or after washing three times with 0.25 M sucrose or TKM buffer or TAM buffer. Aliquots of 0.5 ml (5.6 mg of protein) were incubated for 60 min at 37° in a final volume of 1 ml containing TKM buffer or TAM buffer and enzyme activity subsequently determined. Theophylline (5mM) was added to all incubations containing cAMP and to controls. Other additions are shown in the table. Enzyme activity in micromoles of PHPP per hour.

The subfractionation of liver polysomes into membrane-bound and free polysomes is presented in Table VI which shows that the effect of cAMP and ATP is confined to the bound polysomes. In this experiment, the subfractions were washed in unbuffered sucrose by their passage through the gradient and no enzyme activity was present either before or after incubation in the absence of cAMP. However, since the free polysomes may lose some required factor in such preparative procedures, some experiments also combined the supernatant with both fractions. The free polysome fraction showed no increase in tyrosine aminotransferase activity over that added in the supernatant after incubation with cAMP or ATP, while the bound polysomes showed the expected increase.

The effect of ATP on the unwashed microsome–polysome fraction of postnatal rat liver is completely abolished by the inclusion of cAMP phosphodiesterase in the incubation mixture (Table VII). ATPase activity was not detected in the phosphodiesterase preparation. In a second experiment the total polysomes of postnatal liver were prepared by treatment with sodium deoxycholate and passage through the discontinuous sucrose gradient in TKM buffer. This treatment removed a

TABLE VI: Fractionation of the Rat Liver Tyrosine Aminotransferase System.[a]

Liver Fraction	Enzyme Act. (μmoles of PHPP/hr) 1 mM cAMP	1 mM ATP
Bound polysomes (6.4 mg)	3.07	3.06
Free polysomes (3.6 mg)	0.00	0.00
Bound + free polysomes (0.5:0.5) (5.0 mg)	1.54	1.53
Bound + free polysomes (0.5:0.5) (5.0 mg) (no cAMP)	0.00	
Liver supernatant (100 μl)	5.85	5.85
Bound polysomes (6.4 mg) + supernatant (100 μl)	8.92	8.90
Free polysomes (3.6 mg) + supernatant (100 μl)	5.83	5.83

[a] Bound and free polysomes were prepared from inducible 2-day postnatal rats as described in Methods (Blobel and Potter, 1967). Each fraction (0.5 ml) was incubated in a final volume of 1 ml in TKM buffer–5 mM theophylline for 1 hr at 37° with the additions shown in the table. Enzyme activity was subsequently assayed on 100-μl aliquots of the incubate.

TABLE VII: Nature of ATP Effect in Microsomal Tyrosine Aminotransferase System.[a]

Expt	Additions	Enzyme Act. (μmoles of PHPP/hr)
1	None	0.50
	1 mM ATP	2.74
	1 mM ATP, cAMP phosphodiesterase (boiled)	2.74
	1 mM ATP, cAMP phosphodiesterase (0.7 IU, 2.5 mg)	0.50
2 (Deoxycholate treatment)		
	None	0.00
	1 mM cAMP	0.00
	1 mM cAMP + microsome extract (100 μl, 0.66 mg of protein)	2.18
	Microsome extract (100 μl)	0.00
	1 mM ATP	0.00
	1 mM ATP + microsome extract (100 μl)	0.00

[a] In expt 1 the liver microsome–polysome fraction (0.5 ml, 5 mg of protein) was prepared by procedure 1, and incubated for 1 hr at 37° in TKM buffer–5 mM theophylline with the additions shown. Enzyme activity was subsequently determined. In expt 2, the total polysome fraction from liver of 2-day-old inducible rats was isolated on a discontinuous sucrose gradient with deoxycholate as described in Methods. The resuspended polysomes (0.5 ml, 6.3 mg) were incubated at a final volume of 1 ml in TKM buffer–5 mM theophylline, for 1 hr at 37° with the additions shown. The preparation of the microsomal extract is outlined in Methods. Enzyme activity was assayed in both experiments on 100-μl aliquots of the incubates.

factor required for the activity of cAMP, but an active fraction can be obtained by extraction of sucrose-washed microsomes from adult liver with 0.5 M NH_4Cl. Addition of this factor to the deoxycholate polysomes restores the cAMP effect, but ATP is still ineffective in the system.

In Table VIII, puromycin is seen to promote release of enzyme from liver fractions which also respond to cAMP. However, the polysomes prepared by deoxycholate treatment do not require the addition of the microsomal fraction to respond to puromycin.

Table IX shows that both the increased activity of tyrosine aminotransferase that results from incubation of the unwashed liver microsome–polysome fraction and the endogenous activity, cannot be sedimented under the centrifugal conditions used to harvest the original particulate preparation. In addition, incubation of the 400,000*g* (hr) supernatant of postnatal rat liver with cAMP produces no increase in activity.

Figure 3 shows that the enzyme obtained in the supernatant after incubation of rat liver microsomes with cAMP, has the same zone sedimentation behavior as the enzyme obtained from liver supernatants of epinephrine-induced animals after centrifugation at 400,000*g* (hr).

Figure 4 shows a complete sedimentation profile of the microsomal incubation mixture after treatment with cAMP. The absorbancy profile at 260 mμ allows location of the microsome zone at the discontinuity in the sucrose gradient and it can be seen that the protein profile (at 290 mμ) follows this closely. Near the zone of application the 260-mμ profile shows evidence of free cAMP and/or theophylline, and there is suggestive evidence that some 260-mμ-absorbing material has become protein bound, since the profile shows a shoulder in advance of the application zone. Subbands of microsomal material are clearly seen; these are visible to the naked eye in routine preparations of microsomes by the discontinuous gradient technique.

Tyrosine aminotransferase activity is clearly seen only in an intermediate position in the gradient and there is no activity in the control preparation incubated without cAMP. The position of the enzyme activity band corresponds to a molecular weight of approximately 100,000. There is no activity in the microsome region, indicating clearly that under maximal conditions, incubation with cAMP releases all enzyme activity in a form which has sedimentation behavior identical with the supernatant enzyme.

The results of Table X suggest that a protein which binds cAMP is required in the enzyme release system. After addition of 1 mM cAMP to the microsomal extracts, dialysis against 100 volumes of buffer was performed twice. Thus if no binding took place the concentration of cAMP could not exceed 10^{-7} M in the microsome extract. A tenfold dilution of the extract was then made on addition to the microsome incubation. Thus the final concentration of free cAMP could not exceed 10^{-8} M. At 10^{-6} cAMP, no response is obtained in the microsome–polysome system (see Figure 1B). The microsomal extract treated with cAMP and then dialyzed is effective in

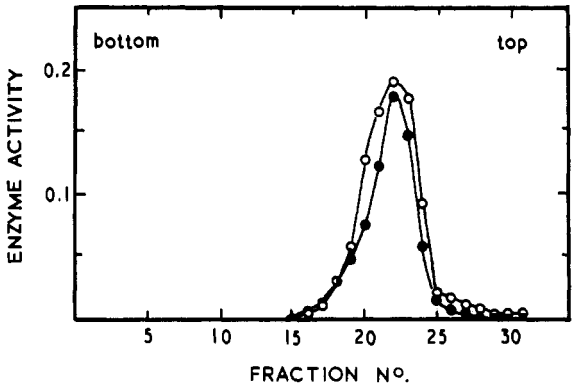

FIGURE 3: Zone centrifugation of tyrosine aminotransferase released from the rat liver microsome–polysome fraction by cAMP *in vitro*. The liver microsome–polysome fraction was prepared from 2-day inducible postnatal rats by procedure 1 and washed three times with 0.25 M sucrose. An aliquot (0.5 ml, 10 mg of protein) was incubated in TKM buffer, 5 mM theophylline, and 1 mM cAMP for 1 hr at 37°. The incubate was then centrifuged at 400,000*g* (hr) and the supernatant collected. This supernatant (100 μl) was layered on 4.2 ml of a linear sucrose concentration gradient (20–30% sucrose) in potassium phosphate (5 mM)–EDTA (5 mM) (pH 7.4). The tubes were centrifuged for 12 hr at 2–4° in the SW56 rotor of the Spinco L2-65 ultracentrifuge at 56,000 rpm. A preparation of tyrosine aminotransferase from the 400,000*g* (hr) supernatant from epinephrine-induced 2-day rat liver was also zone centrifuged in the same way. Fractions were collected through the tube bottom (8 drops each) and incubated for enzyme assay for 1 hr (micromoles of PHPP per hour). (●) Enzyme from microsomal incubate; (○) enzyme from epinephrine induction. Points were plotted only for tubes which showed enzyme activity.

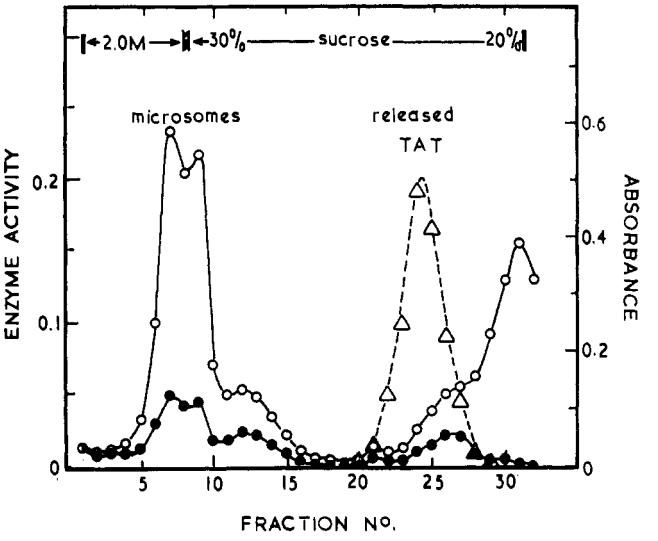

FIGURE 4: Release of tyrosine aminotransferase from rat liver microsomes by cAMP *in vitro*. Liver microsomes were prepared by procedure 2 from 2-day inducible rats and washed three times with 0.25 M sucrose. An aliquot of 0.5 ml (10 mg of protein) was incubated in a final volume of 1 ml in TKM buffer, 5 mM theophylline, and in the presence or absence of 1 mM AMP at 37° for 60 min. Sucrose concentration gradients consisted of 1.2 ml of 2.0 M sucrose followed by a linear gradient (3 ml) of 30–20% sucrose. Aliquots (100 μl) of the incubates above were layered on the top of the gradients and the tubes centrifuged in the SW56 rotor of the Spinco L2-65 ultracentrifuge for 12 hr at 2–4°. Fractions were collected by puncturing the bottom of the tubes (8 drops each) and assayed from duplicate tubes for enzyme activity (△) (micromoles of PHPP per hour) (60-min incubation), or after dilution with 1.0 ml of 0.1 N KOH, for absorbance at 260 mμ (○) and at 290 mμ (●). Points for tyrosine aminotransferase were plotted only for tubes which showed activity. No activity was found after incubation in the absence of cAMP. The absorbance profiles are for the preparation incubated with cAMP.

the release function and the response is not increased by addition of free cAMP. On the other hand, no release is obtained with the untreated, dialyzed microsomal protein unless cAMP is added to the incubation mixture.

TABLE VIII: Effect of Puromycin and cAMP on Tyrosine Aminotransferase Activity in Various Fractions of Rat Liver.[a]

Liver Fraction	Enzyme Act. (μmoles of PHPP/hr)		
	Control (−c-AMP)	1 mM cAMP	1 mM Puromycin
Microsomes (3.8 mg)	0.0	2.13	2.15
Free polysomes (3.6 mg)	0.0	0.0	0.0
Total DOC Polysomes (3.6 mg)	0.0	0.0	2.10
Total DOC polysomes + microsomal extract (0.44 mg)	0.0	2.16	2.16
Microsomal extract (0.44 mg)	0.0	0.0	0.0

[a] One-half of a pooled liver homogenate from 2-day-old inducible rats was fractionated as in procedure 2 to yield the microsome and free polysome fractions and the other half treated with DOC and fractionated to yield the total DOC polysome fraction. All fractions were incubated in a final volume of 1 ml in TAM buffer–5 mM theophylline for 1 hr at 37° with the additions shown. Enzyme activity was assayed on 100-μl aliquots before and after the incubation and the zero-time value subtracted where necessary.

In Table XI it is seen that cycloheximide has no inhibitory effect on the release of tyrosine aminotransferase from washed rat liver microsomes that is dependent on the addition of either puromycin or cAMP. The presence of GTP does not alter this result and cycloheximide alone is not effective in releasing the enzyme.

The results summarized in Table XII show that cAMP-potentiated release of the enzyme can be elicited in rat liver preparations from very young postnatal animals provided that the criterion of puromycin (or epinephrine) inducibility *in vivo* is satisfied. No responses have yet been obtained in preparations from fetal or adult animals but the experiment has been done only six and four times, respectively.

The second experiment in Table XII summarizes the results of a search for the occurrence of the cAMP-dependent release factor in rat liver at different stages of development. The release factor is obviously present in fetal, postnatal, and adult liver although the microsome–polysome fraction from fetal and adult liver did not release tyrosine aminotransferase activity *in vitro*.

Discussion

A general theory for the modulation of specific gene expression in higher organisms has recently been formulated by Tomkins *et al.* (1969). The experiments on which the theory is based have described the induction of tyrosine aminotransferase synthesis by glucocorticoids in HTC cells, a line of rat

TABLE IX: Release of Tyrosine Aminotransferase from the Rat Liver Microsome–Polysome Fraction by cAMP.[a]

Additions	Fraction Analyzed	Enyzme Act. (μmoles of PHPP/hr)
None	Microsome–polysome (0 min)	0.60
	Microsome–polysome (60 min)	0.60
	Supernatant	0.58
	Pellet	0.00
1 mM cAMP	Microsome–polysome	3.67
	Supernatant	3.44
	Pellet	0.00
None	Postpolysomal supernatant	3.78
1 mM cAMP	Postpolysomal supernatant	3.78

[a] The liver microsome–polysome fraction was prepared by procedure 1 from inducible 2-day postnatal rats and the postpolysomal supernatant retained. The microsome–polysome suspension (5 mg of protein; 0.5 ml) was incubated at 37° for 60 min in TKM buffer–5 mM theophylline with and without the addition of cAMP. The incubates were chilled and centrifuged at 400,000*g* (hr). The supernatant was removed for assay of tyrosine aminotransferase and the pellet washed three times by resuspension and centrifugation in TKM buffer. The washed pellet was resuspended in TKM buffer and assayed for enzyme activity. The postpolysomal supernatant was incubated in TKM buffer for 60 min at 37° in presence and absence of cAMP and assayed for enzyme activity.

TABLE X: Release of Tyrosine Aminotransferase from Rat Liver Microsomes by a cAMP Binding Protein.[a]

Expt	Additions	Enzyme Act. (μmoles of PHPP/hr)
1	None	0.00
	Microsomal protein (prebound)[b] (100 μl, 0.66 mg)	2.63
	Microsomal protein (prebound) (100 μl, 0.66 mg) + 1 mM cAMP	2.64
2	Microsomal protein[c] (100 μl, 0.69 mg)	0.00
	Microsomal protein (100 μl, 0.69 mg) 1 + mM cAMP	2.64

[a] Rat liver microsomes were prepared by procedure 2 from inducible 2-day postnatal animals, washed in TKM buffer three times, and suspended in TKM buffer. Microsomes (0.5 ml, 5.9 mg of protein) were incubated at 37° for 15 min in a final volume of 1.0 ml of TKM buffer–5 mM theophylline in presence of the additives shown, and enzyme activity subsequently assayed. [b] The microsomal protein solution (see methods for preparation) was made 1 mM in cAMP and then dialyzed against two changes of 100 volumes of TKM buffer–5 mM theophylline, for a period of 8 hr each on a rotating dialyzer at 4° (prebound). [c] The microsomal protein solution was dialyzed as above but without addition of cAMP.

hepatoma cells cultured *in vitro*. Paradoxical effects of actinomycin D on enzyme levels preinduced by steroids together with other properties of the system (see Tomkins *et al.*, 1969, for review) have led the authors to suggest the existence of a repressor acting at a posttranscriptional level, perhaps by binding to enzyme-specific mRNA preventing its translation. The repressor thus controls the *initial* step of polypeptide assembly and is deduced to be inactivated by interaction with the inducing glucocorticoid. The results of more recent experiments with the same cell line using insulin and bovine serum have also been interpreted in terms of posttranscriptional control phenomena (Gelehrter and Tomkins, 1970) although the mechanisms are probably different. Post transcriptional control of steroid-induced tyrosine aminotransferase synthesis in adult rat liver has also been suggested on the basis of effects of azaguanine (Levitan and Webb, 1970).

The data reported in this paper offer confirmation of previous suggestions (Holt and Oliver, 1969a; Chuah *et al.*, 1971) for the operation of a different posttranscriptional control mechanism which is mediated by 3′,5′-cAMP, and is located at the *terminal* point in enzyme synthesis, the release of enzyme (or polypeptide subunits) from the polysome. The report supports previous work of Khairallah and Pitot (1967) in which 3′,5′-cAMP was shown to promote release of radioactively labeled polypeptides from polysomes in a cell-free system. Such a system is apparently not required for the steroid induction of tyrosine aminotransferase in HTC cells since it has been reported that such cells contain neither adenylylcyclase nor cAMP, and that dibutyryl-cAMP was ineffective as an inducer (Granner *et al.*, 1968). These facts alone, however, suggest that HTC cells are markedly deviant from normal rat liver cells.

TABLE XI: Effect of Cycloheximide on cAMP-Dependent Release of Tyrosine Aminotransferase from Neonatal Rat Liver Microsomes.[a]

Additions	Enzyme Act. (μmoles of PHPP/hr)
None	0.05
1 mM cAMP	2.81
1 mM cAMP − GTP	2.79
1 mM puromycin	2.79
1 mM puromycin − GTP	2.79
3.5 mM cycloheximide	0.05
3.5 mM cycloheximide + 1 mM cAMP	2.78
3.5 mM cycloheximide + 1 mM puromycin	2.78

[a] Liver microsomes were prepared from inducible 2-day rats by procedure 2 and washed three times with 0.25 M sucrose. Microsomes (0.5 ml, 5.7 mg of protein) were incubated in a final volume of 1 ml of TAM buffer, 5 mM theophylline, 5 mM GSH, 5 mM PEP, 10 IU of PK (pH 8.0), and 0.25 mM GTP. Incubation was at 37° for 60 min, and enzyme activity was then measured.

TABLE XII: Release of Tyrosine Aminotransferase from Liver Microsomes by cAMP as a Function of Age.[a]

Expt	Additions	Enzyme Act. Released from Microsomes (μmoles of PHPP/hr)			
			6-hr Postnatal		
		Fetus	Puromycin Inducible	Non-inducible	Adult
1 Sucrose-washed microsomes	1 mM cAMP	0.00	1.72	0.00	0.00
		Fetus	2-Day Postnatal		Adult
2 TMK-washed microsomes	1 mM cAMP	0.00	0.00		0.00
	1 mM cAMP + fetal liver supernatant	0.00	2.58		0.00
	1 mM cAMP + 2-day liver supernatant	0.00	2.60		0.00
	1 mM cAMP + adult liver supernatant	0.00	2.60		0.00

[a] Liver microsomes were prepared from rats of various ages by procedure 2 and washed three times either in 0.25 M sucrose or TKM buffer. Postpolysomal supernatants were also prepared from each donor animal. Microsomes (0.5 ml, 5.0 mg of protein) were incubated for 60 min at 37° in TKM buffer–5 mM theophylline with the additions shown and tyrosine aminotransferase activity assayed after 0- and 60-min incubation. Controls were incubated with no additions and subtracted from the other values to calculate the enzyme activity released. The addition of 2-day and adult liver supernatants produced control values as high as 9.5 but such values did not change on incubation in the absence of the required release factors.

In neonatal rat liver, a posttranscriptional control in the synthesis of tyrosine aminotransferase was first suggested by Holt and Oliver (1969a) since during postnatal *de novo* synthesis of the enzyme, actinomycin D fails to prevent the elevation of enzyme activity that results from injection of epinephrine or 3′,5′-cAMP in the intact animal. Secondly, Chuah *et al.* (1970) showed that the liver of neonatal animals in which enzyme activity was inducible with epinephrine or puromycin, yielded a microsome–polysome fraction which released tyrosine aminotransferase upon incubation with high concentrations of RNase *in vitro*. The same liver fraction isolated from noninducible animals or from animals previously treated with puromycin or epinephrine failed to release enzyme. A control effect of cAMP at translation on the polysome has also been suggested to occur in the synthesis of tryptophanase in *Escherichia coli* (Pastan and Perlman, 1969).

In experiments reported in this paper, preparations of the unwashed microsome–polysome fraction prepared from the liver of 2-day inducible rats showed a two- to threefold elevation in tyrosine aminotransferase activity following incubation *in vitro* with cAMP or ATP, but neither effect required GTP or a nucleotide triphosphate generating system. Theophylline potentiated the effect presumably due to inhibition of cAMP phosphodiesterase activity. It is important to note that no effect was obtained in the same liver fraction prepared from noninducible animals of the same age (Table I). No other cyclic nucleotides were effective; 5′-AMP and 2′,3′-cAMP were without effect but dibutyryl-3′,5′-cAMP was more effective than the unsubstituted compound in the absence of theophylline. cIMP and GMP clearly showed antagonistic effects to cAMP when present at a tenfold concentration excess (Tables I and II) but did not replicate the cAMP effect. Thus even in this minimally fractionated system the effect was highly specific to cAMP.

The cAMP effect is dependent on concentration and time of incubation (Figure 1) but some effect is still obtained at 10^{-5} M cAMP which is close to physiological concentrations in liver tissue *in vivo* (*e.g.*, Turtle and Kipnis, 1967).

The pH optimum of the effect is similar to that reported by Khairallah and Pitot (1967) for the release of polysome-bound protein mediated by cAMP, and it is temperature dependent (Table III).

The microsome–polysome fraction can be washed free from detectable enzyme activity with either TKM buffer or unbuffered sucrose but in the latter system, enzyme activity reappears after incubation with cAMP, while TKM buffer removes the response capacity (Table IV). This finding indicates the necessity for a factor which is readily removed from the liver fraction by ionic media and Figure 2 shows that such a factor can be found in the postmicrosomal supernatant. Addition of increasing amounts of supernatant, even to the unwashed microsome–polysome fraction results in an increased rate of response to cAMP but the increment of enzyme activity always remains the same.

Under certain conditions described by Chuah *et al.* (1970), puromycin administration to intact neonatal rats results in increased activity of tyrosine aminotransferase in the liver. Incubation of the liver microsome–polysome fraction with puromycin also results in elevation of enzyme activity and the effect is stable to washing either with sucrose or buffers. Ammonium ion is required for the puromycin effect on enzyme activity and potassium will not substitute. This is in accord with other data on the ionic requirements for puromycin release of polypeptides from polysomes *in vitro* (Moldave *et al.*, 1968). The cAMP effect can be elicited in either of the ionic media (Table V).

The experiments so far discussed were all carried out with preparations from liver similar to those originally described

by Chuah *et al.* (1971), in which enzyme activity could be increased *in vitro* by incubation with RNase. No attempts at fractionation were made until both the effect of cAMP and its specificity were established.

The subfractionation of liver polysomes into free and membrane-bound forms shows that the effect of cAMP is confined to the bound polysomes (microsomes). Mixing experiments and addition of liver supernatant to each fraction, reveals that no essential factors have been lost by the fractionation procedure and confirm that the effect is localized in elements of the endoplasmic reticulum of liver cells (Table VI). Since the effect is so confined, the fact that ATP can replace cAMP in the unwashed polysome–microsome fraction (Table I), suggested that ATP functions in the system through the formation of cAMP by adenylylcyclase located in the membranous microsome fraction. Inclusion of purified cAMP phosphodiesterase in the incubation mixture abolishes the ATP effect and the removal of membranous elements from microsomes by deoxycholate treatment has the same effect (Table VII). The use of TKM buffer in the deoxycholate experiment also removes the factor required for the cAMP effect, and this factor was thus replaced by addition of a microsomal extract. These experiments show that the effect of ATP is in fact due to adenylylcyclase activity in the microsome fraction but further experiments will be needed to localize the enzyme to the smooth or rough surfaced elements of the endoplasmic reticulum. The effects of puromycin and cAMP *in vitro* suggest that there is a common pool of tyrosine aminotransferase located in the membrane-bound polysomes and this is confirmed by the results in Table VIII. Puromycin and cAMP both promote increase in enzyme activity in both the microsome and DOC–polysome fractions but not in free polysomes. Both agents have no effect on the microsomal extract which is not required for the puromycin effect on DOC–polysomes. This finding is as predicted from the stability of the puromycin effect to washing of microsomes.

The results so far discussed could be explained in several ways. (1) cAMP together with an extractable microsomal factor or supernatant factor activates tyrosine aminotransferase which is bound to microsomes in a nonextractable, inactive form. (2) cAMP and another factor promote release of nascent tyrosine aminotransferase from "preloaded" polysomes as previously suggested by Chuah *et al.* (1971). (3) cAMP and the same factor promote translation of enzyme-specific polysomes *in vitro* and the release of the finished enzyme, or the translation process only, with release under the control of a factor not requiring cAMP.

The latter possibility appears remote since the increase in enzyme activity requires neither GTP, ATP, or an ATP regenerator, and can be elicited in washed microsomes which should be free from amino acids, tRNAs, and the aminoacyl synthetases and thus incapable of polypeptide chain elongation.

The second alternative appears to be the correct interpretation from the following facts. (1) The enzyme activity that results from incubation of the unwashed microsome–polysome fraction with cAMP cannot be sedimented under the centrifugal conditions used to harvest the original fraction (Table IX) and the sedimentation profile of such activity is identical with that of the enzyme from the original high-speed supernatant (Figure 3). (2) Direct zone sedimentation of the cAMP-treated microsomal suspension in a discontinuous sucrose gradient locates the microsomes at the discontinuity, while the enzyme activity sediments independently of the microsomes, in the approximate position expected in these gradients for a protein of molecular weight approximately 100,000 (Figure 4). These results indicate the release of enzyme in an active form from preloaded polysomes. (3) Cycloheximide has no effect on the yield of enzyme activity resulting from incubation of microsomes with cAMP or puromycin, even when GTP and a regenerator are present (Table XI). Cycloheximide has recently been shown to inhibit translocation of peptidyl-tRNA from the aminoacyl site to the peptidyl site on the ribosome (McKeehan and Hardesty, 1969; Baliga *et al.*, 1970). Thus its failure to reduce the yield of released TAT is further evidence against the occurrence of polypeptide chain elongation in this system. (4) Incubation of enzyme found in the high-speed supernatant of neonatal liver with cAMP results in no increase in activity (Table IX). These results do not completely eliminate the possibility of enzyme activation by cAMP, since the supernatant enzyme may be already fully activated, but such an activation would be an additional function of the nucleotide, since release of enzyme is demonstrated in the experiments described under points 1 and 2 above.

The characteristics of the system so far described also indicate that only completed enzyme molecules have activity when they are released either by cAMP or puromycin (see also Chuah *et al.*, 1971).

Extracts of adult rat liver microsomes contain a protein species which strongly binds [^{3}H]cAMP and retains the label even after exhaustive dialysis. The binding constant determined by equilibrium dialysis is about 10^8 M. In zone sedimentation, this species can be monitored by the radioactivity bound to it and has a molecular weight of about 40,000. A small amount of the protein is also present in the supernatant fraction (G. R. Donovan and I. T. Oliver, unpublished experiments). Gill and Garren (1969) previously reported evidence for a cAMP binding protein in adrenal cortical microsomes.

When the microsomal extract is mixed with 1 mM cAMP and then dialyzed exhaustively, the extract promotes release of tyrosine aminotransferase from TKM-washed microsomes and the addition of 1 mM cAMP to the system is without effect. The extract without pretreatment is inactive in the absence of cAMP (Table X). Although it is possible that the preincubation of the microsomal factor with cAMP converts it irreversibly into an active form without binding, these findings constitute circumstantial evidence that a cAMP binding protein of the microsomes is required for the release of tyrosine aminotransferase from the preloaded polysome. Definite proof of this suggestion must await further purification of the factor.

Preloaded polysomes were not detected in either fetal or adult rat liver using the techniques described in this paper but in the 6-hr postnatal animals delivered by uterine section, preloaded polysomes were detected (Table XII). As previously reported (Chuah *et al.*, 1971) anomalous inductive effects of puromycin can be elicited in newborn animals soon after tyrosine aminotransferase synthesis has been initiated and in the experiments reported, liver microsomes prepared from puromycin-inducible animals released enzyme when incubated in cAMP *in vitro*, while those from a noninducible group of animals did not. These results, however, might be accounted for by absence of the release factor from either fetal or adult liver. Accordingly in the second experiment, TKM-washed microsomes from inducible 2-day-old animals were used to assay for the factor. The data show clearly that the release factor is present in fetal, 2-day postnatal, and adult animals. The delay in the synthesis of tyrosine aminotransferase in rat liver until the immediate postbirth period (Sereni *et al.*, 1959; Holt and Oliver, 1968) is thus not due to absence

or to postnatal synthesis of the release factor, but is due to absence of the initial inductive signal or signals for enzyme synthesis until after birth.

Tyrosine aminotransferase of rat liver has been shown to occur in multiple forms, and evidence has been presented that each form may be under different inductive control (Holt and Oliver, 1969b). Only one form (C) appears to be under the control of cAMP *in vivo* and this form is the first to appear postnatally (Holt and Oliver, 1971).

Some of the results presented in this paper were in a sense, anticipated by Tryfiates and Litwack (1964), who showed an increment of tyrosine aminotransferase activity in a cell-free system from liver following incubation with ATP and cAMP *in vitro*. The cell-free liver fraction was the supernatant after centrifugation at 15,000*g* for 15 min and would thus contain microsomes together with the soluble enzyme. The small increments in activity that were observed after incubation were almost certainly due to the release of enzyme from preloaded polysomes that has been described here. Puromycin was not inhibitory (see also Tables II and V, this paper) and although addition of cAMP gave only a small increment over the ATP level, this effect could be expected from a system without theophylline. Tryfiates (1969) also reported data on tyrosine aminotransferase synthesis *in vitro* using a cell-free protein-synthesizing system from rat liver. These data may be best interpreted in terms of partial completion of synthesis of the enzyme on preloaded polysomes since puromycin and cycloheximide both were inhibitory.

The existence of a control system operating at the terminus of enzyme synthesis raises several specific issues of significance for the synthesis of tyrosine aminotransferase in rat liver and more general problems for the control of protein synthesis in eucaryotes. Firstly there is the question of intracellular logistics. Using earlier data of our own on RNA and ribosome concentrations in neonatal rat liver (Oliver *et al.*, 1963) together with more recent data of Blobel and Potter (1967) on adult liver to refine the accuracy of the calculations, the total number of ribosomes in an average neonatal parenchymal cell can be calculated at about 3×10^6. The purification of tyrosine aminotransferase of rat liver by Valeriote *et al.* (1969) to a state of near homogeneity enables the calculation of a turnover number for the enzyme. The experiments on release of enzyme in this paper allows the calculation of the maximum number of enzyme molecules released per liver cell (about 15,000) and assuming a mean polysome size of 10 ribosomes, the number of polysomes preloaded with enzyme can be calculated. Assuming that only one tyrosine aminotransferase molecule is released from each polysome by cAMP in the *in vitro* system, about 5% of the total polysomes in an average liver cell would be in the preloaded condition. While this proportion might appear to be high it should be emphasized that in the neonatal liver the enzyme is synthesized *de novo* and it is not unreasonable then, to expect a relatively large number of polysomes to be engaged in this specific synthesis. Similar logistic expectations would be also reasonable for any enzyme showing a rapid *de novo* synthetic rate or turnover rate in the steady state.

One of the assumptions above is that only the completed polypeptide chain is released from the polysome by the cAMP mechanism. Since the zone sedimentation patterns of the released enzyme show that only complete or near complete molecules have activity, it is not possible to specify that the mechanism operates exclusively at the C terminus of the enzyme, but in the washed microsome system, polypeptide chain elongation does not take place. In addition it is not yet known whether tyrosine aminotransferase is composed of subunits (see Valeriote *et al.*, 1969), but if this is so, then C-terminal release of the enzyme subunits must be followed by spontaneous acquisition of quaternary structure. cAMP might also function as an obligatory effector of such an association process.

If the cAMP mechanism is confined to C-terminal release (and such a function would appear to be more attractive *in vivo* than an indiscriminate puromycin-like release of all nascent polypeptide on a polysome) then such a control mechanism would have quite different consequences in intact cells from those in isolated microsomes. In intact cells, a sudden availability of cAMP following hormone stimulation of adenylyl cyclase would open the release "gate" and lead to a cascade phenomenon, in which the release of the C-terminal polypeptide would result in the completion of translation and the release of all the partially assembled polypeptides on the polysome, since all the substrates and cofactors of protein synthesis are presumably available in the organized cell. This cascade phenomenon would not be inhibited by actinomycin D since no new mRNA is required, but it would be inhibited substantially by both puromycin, due to abortive release of the majority of nascent polypeptide molecules in an inactive form, and by cycloheximide which would inhibit translocation on the ribosome. However, an uninhibited cascade phenomenon would result in incorporation of radioactive amino acid into protein, just as though *de novo* protein synthesis had occurred. (In this sense, *de novo* is taken to mean initiation of complete synthesis from N terminus to C terminus of the particular polypeptide as distinct from completion of partial chains). The results of Wicks (1968, 1969) on fetal liver explants in organ culture in which cAMP and epinephrine induction of tyrosine aminotransferase is blocked by cycloheximide are explicable in the above terms. It should be noted that actinomycin D blocked only about 50% of the cAMP-potentiated synthesis of tyrosine aminotransferase in this system. In washed microsomes *in vitro* on the other hand no such cascade effect can take place, even if the cAMP mechanism operates in a nondiscriminative fashion at each peptidyl-tRNA fragment on the polysome, since incomplete peptides are enzymatically inactive.

A cascade effect operating *in vivo* would explain the occurrence of inducible and noninducible animals in the sense of the terms used in this paper. Any animal in which environmental stimuli have recently caused elevation of hepatic levels of cyclic AMP will be noninducible by puromycin and epinephrine, since polysomes loaded with tyrosine aminotransferase will have released their enzyme. Inducible animals, on the other hand, will be those in which recent enviornmental events have not resulted in hormonal or other stimulation of cAMP levels and thus hepatic polysomes loaded with the transaminase will be identifiable.

The cAMP-release mechanism may be of more general importance in the control of protein synthesis than is suggested by the results on tyrosine aminotransferase in this paper, since Khairallah and Pitot (1967) have already demonstrated the release of bound polypeptides from polysomes *in vitro* which requires cAMP. Two other enzymes which are known to be synthesized postnatally in rat liver, were also surveyed in this laboratory for effects (D. Yeung and C. C. Chuah, unpublished experiments). Microsome–polysome fractions from liver of newborn animals (3- to 6-hr old) were tested for release of phosphopyruvate carboxylase and serine dehydratase with negative results. cAMP has been implicated in the postnatal induction of both these enzymes (Yeung and Oliver,

1968; Greengard and Dewey, 1967). This particular question thus remains unanswered at this time.

cAMP binding proteins have been found in a variety of tissues and biochemical activity, which is dependent on cAMP, has been defined for some of them (Walsh *et al.*, 1968; Corbin and Krebs; 1969; Gill and Garren, 1969, 1970). Emmer *et al.* (1970) have presented evidence for the function of a cAMP binding protein in the induced synthesis of enzymes in bacteria.

The experiments in this paper implicate a cAMP binding protein in the release of a specific enzyme from polysomes and this raises the further general question of the multiplicity of control points in protein synthesis and genetic expression in cells of higher organisms. It appears that in developmental processes, control mechanisms can be expected at the level of DNA transcription, mRNA translation (initiation), and at the termination or release step of polypeptide synthesis.

The unique cascade properties of the effector-mediated polypeptide release mechanism and the possibility of its widespread operation in the synthesis of specific proteins make necessary the cautious interpretation of experiments in which inhibitors acting at translational levels are used. As we have shown, the properties of a protein synthetic system which includes a controlled release step, are in most cases, nearly indistinguishable from those of a system in which the initiation of translation is the locus of control.

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