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STIMULATORY EFFECTS OF AMINOPHYLLINE ON AMINO ACID INCORPORATION INTO PROTEIN BY CELL-FREE SYSTEMS

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Abstract—Aminophylline, over a concentration range of 1–6 mM, strongly stimulates amino acid incorporation into proteins by rat liver and brain microsomal and ribosomal systems. At least two different steps, namely formation of aminoacyl tRNA and the transfer of amino acid from amino acid tRNA, are affected by aminophylline. Activation of amino acids is not influenced by aminophylline. The aminophylline stimulation is observed in the presence of optimal or greater than optimal amounts of the various cofactors needed for these reactions. The effect of aminophylline can also be perceived in the presence of a complete amino acid mixture or of mercaptoethanol. PolyU-dependent stimulation of polyphenylalanine synthesis in ribosomal systems is greatly enhanced in the presence of aminophylline. This stimulatory effect cannot be reversed by addition of increasing amounts of polyU. The effects of aminophylline cannot be duplicated with several other methylxanthines that were tested. Theophylline glycinate and theophylline cholate stimulate the incorporation of amino acid into tRNA and the transfer reaction by about the same degree as aminophylline.

IT IS well known that methylxanthines stimulate the CNS and possess many other important pharmacological properties.[1] However, the precise effects of these compounds on many biochemical reactions are still to be determined. The recent studies on the effects *in vitro* of methylxanthines in general and of theophylline in particular[2–4] have been prompted by the findings that these compounds inhibit cyclic-3′,5′-nucleotide phosphodiesterase activity *in vitro*[5] and thus may serve to raise the intracellular levels of cyclic AMP.

We observed, in the course of studies on the role of cyclic AMP in brain metabolism, that aminophylline stimulates the incorporation of ^{14}C-labeled amino acids into protein by cerebral microsomal and ribosomal preparations.* Stimulation by aminophylline could also be demonstrated with liver ribosomal preparations, suggesting that it may have a general effect on protein synthesis in mammalian microsomal-ribosomal systems. The experiments reported in the present paper were performed to elucidate the stage or stages at which aminophylline affects protein synthesis and to determine the extent to which this effect is specific for aminophylline.

It is shown that aminophylline stimulates the incorporation of ^{14}C-labeled amino acid into tRNA as well as the transfer of [^{14}C]amino acids from [^{14}C]aminoacyl tRNA to protein. It is further shown that aminophylline stimulates the polyU-directed incorporation of [^{14}C]phenylalanine into polyphenylalanine. Evidence is presented

* A preliminary account of our findings was presented at the 1969 FASEB meeting in Atlantic City (*Fedn Proc.* **28**, 473, 1969).

which suggests that the aminophylline stimulation operates, in part at least, through a ribosomal mechanism.

MATERIALS AND METHODS

Chemicals

Aminophylline and caffeine were products of Mann Research Laboratory, New York, N.Y. Theophylline and ethylenediamine were obtained from K & K Laboratories, Plainview, N.Y. Puromycin dihydrochloride, methylaminopurine, dimethylaminopurine and ribonuclease were purchased from Sigma Chemical Company, St. Louis, Mo. Homogentisic acid was obtained from Eastman Organic Chemicals, Rochester, N.Y. ATP, GTP and creatine phosphate (all sodium salts), polyuridylic acid (potassium salt) creatine phosphokinase and soluble ribonucleic acid were purchased from Calbiochem, Los Angeles, Calif. Theophylline cholate was a gift from Warner–Lambert Research Institute, Morris Plains, N.J. Theophylline glycinate was kindly provided by The Central Pharmacal Company, Seymour, Ind.

Radioactive compounds

Uniformly labeled [^{14}C]amino acids and [^{14}C]amino acids-sRNA were obtained from New England Nuclear Corp., Boston, Mass. [^{14}C]polyU was obtained from Schwarz BioResearch, Van Nuys, Calif.

Preparation of subcellular fractions

Sprague–Dawley rats of both sexes (200–300 g) were lightly anesthetized with ether and then killed by decapitation. Livers and brain cortices were removed, rinsed with ice-cold medium A[6] and then minced. The minced tissue was homogenized with 8 vol of medium A in a Potter–Elvehjem homogenizer. Microsomes and pH 5 enzyme fractions were prepared essentially as described by Zomzely *et al.*[7] and stored until used. Mixed ribosomes were prepared as described previously.[8]

Assay procedures

Formation of aminoacyl tRNA. The ^{14}C-labeled amino acids were incubated for 30 min with 1 mM ATP and 500 μg sRNA with the pH 5 enzyme fraction as a source of aminoacyl tRNA synthetases. The final volume was 1·7 ml. The reaction was terminated by addition of 10% TCA, and the RNA was then extracted and assayed for its ^{14}C content as described by von der Decken.[9]

Adenosine triphosphate–pyrophosphate exchange reaction. Incubation conditions used for the assay of the ATP–pyrophosphate exchange reaction were the same as those described by Stulberg and Novelli.[10] After incubation, proteins were precipitated with TCA, and ATP was isolated from the supernatant and hydrolyzed. Aliquots of the final acid hydrolysate were removed for determination of inorganic phosphorus and for assay of ^{32}P.

Amino acid incorporation into protein. The system used for the incorporation of labeled amino acids into protein by microsomes and ribosomes was essentially as described by us elsewhere.[11] Incubation mixtures contained the following substances (final concentrations): 250 mM sucrose, 50 mM tris (pH 7·5), 4 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, 10 mM creatine phosphate, 100 μg creatine phosphokinase, 0·25

mM GTP and [^{14}C]amino acid (0·5 μc in microsomal systems; 0·125 μc in ribosomal systems). Various amounts of microsomal, ribosomal and pH 5 enzyme protein were used in different experiments. The final volume of the incubation mixture was 1·7 ml for experiments with microsomes and 0·85 ml for experiments with ribosomes.

The incubations were carried out at 37° and usually for 35 min. The reactions were stopped by the addition of 1 ml of 10% TCA containing 0·2% of the unlabeled amino acid. The TCA-insoluble material was washed twice with the TCA solution and then suspended in 2 ml of 10% TCA and heated at 90° for 10 min. The hot TCA-insoluble protein was washed twice with TCA and then dissolved in 0·5 ml of 88% formic acid and assayed for ^{14}C content.

Polyphenylalanine synthesis. Incubation mixtures containing ribosomes, pH 5 enzymes and the various cofactors were incubated for 30 min at 37°. The mixtures were cooled in ice, and polyU and [^{14}C]-labeled phenylalanine were then added. Incubation at 37° was resumed for another 30 min, after which the reactions were terminated with TCA and the proteins were processed as described above. In some experiments, the preincubation procedure was omitted.

Transfer of amino acid from aminoacyl tRNA to protein. The transfer of [^{14}C]amino acids from [^{14}C]aminoacyl tRNA to ribosomes was measured using the same incubation system as described above for the incorporation of amino acids into protein, except that 11·5 μg (about 3800 counts/min) of [^{14}C]aminoacyl tRNA was used instead of the [^{14}C]amino acid.

Adenosine triphosphatase assay. ATPase was assayed by measuring the release of P_i from ATP.[12] Rat brain ribosomes were incubated with 5 mM ATP in medium A in the presence or absence of cell sap. The P_i released during a 5-min incubation period was determined.

Ribonuclease assay. RNase activity in the various fractions was assayed by the method of Barondes and Nirenberg.[13] The tissue preparation (postmitochondrial supernatant or microsomes) was incubated for periods ranging from 0 to 60 min in a medium which contained the following: 50 mM tris–HCl buffer, pH 7·5; 10 mM Mg^{2+}; 100 mM KCl; and 50 mμmoles [^{14}C]polyU. Aliquots were removed from the incubation medium and the undegraded polyU was precipitated by the addition of 2 vol. of ethanol and 1 vol. of 0·1 M magnesium acetate. The precipitation was complete in 30 min at 0°, after which 2 ml of ice-cold 0·05 M magnesium acetate in 50% ethanol was added. The precipitates were collected on millipore filters, washed with the ethanol–magnesium acetate solution, air-dried on the filters and assayed for radioactivity.

Chemical analyses

Proteins were determined by the biuret method of Gornall *et al.*[14] and inorganic phosphorus by the method of Fiske and Subbarow.[15] RNA was estimated by the orcinol procedure.[16]

RESULTS

Effects of aminophylline on the incorporation of ^{14}C-amino acids into proteins by liver and brain microsomes

Table 1, which shows the results of representative experiments, demonstrates that aminophylline, at a concentration of 3 mM, markedly stimulated the incorporation of

^{14}C-labeled leucine, isoleucine and valine into proteins by liver microsomes, and stimulated the incorporation of [^{14}C]phenylalanine and leucine into protein by brain microsomes. The incorporation of amino acids was more than doubled by aminophylline in each case.

TABLE 1. STIMULATORY EFFECTS OF AMINOPHYLLINE ON THE INCORPORATION OF [^{14}C]AMINO ACIDS INTO PROTEIN BY LIVER AND BRAIN MICROSOMES*

Source of microsomes and pH 5	[^{14}C]amino acid	Specific activity of protein (counts/min/mg) Control	Aminophylline (3 mM)	Increase due to aminophylline (%)
Liver	Leucine	2530	5610	122
	Isoleucine	2120	4470	111
	Valine	1270	2610	106
Brain	Leucine	2750	6340	131
	Valine	3470	7920	128

* Microsomal protein (2·0 mg) and pH 5 enzyme protein (1·5 mg) were incubated with [^{14}C]amino acid under the conditions described in the text. Each value is the mean of three observations.

Of a number of methylxanthines tested (Table 2), only aminophylline and the two other salts of theophylline, namely theophylline glycinate and theophylline cholate, increased incorporation. Neither theophylline nor ethylenediamine alone had any

TABLE 2. EFFECTS OF METHYLXANTHINES ON INCORPORATION OF [^{14}C]PHENYLALANINE INTO PROTEINS BY BRAIN MICROSOMES*

Addition	Specific activity of protein (counts/min/mg)	Effect (%)
None	2170	
Aminophylline	5070	+135
Theophylline	2190	+1
Caffeine	2020	−7
Methylaminopurine	2220	+3
Dimethylaminopurine	2270	+5
Ethylenediamine	1870	−13
Theophylline + ethylenediamine	1920	−11
Theophylline glycinate	4960	+130
Theophylline cholate	4660	+115

* Incubation conditions as in Table 1. All the compounds were included in the incubation mixture at 3 mM concentration. Each value is the mean of four observations.

stimulatory effect. In fact, ethylenediamine consistently inhibited the extent of incorporation. It may be noted that theophylline and ethylenediamine, when added together, did not increase the incorporation.

Aminophylline and amino acid activation

Aminophylline had no effect on the activation of amino acids by ATP. In a rat liver pH 5 enzyme system, the ATP–pyrophosphate exchange ranged between 1·62 and 2·16 per cent in the presence of a complete amino acid mixture. In the presence of 3–6 mM aminophylline, the range was 1·58–1·96 per cent. Essentially similar results were obtained when the complete amino acid mixture was omitted or was replaced by the mixtures described by Hradec.[17]

Aminophylline and incorporation of amino acids into tRNA

Addition of 3 mM aminophylline to mixtures containing pH 5 enzymes, ATP and tRNA resulted in a substantial increase in the formation of some but not all of the [^{14}C]aminoacyl tRNA's (Table 3). The incorporation of [^{14}C]isoleucine, leucine and phenylalanine into the respective aminoacyl tRNA's was increased 2-fold or more by aminophylline. On the other hand, incorporation of proline and lysine were essentially unaffected, while that of alanine and arginine was actually inhibited. In other experiments (results not shown here) it was observed that while the incorporation of [^{14}C]isoleucine and leucine were increased by aminophylline, theophylline and ethylenediamine, added either singly or in combination, did not affect the incorporation of these amino acids into the respective tRNA's.

TABLE 3. EFFECT OF AMINOPHYLLINE ON THE INCORPORATION OF [^{14}C]AMINO ACIDS INTO tRNA BY LIVER AND BRAIN ENZYMES*

	Effect of aminophylline (%)	
[^{14}C]Amino acid	Liver	Brain
Alanine		−73
Arginine	−30	−36
Aspartic acid		+77
Glycine		+69
Histidine	+15	+51
Isoleucine	+116	+98
Leucine	+146	+112
Lysine	−10	
Methionine	+21	
Phenylalanine	+97	+110
Proline	0	0
Serine		+28
Threonine	+52	+40
Valine	+44	

*Experimental conditions as described in the text. The values represent the mean obtained from four different experiments. Aminophylline concentration, 3 mM.

Some characteristics of aminophylline-stimulated incorporation of [^{14}C]leucine into [^{14}C]leucyl tRNA by the pH 5 enzyme fraction derived from brain are demonstrated in Figs. 1–3. Enzyme systems derived from liver responded to aminophylline in an identical manner. A stimulatory effect became clearly evident at an aminophylline concentration of 0·6 mM (Fig. 1). At this concentration, the incorporation of leucine into tRNA was increased by 29 per cent. At a concentration of 3 mM,

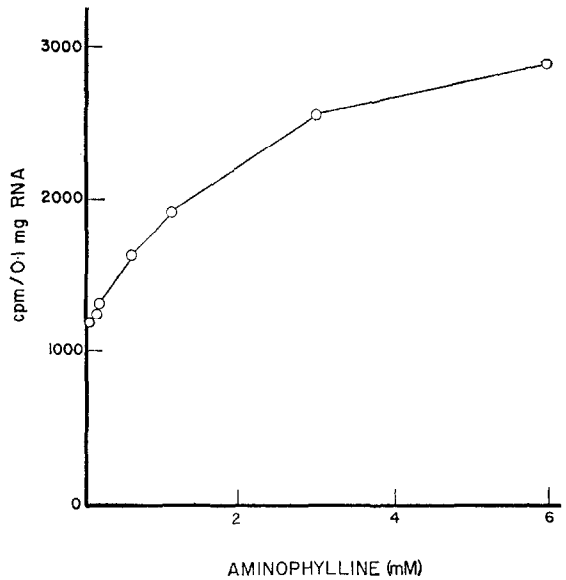

FIG. 1. Relationship between aminophylline concentration and stimulation of incorporation of [^{14}C]leucine into tRNA by brain pH 5 enzymes. ^{14}C recovered in 100 μg tRNA, ○—○.

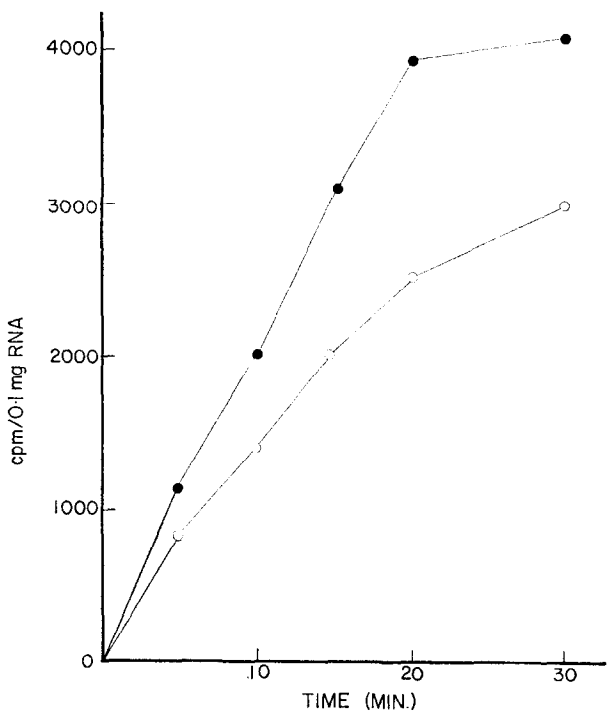

FIG. 2. Time course of aminophylline stimulation of [^{14}C]leucine incorporation into tRNA by liver pH 5 enzymes. Control, ○—○; aminophylline (3 mM), ●—●.

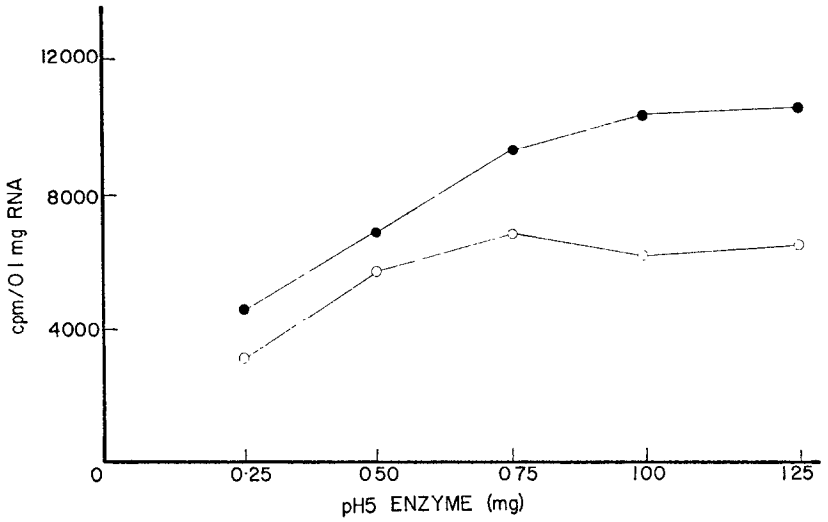

FIG. 3. Stimulatory effects of aminophylline on the incorporation of [^{14}C]valine into tRNA at various liver pH 5 enzyme concentrations. Incubation conditions as described in text. Control, ○—○; aminophylline (3 mM), ●—●.

aminophylline approximately doubled the leucine incorporation, while at 6 mM the incorporation was increased by 144 per cent. The results of experiments carried out with [^{14}C]phenylalanine and [^{14}C]valine were essentially similar to those reported here for leucine, although the magnitude of stimulation varied.

The time course of incorporation of [^{14}C]leucine into tRNA by rat liver pH 5 enzymes (Fig. 2) clearly showed that more radioactivity was found in the tRNA of mixtures containing aminophylline (3 mM) at all time intervals studied. In the presence of aminophylline maximum specific activity was reached after 20 min of incubation. These results indicate that the tRNA was more rapidly charged with leucine in the presence of aminophylline. This was found also to be true when labeled isoleucine or phenylalanine was employed as substrate.

The results shown in Fig. 3 indicate that the stimulatory effects of aminophylline were not reversed when the pH 5 enzyme concentration was increased. Even at saturating levels of pH 5 enzymes, aminophylline stimulated the incorporation of [^{14}C]valine into tRNA by about 50 per cent.

In his studies on the effects of polycyclic hydrocarbons on protein synthesis, Hradec[17] observed that the carcinogenic hydrocarbons stimulated the incorporation of [^{14}C]amino acids into tRNA by rat liver pH 5 enzymes and this stimulatory effect could be reduced or even abolished by preincubating pH 5 enzymes and ATP in the presence of the hydrocarbons before adding the labeled amino acids. Similar experiments carried out with aminophylline showed that preincubation of rat liver pH 5 enzymes and ATP with 3 mM aminophylline did not abolish the stimulatory effect of aminophylline. After preincubation periods ranging from 5 to 15 min, the stimulatory effect of aminophylline was clearly persistent. It was further observed that preincubation did not result in any loss of the ability of control mixtures to incorporate [^{14}C]amino acids into tRNA.

The possibility was considered that aminophylline may increase the formation of

aminoacyl tRNA by enhancing ATP regeneration. When ATP was omitted from the incubation mixture, little or no incorporation took place either in the presence or absence of aminophylline. Moreover, aminophylline stimulated incorporation equally at several ATP levels, and the stimulation could be demonstrated both in the presence (Table 1) and absence of an ATP-regenerating system (Table 4). Further, as can be seen from the data presented in Table 5, aminophylline had no effect on the ATPase

TABLE 4. STIMULATORY EFFECTS OF AMINOPHYLLINE ON [^{14}C]PHENYLALANINE INCORPORATION INTO PROTEIN BY RAT BRAIN MICROSOMES IN THE ABSENCE OF ATP-REGENERATING SYSTEM*

Aminophylline concn (mM)	Specific activity of protein (counts/min/mg)	Increase due to aminophylline (%)
None	920	
1·5	1560	69
3·0	2120	130

* Rat brain microsomes and pH 5 enzyme fractions were incubated under the conditions described in the text with the exception that creatine phosphate and creatine phosphokinase were not included. Each value is the mean of four observations.

TABLE 5. LACK OF EFFECT OF AMINOPHYLLINE ON ATPASE ACTIVITY OF RAT BRAIN FRACTIONS*

Fraction	Addition	ATPase (nmoles P_i/min)
Ribosomes (0·5 mg protein)	None	8·4
	Aminophylline (3 mM)	8·3
Ribosomes + cell sap (0·5 mg protein each)	None	9·1
	Aminophylline (3 mM)	9·4

* ATPase activity of rat brain ribosomes and cell sap was measured as described in the text. Each value is the mean of three observations.

activity of rat brain ribosomes and the soluble fraction. These findings rule out the possibility that aminophylline stimulation is secondary to an effect on ATP formation.

Aminophylline effects on the transfer of amino acid from aminoacyl tRNA to microsomes and ribosomes

The transfer of [^{14}C]valine from [^{14}C]valyl tRNA into proteins by liver microsomal preparations was increased by aminophylline (Table 6). The transfer was stimulated by 45 per cent at 1 mM aminophylline concentration, by 80 per cent at 3 mM, and by about 120 per cent at 6 mM concentrations. It is not likely that this stimulation was secondary to an alteration in the permeability of the microsomal membrane, since the stimulatory effects of aminophylline could be observed with ribosomal preparations as well. The ribosomal system, in fact, was more sensitive to the action of aminophylline than the microsomal systems (Table 6). Thus, at each aminophylline

TABLE 6. AMINOPHYLLINE STIMULATION OF THE TRANSFER OF [^{14}C]VALINE FROM [^{14}C]VALINE tRNA TO PROTEIN BY LIVER MICROSOMES AND RIBOSOMES*

Addition	Transfer of [^{14}C]valine to protein by			
	Microsomes		Ribosomes	
	Sp.act. of protein (counts/min/mg)	Increase (%)	Sp.act. of protein (counts/min/mg)	Increase (%)
None	140		200	
Aminophylline (1 mM)	210	45	330	70
Aminophylline (3 mM)	260	80	420	116
Aminophylline (6 mM)	320	119	550	183

* Liver microsomes (1·7 mg protein) and ribosomes (0·8 mg protein) were incubated with pH 5 enzyme protein (1·7 and 0·8 mg protein respectively) with [^{14}C]valine tRNA under conditions described in the text. The values are corrected for zero-time blanks and represent the mean of three observations.

concentration tested, the stimulatory effect on the transfer of [^{14}C]valine from [^{14}C]valyl tRNA was greater in the ribosomal system than in the microsomes.

PolyU-directed incorporation of [^{14}C]*phenylalanine and transfer of* [^{14}C]*phenylalanine from* [^{14}C]*phenylalanyl tRNA into protein*

The effect of aminophylline on polyU-directed incorporation of [^{14}C]phenylalanine into polyphenylalanine was studied using rat liver ribosomes. Two different incubation conditions were employed. In one, polyU was added to the complete incubation medium and the incorporation of [^{14}C]phenylalanine into protein was measured. In the other, ribosomes and pH 5 enzymes were first preincubated for 30 min with GTP, ATP and an ATP regenerating system. This latter procedure served to destroy much of the endogenous messenger activity and render the system more dependent on the added polyU. After the preincubation, polyU, aminophylline and [^{14}C]phenylalanine were added and the incorporation into protein was measured. The results of two representative experiments (Table 7) demonstrate that both systems responded to the addition of polyU. Preincubation decreased the extent of incorporation in control mixtures by about one-half. Addition of aminophylline alone increased the incorporation by about 55 per cent in both the systems. In mixtures that were not preincubated, addition of both polyU and aminophylline increased the incorporation by about 240 per cent, indicating that the effects of these compounds are virtually additive in this system. In contrast, the addition of polyU and 3 mM aminophylline to preincubated mixtures stimulated the incorporation by about 330 per cent. This extent of increase is much greater than the sum of the individual effects of the two compounds. In experiment 2, in which less polyU was used, essentially similar results were obtained, even though the actual extents of stimulation by aminophylline and by polyU were not the same as in experiment 1. The possibility that these effects of aminophylline are indirect ones resulting from inhibition of endogenous ribonuclease activity is ruled out by the results presented in Table 8, which show that aminophylline had no effect on the ribonuclease activity of postmitochondrial and ribosomal preparations of adult rat brain. These data suggest that

TABLE 7. AMINOPHYLLINE STIMULATION OF POLYU-DIRECTED INCORPORATION OF [^{14}C]PHENYLALANINE INTO PROTEIN BY LIVER RIBOSOMES*

Expt No.	Addition	No preincubation		Preincubation	
		Sp.act. of protein (counts/min/mg)	Increase (%)	Sp.act. of protein (counts/min/mg)	Increase (%)
1	None	950		430	
	PolyU (100 μg)	2420	154	1030	144
	Aminophylline (3 mM)	1510	58	650	53
	PolyU (100 μg) + aminophylline (3 mM)	3210	237	1830	329
2	None	1240		440	
	PolyU (50 μg)	1770	43	810	85
	Aminophylline (3 mM)	1930	56	540	23
	PolyU (50 μg) + aminophylline (3 mM)	2470	99	1570	258

* Details of the experimental conditions are described in the text. Liver ribosomes (0·4 mg protein) and pH 5 enzymes (0·6 mg protein) were used with or without the preincubation procedure (see text). Values are means of three observations.

TABLE 8. LACK OF EFFECT OF AMINOPHYLLINE ON RIBONUCLEASE ACTIVITY OF POSTMITOCHONDRIAL AND RIBOSOMAL PREPARATIONS OBTAINED FROM ADULT RAT BRAIN*

Fraction	Incubation time (min)	[^{14}C]poly Udegraded (%)	
		Control	Aminophylline (3 mM)
Postmitochondrial Supernatant	0	4	3
	20	46	47
	60	72	70
Ribosomes	0	3	3
	20	16	17
	60	29	27

* Assay conditions were as described in the text. Approximately 1 mg of the postmitochondrial or ribosomal protein was used in each incubation. Each value is the mean of five observations.

aminophylline exposes more of the existing sites on the ribosomes, consequently resulting in an increase in protein synthesis.

The effects of aminophylline and several related compounds on polyU-directed transfer of [^{14}C]phenylalanine are presented in Table 9. Among the various compounds tested, only aminophylline, theophylline glycinate and theophylline cholate stimulated the transfer. As in the experiments on the incorporation of amino acids into protein by microsomes, addition of theophylline and ethylenediamine, either alone or together, did not increase the transfer. It is of interest to note that the effects of aminophylline and polyU are not additive.

The results of experiments on the effect of increasing amounts of polyU on the aminophylline stimulation are presented in Table 10. Increasing the polyU concen-

TABLE 9. EFFECT OF METHYLXANTHINES ON THE POLYU-DIRECTED TRANSFER OF [^{14}C]PHENYLALANINE FROM [^{14}C]PHENYLALANINE tRNA INTO PROTEINS BY LIVER RIBOSOMES*

Addition	Sp.act. of protein (counts/min/mg)	Increase (%)
None	170	
Aminophylline	210	24
PolyU (100 μg)	430	161
PolyU (100 μg)+		
Aminophylline	750	356
Theophylline	440	167
Caffeine	420	155
Methylaminopurine	420	155
Dimethylaminopurine	350	114
Ethylenediamine	450	171
Theophylline + Ethylenediamine	440	165
Theophylline cholate	650	290
Theophylline glycinate	690	321

* Preincubated systems were used as described in the text; other conditions as in Table 5. Each value is the mean of three observations. Values are corrected for zero-time controls. Methylxanthines were tested at 3 mM concentrations.

tration resulted in increased incorporation of phenylalanine into polyphenylalanine in preincubated liver ribosomal systems. The aminophylline stimulation was present at all polyU concentrations employed.

Relationship between Mg^{2+} concentration and aminophylline effect

The results shown in Table 11 demonstrate that the stimulatory effects of aminophylline could be observed at all Mg^{2+} concentrations that sustained protein synthesis. Cerebral microsomes used in these studies were prepared in the usual manner and washed with Mg^{2+}-free medium A. In the absence of Mg^{2+} and at 1 mM Mg^{2+} concentration, the incorporation of phenylalanine was low and even this incorporation was perhaps due to the presence of low amounts of Mg^{2+} in the pH 5 enzymes and ribosomal fractions. At all higher concentrations of Mg^{2+}, the stimulatory effects of aminophylline persisted. At the highest Mg^{2+} concentration, namely 8 mM, incorporation in both the control and aminophylline-containing mixtures was below maximal, but the stimulatory effect of aminophylline was still apparent.

TABLE 10. PERSISTENCE OF AMINOPHYLLINE EFFECT ON POLYPHENYLALANINE SYNTHESIS AT DIFFERENT CONCENTRATIONS OF POLYU*

PolyU (μg)	Sp.act. of protein (counts/min/mg)		Increase due to aminophylline (%)
	Control	Aminophylline (3 mM)	
0	290	370	29
50	1010	1890	87
100	1440	2750	91
200	1870	3400	82
500	1920	3480	81

* The preincubated liver ribosomal system was used under conditions described in the text and in Table 5. Each value is the mean of three observations.

TABLE 11. EFFECT OF Mg^{2+} CONCENTRATION ON AMINOPHYLLINE STIMULATION OF [^{14}C]PHENYLALANINE INCORPORATION INTO PROTEIN BY BRAIN MICROSOMES*

Mg^{2+} concn (mM)	Sp.act. of protein (counts/min/mg)		Increase due to aminophylline (%)
	Control	Aminophylline (3 mM)	
0	490	490	
1	510	560	10
2	760	1430	88
3	1500	3480	132
4	3870	8020	107
8	2220	4510	104

* Brain microsomes were (1·5 mg protein) washed with Mg^{2+}-free medium A incubated with 1·5 mg of pH 5 enzyme protein and [^{14}C]phenylalanine under the conditions described in the text. Each value is the mean of two observations.

Relationship between GTP and aminophylline effect

GTP is the only essential nucleotide requirement for the transfer reaction in the ribosomal system. The liver ribosomal system which we employed showed strict requirement for GTP (Table 12). Addition of GTP resulted in increased incorporation of phenylalanine into protein, but aminophylline clearly enhanced the transfer of

TABLE 12. EFFECT OF GTP ON [^{14}C]PHENYLALANINE INCOPORATION INTO PROTEIN BY LIVER RIBOSOMES*

GTP concn (mM)	Sp.act. of protein (counts/min/mg)		Increase due to aminophylline (%)
	Control	Aminophylline (3 mM)	
0	330	460	40
0·125	1510	2840	88
0·25	1710	3230	88
0·5	1710	2920	71
1·00	1400	2650	89

* Liver ribosomes (0·4 mg potein) were incubated with 0·8 mg of pH 5 enzyme protein under conditions described in the text. Each value is the mean of two observations.

amino acid to ribosomal protein in the presence of optimal or even greater than optimal amounts of added GTP. A similar independence of aminophylline effect was observed in the transfer reaction as well.

Effects of inhibitors of protein synthesis on aminophylline stimulation

To verify that aminophylline stimulation was not due to an increase in artifactual incorporation reactions, the effects of some classical inhibitors of protein synthesis on aminophylline stimulation were tested. Puromycin, cycloheximide and ribonuclease practically abolished the stimulation due to aminophylline (Table 13), thus indicating that aminophylline affects true protein (RNA-dependent) synthesis. The data in

TABLE 13. EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON AMINOPHYLLINE STIMULATION OF [^{14}C]PHENYLALANINE INCORPORATION INTO PROTEIN BY LIVER RIBOSOMES

Addition	Sp.act. of protein (counts/min/mg)	
	Control	Aminophylline (3 mM)
None	690	1160
Puromycin (0·2 mM)	100	110
Cycloheximide (1·0 mM)	240	270
Ribonuclease (50 μg/ml)	80	70
Homogentisic acid (0·2 mM)	100	100

Table 13 also show that the stimulatory effect of aminophylline was abolished by homogentisic acid. The inhibitory effects of this compound on cell-free protein synthesis have been described.[11,18]

Lack of effect of aminophylline on the incorporation of arginine into protein by rat liver soluble system

Rat liver supernatant fractions are known to actively incorporate labeled arginine into protein in the absence of microsomes.[19,20] This reaction requires ATP, but is not enhanced by an ATP regenerating system, nor is it affected by many agents (e.g. GTP, puromycin) which have their primary influences on protein synthesis at the ribosomal level. From their data, Soffer[19] and Gill[20] infer that this incorporation takes place by a mechanism not related to protein synthesis. The results presented in Table 14 confirm the general characteristics of the rat liver cytoplasmic system and further establish that in this system aminophylline did not increase the incorporation of arginine into protein.

TABLE 14. LACK OF EFFECT OF AMINOPHYLLINE ON THE INCORPORATION OF [^{14}C]ARGININE INTO PROTEIN BY RAT LIVER SUPERNATANT ENZYMES*

Incubation	Sp.act. of protein (counts/min/mg)
Complete	4260
− ATP	940
− GTP, creatine phosphate, creatine phosphokinase	4130
Aminophylline (3 mM)	4200
Aminophylline (6 mM)	4190

*Liver pH 5 enzyme (1·5 mg protein) was incubated with 1 mM ATP, 10 mM creatine phosphate, 100 μg creatine phosphokinase, 0·25 mM GTP and 0·5 μc[^{14}C]-arginine, all in 1·8 ml medium A.

DISCUSSION

The results of the present studies clearly demonstrate that aminophylline, over a concentration range of 1–6 mM, increases the incorporation of [^{14}C]amino acids into protein by rat liver and brain microsomal and ribosomal systems. The mechanism of stimulation is apparently a complex one. At least two reactions in the protein synthetic mechanism are enhanced by aminophylline: namely, the incorporation of amino acid into tRNA and the transfer of amino acid from aminoacyl tRNA to protein. Although amino acid activation and charging of tRNA with amino acids are generally believed to represent two aspects of the same reaction,[17,21] aminophylline stimulates only the latter.

The stimulatory effect of aminophylline on the formation of aminoacyl tRNA might result from either of the following two mechanisms: (1) an effect on the specific aminoacyl tRNA synthetases, or (2) an effect on the capacity of tRNA to accept the individual amino acid.

The stimulatory effects of aminophylline in systems containing microsomes and ribosomes appear to involve true protein synthesis, since they can be overcome by the addition of puromycin and cycloheximide. Our data rule out certain explanations for the effect of aminophylline on protein synthesis. The aminophylline effect is not secondary to an effect on ATP generation since: (1) stimulation of incorporation of ATP can be observed both in the presence and absence of ATP-regenerating system, and (2) the stimulation persists even at elevated ATP levels. Further, aminophylline has no effect on the ATPase activity of the cell-free preparations used in the present study. The aminophylline effect is also evident in the presence of a complete amino acid mixture, mercaptoethanol or saturating levels of GTP, thus indicating that it is not dependent on the availability of these factors. Alteration in membrane permeability to amino acids or to tRNA-bound amino acids can also be ruled out, since the stimulatory effects of aminophylline can be demonstrated in microsomal as well as ribosomal systems. Furthermore, the stimulation of protein synthesis by aminophylline is essentially unaltered at various magnesium concentrations, thereby indicating that aminophylline does not act at magnesium binding sites.

Simon *et al.*[22] have shown that ribaminol, a salt of sRNA and diethylaminoethanol, stimulates brain protein synthesis *in vivo* and *in vitro*. In their experiments, the individual components, RNA and diethylaminoethanol, were without effect. In our studies, while aminophylline clearly stimulated the incorporation of amino acids into tRNA or protein, the individual components, namely theophylline and ethylenediamine, had no effect. The precise mechanism by which aminophylline exerts its specific effect is still unclear.

Despite the temptation to relate the stimulatory effects of aminophylline to the recently established effects of methylxanthines on cyclic-3′,5′-nucleotide phosphodiesterase activity and on the intracellular levels of cyclic AMP, the evidence presented in this paper, as well as other unpublished data, does argue against an involvement of cyclic AMP. Aminophylline stimulates the incorporation of [^{14}C]amino acids into tRNA by the pH 5 enzyme system. It is hard to conceive of an involvement of cyclic AMP in this reaction, since adenyl cyclase, the enzyme that forms cyclic AMP, is strictly a particulate one.[23] Further, several other methylxanthines (whose effects on the cyclic nucleotide phosphodiesterase have been well documented) do not stimulate polypeptide synthesis from free or tRNA-bound amino acids. Finally, neither cyclic

AMP nor its dibutyryl ester has a stimulatory effect on the above-mentioned reactions. The results of Khairallah and Pitot[24] are of particular interest in this context. In their studies on the effect of cyclic AMP on the release of polysome-bound protein *in vitro*, they observed that cyclic AMP or dimethylaminopurine added separately could release nascent protein, but that the effect of addition of both of these compounds was more than additive. The latter finding would imply that dimethylaminopurine might have an independent effect on polysomal protein synthesis.

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