Inhibition of Hepatic Protein Synthesis by α-Methyl-DL-tryptophan *in Vivo*. Further Studies on the Glyconeogenic Action of α-Methyltryptophan*

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ABSTRACT: Treatment of rats with α-methyl-DL-tryptophan causes initially (2–20 min) a slight stimulation of the incorporation of [^{14}C]leucine into protein *in vitro*. However, an inhibition of [^{14}C]leucine incorporation soon develops and is maximal at 60 min after injection of α-methyltryptophan; there is little further change up to 16 hr. At 16 hr, the ability of brain to incorporate [^{14}C]leucine into protein is diminished. α-Methyltryptophan causes disaggregation of the hepatic polyribosomes to the lighter species. An intraperitoneal injection of L-tryptophan, given 1 hr prior to death, reverses the polyribosomal disaggregation and inhibition of protein synthesis caused by the α-methyl analog. Addition of tryptophan or a complete amino acid mixture to a hepatic cell-free amino acid incorporating system prepared from rats treated with α-methyltryptophan does not overcome the inhibition of [^{14}C]leucine incorporation into protein. Both microsomes and cell sap isolated from livers of the treated rats are less effective in synthesizing protein than corresponding fractions from control animals. A defect in the cell sap accounts for a greater proportion of the inhibitory effect of the analog. α-Methyltryptophan has no action *in vitro*. Treatment of rats with α-methyltryptophan does not diminish the overall rate of amino acid activation as measured by ATP–pyrophosphate exchange; however, the incorporation of radioactivity from [^{14}C]Leu-tRNA into protein is diminished by this treatment.

The concentration of tryptophan in the blood begins to fall 15 min after injection of α-methyltryptophan and continues to do so for a further 30 min. The inhibition of [^{14}C]-leucine incorporation into protein occurs at the time when blood tryptophan is decreasing. Tryptophan oxidation *in vivo* is stimulated as early as 5 min after administration of the α-methyl compound. These data suggest that α-methyltryptophan, by its stimulatory action on pyrrolase activity *in vivo*, can induce a tissue deficit of tryptophan which then could result in a diminished rate of synthesis of protein. This in turn would spare amino acids for oxidation and their subsequent conversion into glycogen.

Recently studies in our laboratory (Oravec and Sourkes, 1967, 1968) have shown that α-methyltryptophan stimulates glyconeogenesis in rat liver. In those experiments, the conversion of amino acids, acetate, and pyruvate into glycogen was strongly accelerated in rats treated with the tryptophan analog. Accompanying this phenomenon there was an enhanced rate of (a) oxidation of these substances (except acetate) to carbon dioxide, and (b) excretion of urea (Oravec and Sourkes, 1968).

The fact that the increased activity of tryptophan pyrrolase caused by AMTP[1] in intact (Sourkes and Townsend, 1955; Sankoff and Sourkes, 1962) or adrenalectomized (Civen and Knox, 1960; Madras and Sourkes, 1968; Sourkes and Missala, 1969) rats is prolonged (Sankoff and Sourkes, 1962; Moran and Sourkes, 1963; Madras and Sourkes, 1968) suggested the possibility that this substance promotes excessive oxidation of dietary and endogenous tryptophan in the liver. If the net loss of free tryptophan is not replenished from other sources there will be a deficit of this substance in the amino acid pool, with serious consequences for the nitrogen balance of the animal. Indeed it now has been found that treatment of rats with AMTP significantly decreases the level of free tryptophan in the liver, brain, and blood (Sourkes *et al.*, 1970). This deficit of tryptophan could serve to reduce the synthesis of protein, for in normal animals tryptophan is the least abundant amino acid in the free amino acid pool and in proteins formed from that pool (Munro, 1968); hence the concentration of tryptophan is probably rate limiting for protein synthesis and thus determines the state of polyribosomal aggregation in the liver of the intact animal. Such an inhibition in AMTP-treated rats could spare amino acids for catabolism and for conversion into glycogen, and would perhaps provide a sufficient explanation for the glyconeogenic action of AMTP. This paper describes experiments designed to test this hypothesis concerning the mode of action of AMTP. A preliminary report has been presented (Oravec and Sourkes, 1968b).

Materials

L-[*U*-^{14}C]leucine (180 mCi/mmole) and L-[*U*-^{14}C]isoleucine (158 mCi/mmole) were obtained from Schwarz BioResearch

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[1] The abbreviation used is: AMTP, α-methyl-α-amino-β-(indolyl-3′)-propionic acid.

TABLE I: Effect of Intraperitoneally Administered AMTP on the Incorporation of [^{14}C]Leucine into Protein *in Vitro*.[a]

Organ	Treatment	n	[^{14}C]Leucine Incorpn: dpm into Protein/Sample	[^{14}C]Leucine Incorpn: % of Control
Liver	Control	6	22,372 ± 1648[b]	100
Liver	AMTP	6	12,286 ± 1726[c]	55
Brain	Control	6	17,712 ± 448	100
Brain	AMTP	7	14,330 ± 222[c]	81

[a] Animals received by intraperitoneal injection 100 mg of AMTP/kg; they were then fasted for 16 hr (overnight) and killed. For the incorporation studies the final incubation medium, in a total volume of 1.1 ml, contained 0.5 μmole of ATP, 0.2 μmole of GTP, 5 μmoles of phosphoenolpyruvate, 3.15 μg of pyruvate kinase, and 0.5 μCi of [*U*-^{14}C]leucine. The medium also contained 0.03 M Tris (pH 7.5), 0.15 M NH_4Cl, and 0.0035 M $MgCl_2 \cdot 6H_2O$. The reaction was initiated by the addition of an amount of postmitochondrial supernatant fraction corresponding to 45 mg of fresh liver or brain. After 30 min at 37°, the reaction was terminated by the addition of 1.2 ml of 0.2 N NaOH, with further incubation at 37° for 30 min. Proteins were precipitated with 5 ml of 10% trichloroacetic acid, and the precipitate was prepared for counting of radioactivity as described under Methods. [b] Mean ± std error. [c] Differences between the pairs of means are statistically significant ($P < 0.01$).

Inc., Orangeburg, N. Y. DL-[2-^{14}C]Tryptophan (label in pyrrole ring) was purchased from Mallinckrodt Co., Orlando, Fla. [^{32}P]Sodium pyrophosphate (1990 mCi/mmole) and [*U*-^{14}C]leucyl-tRNA (0.357 mCi/mg) were purchased from New England Nuclear Corp., Boston, Mass. Semipurified *Escherichia coli* tryptophanase was obtained from Sigma Chemical Co., St. Louis, Mo. Phosphoenolpyruvate (tricyclohexylammonium salt) and crystalline pyruvate kinase were products of Calbiochem, Los Angeles. DL-AMTP·H_2O was a gift of Merck Sharp and Dohme Research Laboratories, Inc., Rahway, N. J.

Methods

Male Sprague–Dawley rats weighing approximately 150 g were used throughout. The animals usually received 100 mg of AMTP/kg by intraperitoneal injection at about 5 pm, were fasted overnight for 16 hr, and were then killed. Control animals received an injection of saline and were fasted overnight.

Measurement of the rate of incorporation of [^{14}C]leucine into protein *in vitro* in the 15,000*g* supernatant of rat liver or brain was performed by the method of Sidransky *et al.* (1967). In some experiments the postmitochondrial supernatant fraction was further fractionated in a Beckman Model L ultracentrifuge for 60 min at 105,000*g* (average) to obtain the microsomal fraction and cell sap. The conditions of incubation and measurement of radioactivity in protein were the same as above.

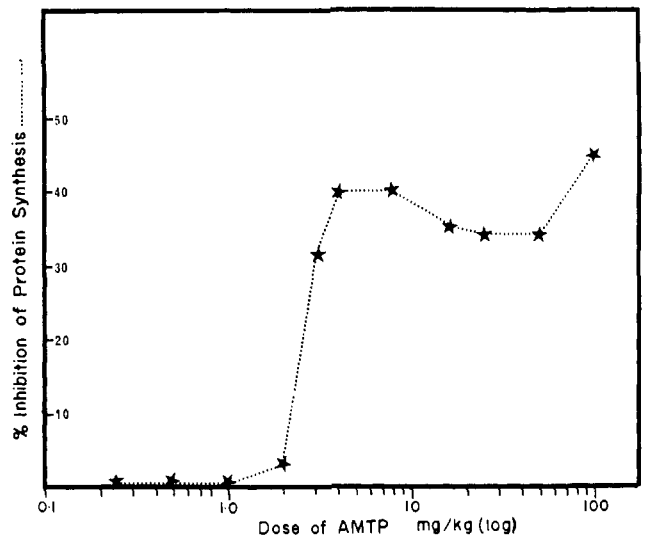

FIGURE 1: Effect of different dosages of AMTP on the synthesis of protein. Animals received AMTP by intraperitoneal injection, were fasted overnight (16 hr), and then killed. Incorporation of [^{14}C]-leucine into protein *in vitro* was performed as outlined in Table I.

An estimate of the rate of amino acid activation in the cell sap of the liver was obtained by measuring the rate of exchange of [^{32}P]pyrophosphate with ATP by the procedure of Stulberg and Novelli (1962).

Polyribosomes were prepared and analyzed on sucrose gradients by the method of Kwan and Webb (1967). P_i was measured by the method of Sumner (1944). In some experiments, the free amino acid content of the postmitochondrial fraction of the liver was analyzed with a Beckman-Spinco automatic amino acid analyzer. For these experiments, a protein-free preparation of the postmitochondrial supernatant was prepared with sulfosalicylic acid.

Free tryptophan was determined in the blood in the presence of AMTP by a recently developed enzymic technique involving the action of a crude preparation of *E. coli* tryptophanase on tryptophan (Sourkes *et al.*, 1970). AMTP does not interfere with the enzymatic degradation of tryptophan to indole, pyruvic acid, and ammonia, nor does it act as a substrate for the enzyme. The indole is extracted and estimated colorimetrically.

Oxidation of tryptophan labeled in the 2 position of the pyrrole ring was followed *in vivo* as previously described (Moran and Sourkes, 1963; Oravec and Sourkes, 1968a). The formation of [^{14}C]CO_2 from injected [2-^{14}C]tryptophan (labeled in the pyrrole ring) gives an indication of the activity of tryptophan pyrrolase in the liver of the animal (Knox, 1967; Yamaguchi *et al.*, 1967; Madras and Sourkes, 1968). This does not hold for tryptophan labeled in the side chain (Kim and Miller, 1969). In some experiments, the rate of oxidation of uniformly labeled leucine to [^{14}C]CO_2 was determined after administration of AMTP.

Results

Effect of AMTP on the Synthesis of Protein. The data of Table I depict the influence of AMTP on the synthesis of liver and brain protein, as measured *in vitro*. Treatment of

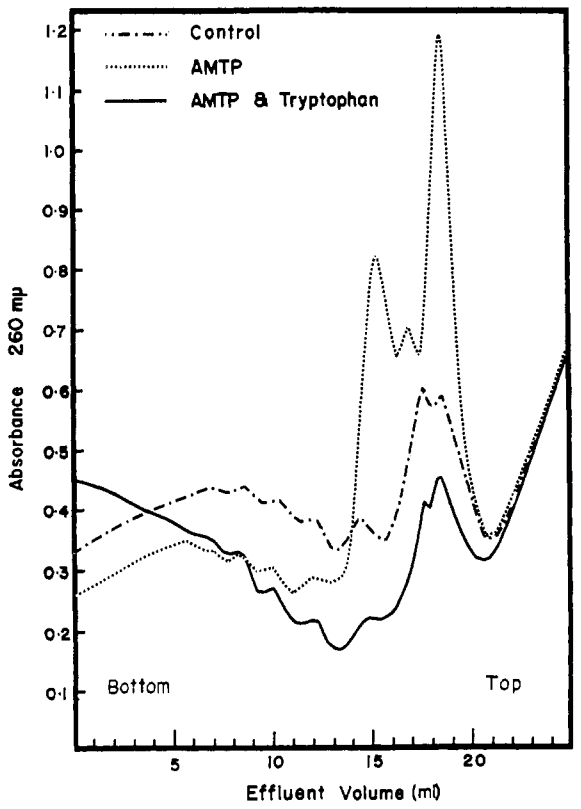

FIGURE 2: Sucrose gradient patterns of hepatic polyribosomes from rats. Rats received an intraperitoneal injection of 100 mg of AMTP/kg and were fasted overnight for 15 hr. At the end of this period, animals received either an intraperitoneal injection of 500 mg of L-Trp/kg or an injection of saline. They were killed 1 hr later. Control animals received saline injections at both times. The liver of the rat was removed under light ether anaesthesia, minced, and homogenized in 0.25 M sucrose buffer (1 g/2 ml) containing 0.05 M Tris (pH 7.5), 0.025 M KCl, and 0.005 M $MgCl_2$. A postmitochondrial supernatant fraction was obtained by centrifugation, treated with deoxycholate (0.7% final concentration), and finally analyzed on a sucrose gradient by the method of Kwan and Webb (1967). An amount of postmitochondrial fraction corresponding to 104 mg of liver was layered over 25 ml of a 10–40% exponential sucrose gradient and centrifuged for 4.5 hr at 63,000*g* in a SW25.1 rotor of a Beckman L-2 ultracentrifuge at 4°. Following centrifugation, the bottom of the tube was punctured and the size distribution of the polyribosomes was monitored by passing the effluent through a flow cell (1.0-cm light path) in a Beckman DB spectrophotometer recording at 260 mμ. A ferritin correction was not applied to the monomer peak in the patterns as relative, rather than absolute, concentrations were of interest. The small ribosomal aggregates are located toward the top of the gradient.

rats 16 hr before death with 100 mg of AMTP/kg depressed the rate of incorporation of [^{14}C]leucine into liver protein by 45%, and into brain protein by 19%. Substitution of [^{14}C]-isoleucine for [^{14}C]leucine in the cell-free amino acid incorporating system gave essentially the same results. The concentration of free leucine in the liver was 0.14 ± 0.01 μmole/g (mean ± std error, n = 5, individual rat livers) in control animals. Rats injected with 100 mg/kg of AMTP had a hepatic concentration of 0.15 ± 0.01 μmole/g. Thus, the decreased incorporation of [^{14}C]leucine into protein does not result from a change in the leucine content of the livers of rats receiving AMTP, nor can the decreased incorporation be explained by such a change.

TABLE II: Effect of AMTP and L-Tryptophan Given *in Vivo* on the Incorporation of [^{14}C]Leucine into Protein *in Vitro*.[a]

Treatment	n	[^{14}C]Leucine Incorpn: dpm into Protein/Sample	[^{14}C]Leucine Incorpn: % of Control
1. Control	2	15,982; 19,450	100
2. AMTP	2	8,979; 10,606	54
3. AMTP + tryptophan (ip, 1 hr)	4	22,795 ± 2,331[b]	129
4. Tryptophan (ip, 1 hr)	4	24,547 ± 2,074	138
5. AMTP + tryptophan (ip, 3 hr)	3	9,137 ± 317	52
6. Tryptophan (ip, 3 hr)	3	20,660 ± 3,432	117
7. AMTP + tryptophan (sc, 3 hr)	3	17,009 ± 1,128	97
8. Tryptophan (sc, 3 hr)	3	17,532 ± 356	99

[a] Rats received an intraperitoneal injection of 100 mg of AMTP/kg; they were fasted 16 hr (overnight) and then killed. Some rats received an intraperitoneal injection (ip) of 500 mg of L-tryptophan/kg 1 hr (15 hr after AMTP) or 3 hr (13 hr after AMTP) before death. In other cases, a subcutaneous (sc) injection of the same amount of tryptophan was given 3 hr before death. The incorporation of [^{14}C]leucine into protein was performed as in Table I. [b] Mean ± std error.

Treatment of rats with AMTP for 16 hr did not alter the liver and brain weights of these animals, nor did AMTP alter the yield of postmitochondrial protein per unit weight of fresh tissue. Thus, if the measures of incorporation in Table I were expressed per mg of protein or per total organ, the relation between control and experimental values would be unchanged.

The effect of AMTP on incorporation of [^{14}C]leucine into liver protein is dose dependent, as illustrated in Figure 1, with a very steep slope between doses of 2 and 4 mg per kg.

Effect of AMTP on Hepatic Polyribosomes. Figure 2 depicts the analysis of hepatic ribosomes on a sucrose gradient. Ribonucleoprotein particles prepared from livers of rats treated with AMTP contain a much higher proportion of mono-, di- and triribosomes, and a lower proportion of heavy ribosomal aggregates than those from control animals. These changes in the pattern are consistent with the observed decrease in the rate of protein synthesis (Table I) and the decreased ability of microsomes from AMTP-treated animals to support protein synthesis *in vitro* (Table III).

Reversal by Tryptophan of the Effect of AMTP on Protein Synthesis and Polyribosomal Integrity. Because AMTP diminishes the level of free tryptophan in the liver, blood, and brain (Sourkes *et al.*, 1970), it was of interest to determine what effects tryptophan administration might have on the synthesis of protein in AMTP-treated rats. Treatment of rats with 100 mg of AMTP/kg 16 hr before sacrifice depressed the rate of [^{14}C]leucine incorporation into protein (Table I, and first two lines of Table II), but when the animals received 500 mg of L-tryptophan intraperitoneally 1 hr prior to sacrifice (*i.e.*, 15 hr after AMTP), there was no decrease in the rate of [^{14}C]leucine incorporation into protein (Table II, line 3).

TABLE III: Effect of AMTP on the Incorporation of [^{14}C]Leucine into Protein *in Vitro* by a System Containing Isolated Microsomes and Cell Sap.[a]

Source of:		[^{14}C]Leucine Incorpn			
		Expt I		Expt II	
Cell Sap[b]	Microsomes[b]	dpm/mg of Microsomal Protein	% of Control	dpm/mg of Microsomal Protein	% of Control
Normal	Normal	16,358	100	18,691	100
AMTP	AMTP	11,077	68	14,547	78
Normal	AMTP	14,972	92	15,871	85
AMTP	Normal	11,891	73	13,935	75
AMTP + Trp[c]	Normal	11,327	69	10,765	58

[a] Animals were treated with 100 mg of AMTP/kg, fasted for 16 hr (overnight), and then killed. The conditions for assaying the incorporation of [^{14}C]leucine into protein were the same as in Table I except that in place of the postmitochondrial supernatant fraction, microsomes corresponding to 1.3 mg of protein and cell sap corresponding to 2.5 mg of protein were employed. Preliminary experiments established that the amount of cell sap was in excess; hence only the microsomes were rate limiting. [b] The sources were livers of saline-injected (normal) rats or of AMTP-injected rats. [c] Tryptophan (5 μmoles) added to the incubation medium.

In fact, the rate of incorporation increased somewhat above the control values. However, when AMTP-treated rats received the same amount of tryptophan intraperitoneally 3 hr prior to sacrifice, the inhibitory effect of the analog was not alleviated (Table II, line 5), despite the fact that intraperitoneal injection of tryptophan alone 3 hr prior to death stimulated the incorporation of [^{14}C]leucine into protein (Table II, line 6). The failure of tryptophan administered with a 3-hr delay to prevent the action of AMTP is presumably due to the elevated level of tryptophan pyrrolase activity in these animals; the increased enzyme activity promotes a rapid breakdown of the administered tryptophan, so that by 3 hr relatively little of the injected tryptophan is available in the liver to oppose the action of AMTP on the synthesis of protein. In similar experiments rats treated with AMTP were given an injection of L-tryptophan (500 mg/kg) by the subcutaneous route 13 hr later, with excision of the liver at the 16th hr for measurement of [^{14}C]leucine incorporation. Administration of tryptophan in this manner now prevented the depression in [^{14}C]leucine incorporation caused by AMTP (Table II, line 7). Subcutaneous injection probably affords a slower flow of tryptophan to the liver, but with maintenance of tissue concentration sufficiently high to reverse the action of AMTP.

Addition of L-tryptophan to an amino acid incorporating system prepared from the livers of AMTP-treated rats did not restore the rate of [^{14}C]leucine incorporation to normal. In other experiments substitution of a complete amino acid mixture for the tryptophan in the cell-free system also failed to overcome the inhibition of [^{14}C]leucine incorporation into protein.

The effect of tryptophan on the integrity of hepatic polyribosomes of AMTP-treated animals was also studied (Figure 2). The polyribosomal disaggregation caused by AMTP was not observed when L-tryptophan (500 mg/kg) was administered to these animals 1 hr before they were killed. This is evident from the large diminution in the mono- and diribosomes, and relative increases in the heavy ribosomal aggregates (Figure 2).

In a recent report, Sidransky *et al.* (1968) have shown that tryptophan administered to adrenalectomized mice stimulates protein synthesis and promotes the aggregation of polyribosomes. Thus, the apparent reversal by tryptophan of the AMTP-mediated inhibition of [^{14}C]leucine incorporation into protein and polyribosomal disaggregation (Table II and Figure 2) cannot be attributed to increased glucocorticoid secretion caused by administration of tryptophan.

Mode of Action of AMTP on the Inhibition of Protein Synthesis. AMTP was tested for its ability to interfere directly with the incorporation of [^{14}C]leucine into protein in a cell-free amino acid incorporating system prepared from the livers of normal fasted animals. At a wide variety of concentrations (0.21–6.66 mM) AMTP had no effect. Thus, it is unlikely that this analog interferes directly with tryptophan in the mechanism of protein synthesis.

In an attempt to localize the site of action of AMTP on the synthesis of protein the postmitochondrial supernatant, as used in previous incorporation studies, was further fractionated into microsomes and cell sap. Various combinations of these fractions from normal and AMTP-treated rats were tested (Table III). In all experiments sufficient cell sap was used so that the microsomes were rate limiting in amount. In two separate experiments, a combination of microsomes and cell sap from AMTP-treated animals incorporated 32 and 22% less [^{14}C]leucine, respectively, than did a similar combination from normal animals (Table III, line 2). A combination of normal cell sap and AMTP microsomes incorporated 8 and 15% less [^{14}C]leucine, respectively, than did the control preparation in the two experiments (Table III, line 3). However, a combination of AMTP cell sap and normal microsomes was as inefficient in incorporating [^{14}C]leucine as was the AMTP cell sap and AMTP microsome combination (Table III, line 4). These results suggest that AMTP treatment results in a defect in both the cell sap and the microsomes.

TABLE IV: Effect of AMTP on the Activation of Amino Acids.[a]

Treatment of Rats	Addn to Incubn Medium	n	% Exchange/ Sample	Rate of Exchange (μmoles/hr)
Control		5	21.9 ± 0.9[b]	3.7 ± 0.1
AMTP		5	23.9 ± 1.3	4.0 ± 0.2[d]
Control	*c*	5	30.3 ± 0.7	5.4 ± 0.1
AMTP	*c*	5	34.8 ± 1.7	6.4 ± 0.3
Control	AMTP	4	19.3 ± 1.9	3.2 ± 0.3

[a] Animals received 100 mg of AMTP/kg or saline and were killed 16 hr later. The livers were removed and homogenized in 0.05 M KCl (1 g/ml of medium). The cell sap was obtained by centrifuging the homogenate at 105,000*g* for 60 min, and was assayed for its ability to activate amino acids. The incubation medium (final volume of 1.0 ml) contained 100 μmoles of Tris (pH 7.5), 5 μmoles of ATP, 5 μmoles of sodium pyrophosphate, a trace amount of [^{32}P]pyrophosphate containing 200,000–300,000 cpm, 5 μmoles $MgCl_2 \cdot 6H_2O$, 50 μmoles KF, and in some cases 2 μmoles of each of the natural L-amino acids or 1 μmole of AMTP. After a short preincubation, the reaction was initiated by the addition of 0.4 ml of the high-speed supernatant and 10 min later the reaction was terminated by the addition of 3.0 ml of 10% trichloroacetic acid. Labeled ATP was isolated from the supernatant by charcoal adsorption (acid-washed Norit A) and estimated by the method of Stulberg and Novelli (1962). The per cent exchange and rate of exchange in micromoles per hour were also calculated as outlined by Stulberg and Novelli (1962). [b] Mean ± std error. [c] Complete amino acid mixture. [d] Not significantly different from control, $P > 0.05$.

TABLE V: Effect of AMTP on the Incorporation of Label from [^{14}C]Leu-tRNA or [^{14}C]Leucine into Protein *in Vitro*.[a]

Treatment	Experiment I: dpm Incorpd from [^{14}C]Leu-tRNA into Protein/Sample	Experiment I: % of Control	Experiment II: dpm Incorpd from [^{14}C]-Leu into Protein/ Sample	Experiment II: % of Control
Control	25,247 ± 855[b]	100	13,702 ± 936[b]	100
AMTP	21,874 ± 465[c]	86	10,140 ± 676[c]	74

[a] The animals received 100 mg of AMTP/kg, were fasted overnight, and then killed. The components and conditions of the incubation were the same as described for the incorporation of [^{14}C]leucine into protein (Table I) except that in some experiments approximately 50,000 dpm of [^{14}C]-Leu-tRNA was added in place of the [^{14}C]leucine. [b] Mean ± std error (n = 4). [c] Difference from control is significant, $P < 0.02$.

Supplementation of the AMTP cell sap and normal microsome combination with tryptophan did not overcome the inhibition of [^{14}C]leucine incorporation (Table III, line 5).

Because of the apparent defect in the cell sap it was of interest to measure the rate of activation of amino acids there. The data of Table IV indicate that this process was not affected by pretreatment of the animals with AMTP. When a complete amino acid mixture was included in the incubation medium, the rate of [$^{32}P_i$ $^{32}P_i$]ATP–pyrophosphate exchange in the control and experimental preparations both increased to the same extent (Table IV, lines 3 and 4). Addition of a relatively large amount of AMTP (1 μmole) to the incubation medium containing normal cell sap did not significantly alter the rate of exchange (Table IV, line 5). This suggests that AMTP is not activated by the tryptophan-activating enzyme.

Allen *et al.* (1969) have shown that force feeding of rats with amino acid mixtures deficient in tryptophan lowered the charging level of tryptophan (*i.e.*, Trp-tRNA), but not that of the other amino acids. Because the level of free tryptophan is diminished in the livers of rats receiving AMTP (Sourkes *et al.*, 1970), it is reasonable to assume that the level of Trp-tRNA is diminished in this situation also. Such a change would not be detected by the ATP–pyrophosphate-exchange assay, as this procedure gives a measure of the overall rate of activation of all the amino acids together. Studies are in progress to determine whether AMTP might alter the activity of the Trp-tRNA synthetase itself.

The data of Table V (expt 1) show that treatment of rats with AMTP decreases the incorporation of radioactivity from [^{14}C]Leu-tRNA into protein in the standard cell-free amino acid incorporating system. The inhibition was significant but, nevertheless, not as great as the 26% inhibition of [^{14}C]-leucine incorporation into protein observed in a parallel experiment (Table V, expt 2). The reason for this discrepancy is not clear. These results indicate that in AMTP-treated animals there may be a defect in one or both of the transfer factors (AA-tRNA binding enzyme and translocase) or in the ribosome-associated peptidyl transferase. Experiments are under way to isolate the soluble cell sap factors from the livers of normal and AMTP-treated rats and to compare activities.

Time Course of Action of AMTP. During the first 20 min of its action *in vivo*, AMTP appeared to stimulate the incorporation of [^{14}C]leucine into protein (Figure 3). However, by 40 min the rate of incorporation had decreased below control values; the inhibition developed steadily for a further 20 min, and changed little thereafter, at least up to 16 hr. The initial stimulation of incorporation of [^{14}C]leucine is difficult to explain; perhaps it reflects a stimulation of the synthesis of protein by amino acids liberated from some labile pool of liver protein.

In Figure 3 the changes in blood tryptophan concentrations following administration of AMTP are shown. By 15–20 min after injection of AMTP, the level of tryptophan begins to fall and continues to do so for at least 30 min more, after which time no further change is observed. This event seems to parallel the inhibition of protein synthesis (Figure 3). It is conceivable that these changes in blood tryptophan levels reflect alterations in the typtophan level and tryptophan pyrrolase activity of the liver. It has already been shown that AMTP lowers the level of tryptophan in the liver (Sourkes *et al.*, 1970).

Changes in the rate of oxidation of [^{14}C]tryptophan (labeled in the 2 position of the pyrrole ring) *in vivo* are also depicted in Figure 3. In order to minimize problems of absorption of the amino acid, the labeled tryptophan was injected 30 min before the AMTP. An increase in the rate of oxidation occurs as early as 5 min after injection of AMTP, presumably as a result of the AMTP-induced stimulation of pyrrolase activity of the liver. This phenomenon clearly precedes both the inhibition of protein synthesis and the fall in blood tryptophan in these animals (Figure 3). In contrast to this, the rate of oxidation of leucine is increased above control levels only 5 hr after the injection of AMTP, and this stimulation persists for many hours (M. Oravec, unpublished work). Probably the oxidation of other amino acids is also stimulated at this time.

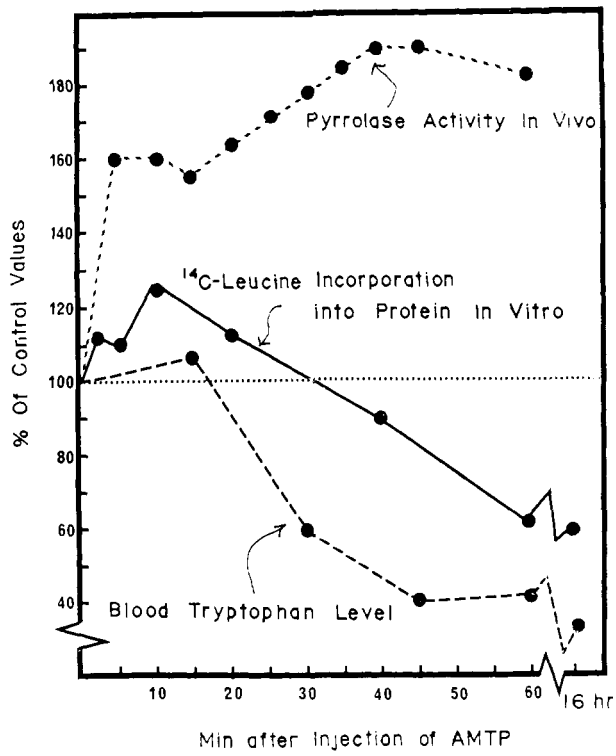

FIGURE 3: Time course of action of AMTP on the synthesis of protein and catabolism of tryptophan. Fasted animals received 100 mg of AMTP/kg and were killed at varying times thereafter, as indicated by the abscissa of the graph. The livers were quickly removed and homogenized and a postmitochondrial fraction was prepared and then assayed for its ability to incorporate [^{14}C]leucine into protein as described in Figure 1. For the estimation of tryptophan, 2 ml of blood was diluted 2-fold with 0.14 M KCl and then heated in a boiling-water bath for 2 min. The precipitated protein was centrifuged down and the supernatant was analyzed for the presence of tryptophan (Sourkes *et al.*, 1970). The reaction mixture contained 1.0 ml of 0.3 M phosphate buffer (pH 8.3), 1.0 ml of tryptophanase solution (1 mg of crude enzyme/ml of 1.6 M KCl), and 0.1 ml of pyridoxal phosphate (30 μg). After a brief preincubation at 37°, the reaction was initiated by the addition of the supernatant obtained after heating the blood. At the end of 1.5 hr, termination was achieved by the addition of 5 ml of 10% trichloroacetic acid and the protein was sedimented by centrifugation. The indole was extracted from the supernatant and estimated colorimetrically (Sourkes *et al.*, 1970). The concentration of tryptophan in the blood of control rats was 1.0 mg/100 ml of whole blood. For the measurement of tryptophan pyrrolase activity *in vivo*, the rate of liberation of [^{14}C]carbon dioxide from [2-^{14}C]tryptophan (label in 2 position of pyrrole ring) was taken as an index of this measurement. Fasted animals received by injection 0.5 μCi of [2-^{14}C]tryptophan. Thirty minutes later they received an intraperitoneal injection of 100 mg of AMTP/kg and were placed immediately into separate metabolic cages. The collection of [^{14}C]CO_2 and estimation of radioactivity were performed as previously described (Moran and Sourkes, 1963; Oravec and Sourkes, 1968a). Each point is the average of two or three experiments.

Discussion

The experiments described in this paper were designed to determine whether the stimulation of hepatic glyconeogenesis and of amino acid catabolism (Oravec and Sourkes, 1967, 1968a,b) in the rat by AMTP can be related to the stimulation of the activity of hepatic tryptophan pyrrolase evoked by this compound. The data presented in this paper and other recent findings in this laboratory (Oravec and Sourkes, 1967; Sourkes *et al.*, 1970) support such a relationship. Thus, (1) within 5 min of administration of AMTP to rats, the degradation of tryptophan *in vivo* is enhanced (Figure 3), a phenomenon that has been attributed to increased activity of tryptophan pyrrolase in liver (Yamaguchi *et al.*, 1967; Madras and Sourkes, 1968). This effect probably accounts for the decreased level of tryptophan in blood which develops 20–30 min after injection of the α-methylamino acid (Figure 3). AMTP treatment also lowers the level of free tryptophan in the brain and liver of rats (Sourkes *et al.*, 1970). (2) Stimulation of pyrrolase activity clearly precedes the inhibition of protein synthesis in rats receiving AMTP (Figure 3); the rate of incorporation of [^{14}C]leucine into protein is diminished below control values only 30–40 min after injection of the α-methylamino acid. The lag between the stimulation of pyrrolase activity and inhibition of [^{14}C]leucine incorporation presumably represents the time for the concentration of tryptophan to diminish to the level where the synthesis of protein is jeopardized; the level of tryptophan in tissues is normally low (Munro, 1968) relative to other amino acids and, hence, any substantial reduction in its concentration could render the amino acid rate limiting for the synthesis of protein. In this work only liver was examined, but it is conceivable that AMTP has the same effects on the synthesis of protein in other tissues as well. (3) Approximately 5 hr after the injection of AMTP the oxidation of amino acids is enhanced significantly. Paralleling this oxidative stimulation, there is increased synthesis of glycogen in the liver (Oravec and Sourkes, 1967). Thus, amino acid catabolism and glyconeogenesis are stimulated only many hours after the changes in the rate of protein synthesis and in pyrrolase activity have commenced. AMTP also causes a weight loss of approximately 10% (Sankoff and Sourkes, 1962) during the first 24 hr after its administration; probably loss of body protein contributes to this, the constituent amino acids providing carbon skeletons for glyconeogenesis at this later time. There are other results that lend support to the proposed mode of action of AMTP. (4) AMTP, acting *in vitro*, does not inhibit the incorporation of [^{14}C]leucine into protein. Hence, the analog does not interfere directly with any of the steps in the synthesis of protein. AMTP is without effect in this situation presumably because it cannot stimulate tryptophan pyrrolase activity *in vitro* (Sourkes and Townsend, 1955; Civen and Knox, 1960). Also, AMTP does not appear to be activated (Table IV), and would then not be incorporated into protein. (5) The polyribosomal disaggregation and inhibition of protein synthesis mediated by AMTP can be reversed by administration of tryptophan to the animals

(Table II and Figure 3). The effect of tryptophan is short-lived, for within 3 hr of its intraperitoneal injection into AMTP-treated rats, inhibition of [^{14}C]leucine incorporation into protein is evident. It is possible that the elevated pyrrolase activity facilitates the catabolism of the administered tryptophan to such an extent during the 3 hr that at the end of this time insufficient amino acid remains to overcome the action of AMTP. On the other hand, reversal occurs within 1 hr of an intraperitoneal injection of tryptophan or even within 3 hr if the amino acid is given subcutaneously (Table II), *i.e.*, by a route providing slower absorption than from the peritoneal cavity. These experiments support the involvement of pyrrolase and of a tryptophan deficit in the action of AMTP.

Recent studies suggest that tryptophan plays a special role in the regulation of protein synthesis in rat and mouse livers (Sidransky *et al.*, 1967; 1968; Pronczuk *et al.*, 1968). For example, force-feeding rats an amino acid mixture devoid of tryptophan decreases the protein-biosynthetic capacity of the liver and this is accompanied by a disaggregation of hepatic polyribosomes (Fleck *et al.*, 1965; Wunner *et al.*, 1966). Amino acid mixtures containing tryptophan but deficient in other amino acids do not cause these changes (Pronczuk *et al.*, 1968). The inhibition of protein synthesis (Table I) and disaggregation of polyribosomes (Figure 2) in the livers of animals treated with AMTP clearly resemble the effects on these parameters of force feeding a tryptophan-deficient amino acid mixture.

The effects of tryptophan deficiency in rats are effectively reversed by feeding the animals an amino acid mixture containing tryptophan (Wunner *et al.*, 1966). Related to this is the finding by Sidransky *et al.* (1967, 1968) that fasted mice, force-fed tryptophan alone or with an amino acid mixture which is complete or incomplete with respect to other amino acids, exhibit an increased rate of protein synthesis and greater polyribosomal aggregation in the liver; other amino acids do not mimic this effect. Similarly, the effects of AMTP are reversed by the administration of tryptophan (Table II and Figure 2).

As to the manner whereby AMTP inhibits the synthesis of protein and causes polyribosomal disaggregation, it is possible that these changes are the consequence of the deficit of tryptophan caused by AMTP (Sourkes *et al.*, 1970). In the normal whole animal, tryptophan is probably rate limiting for protein synthesis and polyribosomal aggregation because it is the least abundant amino acid in the free amino acid pool (Munro, 1968). Hence, diminution of the tryptophan level by AMTP could result in insufficient Trp-tRNA at the sites of protein synthesis on the ribosome. It has recently been shown by Allen *et al.* (1969) that the level of Trp-tRNA is actually diminished in rats force-fed a complete amino acid mixture devoid of tryptophan. In the liver of the intact animal it does not seem possible to reduce the intracellular concentration of amino acids to the point at which they become more rate limiting in protein synthesis than tryptophan (Munro, 1968). In a cell-free system where all endogenous amino acids had been removed (Baliga *et al.*, 1968), reaggregation of ribosomes to form a polyribosome structure could be observed if a complete amino acid mixture was added to the incubation medium. However, omission of tryptophan or any one of a number of other amino acids from the complete amino acid mixture prevented polyribosome formation. Thus in this situation, a number of amino acids were equally rate limiting for polyribosome formation.

Tryptophan, when added directly to a cell-free amino acid incorporating system containing cell sap prepared from AMTP-treated rats (Table IV, line 5), failed to prevent the inhibition of leucine incorporation. This could mean that the depression of protein synthesis caused by AMTP is accompanied by a change in one or more of the factors or enzymes involved in synthesizing protein. Because of the apparent defect in the cell sap (Table III), further studies are in progress to isolate the soluble cell sap factors (binding enzyme and translocase) and to determine whether AMTP treatment of rats causes changes in activity.

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