Effect of Hypobaric Stress on Enzymes of Tryptophan Metabolism

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1. On exposure of rats to hypobaric stress the tryptophan pyrrolase and tyrosine aminotransferase activities of the liver increased about threefold in 4 h. 2. The tryptophan hydroxylase activity increased about 50% on exposure for 24 h or more. 3. The increased activities reverted to the basal value on removal of the stress. 4. Treatment with cycloheximide inhibited the increase in the enzyme activities when the time of exposure was short (4 h). However, the inhibitor-treated animals showed paradoxically high tyrosine aminotransferase activity on prolonged exposure (24 h). 5. The pattern of haematin saturation indicated that the increase in pyrrolase activity under low pressure resembled that obtained with cortisol and not with tryptophan. 6. Repeated administration of cortisol or tryptophan did not have any effect on the activity of tryptophan hydroxylase. 7. The stress-induced increase in hydroxylase activity was not eliminated by the prior administration of 5-hydroxytryptophan to the animals.

Human and animal behaviour is known to change on exposure to low atmospheric pressure (Folk, 1966). Since one of the important factors involved in the regulation of the mental state, namely 5-hydroxytryptamine (serotonin), is derived from tryptophan, the metabolism of this amino acid under conditions of stress assumes importance. Excluding protein synthesis, the major pathway for the metabolism of tryptophan involves the enzyme tryptophan pyrrolase (L-tryptophan-oxygen oxidoreductase, EC 1.13.1.12). Lovenberg *et al.* (1967) found that tryptophan hydroxylase is the rate-limiting enzyme in the conversion of the amino acid into serotonin. Tryptophan hydroxylase activity is present both in the liver and in the brain whereas the pyrrolase activity is confined to the liver. However, the concentration of brain serotonin has been found to bear an inverse relationship to hepatic pyrrolase activity (Green & Curzon, 1968).

Since both tryptophan pyrrolase and tryptophan hydroxylase are oxygen-dependent enzymes, the exposure of animals to low atmospheric pressure and consequent hypoxia may be expected to change the activities of the two enzymes. The results presented in the present paper show that both the enzymes undergo adaptive changes under hypobaric stress. A preliminary report of some of these findings has been published (Inamdar *et al.*, 1970).

Materials and Methods

Animals and exposure to low pressure

Male albino rats weighing 250–300 g from the stock colony of this Institute were exposed to a pressure of 0.05 MN/m² (corresponding to an altitude

of about 6100 m) in a decompression chamber. The flow of air inside the chamber was maintained by evacuation from one side and a leak on the other. Low pressure was obtained in the chamber in less than 3 min after the start of evacuation and was maintained at a constant value (Ramasarma, 1965).

Preparation of cytosol

At the end of the experimental period, atmospheric pressure was restored in the chamber and the animals were removed and killed by stunning and decapitation. The livers were removed, chilled in ice and then homogenized with 1% (w/v) KCl (3 ml/g of liver) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged in a Servall RC 2 refrigerated centrifuge at 20000g for 35 min and the supernatant fraction was used as the source of enzymes.

Assay of enzymes

Tryptophan pyrrolase activity was assayed essentially as described by Knox & Auerbach (1955). The reaction mixture contained 200 μ mol of potassium phosphate buffer, pH 7.0, 10 μ mol of L-tryptophan and about 30 mg of the supernatant protein in a total reaction volume of 4 ml. Haematin (50 μ g) was added except where indicated. The reaction mixture was incubated in a metabolic shaker at 37°C and the reaction was initiated by the addition of the enzyme. After 20 min, 1 ml of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation the clear supernatant solution was collected, neutralized with 1 M-NaOH and the concentration of

kynurenine formed was calculated from the E_{365} by using a molar extinction coefficient of 4540 (1 mol/litre, 1 cm light-path).

Tryptophan hydroxylase activity was assayed by the method of Freedland *et al.* (1961) with some modifications. The reaction mixture contained 60 μ mol of potassium phosphate buffer, pH 6.7, 840 nmol of NAD, 10 μ mol of nicotinamide, 2 μ mol of FeSO_4 , 1.16 μ mol of Kaufman's cofactor (6,7-dimethyl-5,6,7,8-tetrahydropterine; Kaufman & Levenberg, 1959), 40 μ mol of L-tryptophan and about 50 mg of the supernatant protein in a total reaction volume of 2.5 ml. The reaction mixture was incubated in a metabolic shaker at 37°C for 60 min after the initiation of the reaction by the addition of the enzyme. The reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. After centrifugation, the concentration of 5-hydroxytryptophan in the supernatant liquid was measured colorimetrically by diazotization with sodium nitrite and coupling with 1-nitroso-2-naphthol as described by Udenfriend *et al.* (1955). The E_{540} was read in a Beckman DB spectrophotometer.

Tyrosine aminotransferase (L-tyrosine-2-oxoglutarate aminotransferase, EC 2.6.1.5) was assayed as described by Lin *et al.* (1958). The reaction system

contained 200 mmol of potassium phosphate buffer, pH 7.4, 120 nmol of pyridoxal phosphate, 7.6 μ mol of sodium diethyldithiocarbamate, 20 μ mol of L-tyrosine, 20 μ mol of 2-oxoglutarate and about 5 mg of the supernatant protein in a total reaction volume of 2 ml. The reaction was started by the addition of the protein and was continued for 5 min at 37°C. The reaction was then stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid and, after centrifugation, the concentration of *p*-hydroxyphenylpyruvate was determined by noting the E_{310} after appropriate dilution and using a standard curve for an authentic sample of the chemical.

In all experiments six rats were used unless otherwise stated. At each time two experimental and two control animals (kept at atmospheric pressure outside the chamber) were killed. The animals were killed about 10 a.m. to avoid changes due to circadian variations in the activity of the enzymes. In spite of these precautions substantial variations in the enzyme activities were sometimes encountered within the animals of the same group (including controls) killed on different days. This was particularly so in the case of tyrosine aminotransferase. The standard deviations in Fig. 1 and Table 1 are large in some cases because of this. However, within the group killed at

Fig. 1. Effect of exposure to low pressure on the activities of tryptophan pyrrolase and tryptophan hydroxylase in rat liver

Animals were exposed to a pressure of 0.5 atm (0.05 MN/m²) for the time indicated. The methods of enzyme assay are described in the Materials and Methods section. The points indicate the mean of six independent determinations (animals) $\pm$ S.D. *P* values are calculated by the *t* test and are indicated in the figure; N.S., not significant. Tryptophan pyrrolase: ○, in control animals; ●, in exposed animals. Tryptophan hydroxylase: △, in control animals; ▲, in exposed animals.

the same time the differences between controls and experimental animals were always consistent and reproducible.

Results

Effect of exposure to low pressure on tryptophan pyrrolase and hydroxylase

The changes in the activities of hepatic tryptophan pyrrolase and tryptophan hydroxylase when rats were exposed to hypobaric conditions (0.05 MN/m², 0.5 atm) are shown in Fig. 1. The pyrrolase activity increased by 49% after 2 h exposure. On continued exposure the activity increased two- to three-fold and remained at this high value even after 50 h of exposure. In contrast, the hydroxylase activity showed no significant change up to 12 h of exposure. However, it increased by 50% on exposure for 24 h or more. The activity of hydroxytryptophan decarboxylase (5-hydroxy-L-tryptophan carboxylase, EC 4.1.1.28) was not affected by exposure for up to 50 h (Inamdar *et al.*, 1970).

Reversibility of the increase in enzyme activity

To determine whether the increased pyrrolase activity reverts to the normal value on removal of the stress, rats were first exposed to low pressure and

then restored to atmospheric pressure. The results in Fig. 2 indicate that the pyrrolase activity decreased rapidly on the withdrawal of the animals from the stress and reverted to the basal value in due course.

It is noteworthy that liver tyrosine aminotransferase, which like tryptophan pyrrolase shows circadian oscillations in activity (Fuller, 1970), has a short half-life and is inducible by hormones (Schimke & Doyle, 1970), also increased two- to three-fold on subjecting the animal to hypobaric stress (Fig. 2). This activity also decreased rapidly on withdrawal of stress and in due course reverted to the basal value (Fig. 2). The rate of decrease of the enzyme activity in both cases ($t_{1/2}$, 3 h) agrees well with the values quoted by Schimke & Doyle (1970).

Effect of cycloheximide on the increase in enzyme activity

To determine whether the increase in enzyme activity on exposure of the animal to low pressure reflects an increase in the synthesis of the enzyme protein, the effect of cycloheximide, which is known to inhibit protein synthesis in the intact animal (Young *et al.*, 1963), was tested. The results in Table 1 indicate that the pyrrolase and transaminase activities did not show the response when the inhibitor-treated animals were exposed to the stress,

Fig. 2. Effect of re-exposure to atmospheric pressure on the activities of liver tryptophan pyrrolase (●) and tyrosine aminotransferase (○)

Rats were exposed to low pressure (0.5 atm, 0.05 MN/m²) for 12 h and restored to atmospheric pressure for the time indicated. Other details are given in the Materials and Methods section.

suggesting thereby that the increase in activity was due to stimulation of protein synthesis. However, when cycloheximide-injected rats were exposed to low pressure for longer periods of time (24h) the increase in enzyme activity was not inhibited by the antibiotic. This is in agreement with reports that the inhibitory effect exerted by the antibiotic is reversible and does not last for more than a few hours in the intact animal (Young *et al.*, 1963). Cycloheximide at a dosage of 200 µg/rat effectively inhibited the stress-induced increase in enzyme synthesis when the duration of exposure was short (4h). Dosages in excess of 500 µg of the antibiotic/rat were found to be lethal.

Synthesis of apo- and holo-tryptophan pyrrolase under hypobaric stress

Exposure of animals to low atmospheric pressure even for short periods of time resulted in considerable increases in the activities of liver tryptophan pyrrolase and tyrosine transaminase (Fig. 1 and Table 1).

Haematin, which is the prosthetic group of tryptophan pyrrolase, has been shown to be easily dissociable from the enzyme and to remain associated with the microsomal fraction of the liver (Greengard & Fiegelson, 1960; Fiegelson & Greengard, 1961a). A 'double assay' of the activity with and without externally added haematin would therefore provide a measure of the degree of saturation of the enzyme

Table 1. *Effect of cycloheximide on the increase in hepatic enzyme activity on exposure to low pressure*

Rats weighing 270–300 g were injected with cycloheximide (500 µg/rat in 0.5 ml of 0.9% NaCl) intraperitoneally, kept at atmospheric pressure for 30 min, transferred to the decompression chamber (0.5 atm, 0.05 MN/m²) and kept for the period indicated. Control animals were injected with 0.9% NaCl and kept at atmospheric pressure. Other experimental details were as given in the Materials and Methods section. The values given are the means of the enzyme activities (µmol of product formed/h per g fresh wt. of liver) ± S.D. for four animals. Two animals in each group were killed at a time.

Time of exposure (h)	Treatment	Tryptophan pyrrolase		Tyrosine aminotransferase		Tryptophan hydroxylase	
		Control	Low pressure	Control	Low pressure	Control	Low pressure
4	NaCl	3.4 ± 0.4	10.0 ± 2.7	93 ± 23	182 ± 3	—	—
	Cycloheximide	1.6 ± 0.2	2.4 ± 0.2	86 ± 18	78 ± 18	—	—
24	NaCl	3.7 ± 0.2	9.4 ± 3.9	45 ± 6	129 ± 52	0.57 ± 0.16	1.00 ± 0.23
	Cycloheximide	2.8 ± 0.2	8.0 ± 1.8	33 ± 10	260 ± 80	—	1.10 ± 0.22

Table 2. *Effect of administration of cortisol and tryptophan on the activity of liver enzymes*

In Expt. 1 the animals were dosed with aqueous suspensions of cortisol (3 mg/rat) or tryptophan (120 mg/rat) intraperitoneally and killed 4 h later. In Expt. 2 the animals were injected with cortisol (3 mg/rat) and tryptophan (120 mg/rat) intraperitoneally four times in 24 h and then killed. Other details were as given in the Materials and Methods section. The enzyme activities are expressed as µmol of product formed/h per g fresh wt. of liver.

Expt.	Treatment	Tryptophan pyrrolase			Tryptophan hydroxylase	Tyrosine aminotransferase
		Without haematin	With haematin	Holoenzyme (% of total activity)		
1	0.9% NaCl	0.15 ± 0.07	3.4 ± 0.5	5	0.72 ± 0.11	50 ± 12
	Cortisol	1.9 ± 1.0	10.8 ± 3.7	17	0.72 ± 0.02	147 ± 11
	Tryptophan	6.4 ± 1.8	14.1 ± 0.0	46	0.80 ± 0.10	212 ± 16
2	0.9% NaCl	0.13 ± 0.05	2.9 ± 0.4	4	0.64 ± 0.06	51 ± 2
	Cortisol	0.63 ± 0.2	8.4 ± 3.4	8	0.64 ± 0.10	158 ± 12
	Tryptophan	16.0 ± 2.0	45.8 ± 5.5	35	0.53 ± 0.11	—
	Cortisol + tryptophan	27.8 ± 5.0	39.0 ± 5.0	71	0.50 ± 0.08	119 ± 2

with the cofactor (Fiegelson & Greengard, 1961*b*). By this technique it was shown that glucocorticoid-induced enzyme increase was confined almost exclusively to the apoprotein whereas tryptophan induced the formation of the holoenzyme (Greengard & Fiegelson, 1961; Fiegelson *et al.*, 1962).

In normal animals holo-tryptophan pyrrolase represents only about 5% of the total pyrrolase activity (Table 2). However, in a few instances activities as high as 30% and as low as 0% have been encountered. The pattern remained essentially the same when cortisol was injected either once or repeatedly for 24 h indicating that the hormone-induced synthesis of the protein is largely confined to the apoprotein. On the other hand, when tryptophan was administered, the holoenzyme content increased substantially and about 40–50% of the total enzyme activity could be elicited without the addition of haematin. When both cortisol and tryptophan were injected together, the pattern resembled that obtained with the amino acid alone. The tyrosine aminotransferase activity increased under both treatments. However, the hydroxylase activity did not show any significant change with the administration of either the hormone or the amino acid.

The pattern of cofactor-saturation of tryptophan pyrrolase given in Table 3 resembles more the hormone-mediated enzyme increase than that obtained with the amino acid.

Effect of 5-hydroxytryptophan on the liver enzyme

On exposure of animals to hypobaric stress a rapid increase in the activity of tryptophan pyrrolase was followed by a slow increase in the activity of tryptophan hydroxylase (Fig. 1). Neither cortisol nor tryptophan showed any ability to induce the hydroxylase (Table 2). This suggested the possibility that the increase in hydroxylase activity on exposure to low pressure may be a secondary adaptive change to

compensate for the decrease in pool tryptophan, consequent on the increase in the pyrrolase activity, so as to maintain the tissue concentration of serotonin. If this were so, an artificial increase in the tissue concentration of 5-hydroxytryptophan resulting from the administration of the compound to the animal should abolish the stress-induced increase in the hydroxylase activity. The results of such an experiment (Table 4) show that the hydroxylase activity increased on exposure to low pressure even in animals in which the tissue concentration of hydroxytryptophan may not be limiting, assuming that the exogenous supply increased the concentration of the compound in the tissue for the duration of the experiment.

Discussion

The results presented in this paper show that both tryptophan pyrrolase and tyrosine aminotransferase in the liver of the rat increased rapidly when the animal was subjected to hypobaric stress. With mice as the experimental animal, Berry *et al.* (1968) found that the pyrrolase activity of the liver did not show any change when the animals were exposed for 17 h to simulated altitudes up to 6700 m. Exposure to lower pressures resulted in a twofold increase in the activity of the enzyme. Francesconi & Mager (1970), on the other hand, observed that exposure of mice to a simulated altitude of 5800 m for a few hours was enough to increase the enzyme activity substantially. The activity was maximum (two- to three-fold increase over the controls) after about 18 h of exposure. Prolonged exposure thereafter tended to decrease the activity of the enzyme in mice and after 30 h of exposure it reverted to the normal value. In contrast, enzyme activity in rats remained at a higher value even after 50 h of exposure. Continued whole-body irradiation of the rat increases the pyrrolase activity initially, which reverts to the normal value

Table 3. *Pattern of increase of liver tryptophan pyrrolase under hypobaric stress*

The animals were exposed to low pressure (0.5 atm, 0.05 MN/m²) for the time-period indicated. The enzyme activity is expressed as μ mol of kynurenine formed/h per g fresh wt. of liver. Other details are as given in the Materials and Methods section.

Treatment	Tryptophan pyrrolase activity		Holoenzyme (% of total activity)
	Without haematin	With haematin	
Exposed to low pressure for 4 h	2.1 $\pm$ 0.1	10.3 $\pm$ 1.0	22
Exposed to low pressure for 12 h	2.4 $\pm$ 0.1	13.6 $\pm$ 1.5	18
Exposed to low pressure for 24 h	1.9 $\pm$ 0.7	12.7 $\pm$ 0.9	15
Exposed to low pressure for 12 h and withdrawn to atmospheric pressure for 4 h	0.9 $\pm$ 0.1	4.7 $\pm$ 0.5	19

Table 4. *Effect of 5-hydroxytryptophan on the liver enzyme activity*

The enzyme activities are expressed in μmol of product formed/h per g fresh wt. of liver. Animals were exposed to low pressure (0.5 atm, 0.05 MN/m²) for 25 h without or after a single intraperitoneal injection of 5-hydroxytryptophan (35 mg/rat) in 1 ml of water as indicated. The values are the mean of two independent experiments.

Treatment	Tryptophan pyrrolase	Tryptophan hydroxylase	Tyrosine aminotransferase
None	4.0	0.62	82
Low pressure	10.3	0.99	117
5-Hydroxytryptophan + low pressure	6.9	1.12	392

by about 27 h of exposure. A further increase was observed after 72 h of irradiation (Popov & Ankov, 1970). These results would indicate that the reaction of the animal to the stress is dependent not only on the animal species but also on the nature of the stress.

When rats exposed to hypobaric stress were re-exposed to atmospheric pressure the enzyme activities readily reverted to the basal value (Fig. 2). In this respect hypobaric stress resembles whole-body irradiation (Thomson & Mikuta, 1954; Popov & Ankov, 1970).

A question that arises is whether the increase in the enzyme activities under hypobaric hypoxia is the effect of low pressure, low oxygen or both. Preliminary experiments in the laboratory indicated that hypoxia alone (that is, under conditions where the pressure is kept normal but the oxygen partial pressure is decreased to half) did not cause an increase in the pyrrolase activity even after 12 h exposure. This would indicate that the increase observed under hypobaric hypoxia is not an 'oxygen effect'.

The elegant technique of 'double assay' developed by Fiegelson & Greengard (1961b) has enabled us to obtain information on the probable mechanism by which the pyrrolase activity increases under hypobaric stress. The observation that increase in enzyme activity is a result of the increased synthesis of the apo-enzyme (Tables 1 and 3) would point to the mediation of steroid hormones. This is further supported by the observation that a variety of hormones, especially glucocorticoids, induced these enzymes (Schimke & Doyle, 1970) and that these enzymic responses to stress were inhibited in adrenalectomized animals (Thomson & Mikuta, 1954). Moreover, exposure to environmental stress has been demonstrated to result in prompt increases in the concentration of corticosterone both in the adrenal glands and in plasma (Rosecrans *et al.*, 1966; Smookler & Buckley, 1970). The above explanation, however, does not hold good for the increase in tryptophan hydroxylase activity observed under hypobaric stress.

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References

- Berry, L. J., Smythe, D. S., Colwell, L. S. & Chu, P. H. C. (1968) *Amer. J. Physiol.* **215**, 587
- Fiegelson, P. & Greengard, O. (1961a) *J. Biol. Chem.* **236**, 153
- Fiegelson, P. & Greengard, O. (1961b) *J. Biol. Chem.* **236**, 158
- Fiegelson, P., Fiegelson, M. & Greengard, O. (1962) *Recent Progr. Horm. Res.* **18**, 491
- Folk, G. E., Jr. (1966) *Introduction to Environmental Physiology*, p. 213, Lea and Febiger, Philadelphia
- Francesconi, R. P. & Mager, M. (1970) *Amer. J. Physiol.* **218**, 1342
- Freedland, R. A., Wadzinski, I. M. & Waisman, H. A. (1961) *Biochem. Biophys. Res. Commun.* **5**, 94
- Fuller, R. W. (1970) *Proc. Soc. Exp. Biol. Med.* **133**, 620
- Green, A. R. & Curzon, G. (1968) *Nature (London)* **220**, 1095
- Greengard, O. & Fiegelson, P. (1960) *Biochim. Biophys. Acta* **39**, 191
- Greengard, O. & Fiegelson, P. (1961) *Nature (London)* **190**, 446
- Inamdar, A. R., Kurup, C. K. R. & Ramasarma, T. (1970) *Biochem. Biophys. Res. Commun.* **38**, 220
- Kaufman, S. & Levenberg, B. (1959) *J. Biol. Chem.* **234**, 2683
- Knox, W. E. & Auerbach, V. H. (1955) *J. Biol. Chem.* **214**, 307
- Lin, E. C. C., Pitt, B. M., Civen, M. & Knox, W. E. (1958) *J. Biol. Chem.* **233**, 668
- Lovenberg, W., Jequier, E. & Sjoerdsma, A. (1967) *Science* **155**, 217
- Popov, P. G. & Ankov, V. K. (1970) *Radiobiologiya* **10**, 32
- Ramasarma, T. (1965) *Proc. Indian Armed Forces Med. Services Symp. High Altitude* 82
- Rosecrans, J. A., Watzman, N. & Buckley, J. P. (1966) *Biochem. Pharmacol.* **15**, 1707
- Schimke, R. T. & Doyle, D. (1970) *Annu. Rev. Biochem.* **39**, 929
- Smookler, H. H. & Buckley, J. P. (1970) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **29**, 1980
- Thomson, J. F. & Mikuta, E. T. (1954) *Proc. Soc. Exp. Biol. Med.* **85**, 29
- Udenfriend, S., Weissbach, H. & Clark, C. T. (1955) *J. Biol. Chem.* **215**, 337
- Young, C. W., Robinson, P. F. & Sacktor, B. (1963) *Biochem. Pharmacol.* **12**, 855