Biochemical Pharmacology, Vol. 31, No. 13, pp. 2251–2255, 1982.
Printed in Great Britain.

0006–2952/82/132251-05 $03.00/0

EFFECT OF BENSERAZIDE ON THE LEVELS OF PINEAL 5-HYDROXYTRYPTAMINE, MELATONIN SYNTHESISING ENZYMES AND SERUM MELATONIN

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(*Received* 16 *October* 1981; *accepted* 10 *February* 1982)

Abstract—The effect of benserazide, an aromatic L-amino acid decarboxylase inhibitor, has been investigated on pineal 5-hydroxytryptamine content, melatonin synthesising enzyme activities and serum melatonin concn. Increasing doses of benserazide caused increasing reductions in the pineal content of 5-hydroxytryptamine. Dark phase serum melatonin concns were also greatly reduced. The drug abolished the diurnal rhythm of hydroxyindole-*O*-methyltransferase by increasing the light period activities and decreasing the night levels of this enzyme. Pineal *N*-acetyltransferase was unaffected. It is concluded that benserazide probably inhibits melatonin synthesis by preventing the formation of the substrate, 5-hydroxytryptamine.

In the synthesis of melatonin (*N*-acetyl-5-methoxytryptamine; MT), pineal 5-hydroxytryptamine (5-HT) is acetylated by *N*-acetyltransferase (NAT) EC 2.3.1.5) using acetyl CoA and then converted to MT by hydroxyindole-*O*-methyltransferase (HIOMT) (EC 2.1.1.4), *S*-Adenosyl-methionine acting as methyl donor. The activity of both these enzymes exhibit a diurnal rhythm. NAT in particular is sensitive to light [1] such that the dark phase activity of the enzyme is many-fold greater than daytime levels in most species studied. However, HIOMT exhibits only a 1–2-fold amplitude between the dark period and the daytime activities [2]. Pineal 5-HT content also shows a diurnal rhythm which is 180° out of phase with those of the enzyme activities. Thus 5-HT concns are high in the light period and decrease rapidly with the onset of darkness as MT is synthesised [3]. Consequently, the serum MT concns also exhibit a diurnal rhythm such that dark phase levels are several-fold higher than daytime concns. The pineal is innervated by nerves originating in the superior cervical ganglion. MT synthesis is thought to be induced by darkness which stimulates the release of L-noradrenaline from postganglionic nerve endings. The neurotransmitter acts on the pineal β-adrenergic receptors thus inducing NAT activity [4]. HIOMT activity may also be influenced since noradrenaline *in vitro* inhibits the action of HIOMT [5] and stimulation of the preganglionic fibres of the superior cervical ganglion also decreases HIOMT activity [6]. Recently, it has been shown by the use of benserazide (R04-4602), an aromatic L-amino acid decarboxylase (AAD) inhibitor, that pineal 5-HT levels are reduced by some 80% [7] by preventing its formation from 5-hydroxytryptophan. Moreover, as 5-HT concns in the pineal are reduced, so the dark phase pineal MT concns are decreased by up to 95% [7]. Simultaneously, hypothalamic 5-HT levels are unaffected, thus confirming that the rat pineal is outside the blood–brain barrier. Since in the synthesis of MT, the substrate 5-HT and enzymes NAT and HIOMT are coupled together, the effect of benserazide on the NAT and HIOMT enzyme activities as well as on pineal 5-HT and serum MT concns was investigated.

MATERIALS AND METHODS

Benserazide hydrochloride was donated by Roche Products Ltd. (Welwyn Garden City, U.K.). 5-Hydroxytryptamine creatine sulphate (5-HT), acetyl coenzyme A and tryptamine hydrochloride were purchased from Sigma Chemical Co. (London, U.K.). *S*-Adensoyl-L-[methyl-^{14}C]methionine (sp. act. = 57 mCi/mmole) and [^{14}C]acetyl coenzyme A (sp. act. = 56.6 mCi/mmole) was purchased from the Radiochemical Centre (Amersham, U.K.). *N*-[2-aminoethyl-2-^{3}H]acetyl-5-methoxytryptamine ([^{3}H]-MT) (50 Ci/mmole) was purchased from New England Nucleur (Dreieich, F.R.G.). All other reagents were commercially available and were analytical grade.

The 5-HT assays were performed using a modification of the method reported earlier [8]. Pineal homogenate (1 ml) was washed twice with 0.1 ml KCl-saturated 2 M phosphate buffer pH 10. Homogenate (0.8 ml) was shaken with 2 ml of cyclohexane and 1 ml of 0.1 M HCl. After centrifugation, the organic phase was aspirated and 0.8 ml of the aq. phase was added to 0.4 ml of 0.625 M Na_2HPO_4 and 0.1 ml of 0.1 M ninhydrin. The mixture was incubated at 70° for 30 min. Fluorescence of the samples was determined at 60 min in a spectrophotofluorometer (Perkin–Elmer MPF3). Excitation was 385 nm and emission 490 nm, both uncorrected.

The NAT assay was a modification [9] of an earlier method [10]. Pineals were homogenised in a Teflon homogeniser in 0.1 ml of reaction mixture containing 50 nmole of acetyl coenzyme A (originally labelled acetyl CoA; sp. act. = 56.6 mCi/mmole; diluted with

unlabelled acetyl CoA to give a sp. act. of 1.42 mCi/mmole) and tryptamine (1 μmole base) in phosphate buffer pH 6.8 (0.5 M). The homogenate was incubated for 20 min and 0.075 ml was transferred into 1 ml water-saturated chloroform, mixed for 30 sec and centrifuged. The aq. layer was removed and the chloroform was washed with 0.2 ml of buffer pH 6.8 (0.5 M phosphate); 0.5 ml was evaporated to dryness, redissolved in 0.5 ml ethanol and after addition of 9.5 ml of scintillant was counted in a Tricarb scintillation counter.

The HIOMT assay was as described earlier [11] with the following modifications. The pineal was homogenised in 0.15 ml of phosphate buffer (0.1 M, pH 7.9) and 0.075 ml of homogenate was incubated in the final vol. of 0.125 ml of buffer containing 18.0 μg *N*-acetyl-5-hydroxytryptamine and 50 nCi of *S*-adenosyl-L-[methyl-^{14}C]methionine (sp. act. – 57 mCi/mmole). The reaction was stopped by 1 ml 0.1 M NaOH and extracted into 8 ml chloroform. After washing with water, 4 ml of chloroform extract was evaporated to dryness and the residue redissolved in 0.5 ml ethanol. Scintillant (10 ml) was added and the sample counted as before.

The serum MT was assayed by RIA by a modification of that reported earlier [12]. The antibody (final dilution 1:1400) was raised in rabbits [13] and was specific for MT and exhibited no serious cross-reaction with any major indole (including 6-hydroxymelatonin and *N*-acetyl-5-hydroxytryptamine) nor benserazide. The assay sensitivity was 10 pg/ml of serum and inter- and intra-assay variation were 16 and 11% respectively. Recovery of authentic [^{3}H]MT was 62 ± 8%. All solvents were double distilled. Serum (1 ml) was shaken with 1.5 ml 2 M phosphate buffer (pH 10) saturated with potassium chloride and 25 ml chloroform. The mixture was shaken and centrifuged and the aq. layer was removed. A 20-ml aliquot was evaporated under nitrogen at 37°. The residue was dissolved in 1 ml ethanol transferred to an assay tube, evaporated to dryness and redissolved in 0.05 ml of 0.1% gelatin-phosphate buffer pH 7.1 (0.1 M). Petroleum ether (0.5 ml) was added and after the mixture was shaken and centrifuged, the petroleum ether was removed. Antisera (0.1 ml) and 0.05 ml of [^{3}H]MT (17 nCi/ml ≡ 850 pCi/assay) were added to the assay tube. A vol. of 0.05 ml of a series of MT solutions (equivalent to a final concn of 5–200 pg MT/tube) were used in standard assays. The serum MT was then assayed as described earlier [12].

All animals used in these experiments were male Sprague–Dawley (160–230 g) rats. The animals were individually housed in 14:10 hr light–dark cycles for seven days to acclimatise to the photoperiod. Four groups of rats (n = 5–8) were pretreated with varying doses of benserazide (40, 50 and 80 mg/kg calculated as HCl salt) or the equivalent vol. of saline i.p. 3 hr before sacrifice. Each group was sacrificed 6 hr after the onset of light. Pineal glands were removed immediately, homogenised and assayed for 5-HT content as described above.

Five groups of rats (n = 4) were pretreated with benserazide (80 mg/kg calculated as HCl) at the following times: 30, 60, 120, 240 and 360 min before sacrifice at 6 hr after light. Control groups (n = 4) were saline injected at 60, 120 and 180 min before sacrifice together with the experimental group. The pineals were removed and stored in liquid nitrogen until assay for 5-HT content.

Eight groups of rats (n = 6), four test and four control, were pretreated with benserazide (40 mg/kg calculated as HCl) or the equivalent vol. of saline i.p. 2 hr before sacrifice. Paired groups were sacrificed at 5, 9, 19 and 23 hr after onset of light respectively. The pineals were removed and stored in liquid nitrogen until assay for NAT activity as described. Another eight groups of rats (n = 5), again four experimental and four control animals, were pretreated with benserazide (80 mg/kg calculated as HCl) or the same vol. of saline i.p. Paired groups were repeatedly injected 7, 4 and 1 hr before sacrifice at the following time-points: 3, 11, 16 and 22 hr after the onset of light respectively. The pineals were removed and stored in liquid nitrogen until assayed for NAT activity as above.

Eight groups of male rats (n = 6), four test and four control animals, were pretreated with benserazide (40 mg/kg calculated as HCl) or an equivalent vol. of saline i.p. for 2 hr. Paired groups were sacrificed at 3, 10, 18 and 23 hr after lights-on, respectively. The pineal glands were removed immediately, homogenised and assayed for HIOMT activity as described above. Four groups of rats (n = 5), two experimental and two control animals, were pretreated with benserazide (80 mg/kg calculated as HCl) or saline i.p. Paired groups were repeatedly injected 7, 4 and 1 hr before sacrifice at the following time-points: 9 and 20 hr after the onset of light respectively. The pineals were removed and stored in liquid nitrogen until assayed for HIOMT activity as described above.

Eight groups of rats (n = 4), four experimental and four control animals, were pretreated with benserazide (80 mg/kg calculated as HCl) or saline i.p. Paired groups were injected repeatedly at 7, 4 and 1 hr before sacrifice at the following time-points: 3, 11, 16 and 21 hr after the onset of light respectively. The serum was collected and MT concn assayed as described above.

RESULTS

Table 1 illustrates pineal 5-HT content 6 hr after light. The 5-HT content in control animals was 116 ng/pineal. Benserazide (80 mg/kg) administered 3 hr before sacrifice, resulted in a 70% decrease in pineal 5-HT concn.

Figure 1 shows the effect of a single injection of benserazide (80 mg/kg) over a period of 6 hr. The decrease in pineal 5-HT content in the treated animals was 87% after 1 hr ($P < 0.0002$) and remained significant for at least 3 hr.

Figure 2 summarises NAT activity (nmole/gland/hr) in saline-treated control rats and in animals administered with benserazide (40 mg/kg) at four different time-points. All animals were sacrificed 2 hr after drug treatment; NAT activity in the control rats exhibited a diurnal rhythm. The night levels (9 hr after dark) were some 60–70-fold higher than daytime values (9 hr after light) ($P < 0.0002$). Benserazide administration (40 mg/kg) did not elicit any significant difference in enzyme activities at any of the time-points.

Table 1. Effect of benserazide on 5-hydroxytryptamine content of rat pineal

Dose of benserazide HCl i.p. (mg/kg)	5-HT content (ng/gland ± S.E.)	Number	% Reduction
Saline	116.7 ± 9.9	15	0
40	46.4 ± 3.8	8	60.2
50	38.3 ± 3.5	5	67.2
80	35.2 ± 3.8	5	69.8

Each animal was pretreated with the drug or the equivalent vol. of saline 3 hr before sacrifice at 6 hr after lights-on. (Light, 0700–2100 hr and dark, 2100–0700 hr.)

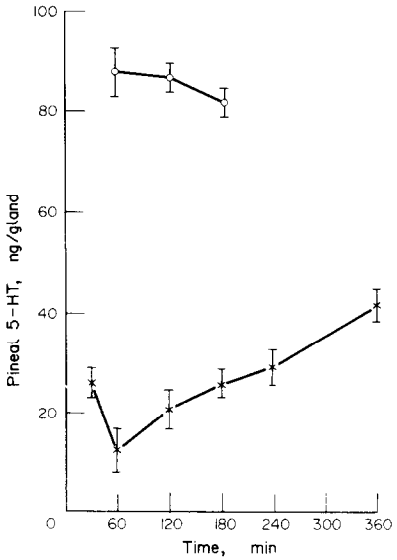

Fig. 1. Variation of the effect of benserazide (80 mg/kg) with time on pineal 5-hydroxytryptamine content. The x-axis represents the time after pretreatment with benserazide HCl (80 mg/kg) before sacrifice ($n = 4$) ± S.E.

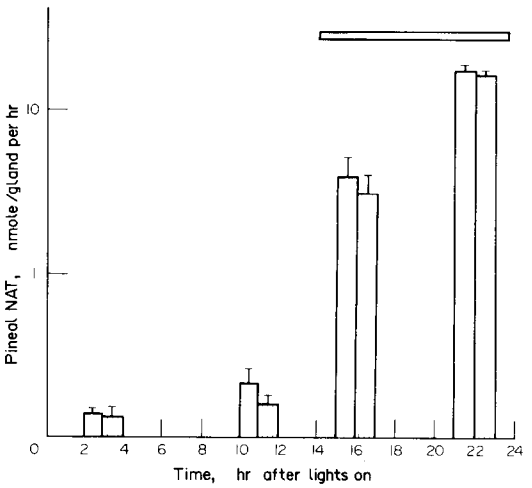

Fig. 3. Effect of benserazide (80 mg/kg) on pineal N-acetyltransferase activity. Plain—controls ($n = 5$) ± S.E.; stripes—each animal is pretreated with benserazide HCl (80 mg/kg) 7, 4 and 1 hr before sacrifice ($n = 5$) ± S.E. Time of sacrifice was at 3, 11, 16 and 22 hr after light (± 10 min). Horizontal bar—dark.

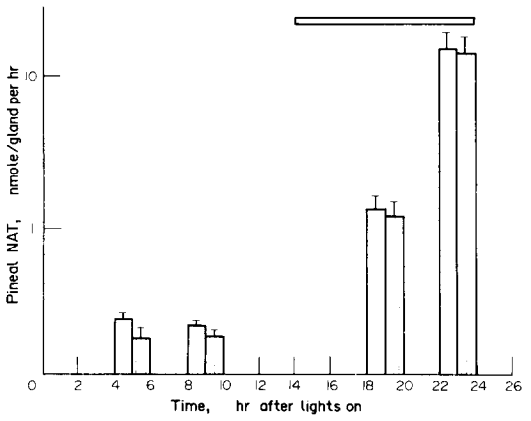

Fig. 2. Effect of benserazide (40 mg/kg) on pineal N-acetyltransferase activity. Plain—controls ($n = 6$) ± S.E.; stripes—each animal is pretreated with benserazide HCl (40 mg/kg) 2 hr before sacrifice ($n = 6$) ± S.E. Time of sacrifice was 5, 9, 19 and 23 hr after light (±10 min). Horizontal bar—dark.

Figure 3 represents NAT activity (nmole/gland/hr) in rats administered saline or benserazide (80 mg/kg) repeatedly at 7, 4 and 1 hr before sacrifice. Control rats exhibited night-time levels (8 hr after dark) 80-fold higher than the equivalent daytime levels (11 hr after light) ($P < 0.002$). The longer term pretreatment of the benserazide at the higher dosage did not significantly reduce the NAT activity at any time-point.

Figure 4 illustrates HIOMT (pmole/gland/hr) in rats treated with saline or benserazide (40 mg/kg) at four time-points. All animals were killed 2 hr later. Control HIOMT levels exhibited a rhythm of small amplitude (comparing 10 and 18 hr after onset of light; $P < 0.05$). Injection of benserazide (40 mg/kg) increased the enzyme activity measured during the light period significantly, hence reducing the amplitude of the light/dark fluctuation. The difference between the control and injected rats during dark phase was not significant.

Figure 5 shows HIOMT (pmole/gland/hr) in rats administered benserazide (80 mg/kg) or saline

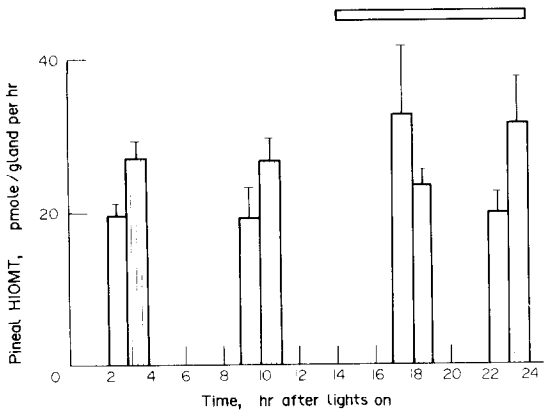

Fig. 4. Effect of benserazide (40 mg/kg) on pineal hydroxyindole-*O*-methyltransferase activity. Plain—controls ($n = 6$) ± S.E.; stripes—each animal is pretreated with benserazide HCl (40 mg/kg) 2 hr before sacrifice ($n = 6$) ± S.E. Time of sacrifice was at 3, 10, 18 and 23 hr after light (±10 min). Horizontal bar—dark.

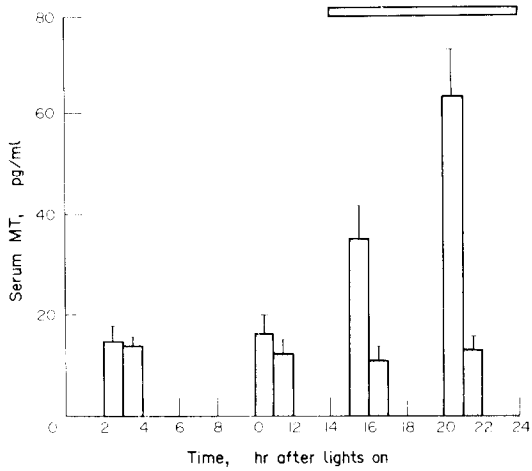

Fig. 6. Effect of benserazide (80 mg/kg) on serum melatonin concn. Plain—controls ($n = 4$) ± S.E.; stripes—each animal is pretreated with benserazide HCl (80 mg/kg) 7, 4 and 1 hr before sacrifice ($n = 4$) ± S.E. Time of sacrifice was at 3, 11, 16 and 21 hr after light (±10 min). Horizontal bar dark.

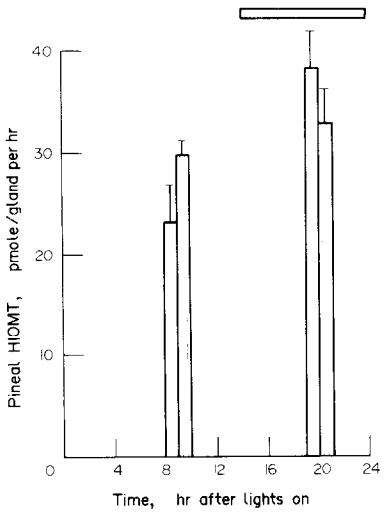

Fig. 5. Effect of benserazide (80 mg/kg) on pineal hydroxyindole-*O*-methyltransferase activity. Plain—controls ($n = 5$) ± S.E.; stripes—each animal is pretreated with benserazide HCl (80 mg/kg) 7, 4 and 1 hr before sacrifice ($n = 5$) ± S.E. Time of sacrifice was at 9 and 20 hr after light (±10 min). Horizontal bar—dark.

repeatedly at 7, 4 and 1 hr before sacrifice. Control HIOMT levels again exhibited a rhythm of small amplitude (comparing 9 and 20 hr after onset of light; $P < 0.05$). However, repeated injection of benserazide (80 mg/kg) at 3-hr intervals abolished the rhythm in HIOMT activity (comparing 9 and 20 hr; $P > 0.05$).

Figure 6 illustrates serum MT (pg/ml) in rats treated with saline or benserazide (80 mg/kg) repeatedly at 7, 4 and 1 hr before sacrifice. Control rats exhibited night-time concns (7 hr after dark) 4-fold higher than daytime levels (11 hr after light) ($P < 0.001$). Repeated treatment of benserazide (80 mg/kg) significantly reduced serum MT at 2 and 7 hr after dark compared to the control animals ($P = 0.014$ and 0.004 respectively).

DISCUSSION

The daytime pineal 5-HT content was found to be 116 ng/pineal which is similar to values reported earlier [3]. A dose of 80 mg/kg was selected as a peripherally acting dose giving a high level of inhibition. Previously, benserazide (80 mg/kg) reduced daytime pineal 5-HT levels by some 80% whilst leaving the hypothalamic 5-HT content unchanged [7]. It was concluded by those workers that benserazide, at 80 mg/kg would have a minimal central effect.

Recently [14], benserazide at a centrally acting dose (500 mg/kg) administered $3\frac{1}{2}$ hr before sacrifice showed that dark phase pineal MT content was reduced to 5% of control values. A drastic reduction in pineal MT concn was also achieved by using peripheral doses of benserazide (80 and 50 mg/kg) administered repeatedly [7]. Since a single injection of benserazide (80 mg/kg) was effective for up to 3 hr in reducing 5-HT it might be anticipated that repeated injections every 3 hr was sufficient to maintain a low pineal 5-HT content and thus produce a serum MT reduction. In this study repeated administration of benserazide (80 mg/kg) at 3-hourly intervals abolished the nocturnal rise in serum MT concn.

Usually, tryptophan hydroxylase is considered to be the rate-limiting step in the synthesis of 5-HT [15]. Thus AAD inhibition would be expected to show only a slight reduction in 5-HT content. However, this does not appear to be the case in the pineal since AAD inhibition by benserazide has reduced pineal 5-HT concns, possibly due to the fact that this amine in the pineal has a high turnover rate [16].

Apart from limiting 5-HT production, the benserazide inhibition of pineal AAD may also cause a reduction in catecholamine content [17]. Any decrease of noradrenaline in the pineal may in turn

cause a reduction in the activity of the β-adrenergically controlled NAT enzyme [2, 4]. It was possible that the benserazide induced reduction of dark phase pineal MT reported earlier [7] and the dark period decrease in serum MT observed here was due to a combined lack of 5-HT substrate and a low activity of NAT enzyme. However, the results reported here suggest that it is probably the reduction in pineal 5-HT content that is responsible for the reduction in night-time serum MT concn. Pretreatment with benserazide (40 mg/kg) reduced pineal 5-HT content by 60% (Table 1) but did not apparently affect NAT activity (Fig. 2). These results suggest that the NAT enzyme is not influenced by the 5-HT concn of the pineal. However, further studies at more time-points may clarify the apparent lack of effect of benserazide on NAT activity.

It was also possible that the catecholamine-controlled NAT activity would require a longer time than 2 hr for a catecholamine-induced reduction of NAT activity to occur. However, no reduction in NAT activity occurred even when 80 mg/kg benserazide was administered repeatedly at 7, 4 and 1 hr before sacrifice. These conditions abolished the nocturnal rise in serum MT and a similar dosage regime has previously been shown to reduce pineal MT content by 95% [7]. Therefore, even if benserazide caused a reduction in the synthesis of noradrenaline, it is possible that NAT activity was maintained by a sufficient release of the neurotransmitter. Information on pineal catecholamine levels after benserazide would be of value.

The control mechanism of HIOMT is more complicated than NAT. The enzyme is believed to exhibit a diurnal rhythm of small amplitude which is synchronous with that of NAT activities [2]. The results here show a significant difference between the night-time and daytime HIOMT activities in the control animals. After injection of benserazide, either as a single dose of 40 mg/kg or repeated injections of 80 mg/kg at 3-hr intervals, the difference between the night-time and daytime enzyme activities became insignificant. This effect of the drug is difficult to explain. It may be caused by the reduction of 5-HT or catecholamine levels in the pineal. However, it was reported that even when pineal noradrenaline was depleted by 6-hydroxydopamine, the activity of HIOMT was not markedly reduced [18]. On the other hand, noradrenaline or stimulation of preganglionic fibres of the superior cervical ganglion decreased HIOMT activity [5, 6]. We cannot rule out the possibility however that benserazide may have other peripheral effects, e.g. on gonadal hormone secretion, which might indirectly cause the changes in HIOMT activity [19, 20]. Further experimentation is required to clarify this point.

Thus we conclude that the inhibitory effect of benserazide on MT synthesis is probably due to the prevention of 5-HT production. The drug does not appear to cause a reduction in the activities of NAT nor apparently are the activities of this enzyme influenced by the low substrate levels. On the other hand, benserazide appears to abolish the diurnal rhythm of the HIOMT activity. Further studies at more time-points should clarify the position. In any event, benserazide has caused the 24-hr cycle of NAT and HIOMT activities to become out of synchronisation with serum MT concns. Such a desynchronisation could possibly have profound effects on the rat's physiology and behaviour.

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