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CONTROL OF THE BROWN FAT RESPIRATORY RESPONSE TO NORADRENALINE BY CATECHOL-*O*-METHYLTRANSFERASE

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Abstract—The possibility that *O*-methylation of noradrenaline (NA) to normetanephrine (NM) by catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) may limit the concentration of NA at the receptors, and therefore control the adrenergic response, was evaluated from measurements of both the respiratory rate ($\dot{M}O_2$) and the rate of NM formation ($\dot{M}NM$) in brown adipose tissue from the rat. $\dot{M}O_2$ and its increase ($\dot{M}O_2$ response) induced in tissue slices by endogenous or exogenous NA were measured in a stop flow respirometer perfused with Krebs–Ringer bicarbonate medium. $\dot{M}NM$ was measured in identical tissue slices incubated in the same medium, from exogenous radiolabelled NA and endogenous *S*-adenosyl methionine. This rate was taken as the apparent activity of the COMT system. It reached maximum values of about 100 pmole/min/g wet wt and was cut down to blank value by tropolone, a specific inhibitor of COMT. For any given sustained NA stimulus, the $\dot{M}O_2$ response reached a steady-state value. $\dot{M}NM$ was constant during one hour at concentrations of added NA up to 3 μM, and during 30 min at higher ones. The $\dot{M}O_2$ response was potentiated (100–200 per cent) through inhibition of COMT by 0.2 mM tropolone. This effect was only transitory, with a maximum at 30 min followed by a 100 per cent inhibition of the $\dot{M}O_2$ response to exogenous NA 60 to 90 min later. Exposure of the stimulated preparations to $5 \cdot 10^{-5}$ M DOPA, which competitively inhibits the extraneuronal uptake–COMT system and whose decarboxylation product competitively inhibits not only COMT but also the neuronal uptake (uptake 1), induces a sustained increase of the $\dot{M}O_2$ response. Under blockage of uptake 1 by desmethyl-imipramine (DMI) 10^{-6} M, potentiation of the $\dot{M}O_2$ response was quantitatively the same with tropolone as with DOPA, for both endogenous and exogenous NA stimuli, and it was sustained. Quantitative analysis of the data suggests that COMT can control the local NA concentration at the site of hormone-receptor interaction, not only by the (NA) gradient to the COMT system as a sink, but also indirectly via active induction of neuronal uptake. Large metabolic responses to nanomolar (NA), observed only when both COMT and uptake 1 were blocked, suggested that such a control might be operative even under basal conditions.

The control exerted by the neuronal uptake on the adrenergic transmitter concentration in the synaptic region is common to all adrenergically innervated tissues and organs studied. In contrast, the possibility that the extraneuronal uptake-catechol-*O*-methyltransferase system [1–4], here called COMT system for simplicity, be of any physiological importance in the permanent control of local transmitter concentration is still largely open to question [5, 6], particularly regarding tissues with a rich adrenergic innervation [7]. The present study was aimed at examining this possibility in brown fat, a tissue with a dense adrenergic innervation [8–10] and in the microsomal fraction of which a high COMT activity was found [11]. Brown adipose tissue strongly responds to noradrenaline (NA) by a sustained increase in respiration associated with thermogenesis, essentially a β-adrenergic response [12, 13].

COMT catalyses meta (and para) *O*-methylation of catechol compounds, with the methyl donor *S*-adenosylmethionine (SAM) as cosubstrate and Mg^{2+} as cofactor. This intracellular enzyme can thus, by continuous metabolic clearance of NA taken up from extracellular space, act as a permanent sink towards which an extracellular concentration gradient of NA ((NA) gradient) is maintained in the steady state. If this is true, inhibition of the COMT system should increase local (NA), with the following two consequences: (a) exposure of adrenergic receptors to an increased stimulus; and (b) increased diffusion of excess transmitter into blood (NA overflow) during nerve stimulation. Bacq *et al.* [14] showed that indeed inhibition of COMT, by either product accumulation or catechol analogues, could possibly increase various α responses to catecholamines both exogenous (injected) and endogenous (nerve stimulation). In contrast, β responses were decreased by COMT inhibition with exogenous NM [15, 16], and the NA overflow during nerve stimulation was not modified by *in vivo* competitive inhibition of COMT in the cat spleen [17]. The very existence of opposite effects of COMT inhibition on adrenergic responses justified further inquiry about the function of the enzyme.

In the present study, fragments of brown adipose tissue were stimulated *in vitro*, either with NA added to the perifusion medium or by potassium-induced transmitter release [13]. The effects of COMT inhibitors on both the respiratory (i.e. thermogenic) response and the apparent activity of the COMT system were examined under steady-state conditions and analysed quantitatively.

METHODS

Preparation. Male Sprague–Dawley rats weighing

from 250 to 380 g, kept at 23° with a 12 hr illumination period per day, fed *ad libitum*, were used. Immediately after decapitation, threadlike pieces of the interscapular brown fat, about 10 mg wet weight each, were excised and incubated at 30°, pH 7.4, in stirred Krebs–Ringer bicarbonate-buffered medium, enriched with 5 mM glucose and gassed with a mixture of 95% O_2 and 5% CO_2.

Measurements. The O_2 uptake rate of the preparations was measured during about five hours, with short interruptions at 10–20 min intervals for the renewal of the medium in the chambers of the respirometer as previously described [13]. The noradrenaline (NA) stimulus was either exogenous (added to the medium) or, in the respiration experiments, endogenous (transmitter released from the tissue's own nerve endings by KCl depolarization). Chemical alteration of NA was prevented by prior complexation of contaminating metal ions in the albumin-free medium with 3,5-pyrocatechol-disulfonate (tiron). COMT activity was assayed in identical preparations, in the same medium and under similar oxygenation conditions, using exogenous radiolabelled NA as substrate and endogenous SAM as cosubstrate [18]. After a 30 min pre-incubation, three fragments were transferred into a final 1 ml volume of fresh medium containing the test pharmaca, and incubated. The COMT activity assay started with the addition of radioactive NA to the medium. Incubation was stopped by addition of 0.5 ml of 0.4 M borate buffer, pH 10, to each vial as it was transferred to an ice bath. The tissue fragments were homogenized and the NM formed extracted with 5 ml of toluene–isoamyl alcohol (3:2). 4 ml of the organic phase were then transferred to counting vials together with 10 ml of toluene–Triton X-100 (1 : 2) plus 0.8% butyl-PBD, and the radioactivity was measured in a Beckman Liquid Scintillation counter. Since incubations in the presence of tropolone, whatever their duration, yielded the same counts as obtained with zero incubation time in the absence of COMT inhibitor, blank values were routinely determined in the presence of 0.2 mM tropolone. All tests were performed in duplicate.

Materials. DL-[7-^{14}C]NA (20–40 mCi/mmole) and DL-[7-^{3}H]NA (5–15 Ci/mmole) were purchased from New England Nuclear, Frankfurt, Germany. L-NA was obtained from Fluka, Buchs, Switzerland. Tropolone was from K & K Laboratories Inc., New York, and DL-DOPA from Sigma, St. Louis, U.S.A. DMI (desipramine) was a gift from Ciba-Geigy, Basel, Switzerland. All other chemicals, reagent grade, were obtained from Fluka, Buchs, Switzerland, or Merck, Darmstadt, Germany.

RESULTS

Respiratory experiments. Each respiratory experiment started with a one to two hours period during which the rate of oxygen uptake ($\dot{M}O_2$) by the brown adipose tissue sample slowly decreased until it reached its steady state basal value. A submaximal NA stimulus was then continuously applied, and again one hour or more elapsed until the $\dot{M}O_2$ response had reached the steady state. Finally, the effect of COMT inhibition on $\dot{M}O_2$ response was observed for 90 to 120 min. Figure 1A presents the mean results (±S.E.M., $n = 13$) obtained on samples from nine different animals, showing

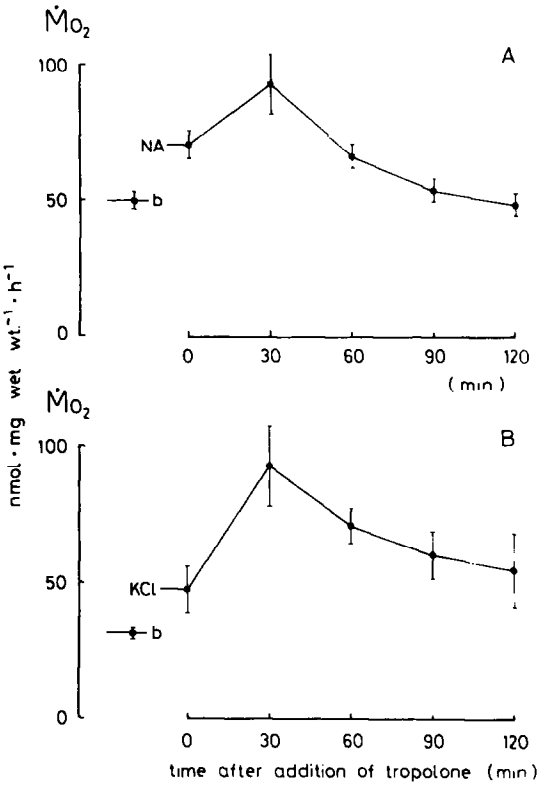

Fig. 1. Time course of the effect of COMT inhibition on the $\dot{M}O_2$ response to exogenous (A) and KCl-induced (B) NA stimuli.

Administration of the inhibitor ($2 \cdot 10^{-4}$ M tropolone) started at time zero, when the $\dot{M}O_2$ response (i.e. the difference between stimulated $\dot{M}O_2$ (NA or KCl) and basal $\dot{M}O_2$ (B) had reached a steady state value. (A): transitory potentiation was followed by complete disappearance of the response to 10^{-8} M NA. (B): the transitory potentiation of the response to endogenous NA (+24.1 mM K^+; −24.1 mM Na^+ in the medium) was relatively larger, as compared to A, and $\dot{M}O_2$ at 90 min was still significantly larger than basal ($P < 0.025$). Values are means ± S.E.M. ($n = 13$).

steady state values of basal and stimulated $\dot{M}O_2$, and the time course of the effect of COMT inhibition by 0.2 mM tropolone. From the steady state of stimulation by 10^{-8} M NA, $\dot{M}O_2$ first increased up to a maximum at about 30 min. The difference between the values at zero and 30 min was highly significant according to the paired t test ($P < 0.0025$). $\dot{M}O_2$ then decreased until disappearance of the response to NA. In these experiments, long term oxidation of NA in the medium was avoided by injection of the catecholamine with a motor-driven plastic syringe from an acidified concentrated solution directly into the respirometer chambers each time these were rinsed with fresh oxygenated medium for 1–2 min every 10 to 20 min. Besides, complexation of exogenous NA was prevented by 10^{-5} M tiron added to the medium (see technical annexe). The results presented in Fig. 1B show that inhibition of COMT by tropolone also induced a transitory increase in $\dot{M}O_2$ ($P < 0.0025$) from the steady state of stimulation obtained with endogenous NA. The potassium concentration of the medium was raised by 24.1 mM (total

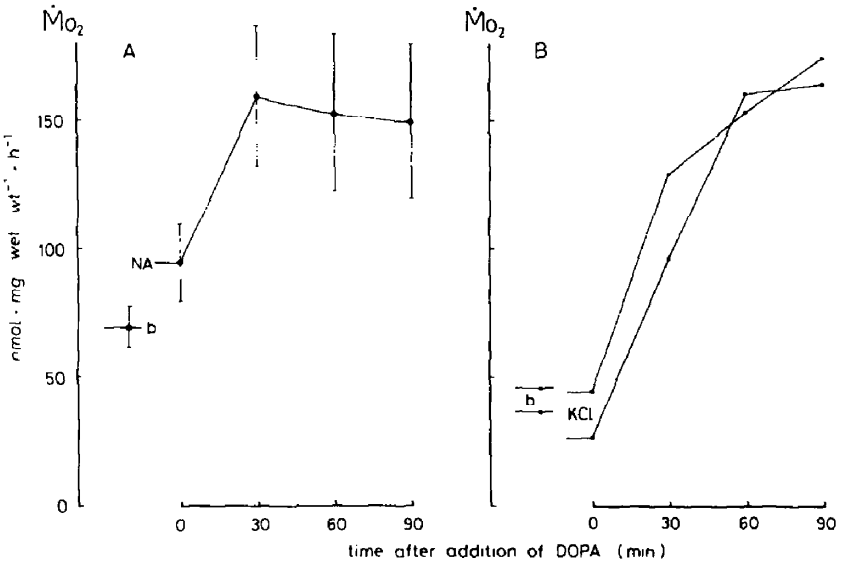

Fig. 2. Time course of the effect of DOPA on the $\dot{M}O_2$ response to exogenous (A) and KCl induced (B) NA stimuli.

Administration of the drug ($5 \cdot 10^{-5}$ M DOPA) started at time zero, when the $\dot{M}O_2$ response to NA had reached a steady state value. (A): potentiation of the response was still significant at 90 min ($P < 0.025$, $n = 10$). Mean $\dot{M}O_2$ values, ±S.E.M. (B): results of two experiments in which undetectable responses to endogenous NA were revealed by DOPA, an effect which progressively developed up to 90 min (see also Fig. 3).

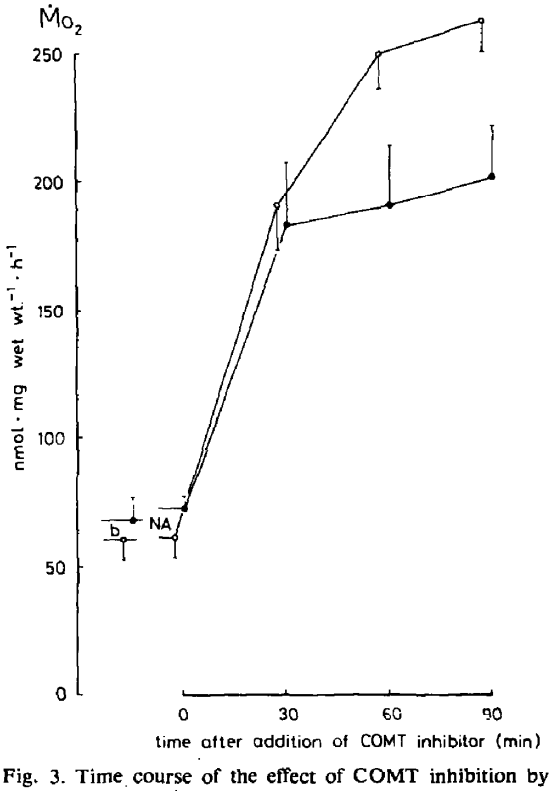

Fig. 3. Time course of the effect of COMT inhibition by tropolone $2 \cdot 10^{-4}$ M (closed circles) and by DOPA $5 \cdot 10^{-5}$ M (open circles) on the $\dot{M}O_2$ response to 10^{-9} M NA, in the presence of 10^{-6} M DMI.

Administration of the inhibitor started at time zero, when the $\dot{M}O_2$ response to NA had reached a steady state value. Means ± S.E.M. ($n = 4$). The effect of DOPA developed progressively up to 90 min (values at 30 and 90 min are significantly different: $P < 0.005$).

K = 30 mM) and sodium was lowered by 24.1 mM; no tiron was added to the medium in these experiments. Basal values were significantly lower than in the first series and the secondary inhibition of the $\dot{M}O_2$ response was smaller. $\dot{M}O_2$ at 90 min after addition of tropolone was indeed still significantly larger ($P < 0.025$) than the basal value. Control experiments showed that tropolone did not affect $\dot{M}O_2$ on its own; the transitory increase in the $\dot{M}O_2$ response to NA by tropolone can thus be considered a potentiating effect. As previously reported [19], DOPA also has a potentiating effect on the $\dot{M}O_2$ response to NA. However, since this effect was later found partly to be due to the protective effect of DOPA against NA complexation, the experiments were repeated here in the presence of tiron. Figure 2A shows the results of ten experiments with exogenous NA ($1–5 \cdot 10^{-8}$ M), in which DOPA was injected as an acidified concentrated solution to a final $5 \cdot 10^{-5}$ M in the respirometer chambers. In contrast with the potentiating effect of tropolone on the response to exogenous NA, the effect of DOPA was sustained. $\dot{M}O_2$ at 90 min after addition of the drug was still significantly higher than the stimulated value at time zero ($P < 0.025$). DOPA had no statistically detectable effect on basal $\dot{M}O_2$ in the presence of tiron ($n = 9$). Figure 2B shows the strong $\dot{M}O_2$ responses revealed by DOPA in two experiments with subthreshold endogenous NA stimuli (total K = 30 mM) in media containing no tiron. Because DOPA or at least its decarboxylation product dopamine can competitively inhibit not only COMT [20] but also the neuronal uptake system [21], experiments were repeated in the presence of 10^{-6} M desmethylimipramine (DMI), a blocker of this catecholamine reuptake [22]. Figure 3 shows the results of eight experiments with 10^{-9} M exogenous NA, a subliminal stimulus, in which COMT was inhibited by

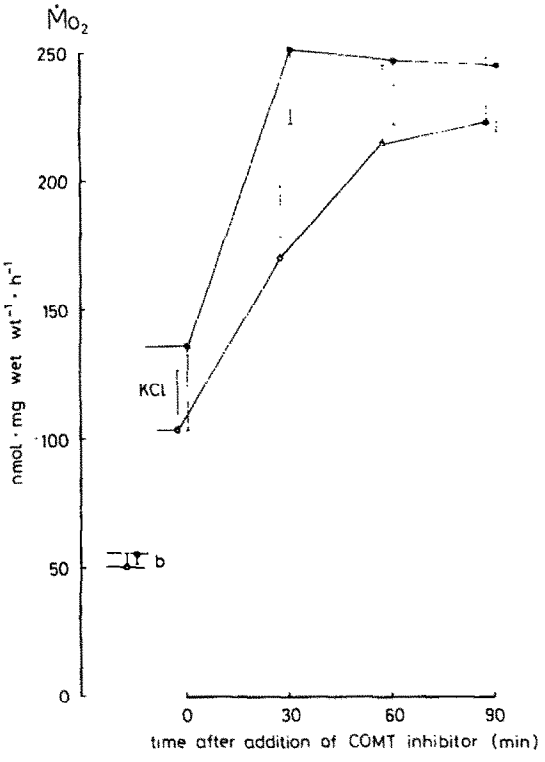

Fig. 4. Time course of the effect of COMT inhibition by tropolone $2 \cdot 10^{-4}$ M (closed circles) and by DOPA $5 \cdot 10^{-5}$ M (open circles) on the KCl-induced $\dot{M}O_2$ response. Administration of the inhibitor started at time zero, when the $\dot{M}O_2$ response to endogenous NA had reached a steady state value. Means ± S.E.M. ($n = 4$ and 6). The effect of DOPA developed progressively up to 90 min (values at 30 and 90 min are significantly different: $P < 0.05$, $n = 6$).

tropolone ($n = 4$) or DOPA ($n = 4$). It is evident that DMI brought both quantitative and qualitative differences with respect to the results obtained in the first series of experiments: $\dot{M}O_2$ reached much higher values under COMT inhibition by either tropolone (closed circles) or DOPA (open circles), and there was no secondary inhibitory effect with tropolone. Control experiments showed that, even in the absence of added NA, tropolone could induce $\dot{M}O_2$ responses probably due to basal endogenous NA. These responses, however, were much smaller than those shown in Fig. 3, which indicates that basal endogenous NA in brown adipose tissue slices with both neuronal uptake and COMT system blocked was less than 10^{-9} M. Figure 3 also shows that whereas the potentiating effect of tropolone had fully developed at 30 min, that of DOPA had not: $\dot{M}O_2$ at 90 min was significantly larger than $\dot{M}O_2$ at 30 min ($P < 0.005$). The same observations were made when increased endogenous NA was the stimulus (Fig. 4). Potassium was raised to only 20 or 25 mM in eight of the nine experiments. Because basal $\dot{M}O_2$ had been found to be lower in the first series of experiments with endogenous NA (see Figs 1B and 2B) as compared to experiments in which tiron was added to the medium (see Figs 1A and 2A), this last series was performed in the presence of 10^{-3} M tiron. Besides, DMI significantly increased basal $\dot{M}O_2$ and the sensitivity to NA (see Table 1). This is why a ten times smaller stimulus was used in these experiments (10^{-9} M added NA instead of the 1 to $5 \cdot 10^{-8}$ M used in the experiments without DMI).

Determinations of apparent COMT system activity. The amount of radioactivity extracted in the presence of 0.2 mM tropolone (about 0.03 per cent of the added radioactivity) was independent of the tissue weight (between 15 and 40 mg) and of the incubation time over 2 hr. This indicated that tropolone 0.2 mM fully inhibited COMT and that no contaminating reaction occurred within the tissue. In the presence of NA concentrations as high as 3 μM, the net radioactivity extracted increased linearly with time during 1 hr. The results could thus be expressed as rates of normetanephrine formation (MNM), that is, as the ratio of the amount of radioactivity extracted after incubations up to 1 hr, over incubation time. Absolute values of MNM will be given in pmole/g wet wt · min ± S.D.

Control experiments showed that the α-adrenergic blocking agents pentolamine (10^{-4} M) and phenoxybenzamine (10^{-5} M), known as inhibitors of the extraneuronal uptake (uptake 2), reduced MNM by 65 and 75 per cent respectively. This confirmed that MNM was a measure of the activity of the uptake 2–COMT system as a whole. Indeed other known effects of phenoxybenzamine besides inhibition of uptake 2, i.e. uptake 1 inhibition and blockage of α-presynaptic in-

Table 1. Effect of neuronal uptake inhibition by 10^{-6} M desmethyl-imipramine (DMI) on basal and noradrenaline stimulated $\dot{M}O_2$

nmole O_2/mg wet wt · h	Control	DMI
Basal $\dot{M}O_2$	49.4 ± 3.1 (17)	64.3 ± 3.1 (17)

Conditions	Basal nmole/mg wet wt · h	Stimulated nmole/mg wet wt · h	% stimulation	(NA) added
Control: pH 6.8	37.7 ± 4.5 (6)	129.8 ± 17.3 (6)	244	10^{-7} M
+DMI; pH 6.8	55.3 ± 2.3 (6)	172.8 ± 18.8 (6)	213	10^{-8} M
+DMI; pH 7.4	62.9 ± 5.3 (8)	194.5 ± 25.6 (8)	209	10^{-8} M

Means ± S.E.M.; number of determinations indicated in parentheses. The best quantitative comparison of stimulated $\dot{M}O_2$ was made in acidic medium (pH 6.8), in which absolute $\dot{M}O_2$ values were known to be smaller [23] and therefore O_2 availability less critical than at normal pH.

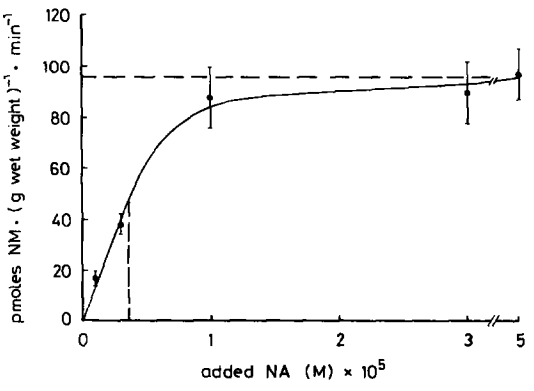

Fig. 5. Rate of normetanephrine formation in BAT slices as a function of the concentration of NA added to the medium. Apparent Vmax and Km values for the COMT system *in situ* are about 100 pmole/g wet wt · min and about 4 μM respectively. Each point is the mean (±S.D.) of at least 6 determinations.

hibitory receptors, would indirectly increase rather than decrease $\dot{M}NM$.

In order to establish the relationship between the apparent activity of the COMT system in the tissue slices and the concentration of added NA, $\dot{M}NM$ was also determined at NA concentrations higher than 3 μM, and over the shorter incubation time (30 min) during which the rate of increase in net radioactivity extracted was constant. Figure 5 shows a plot of $\dot{M}NM$ as a function of NA concentration, from which apparent Vmax and Km values for the COMT system "in situ" could be deduced. These were about 100 pmole/g wet wt · min and about 4 μM respectively. The effects of the COMT inhibitors (tropolone and DOPA) on $\dot{M}NM$ at 10^{-8} M NA were first compared in the absence of DMI. Whereas $\dot{M}NM$ was brought to zero by tropolone, it was 120 ± 25 per cent of control in the presence of $5 \cdot 10^{-5}$ M DOPA. Because of the importance of this point for a sound interpretation of the difference in the potentiating effects of tropolone and DOPA on the $\dot{M}O_2$ responses to NA (Figs. 1 and 2), the effects of DOPA on $\dot{M}NM$ were examined at different NA concentrations, both in the presence and in the absence of DMI. Table 2 summarizes the results. 10^{-6} M DMI increased $\dot{M}NM$ very significantly, as compared to controls, at NA concentrations up to 10^{-7} M, but had no effect at higher ones. Under blockage of the neuronal uptake, DOPA had a significant (36–49 per cent) inhibitory effect on $\dot{M}NM$ at all three NA concentrations examined. Furthermore, at high NA concentration ($3 \cdot 10^{-5}$ M), when the neuronal uptake was likely to be saturated as evidenced by the fact that DMI failed to enhance COMT activity, DOPA diminished $\dot{M}NM$ also in the absence of DMI.

DISCUSSION

Most of the measured $\dot{M}O_2$ values were definitely submaximal with respect to the upper limit (about 300 nmole O_2/mg wet wt · hr) attained by brown adipose tissue slices in our respirometer. It can therefore be considered that to any given $\dot{M}O_2$ value corresponded one and only one local NA concentration. In other words, we postulate a linear relationship between the concentration of NA in the direct vicinity of the receptors involved in the $\dot{M}O_2$ response (local (NA)) and the size of this response.

Results under blockage of neuronal uptake by DMI. Inhibition of COMT strongly potentiated the $\dot{M}O_2$ response to NA when the neuronal uptake was blocked, and this was a steady-state potentiation. With the implicit assumption that the chain of processes between NA-receptor interaction and respiration functioned the same way whether neuronal uptake and/or the COMT system were inhibited or not, the steady-state potentiation must have resulted from an increase of the steady-state local (NA). That such an increase could result from the blockage of any NA "consumption rate" by the tissue and the disappearance of the concomitant local (NA) gradient is supported by many experimental results [24]. The $\dot{M}O_2$ values obtained under blockage of both neuronal uptake and the COMT system were the same with endogenous NA as with the 10^{-9} M exogenous NA. It can thus be assumed that local (NA) was the same in both cases; besides, it equalled 10^{-9} M since in the absence of "consumption" of exogenous NA no concentration gradient of the catecholamine must have occurred between the bulk phase of the medium and the adrenergic receptors. One to three nanomoles per liter is about the NA concentration in the plasma of the resting, undisturbed rat [25, 26]. If our basal $\dot{M}O_2$ values, once corrected for temperature, were not underestimates of the *in vivo* rates this means that the catechol-uptake mechanisms not only remove part of the endogenous NA from the extracellular space but also protect the adrenergic receptors from being in contact with circulating NA, under basal conditions. It must be considered, however, that the potentiating effect of COMT inhibition might have appeared larger *in vitro*, as compared to what it could be *in vivo*. Indeed, the diffusion pathway for NA from circulating blood to

Table 2. Effects of 1 μM DMI and/or 50 μM DOPA on $\dot{M}NM$ in perifused brown adipose tissue

Added (NA)	Control $\dot{M}NM$ (pmole/g wet wt · min)	+DMI (% of control $\dot{M}NM$)	+DMI +DOPA (% of control $\dot{M}NM$)	RI %	+DOPA (% of control $\dot{M}NM$)
10^{-9}	0.010 ± 0.002 (6)	324 ± 35 (6)	206 ± 39 (6)	36	118 ± 8 (6)
10^{-8}	0.10 ± 0.02 (24)	268 ± 23 (8)	170 ± 42 (8)	36	120 ± 25 (8)
10^{-7}	1.6 ± 0.1 (6)	260 ± 36 (6)	—	—	—
10^{-6}	16.5 ± 3.4 (6)	122 ± 20 (6)	—	—	—
3×10^{-6}	38 ± 5 (8)	107 ± 10 (6)	55 ± 10 (6)	49	59 ± 9 (6)

Results are mean ± S.D.; the number of determinations is indicated in parentheses. Column RI gives the per cent inhibition of $\dot{M}NM$ by DOPA in the presence of DMI (see two preceding columns).

adrenergic receptors is expected to be shorter than that from the convective phase of the medium, in our perifused preparations, to the same receptors. Only if the constant wagging of the preparation, maintained in the periphery of the whirl in the vigorously stirred medium, induced significant interstitial convection could the diffusion pathway *in vitro* approach that *in vivo*. If not, local (NA) was more influenced *in vitro* by the COMT system as a sink than it is *in vivo*, and the observed potentiating effect of COMT inhibition in the absence of neuronal uptake was an overestimate. The same reasoning can be held for the experiments with endogenous NA, where the catechol diffuses in the other direction. *In vitro*, COMT is likely to be a relatively more important sink as compared to the bulk phase of medium than it is *in vivo* as compared to blood, and again the effect of COMT inhibition could have been amplified *in vitro*. However, the larger steady state responses to endogenous NA, before COMT inhibition, as compared to those to the exogenous stimulus, indicates that this amplification was less marked than in the experiments with exogenous NA.

The relatively small inhibitory effect of DOPA as compared to tropolone on $\dot{M}NM$ in the presence of DMI was unexpected in view of the similarity of the potentiating effects of the two COMT inhibitors on the $\dot{M}O_2$ response. The most satisfactory explanation of this, as of all the differences between the effects of the two COMT inhibitors on both $\dot{M}O_2$ and $\dot{M}NM$, is that 10^{-6} M DMI did not completely inhibit neuronal uptake. Since dopamine, which is produced by decarboxylation of L-DOPA under the effect of DOPA-decarboxylase in the tissues [20] is an inhibitor of neuronal uptake [21], more complete inhibition of this uptake (uptake 1) could occur under DMI + DOPA as compared to DMI alone. This would inhibit $\dot{M}NM$ relatively less than the COMT system, as the uninhibited part of that system would "see" more NA. This would also make the potentiating effect of DOPA on the $\dot{M}O_2$ response to NA larger than that of tropolone (see Fig. 3). Such a difference was not seen in the experiments with endogenous NA (Fig. 4) probably because both larger extracellular K^+ and lowered Na^+ already contributed to a more complete inhibition of uptake 1 [27]. Our explanation of the differences between DOPA and tropolone is further supported by the observation that the potentiating effect of DOPA in both series of experiments developed more slowly than that of tropolone, which is compatible with a progressive appearance of dopamine in the tissue [28].

Quantitative comparison of $\dot{M}O_2$ and $\dot{M}NM$. Because of the limited O_2 availability in perifused preparations, the present investigation was restricted to largely submaximal $\dot{M}O_2$ responses, and therefore to very low local NA stimuli. The hypothesis that, under these conditions, both suprabasal $\dot{M}O_2$ and $\dot{M}NM$ were proportional to local (NA), with proportionality constants $k1$ and $k2$ respectively, was tested on the basis of our results. Assuming that, in the absence of tiron and DMI, basal $\dot{M}O_2$ was not affected by the tiny amounts of transmitter lost by the unstimulated nerve endings and represented minimum $\dot{M}O_2$ (i.e. unrelated to any effect of NA), then

$$\dot{M}O_2 = k1 \text{ local (NA)} + 44,$$

where 44 nmole/mg wet wt · min ($n = 13$) is taken as the minimum *in vitro* $\dot{M}O_2$ (see also Barde *et al.* [13], and Fig. 6, the basal value corresponding to curve B). The equation can be solved for $k1$ in the very particular case where both neuronal uptake and COMT system were blocked and local (NA) could be considered equal to the concentration of the exogenous catecholamine (i.e. 10^{-9} M in the two sets of experiments represented in Fig. 3). With $\dot{M}O_2 = 200$ (Fig. 6, point E) and (NA) expressed in nanomoles per liter, $k1$ equals 156. In the absence of COMT inhibition, $\dot{M}O_2$ for 10 and 1 nM added NA were 210 and 64 respectively (Fig. 6, curve D). The corresponding local (NA) values computed with $k1$ are 1.06 and 0.13 nM respectively. It is now possible to compute $k2$, the proportionality constant of the relation between local (NA) and $\dot{M}NM$ measured either at 10 or at 1 nM of added NA (see Table 2, +DMI). Computed $k2$ is about the same (0.25) in both cases, which is consistent with the initial hypothesis that $\dot{M}NM$ and $\dot{M}O_2$ are both proportional to local (NA). Dimensional analysis shows that in fact the COMT system in one gram of tissue would effect a clearance of NA of about 0.25 ml of interstitial fluid every minute.

Results with neuronal uptake fully operative. As shown by the experiments illustrated in Fig. 1, the situation was much more complicated when the neuronal uptake was operative. Whereas, according to our quantitative comparison of $\dot{M}O_2$ and $\dot{M}NM$ measured

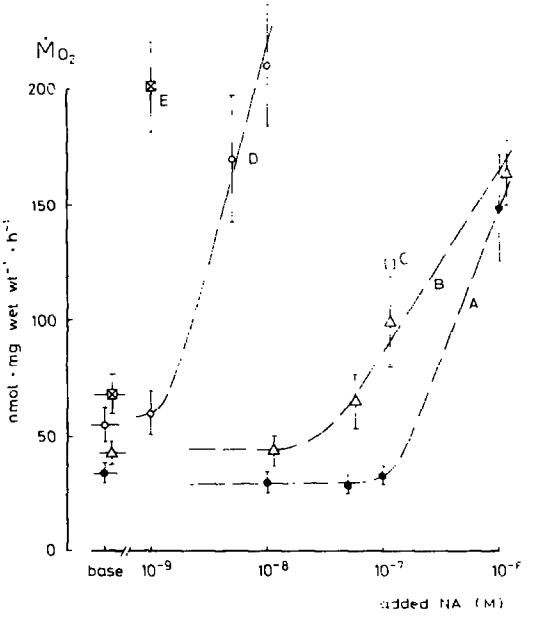

Fig. 6. Rate of oxygen uptake by BAT pieces as a function of the concentration of added NA.

(A) dose–response curve obtained when NA was administered with a metal–glass syringe through a stainless steel needle; (B) responses to the same concentrations of added NA as in A, but the catecholamine had no contact with metal parts; (C) maximum effect of tiron at 10^{-7} M of added NA, relative to the conditions of curve B; (D) dose–response curve obtained in the presence of 10^{-5} M tiron and 10^{-6} M DMI; (E) response to 10^{-9} M NA when both neuronal uptake and the COMT system were blocked. Note the striking divergence of curves B and D. Results are means ± S.E.M. ($n = 5$ in A, B, D and 4 in E).

under blockage of uptake 1, changes in local (NA) did not appear to modify the functioning of the uninhibited COMT system ($\dot{M}NM$ was simply proportional to local (NA)). Figure 1A strongly suggests that the rate of the uninhibited neuronal uptake was definitely changed in a non-monotonous fashion with respect to local (NA) after blockage of the COMT system. According to our postulate, Fig. 1 indicates that local (NA) was first increased, up to 30 min after exposure to tropolone, and then decreased towards basal value. Two alternative possibilities must be considered: (1) uptake 1 changed in proportion to local (NA), and the cause of the late inhibition of the $\dot{M}O_2$ response was unrelated to uptake 1; and (2) neuronal uptake induction was the cause of the secondary decrease of local (NA) and there was, therefore, no simple relationship between this concentration and the rate of uptake 1. An *a priori* explanation, compatible with the first alternative, for the late inhibition of the $\dot{M}O_2$ response is some toxic effect of the intracellular pool of non methylated catechol, or any direct inhibitory effect of a very high interstitial NA concentration. This, however, is unlikely since blockage of uptake 1 by DMI completely suppressed the late inhibition of $\dot{M}O_2$, and no such inhibition was observed in two hours during $\dot{M}O_2$ responses obtained with supramaximal NA stimuli (10^{-8} and 10^{-7} M, in two experiments, not shown). The second alternative is thus favored as an explanation of the late effect of tropolone (Fig. 1A). This effect was slowly reversible, as evidenced by a significant reincrease of $\dot{M}O_2$ which started about one hour after removal of the drug in five out of seven experiments. In the trials with endogenous NA (Fig. 1B) the secondary inhibition of $\dot{M}O_2$ was less marked than in the first series. As already suggested, high K^+ and lowered Na^+ could have partly inhibited uptake 1. As to the inhibition of COMT by DOPA, it did not induce any detectable decrease in $\dot{M}O_2$ probably because of the progressive inhibition of uptake 1 by the decarboxylation product of DOPA.

If the complete disappearance of the $\dot{M}O_2$ response observed already at 90 min in Fig. 1A was due to an increased activity of uptake 1, it must be realized that local (NA) could not have been the signal for such a steady state of high uptake 1 activity and the mechanism by which it occurred is unknown. Other observations are compatible with the idea that the rate of uptake 1 is not simply proportional to local (NA), or more precisely to the local (NA) which determines the $\dot{M}O_2$ response. Comparison of dose–response curves B (without DMI) and D (under 10^{-6} M DMI) of Fig. 6 shows that the higher the $\dot{M}O_2$ (i.e. the higher the local (NA)), the larger the relative activity of uptake 1, that is, the larger the horizontal distance (on the log scale !) between the two curves. It even looks as though, in the absence of DMI, the $\dot{M}O_2$ response to high concentrations of added NA might become limited by the strong induction of neuronal uptake rather than by oxygen availability.

Quantitative comparison of $\dot{M}O_2$ and $\dot{M}NM$. With the proportionality constants $k1$ and $k2$ between local (NA) and the rates ($\dot{M}O_2$ and $\dot{M}NM$ respectively) computed from the results obtained under blockage of uptake 1, the local (NA) can in turn be computed from $\dot{M}O_2$ and $\dot{M}NM$ data in the experiments where neuronal uptake was fully operative. Provided the hypothesis of proportionality between local (NA) and the rate of the process is still valid for both $\dot{M}O_2$ and $\dot{M}NM$ (if not for uptake 1) the results unambiguously show that local (NA) is no more the same in front of the β-receptors as in the vicinity of the uptake 2–COMT system. At 0.1 and 1 μM added NA, local (NA) values computed from $k2$ and measured $\dot{M}NM$ are 6.4 nM and 66 nM respectively, whereas the local (NA) values computed from $k1$ and measured $\dot{M}O_2$ are as low as 0.3 and 0.8 nM respectively, that is, only about 4 and 1 per cent of the local (NA) "seen" by the COMT system. If the brown adipose tissue is mainly under control of its adrenergic innervation [29], β-receptors are likely to be located in the close vicinity of the nerve endings. It is therefore quite possible that these receptors would be exposed to a much lower local (NA) than other parts of the interstitial border when the source of NA is exogenous and the rate of neuronal uptake creates a steep stationary (NA) gradient within the interstitium.

The present results thus suggest that COMT activity would occur mainly at a distance from immediately post-synaptic receptors, or so-called "innervated receptors" [30]. Guimaraes and Paiva [31] have come to the same conclusion, using rabbit intestine preparations as a model. Location of the uptake 2–COMT system out of the synaptic region allows us to see the apparent interplay between the two uptake systems as aimed at maintaining independence of the "innervated receptors" with respect to circulating catecholamines: either saturation or inhibition of the COMT system would entail a rise of interstitial (NA) out of the synaptic cleft, which by some as yet unknown mechanism would actively induce neuronal uptake. Interactions between the two uptake systems have been observed in cat and rabbit hearts [32], the rabbit vas deferencs [33], and in the perfused dog hindlimb [34]. These data indicate that blockage of either uptake increases the rate of the other, supposedly only because local (NA) increases [6]. Although they do not expressly demonstrate any active induction of uptake 1 by inhibition of the COMT system, they are not incompatible with this possibility (e.g. [33]). Thus, the strong induction of neuronal uptake observed after COMT inhibition in brown adipose tissue could occur in other tissues as well. Such an induction could possibly have been responsible for the reduction of the β-responses to catecholamines observed by Bacq and Renson (see Introduction) after COMT inhibition with normetanephrine.

The Km of 4.10^{-6} M as evaluated from the graph of Fig. 5 reflects the affinity of neither uptake 2 nor COMT [32] but of the system as a whole and was therefore called "apparent" Km. It must be realized that it was determined under experimental conditions which entailed local (NA) about ten times lower in the vicinity of the system than in the bulk phase of the medium, in the absence as well as in the presence of an active neuronal uptake. Thus, the apparent Km might well be in the 10^{-7} M range, i.e. an order of magnitude below the lowest estimates [7, 35]. It remains that the affinity of the COMT system for NA is most probably less than that of uptake 1.

CONCLUSIONS

Our first conclusion is that the COMT system can

control the steady-state physiological response of brown adipose tissue to noradrenaline, even at very low concentrations of the transmitter (below 10^{-6} M). This is not incompatible with an apparent Km in the 10^{-7} molar range, as the relatively high capacity of the COMT system can indeed make for its low affinity. Our second conclusion is that the COMT system exerts its control both by its direct effect on local (NA), and indirectly via uptake 1.

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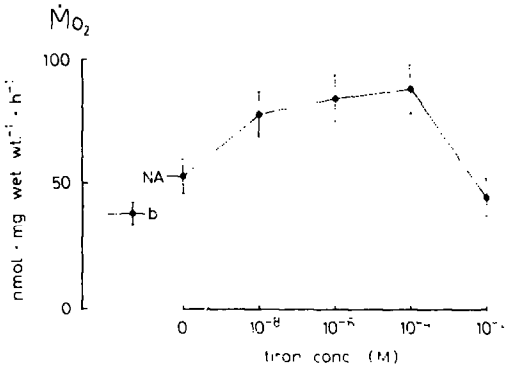

Fig. 7. Effects of various concentrations of tiron on the $\dot{M}O_2$ response to 10^{-7} M of added NA.
Mean steady state $\dot{M}O_2$ values ± S.E.M. (n = 7).

TECHNICAL ANNEXE

Since degradation products of catecholamines, as well as unphysiologically large concentrations of the intact NA itself, might have obscured the physiological functioning of the regulation mechanism under study [24, 36, 37], it was desirable to administrate exogenous NA at concentrations as low as possible, without losing the practical advantage of albumin-free media. No oxidation of NA in the medium was detected over 10–20 min [11], the period during which $\dot{M}O_2$ was measured between two successive rinsings of the chambers with fresh medium and freshly injected NA. More serious was the problem of short term NA complexation with various contaminant metal ions. Curve A in Fig. 6 shows that injection of NA with a metal–glass syringe through a stainless steel needle entailed a change in the lower part of the $\dot{M}O_2$ response curve to added NA, as compared to curve B which was obtained under conditions in which the medium and the injected NA could not have contact with any other material than lucite, polyethylene tubing and gold (heat exchanger). But despite this precaution, Zn^{2+}, Cu^{2+} and other ions can still be present as contaminants of the various analytical grade components of the medium. Complexation of these contaminants with 3,5-pyrocatecholdisulfonate (tiron) added to the medium increased the $\dot{M}O_2$ response to 10^{-7} M NA, provided the tiron concentration did not exceed 10^{-4} M, as shown in Fig. 7. The inhibitory effect of 10^{-2} M tiron on the $\dot{M}O_2$ response to 10^{-7} M added NA was not due to any direct effect of the chelator on the preparation, as it disappeared for a few minutes when the medium containing

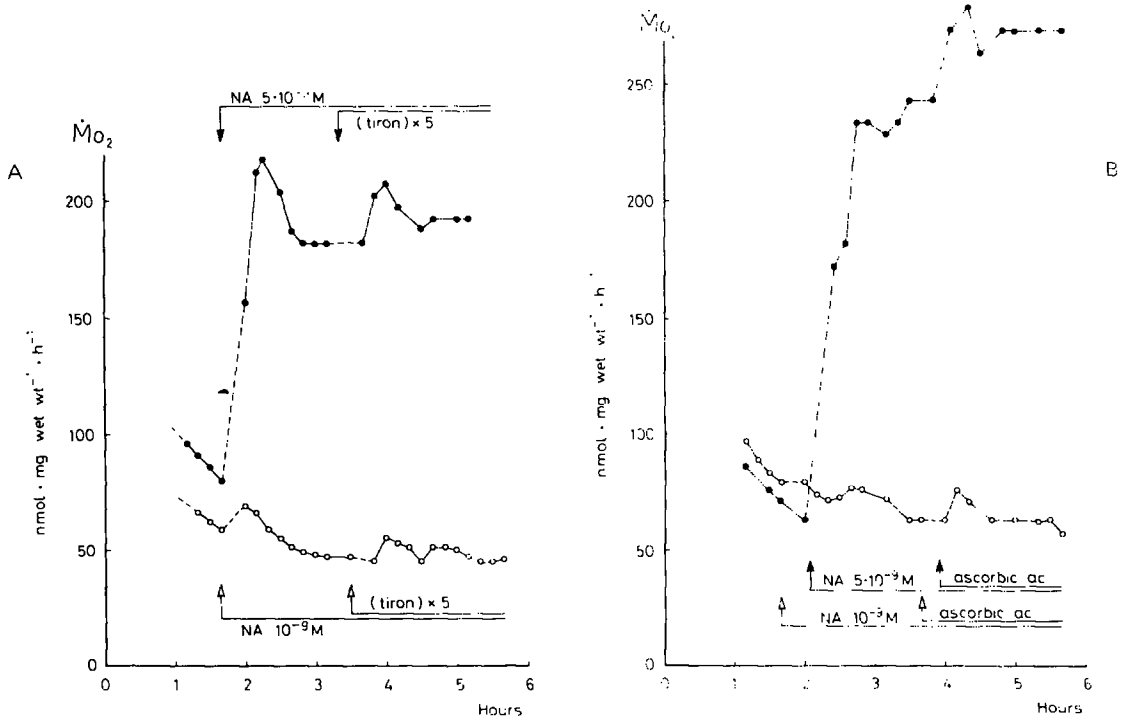

Fig. 8. Effects of $5 \cdot 10^{-5}$ M tiron (A) and of $5 \cdot 10^{-5}$ M ascorbic acid (B) on both a subthreshold and a submaximal NA stimulation of respiration in the presence of 10^{-6} M DMI and 10^{-5} M tiron.
The small transitory increases in $\dot{M}O_2$ might well be only artefacts, due to thorough rinsings of the chambers at transitions from one medium to another (see text).

tiron was thoroughly renewed in the chambers with freshly injected NA. This physicochemical phenomenon has not been analysed yet. 10^{-5} M tiron was considered close enough to the optimum concentration for the protective effect against NA complexation, and was used in all of our experiments with exogenous NA. In a previous series of experiments, addition of 10^{-5} M tiron to the medium increased $\dot{M}O_2$ in the presence of 10^{-7} M NA from 67.6 to 84.1 nmole/mg wet wt·hr ($P < 0.01$), that is, up to approximately the same value as in the present series (85.5); this, however, represented only a 25 per cent increase in $\dot{M}O_2$ whereas, in the present series, it was a 62 per cent increase, suggesting a larger degree of NA complexation in the absence of tiron. In Fig. 6, point C is indicative of what would probably be a maximum effect of tiron, at 10^{-7} M of added NA, relative to the conditions of curve B. At 10^{-6} M of added NA, the effect of tiron was undetectable.

Control experiments were carried out to check that transitions from one medium to another, which entailed complete renewal of the fluid in the chambers (instead of the partial renewal every 10 to 20 min) would not by themselves modify $\dot{M}O_2$ under NA stimulation. The protocol was the same as in the COMT inhibition experiments in the absence of DMI, except that the COMT inhibitor was replaced by 4.10^{-5} M tiron, added to the 10^{-5} M already present from the beginning. A small increase in $\dot{M}O_2$ occurred after the tiron concentration step, with a maximum at 30 min (17.5 per cent, $P < 0.025$, $n = 12$) followed by a gradual disappearance until 90 min. This indicates that degradation of the added NA was probably not completely prevented by tiron and that, therefore, thorough rinsing of the chambers at transitions from one medium to another entailed a slight and transitory increase in the intact NA concentration. The same observation was made in the presence of DMI, under both a subthreshold and a submaximal NA stimulation, as illustrated in Fig. 8A. Ascorbic acid had been found to potentiate the $\dot{M}O_2$ response in media with no tiron [19]. Of eight experiments repeated in the presence of 10^{-5} M tiron, with or without DMI, none showed any significant potentiation of the steady state $\dot{M}O_2$ response. This is illustrated in Fig. 8B, which presents small transitory effects of the addition of $5 \cdot 10^{-5}$ M ascorbic acid, under a subthreshold and a submaximal NA stimulation, with the neuronal uptake blocked. These effects are comparable to those of Fig. 8A and might have the same origin. As to the sustained potentiation effect which had been observed with ascorbic acid in the absence of tiron, it must now be declared artefactual. It can thus be concluded that 50 μM ascorbic acid, as also 50 μM tiron, did not act as an efficient inhibitor of the COMT system in our preparations. Tiron did not modify $\dot{M}NM$ (103 ± 11 per cent of control) and ascorbic acid slightly inhibited it (88 ± 2 per cent of control).

REFERENCES

1. A. J. Eisenfeld, J. Axelrod and L. Krakoff, *J. Pharmac. exp. Ther.* **156**, 107 (1967).
2. A. J. Eisenfeld, L. Landsberg and J. Axelrod, *J. Pharmac. exp. Ther.* **158**, 378 (1967).
3. L. L. Iversen, in *Adrenergic Neurotransmission* (Eds. G. E. W. Wolstenholme and M. O'Connor), CIBA Foundation Study Group No. 33, p. 45. Churchill, London (1968).
4. S. L. Lightman and L. L. Iversen, *Br. J. Pharmac.* **37**, 638 (1969).
5. O. Almgren and J. Jonason, *6th Int. Congress on Pharmacology, Helsinki*; Vol. 2, 105 (1975).
6. S. Kalsner, *Can J. Physiol. Pharmac.* **55**, 315 (1977).
7. U. Trendelenburg, *Life Sci.* **22**, 1217 (1978).
8. N. Weiner, M. Perkins and R. L. Sidman, *Nature, (Lond.)* **193**, 137 (1962).
9. K. Stock and E. O. Westermann, *J. Lipid Res.* **4**, 297 (1963).
10. B. Falck, N. A. Hillarp, G. Thieme and A. Thorp, *J. Histochem. Cytochem.* **10**, 347 (1962).
11. J. Durand, J. P. Giacobino, J. Deshusses and L. Girardier, *Biochem. Pharmac.* **28**, 1347 (1979).
12. C. D.-Joel, *Lipids* **5**, 224 (1970).
13. Y. A. Barde, A. Chinet and L. Girardier, *J. Physiol.* **252**, 523 (1975).
14. Z. M. Bacq, L. Gosselin, A. Dresse and J. Renson, *Science* **130**, 453 (1959).
15. Z. M. Bacq, *C.r. Soc. Biol.* **154**, 2130 (1960).
16. Z. M. Bacq and J. Renson, *Bull. Acad. Roy. Méd. Belg.*, VIe série, **25**, 755 (1960) et *Archs int. Pharmacodyn.* **130**, 385 (1961).
17. Z. M. Bacq, G. L. Brown and C. B. Ferry, *C.r. Acad. Sci. Paris* **250**, 194 (1960).
18. J. Axelrod, *Methods in Enzymology*, Vol. 5. Academic Press, New York (1962).
19. A. Chinet and L. Girardier, *Experientia* **30**, 677 (1974).
20. P. N. Patil, D. D. Miller and U. Trendelenburg, *Pharmac. Rev.* **26**, 323 (1975).
21. A. S. V. Burgen and L. L. Iversen, *Br. J. Pharmac.* **25**, 34 (1965).
22. L. L. Iversen and B. A. Callingham, in *Fundamentals of Biochemical Pharmacology* (Ed. Z. M. Bacq), p. 253. Pergamon Press, Oxford (1971).
23. A. Chinet, C. Friedli, J. Seydoux and L. Girardier, *Experientia, Suppl.* **32**, 25 (1978).
24. U. Trendelenburg, *Handbook of Experimental Pharmacology*, XXXIII, Chap. 16, p. 726. Springer-Verlag, Berlin (1972).
25. F. Depocas and W. A. Behrens, *Can. J. Physiol. Pharmac.* **55**, 212 (1977).
26. C. W. Popper, C. C. Chiueh and I. J. Kopin, *J. Pharmac. exp. Ther.* **202**, 144 (1977).
27. Z. M. Bacq, *J. Physiol., Paris* **72**, 371 (1976).
28. W. D. Horst, W. R. Pool and H. E. Spiegel, *Eur. J. Pharmac.* **21**, 337 (1973).
29. J. Seydoux and L. Girardier, *Experientia, suppl.* **32**, 153 (1978).
30. S. Rosell and E. Belfrage, *Nature* **253**, 738 (1974).
31. S. Guimaraes and M. Q. Paiva, *J. Pharm. Pharmac.* **29**, 502 (1977).
32. K. H. Graefe, H. Bonisch, R. Fiebig and U. Trendelenburg, *6th Int. Congress of Pharmacology, Helsinki*; Vol. 2, 117 (1975).
33. J. Hughes, *Br. J. Pharmac.* **44**, 472 (1972).
34. W. Osswald and D. Branco, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmac.* **277**, 175 (1973).
35. H. C. Guldberg and C. A. Marsden, *Pharmac. Rev.* **27**, 135 (1975).
36. B. B. Wolfe, J. A. Zirrolli and P. B. Molinoff, *Molec. Pharmac.* **10**, 582 (1974).
37. R. W. Colburn and J. W. Maas, *Nature* **208**, 37 (1965).