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Effects of Branched-Chain Fatty Acids on GABA-Degradation and Behavior: Further Evidence for a Role of GABA in Quasi-Morphine Abstinence Behavior

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VAN DER LAAN, J. W., A. W. C. M. JACOBS AND J. BRUINVELS. *Effects of branched-chain fatty acids on GABA-degradation and behavior: Further evidence for a role of GABA in quasi-morphine abstinence behavior.* PHARMAC. BIOCHEM. BEHAV. 13(6) 843–849, 1980.—An increase in GABA-ergic activity has been implicated in the initiation of quasi-morphine abstinence behavior by di-n-propylacetate (DPA). Two structural analogues of DPA, namely, the branched-chain-fatty acid 2-methyl, 2-ethylcaproic acid and 2,2-dimethylvaleric acid have now been used to study this relationship between behavioral and biochemical effects. A correlation appeared to exist between the K_i of these compounds for succinic semi-aldehyde dehydrogenase, the second enzyme in the degradation of GABA, and the doses exerting a maximum effect on behavior. On the other hand concurrent inhibition of GABA-transaminase seemed to suppress the behavioral effects of the fatty acids. This apparent paradox can possibly be explained by supposing a different action of the fatty acids in distinct compartments of the brain, suggesting an important role for increased GABA-ergic activity in the neuronal compartment in the initiation of the quasi-morphine abstinence behavior.

Gamma-aminobutyric acid | di-n-propylacetate | Behavior | Quasi-morphine abstinence
Branched-chain fatty acids | Metabolic compartmentation

ELEVATION of GABA levels by di-n-propylacetate has been shown to occur via inhibition of succinic semialdehyde dehydrogenase (SSA-DH), the second enzyme in the degradation of GABA [12,27]. The resulting accumulation of succinic semialdehyde (SSA) probably increases GABA-concentration by product inhibition of GABA-transaminase (GABA-T). In addition, backward formation of GABA from SSA may occur since the reactions catalysed by GABA-T are strongly biased towards GABA [27].

In order to find more evidence for the involvement of this mechanism in DPA-induced "quasi-morphine abstinence behavior" [8], we have studied the effects of some analogues of DPA both on behaviour and on the enzymes involved in GABA-degradation.

METHOD

Behavioral Studies

Animals. Male albino rats (100–200 g), randomly selected from an inbred Wistar strain (TNO, Zeist, The Netherlands), were used for all experiments. The animals were housed four or five to a plastic cage with food and water ad lib. Lights were kept on from 8.30 a.m. till 20.30 p.m. Behavioral observations were performed between 10.00 a.m. and 16.30 p.m. in a room with constant background noise and a constant temperature of 22 ± 1°C.

Behavior. Observation of behavior was carried out essentially as described by De Boer *et al.* [8]. Briefly, the scoring system of Frederickson and Smits [11] was used, in which scores of 0 (absent), 2 (mild) or 4 (marked) were given to penile erection, penis licking, ptosis, teeth chattering, swallowing, tremor, hunchback posture and piloerection. Escape digging, body shaking, head shaking and foreleg shaking were counted and assigned scores of 2, 4, 6, 8, or 10 if they occurred 2–5, 6–10, 11–20, 21–40 or more than 40 times, respectively, during the observation period of 15 min. Horizontal activity was measured using a Varimex activity meter.

Drug preparation and administration. Sodium di-n-propylacetate (Sodium Valproas, Albic BV, Maassluis, The

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TABLE 1

EFFECT OF SHORT-CHAIN AND BRANCHED-CHAIN FATTY ACIDS ON BEHAVIORAL PARAMETERS

Compound	Dose mmol/kg	n	Counted signs: Escape digging	Head shakes	Swallowing	Checked signs: Hunchback posture	Piloerection	Penis licking	Ptosis	Teeth chattering
DMV	0.6	3	—	1/3	—	1/3	—	—	—	—
	1.2	4	—	—	3/4	1/4	1/4	1/4	2/4	—
	1.8	4	3/4	1/4	3/4	4/4	1/4	3/4	2/4	1/4
	2.4	4	1/4	—	3/4	4/4	2/4	1/4	2/4	2/4
	3.0	4	1/4	2/4	1/4	4/4	1/4	1/4	1/4	1/4
	3.6	4	—	1/4	—	4/4	4/4	—	2/4	—
	5.4	5	—	—	—	2/5	4/5	—	—	—
MEC	0.6	3	—	1/3	—	2/3	1/3	1/3	—	—
	0.9	4	2/4	—	1/4	4/4	2/4	1/4	1/4	—
	1.2	4	—	2/4	—	2/4	4/4	—	—	—
	1.5	4	—	—	—	—	3/4	—	1/4	—
	1.8	4	—	1/4	—	1/4	1/4	—	1/4	—
	2.4	3	—	1/3	—	1/3	2/3	—	1/3	—
DPA	0.6	3	1/3	—	1/3	—	—	1/3	—	1/3
	1.2	4	1/4	2/4	1/4	2/4	3/4	2/4	2/4	—
	1.8	4	3/4	3/4	2/4	4/4	4/4	1/4	2/4	—
	2.4	4	2/4	1/4	2/4	4/4	4/4	—	—	—
	3.6	4	—	2/4	—	4/4	3/4	1/4	—	—
	5.4	4	—	1/4	—	4/4	3/4	—	—	—
Caproate	3.6	3	—	—	—	2/3	2/3	—	1/3	—
	5.4	3	—	—	1/3	3/3	3/3	—	2/3	—
	7.2	3	—	—	1/3	2/3	—	—	—	—
	9.0	3	—	—	1/3	2/3	1/3	—	2/3	—
NaCl	1.8	4	1/4	1/4	1/4	—	—	—	—	—

Survey of the abstinence behavior induced by MEC, DMV, DPA and caproate in rats treated with varying doses and observed for 15 min. For all symptoms the incidence has been given, showing the number of rats in which that particular symptom was observed at the given dose. As a control four rats were treated with NaCl (100 mg/kg). The maximally effective doses for each symptom have been underlined.

Netherlands) was dissolved in deionized water (0.6 mmol/ml). 2-Methyl, 2-ethylcaproic acid (MEC) and 2,2-dimethylvaleric acid (DMV) were a gift from Prof.dr. A. Lespagnol (Lille, France) and were, like caproic acid (Merck, Darmstadt, GFR) dissolved in deionized water, together with an equimolar quantity of sodium hydroxide to obtain a similar solution as for DPA. For intraperitoneal injections, the following concentrations were used: MEC, 0.3 mmol/ml; DMV, 0.4 mmol/ml; and caproate, 1.2 mmol/ml.

Biochemical Studies

Materials. Apart from the drugs mentioned above the following compounds were used: GABA was obtained from Calbiochem; Triton-X-100 and 2-mercaptoethanol were obtained from BDH Chemicals Ltd. SSA was purchased from Sigma Chemical Company. NAD and NADH were obtained from Boehringer Mannheim. (U-^{14}C)GABA (specific radioactivity 224 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. All other chemicals were purchased from Merck, Darmstadt.

Tissue preparation. Randomly selected male Wistar rats (125–175 g) were killed by decapitation and their brains were rapidly removed and weighed. A 10% (w/v) brain homogenate was prepared in an ice-cold solution containing 0.32 M-sucrose and 4.5 mM-2-mercaptoethanol, using a Teflon-glass Potter-Elvehjem homogenizer. One volume of the homogenate was added to 3 vol of ice-cold Triton medium (0.67% w/v Triton-X-100, 50 mM-Tris-HCl (pH 8.5) and 4.5 mM-2-mercaptoethanol) and kept in ice water for 1 hr before use.

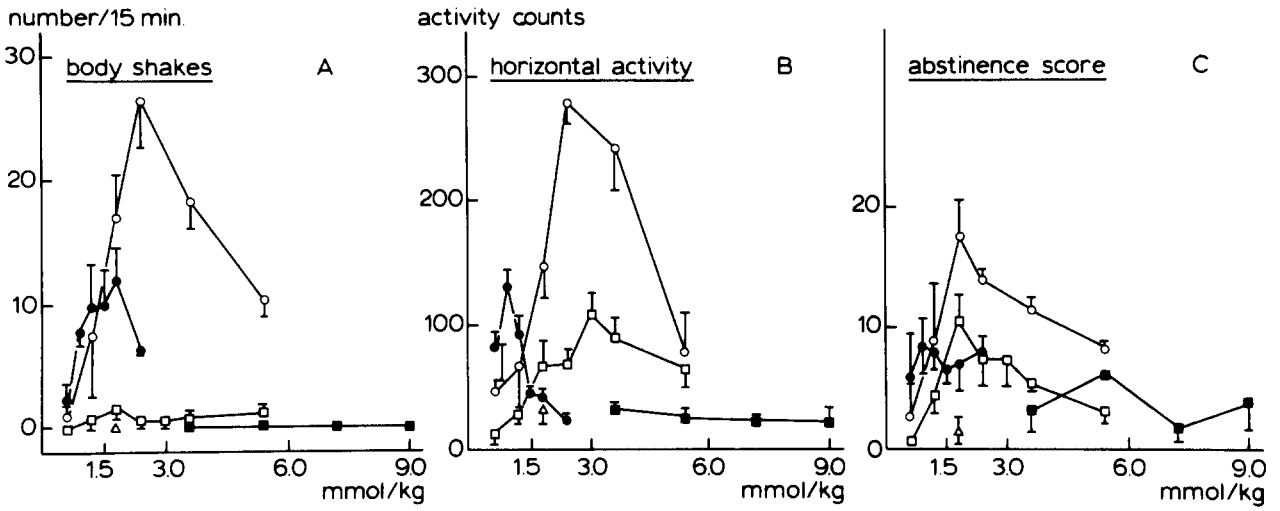

FIG. 1. Dose-effect curves of the behavior observed after injection of some branched-chain fatty acids or caproate. Rats were treated with increasing doses of DMV, MEC, DPA or caproate after which their behavior was observed for 15 min. Bars represent SEM. For each compound the same symbol in all figures was used: ○–○, DPA; ○–○, MEC; □–□, DMV; ■–■, Caproate; △, NaCl.

GABA-T assay. For the assay of GABA-T a radiochemical method was used, as previously described [7], with the following modifications. NAD was omitted from the incubation mixture to exclude interference with SSA-DH. Tissue concentration was 1.11 mg/ml (wet wt.). Drugs, as mentioned above, were used as their sodum salts and, when necessary, sodium isethionate was added to obtain a standard sodium concentration of 20 mM in all incubation media. After preincubation at 22°C for 30 min, the reaction was started by the addition of homogenate and subsequently the mixture was incubated for a further 60 min at the same bath temperature. All experiments were carried out in duplicate.

SSA-DH assay. Measurement of SSA-DH activity was performed as previously described [7] using a tissue concentration of 0.44 mg/ml (wet wt.). In all incubations the sodium concentration used was 10 mM. All incubations were performed in quadruplicate.

Data analysis. The kinetic parameters of GABA-T were calculated from Lineweaver-Burke plots. Since in the assay of SSA-DH more than 5% of the initial concentration of SSA was converted, the apparent kinetic constants were calculated using the integrated form of the Henri-Michaelis-Menten equation [24].

The type of inhibition of the enzymes by the fatty acids was determined graphically. The K_i's were calculated from a replot of the ratio of the apparent K_m and V_{max} versus the concentration of the inhibitor as suggested by Segel [24] for mixed type inhibitions. All constants were calculated from the plots using the method of least squares.

RESULTS

Behavior

As shown in Table 1 and Fig. 1, most behavioral symptoms induced by DPA could also be evoked by the other branched chain fatty acids, MEC and DMV. In contrast, only a few so-called "checked signs", viz. swallowing, hunchback posture, piloerection and ptosis could be observed after injection of the short straight-chain fatty acid, caproic acid.

TABLE 2

EFFECTS OF DPA, MEC, DMV AND CAPROATE ON THE ACTIVITY OF GABA-T AND SSA-DH IN RAT BRAIN HOMOGENATE

	GABA-T K_i (mM)	SSA-DH K_i (mM)	Ratio $(\frac{K_{i\ GABA\text{-}T}}{K_{i\ SSA\text{-}DH}})$
DPA	23.2*	0.43	54
MEC	3.9	0.17	23
DMV	6.0	0.61	10
Caproate	NI	1.89	—

*$K_{i\ GABA\text{-}T}$ value from Van der Laan *et al.* [27]. Values were derived from the replots in the Figs. 2 and 3. The ratio is the quotient of the K_i for GABA-T and the K_i for SSA-DH of any one compound. NI=no inhibition.

As can be seen in Fig. 1, the dose which induced the maximal number of body shakes was, for MEC, 1.2 mmol/kg, whereas with doses higher than 1.8 mmol/kg a decrease was observed. With DMV, a maximal number of body shakes was obtained using 1.8 mmol/kg, while for DPA this was the case with 2.4 mmol/kg (Fig. 1A).

Maximum activity after injection of MEC was found using a dose of 0.9 mmol/kg, while maximal locomotor activity after DPA was obtained using 2.4 mmol/kg and after DMV, when using 3.0 mmol/kg. Caproate did not alter the activity, as compared with NaCl treatment, up to doses of 9.0 mmol/kg. The decrease in motor activity obtained after administration of high doses of the branched chain fatty acids was caused by a loss of righting reflex occurring within the observation period (Fig. 1B).

As was found for body shakes and locomotor activity, so with regard to the total abstinence score (Fig. 1C), MEC was shown to be the most potent compound, eliciting a maximum score after administration of 0.9 mmol/kg. For DMV and DPA the highest abstinence score was evoked using a dose of 1.8 mmol/kg.

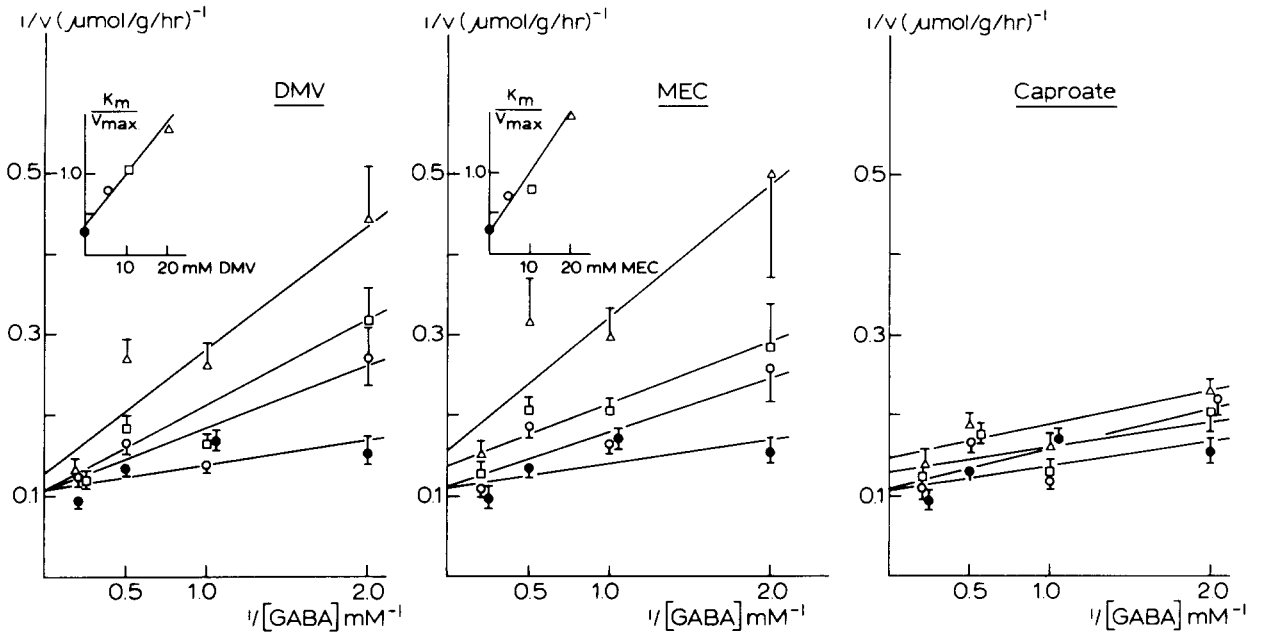

FIG. 2. The effect of DMV, MEC and Caproate on the activity of GABA-T. Reciprocal plots of the velocity vs. the concentration of GABA in the presence of 0.5 mM 2-OG. For each concentration of the inhibitor the same symbol was used in all the figures: ●–●, control in the presence of 20 mM sodium-isethionate; ○–○, 5 mM of a fatty acid; □–□, 10 mM of the fatty acid; △–△, 20 mM of the fatty acid. For DMV and MEC a replot of the ratios of the apparent K_m and V_{max} values vs. the fatty acid concentration is inserted, to calculate the apparent K_i of these compounds. Bars represent SEM (n=3).

Comparing the magnitude of the maximal activity of the various compounds, DPA was found to be the most potent compound in evoking body shakes, horizontal activity and abstinence behavior (Fig. 1). Using MEC, a higher number of body shakes and activity counts was obtained when compared to DMV, whereas the DMV-induced maximal abstinence score was somewhat higher than that found for MEC.

With regard to the checked signs (Table 1), no great differences were apparent between all compounds tested. Therefore, the differences in the total abstinence score can only be ascribed to differences in counted signs, such as body shakes and escape digging. As already mentioned, counted signs were not observed after injection of caproate.

Biochemistry

The kinetics of GABA-T were studied using concentrations of 0.5–5.0 mM GABA and 0.5 mM 2-oxoglutarate. The concentrations of MEC, DMV and caproate were 5, 10 and 20 mM for each compound. Lineweaver-Burke plots are presented in Fig. 2. From these plots the K_i's were calculated using a replot of the ratio of the K_m and V_{max} versus the concentration of the inhibitor and the calculated K_i's are presented in Table 2. MEC was found to be the most potent inhibitor, DMV was slightly less potent, whereas no inhibition could be found with caproate. The inhibition by MEC and DMV had a mixed character, in which the competitive component was the greatest.

To study the effect of the fatty acids on SSA-DH, SSA concentrations of 50, 75 and 150 μM were used. In order to determine control values in the absence of an inhibitor, two additional concentrations were used, namely 25 and 100 μM. The concentrations of the fatty acids varied from 0.25 to 2.0 mM for MEC and from 0.5 to 4.0 mM for DPA, DMV and caproate as can be seen in the replots (Fig. 3). For the determination of the K_m and V_{max} integrated Henri-Michaelis-Menten plots were used. The ratio of these parameters was plotted versus the concentration of the inhibitor to calculate the K_i. The resulting K_i's are presented in Table 2. With respect to SSA-DH, MEC was also found to be the most potent inhibitor while the K_i of DMV was shown to be 1.5 times that found for DPA. The K_i for caproate was found to be 4.5 times that for DPA.

A relation between inhibition of GABA-T and inhibition of SSA-DH is given in Table 2 by expressing the inhibition of the GABA degradating enzymes as the ratio of the K_i's of these compounds.

DISCUSSION

Branched-chain fatty acids have been described as anaesthetic and neurodepressive agents [17,18], while Marcus *et al.* [19], using straight-chain fatty acids, reported an epileptoid rather than an anaesthetic state after administration of these fatty acids.

More recently, however, other behavioral effects of a branched-chain fatty acid, namely DPA, were reported using somewhat lower doses [4,8], e.g. body shakes and enhanced locomotor activity, besides other symptoms, resembling morphine abstinence behavior. Therefore, this behavior was presented as "quasi-morphine abstinence behavior" [1,9] as defined by Collier [3].

In contrast to the data of Lespagnol *et al.* [18], these reports indicate a stimulating rather than a depressive action of this compound. The present results show that these behavioral effects could also be evoked after administration of two other branched-chain fatty acids, MEC and DMV.

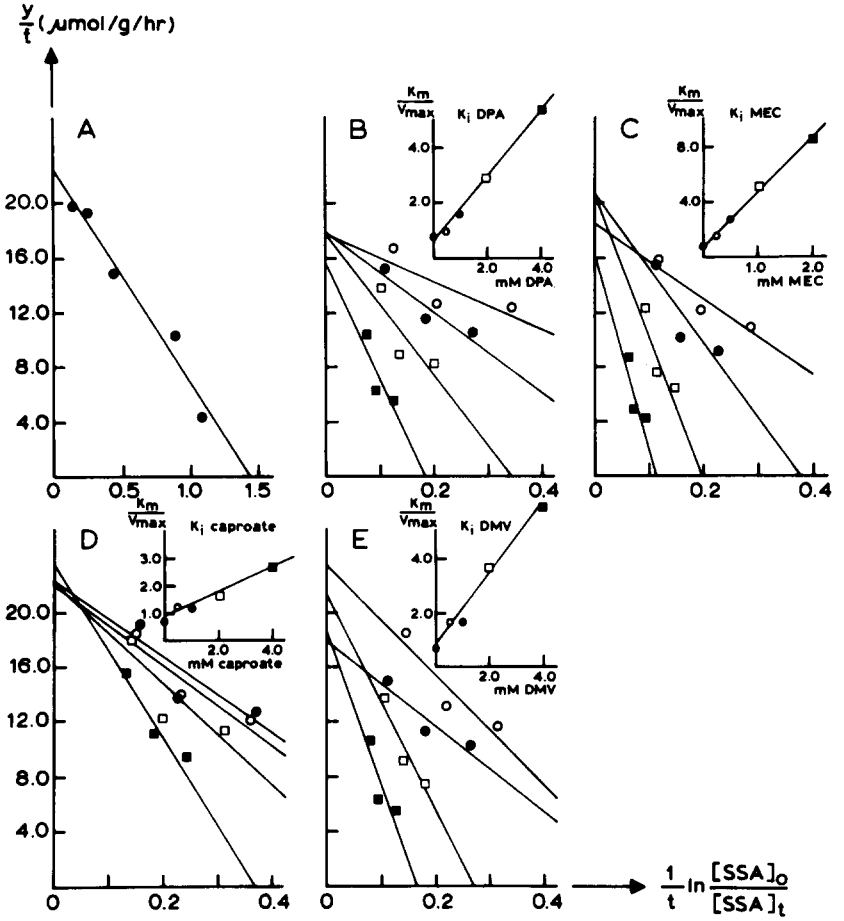

FIG. 3. The effect of DMV, DPA, MEC and Caproate on the activity of SSA-DH. Integrated Henri-Michaelis-Menten curves. Y is equal to the amount of product formed during the incubation time t. $[SSA]_0$ is the initial substrate concentration, whereas $[SSA]_t$ is the substrate concentration at the end of the incubation period. The slope of the lines obtained from these curves equals $-K_m$, while the intercept equals the apparent V_m. A: control in the presence of 10 mM sodium-isethionate. B: ○–○, 0.5 mM DPA; ●–●, 1.0 mM DPA; □–□, 2.0 mM DPA; ■–■, 4.0 mM DPA. C: ○–○, 0.25 mM MEC; ●–●, 0.5 mM MEC; □–□, 1.0 mM MEC; ■–■, 2.0 mM MEC. D: ○–○, 0.5 mM Caproate; ●–●, 1.0 mM Caproate; □–□, 2.0 mM Caproate; ■–■, 4.0 mM Caproate. E: ○–○, 0.5 mM DMV; ●–●, 1.0 mM DMV; □–□, 2.0 mM DMV; ■–■, 4.0 mM DMV. Replots to determine the K_i's of the compounds are inserted, in which the ratio of the apparent K_m and V_{max} as a function of the inhibitor concentration is given. The results are the mean of three experiments.

Comparison of the doses of the compounds used for maximal induction of the behavioral syndrome, revealed that MEC was more potent than DPA or DMV. The dose of MEC inducing the maximal score of body shakes, locomotor activity or total abstinence (1.0 mmol/kg—the mean of 1.2 for body shakes, 0.9 for horizontal activity and 0.9 for the total abstinence score) was found to be lower than those found for DPA or DMV (both 2.2 mmol/kg). On the other hand, the number of body shakes, the locomotor activity and the abstinence score were higher after injection of DPA at its maximally effective dose than after the maximally effective doses of DMV or MEC. After injection of DMV only a few body shakes could be shown, even at the maximally effective dose, whereas the scores for horizontal activity and abstinence were the same as for MEC.

The biochemical data obtained for MEC and DMV with regard to GABA-T are in agreement with those reported by Cash *et al.* [2] and Maitre *et al.* [20]. However, other studies revealed a K_i for these compounds ten-fold lower than that obtained in the present study [5,21]. Possibly, this discrepancy may be due to the use of different methods. Although the K_i's for SSA-DH of the branched-chain fatty acids in the present experiments were eight-fold lower than those reported by Cash *et al.* [2], the potency ranking was found to be the same. The K_i for DPA was shown to be the same as that reported previously [27].

When considering the inhibition pattern of GABA-T by these compounds, it appeared that the compound with an intrinsic butyric acid chain, MEC, was the most potent inhibitor of GABA-T when compared to the compound with a

propionic acid chain (DMV) or with a valeric acid chain (DPA); a conclusion which is in agreement with other reports [10]. When considering inhibition of SSA-DH, the length of the longest chain in the fatty acids studied seems to be more important, as has been reported previously for the straight-chain fatty acids [1].

The present study clearly shows a great similarity in behavioral symptoms evoked by the branched-chain fatty acids, while the short straight chain fatty acid, caproic acid, was inactive in this respect. Furthermore, the present results show that the three branched-chain fatty acids were acting as strong inhibitors of the degradation of GABA, especially by inhibition of SSA-DH.

Combining behavioral and biochemical data, a correlation appeared to exist between the maximally effective doses, evoking the strongest behavioral effects, and the K_i for SSA-DH of the various compounds studied. Thus MEC, which elicits the behavioral syndrome at the low dose of 1.0 mmol/kg, has also been shown to be the strongest inhibitor of SSA-DH with a K_i of 0.17 mM. DPA and DMV, both exhibiting mean dose values evoking maximal behavioral effects higher than that found for MEC, namely 2.2 mmol/kg, are also less potent with regard to inhibition of SSA-DH with K_i values of 0.43 and 0.61 mM, respectively. The much higher K_i for SSA-DH of caproate (1.89 mM) may, therefore, indicate why no similar behavioral effects were observed with this fatty acid.

On the other hand, the number of body shakes, the increase in locomotor activity and the abstinence score appear to be dependent on the ratio of the K_i's for GABA-T and SSA-DH (Table 2). The high ratio [54] found for DPA seems to correlate with the highest behavioral score obtained with this compound, while the moderate ratio found for MEC [23] and the low ratio found for DMV [10] are in agreement with the moderate and low behavioral scores, respectively obtained with these compounds, especially with respect to body shakes.

These results may indicate that inhibition of SSA-DH by these branched-chain fatty acids, thus enhancing GABA levels, is responsible for the initiation of the quasi-morphine abstinence behavior, a conclusion which is in agreement with the findings that the behavior induced by DPA is antagonized by bicuculline, picrotoxine or 3-mercaptopropionic acid, but not by strychnine [8,9]. However, concurrent inhibition of GABA-T by these fatty acids—indicated by a lower ratio of the K_i's for both enzymes—results in a suppression of this abstinence behavior. Intact GABA-T activity seems to be a requirement for full expression of the abstinence behavior since pre-treatment of rats with the GABA-T inhibitor aminooxyacetic acid (AOAA) prevents DPA-induced behavior [9].

Since inhibition of either SSA-DH or GABA-T will both result in an increase in the GABA concentration, the results can be interpreted in favour of a compartmentalized action of DPA, suggesting a preferential inhibition of GABA-degradation, via SSA-DH, in the neuronal compartment as the action responsible for evoking quasi-abstinence behavior, whereas an overflow of GABA to presynaptic areas, possibly GABA-ergic presynaptic receptors, might be responsible for suppression of this behavior [1, 6, 9].

A possible presynaptic action of GABA in inhibiting its own release has been suggested by several authors [22,26]. Recently, Sarhan and Seiler [23] presented evidence for a compartmentalized action of DPA in mouse brain, indicating an increase in synaptosomal GABA during the first 10 min and thereafter, an increase in non-synaptosomal material. This time course for the increase in synaptosomal GABA after DPA is in agreement with the time course of the quasi-abstinence behavior induced by DPA. Iadarola and Gale [16] have also provided evidence that the GABA-increase produced by DPA is associated mainly with GABA-ergic nerve terminals, while AOAA primarily elevates GABA in non-nerve terminal components.

Using the neuronal model as described above, MEC and DMV can be thought to inhibit the quasi-abstinence behavior by enhancement of the concentration of non-synaptosomal GABA (probably located in glial cells and neuronal cell bodies). Release of GABA from the glial compartment will promote an action of GABA on presynaptic GABA-ergic autoreceptors resulting in inhibition of neuronal GABA-release. This explanation is supported by the recent findings of Cunningham *et al.* [5], showing that MEC and DMV are capable of inhibiting GABA-dependent oxygen-uptake in non-synaptosomal mitochondria, while the very weak GABA-T inhibitor DPA was without effect in this system. Thus, DMV and MEC, but not DPA, inhibit GABA-metabolism in the non-synaptosomal compartment.

Although the role for GABA in true morphine-abstinence behavior is still not clear, Ho *et al.* [14,15] have shown that administration of AOAA or bicuculline can affect morphine analgesia, the development of tolerance and the degree of physical dependence.

Studies on the activity of glutamate decarboxylase during morphine dependency and abstinence [13,25] also indicate an enhanced synthesis of GABA (which is localized in the nerve endings) during the abstinence phase when compared to the dependency phase.

In conclusion, the present findings support a role for GABA in quasi-morphine abstinence behavior evoked by branched-chain fatty acids. Inhibition of SSA-DH, resulting in an accumulation of GABA in the synaptosomal compartment, seems to be responsible for evoking quasi-morphine abstinence behavior, whereas inhibition of GABA-T, resulting in an increase in GABA-concentration in the non-synaptosomal compartment, will be responsible for suppression of this behavior. The latter may occur via leakage or release of GABA from this compartment into the extraneuronal space, promoting an action of GABA on presynaptically located autoreceptors.

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