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A quantitative assessment of the reactivity of the fatty alcohols with cetrimide using immersion calorimetry

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The reactivity of the fatty alcohols with cetrimide has been quantitatively assessed using immersion calorimetry. In all cases the reaction was endothermic i.e. it had a positive enthalpy. For the n-alcohols the reactivity, as evaluated by the rate of enthalpy change, decreased with increasing chain length although branching on the tetradecanol and hexadecanol resulted in a higher reactivity. Adding 1-octadecanol to 1-hexadecanol resulted in an increased reactivity rising to a maximum for mixtures containing 20–40% w/w 1-octadecanol. The results have been interpreted in terms of the polymorphic form of the alcohol.

The mixed emulsifier system of cetrimide and a fatty alcohol (specifically cetyl or stearyl or, more commonly, admixtures) is frequently used in the formulation of antiseptic creams. The properties of these creams are related to the presence in the aqueous continuous phase of a gel network formed by the interaction of the surfactant with the alcohol. It has long been realized that a knowledge of this interaction is necessary for the understanding of the network formation (Eccleston 1984) and to this end two techniques, both based on microscopical observation, have been developed. Firstly, a simple observation of the interaction at room temperature and, secondly, the measurement of a temperature at which there is rapid penetration of the surfactant into the fatty alcohol. Unfortunately, it would appear that the former method, although being subjective, is of more importance when considering the kinetics of network formation. Since penetration of the fatty alcohol by the surfactant naturally causes a change in the enthalpy of the system it would appear logical to investigate this by means of immersion calorimetry. It is this technique that has been used in this preliminary study.

Materials and methods

Samples of 1-tridecanol, 1-tetradecanol, 2-tetradecanol, 1-pentadecanol, 1-hexadecanol (cetyl alcohol), 2-hexadecanol, 1-heptadecanol, 1-octadecanol (stearyl alcohol) and 1-eicosanol were obtained from either Fluka A. G. (Buchs, Switzerland) or Aldrich Chemical Co. Ltd (Dorset, UK): all had a measured purity >98%. Mixtures of 1-hexadecanol and 1-octadecanol were prepared by melting and subsequent solidification. Commercial samples of cetostearyl alcohol were obtained from either Efkay Chemicals Ltd (London, UK) or Kelanco Ltd (Formby, UK), but whereas the sample obtained from Efkay Chemical Ltd had been prepared from its natural source (spermaceti), that from Kelanco Ltd was a synthetic mixture (see Patel et al 1985). All samples were carefully sieved through a 720 μm mesh to eliminate possible changes in the surface area. The immersion liquid used was 5% w/w cetrimide solution.

Enthalpy changes were measured using an immersion calorimeter (Model 8700, LKB Instruments Ltd, Croydon, UK). This consisted of a 100 cm^3 glass vessel fitted with a thermistor capable of detecting temperature changes of 10^{-5} °C, and a heater for calibration purposes. A gold stirrer, which also served as a holder for the glass vial containing the powder sample, projected into the vessel. The assembly was contained in a chromium plated brass case and submerged in a water bath maintained at 29 °C by means of a temperature regulator and sensor. An amount of 0·3–0·6 g of sample was sealed into the vial and immersed in 100 cm^3 of 5% w/w cetrimide solution. After thermal equilibrium had been reached, as indicated by a temperature change of less than 10^{-3} °C min^{-1}, the vial was broken by manual depression on to a pointed rod mounted in the floor of the reaction vessel. Temperature changes were then recorded every 15 s until the reaction had ceased.

The method used to evaluate the temperature change during the reaction involved extrapolating the changes that had occurred in the pre- and post-reaction periods and calculating the equal area ordinate. This method was chosen since it takes into account, and corrects for, heat leakage from the system, the heat of stirring, and the heat generated by the thermistor. The corrected temperature was then compared with the calibration in order to evaluate the enthalpy change for the reaction. The time taken for the reaction was evaluated from the temperature change/time curve. All data reported were the mean of two experiments.

Results

In all cases the reaction was endothermic, i.e. it had a positive enthalpy change. For the n-alcohols the reactivity, as evaluated by the rate of enthalpy change, decreased with increasing chain length (Table 1). Branching resulted in a 55% increase in reactivity for the tetradecanol and a 167% increase in reactivity for the hexadecanol. Data for the 1-hexadecanol/1-octadecanol mixtures are shown in Fig. 1. It can be seen that increasing the 1-octadecanol content of the mixture resulted in an increase in reactivity which rises to a maximum for mixtures containing 20–40% w/w octade-

canol and then falls for mixtures containing higher concentrations of octadecanol. Although both batches of commercial cetostearyl alcohol showed similar enthalpy changes (+69·1 J g^{-1} for the synthetic bland, +67·7 J g^{-1} for the natural blend) their measured reaction times were very different (21·0 min for the synthetic blend, 5·9 min for the natural blend) indicating an increased reactivity for the natural blend.

The relevance of these data to the practical situation of the preparation of pharmaceutical antiseptic creams is best discussed by reference to information available on the 1-hexadecanol/1-octadecanol mixes and commercial cetostearyl alcohols. The graphs of enthalpy change and reactivity (Fig. 1) closely mimic those for the viscosity and yield values of emulsion formulations prepared using such mixtures (Fukushima et al 1976), with those mixtures giving high reactivity producing emulsions of high yield and increased viscosity. The same effect between the measured viscosity of a formulation and reactivity can be seen with the data for the two cetostearyl alcohols since previous experience (Patel et al 1985) had shown that ternary gels prepared from the natural source of alcohol (i.e. the most reactive material) were more viscous than those prepared from the synthetic blend.

Since the viscosities of both the emulsion and ternary gel formulations are a direct consequence of the amount and extent of the gel network formed, and since this network is created by the interaction of the cetrimide with the α-polymorph of the alcohol (Fukushima et al 1976), it would appear logical to relate the reactivity data with the polymorphic form of the alcohol. It is now generally accepted that there are three polymorphic forms of the fatty alcohols (Tasumi et al 1964). The β- and γ-polymorphs, which are stable at low temperatures, transform into the α-form at transition points several degrees below their melting points (Table 2). Transition points are lowered in both the presence of homologue impurities (Stewart 1960; Al-Mamun 1974) and water (Lawrence et al 1967; Fukushima et al 1977)

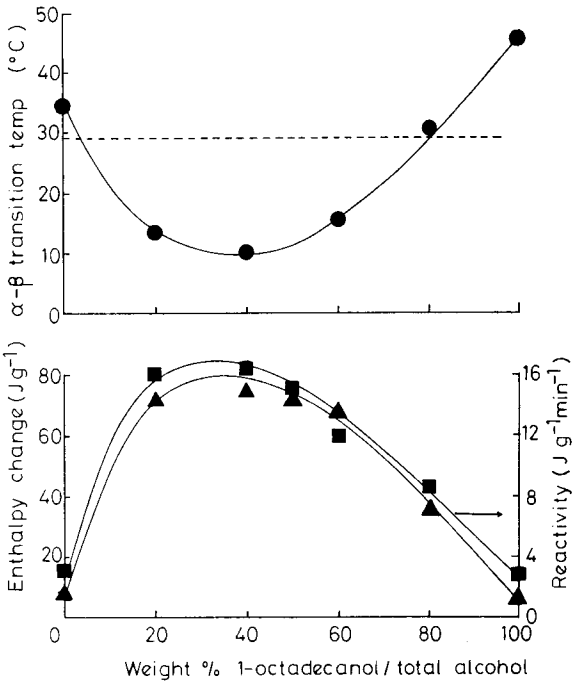

FIG. 1. The effect of increasing 1-octadecanol concentration in 1-hexadeconal/1-octadecanol mixtures on ▲, enthalpy change; ■, reactivity; and ●, transition temperature. (The transition temperatures were taken from Fukushima et al 1977).

and hence will vary with the sample and conditions of measurement. However, some indication of the transition points for the 1-hexadecanol/1-octadecanol mixtures and the standard alcohols can be obtained by considering literature data and allowing for a further lowering due to water. A comparison of the reactivity data with the transition point data for both the 1-hexadecanol/1-octadecanol mixtures (Fig. 1) and the standard alcohols (Tables 1 and 2) certainly shows that

Table 1. Enthalpy change, reaction times and reactivity for the alcohols used.

Alcohol	Enthalpy change (J g^{-1})	Reaction time (min)	Reactivity (J g^{-1} min^{-1})
1-Tridecanol	120·4	4·1	29·5
1-Tetradecanol	103·0	16·1	6·4
2-Tetradecanol	108·7	11·0	9·9
1-Pentadecanol	56·1	17·4	3·2
1-Hexadecanol	6·0	2·0	3·0
2-Hexadecanol	148·9	18·7	8·0
1-Heptadecanol	13·9	5·5	2·5
1-Octadecanol	5·4	2·0	2·7
1-Eicosanol	—	—	—

— no reaction detected.

Table 2. Melting points, α-β transition points, polymorphic form data for the fatty alcohols.

Alcohol	Melting point: Pure alcohol[1] °C	Melting point: Samples used °C	Transition points: Pure alcohol[1] °C	Transition points: Water present[2] °C	Transition points: Impure[3] °C	Polymorphic form at low temp.[1]
1-Tridecanol	30·0	30–32	25	—	—	β
1-Tetradecanol	38·0	38–39	33–36	—	—	βγ
2-Tetradecanol	—	32–34	—	—	—	—
1-Pentadecanol	45·5	45–46	36–41	—	—	β
1-Hexadecanol	49·5	49–50	43–46	30–32	20–35	γ
2-Hexadecanol	—	45–47	—	—	—	—
1-Heptadecanol	54·5	56–58	42–48	—	—	β
1-Octadecanol	58·5	57–58	53–55	40–45	—	γ
1-Eicosanol	65·0	63–65	58–64	—	—	γ

[1] Data taken from Tasumi et al (1964).
[2] Data taken from Fukushima et al (1977).
[3] Data taken from Stewart (1960).
— Unknown.

those mixtures or alcohols with a transition point ≤29 °C (the temperature of the experiment) exhibit a higher reactivity. Such an explanation may also account for the high reactivity seen with the two branched chain alcohols but unfortunately no transition point data for these alcohols exist in the literature.

In conclusion, it can be seen that the method of immersion calorimetry can be used quantitatively to assess the reactivity of the fatty alcohols with cetrimide. The measurements are relatively easy to perform and the results are consistent with the known theories of gel network formation.

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Locomotor activity and contracture of isolated ileum precipitated by naloxone following treatment of guinea-pigs with a single dose of morphine

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Guinea-pigs treated with a single dose of morphine, 15 mg kg^{-1} s.c., exhibited an increase in locomotor activity 2 h later on injection of naloxone, 4 mg kg^{-1} i.p. At the same time, contracture of ileal preparations isolated from morphine-treated guinea-pigs occurred on addition of naloxone 1 μM. Contracture of the ileum was inhibited by the tachykinin antagonist, spantide, and was therefore presumably mediated by a substance P-like agent. This study has established a useful model for the parallel investigation of central and enteric nervous system mechanisms of opiate dependence.

The guinea-pig ileum may be made dependent on opiates by pretreating the animal with morphine (Gintzler 1980) or by incubating the isolated ileum in-vitro with morphine (Collier et al 1981). Dependence may be induced following very brief, 2 min, exposure of ileum to met^5-enkephalin (Chahl 1983) and other opiates (Chahl, unpublished observations). The withdrawal response of ileum following 2 min exposure to met^5-enkephalin was revealed as a contracture following washout of met^5-enkephalin or addition of naloxone. Furthermore, a substance P (SP)-like agent apparently played a major role in the withdrawal contracture since it was inhibited by a tachykinin antagonist (Chahl 1983).

Since opiate dependence of isolated ileum occurred rapidly, signs of withdrawal should be apparent in animals following treatment with a single dose of morphine. The present study was undertaken to determine whether behavioural, as well as gastrointestinal, manifestations of withdrawal occurred in guinea-pigs treated with a single dose of morphine in-vivo, and also to determine whether the withdrawal contracture of the ileum following morphine in-vivo was similar to that following incubation with met^5-enkephalin in-vitro.

Methods

Adult guinea-pigs of either sex, 400–600 g, were used. They were treated subcutaneously with either morphine sulphate, 15 mg kg^{-1}, or an equivalent volume of saline 0·9% w/v (controls), and 2 h later both control and morphine-treated guinea-pigs were given naloxone hydrochloride, 4 mg kg^{-1}. Behavioural signs of withdrawal observed in morphine-treated guinea-pigs

* Correspondence.

included increased locomotor activity, shivering, chewing, clawing at the cage floor and grooming. Preliminary experiments showed that locomotor activity was the most suitable behavioural withdrawal sign in guinea-pigs to quantify, particularly since guinea-pigs caged separately exhibited a low level of basal locomotor activity. Locomotor activity was therefore monitored in an activity cage with two electrical floor contacts at diagonal corners of the cage mounted between a double plastic floor. The cage design allowed only half or full circuits, but not fine movements by the guinea-pigs to be monitored. Cumulative numbers of counts were displayed on a digital counter and a signal corresponding to each count was simultaneously displayed on a chart recorder. Locomotor activity of each guinea-pig was monitored for 1 h before, and for 2 h following injection of morphine or saline, and for a further 1 h after naloxone injection.

For experiments on isolated ileum, guinea-pigs were killed by a blow to the head 2 h after injection of morphine or saline. A segment of distal ileum was removed and placed in a 2 mL organ bath under 1 g tension in Tyrode solution at 37 °C, gassed with oxygen.

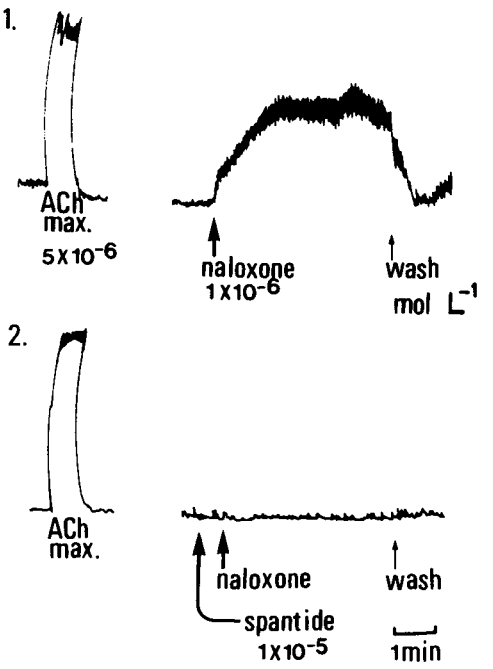

FIG. 2. Responses of ileum preparations from morphine-treated guinea-pigs. The traces show the responses to naloxone (1 μM) of ileum preparations from two different guinea-pigs treated with morphine sulphate 15 mg kg^{-1}. The upper trace shows the response in the absence, and the lower trace in the presence of the substance P antagonist, spantide 10 μM.

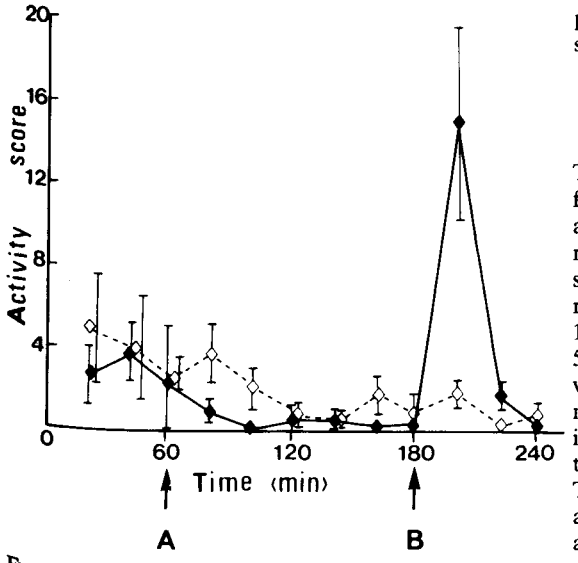

FIG. 1. Locomotor withdrawal responses of guinea-pigs. Points represent mean locomotor activity scores. Key: mean activity scores for control guinea-pigs given saline (at A) (◇), and for animals treated with morphine sulphate 15 mg kg^{-1} subcutaneously (at A) (◆). During the 20 min period after injection of naloxone hydrochloride, 4 mg kg^{-1} i.p. (at B), a significant increase in activity of animals treated with morphine occurred ($0{\cdot}05 > P > 0{\cdot}01$) but not in control animals. Bars are s.e. of means from 7 animals.

To avoid variable periods of washing out of morphine from the tissues, the following sequence of drug additions was adhered to strictly. The height of the maximum response of 5 μM acetylcholine (ACh) was standardized on the Grass polygraph chart. The recovery of sensitivity of the preparation was checked 15 min later by obtaining a response to ACh, 10 μM, and 5 min later naloxone, 1 μM was added to the bath and washed out after 5 min. Finally the sensitivity to SP, 2·5 nM, was tested 10 min later. Similar experiments using ilea from other animals were performed with either the tachykinin antagonist, spantide, (D-Arg^1,D-$Trp^{7,9}$,Leu^{11})-SP (Bachem), 10 μM, or atropine, 5 μM, added to the bath 30 s or 5 min, respectively, before addition of naloxone. Spantide was added 30 s before addition of naloxone since in a previous study this time was found to be adequate to inhibit markedly responses to SP (Chahl 1985).

Results

The mean activity scores for control and morphine-treated guinea-pigs are shown in Fig. 1. Locomotor activity decreased more rapidly in animals given mor-

phine compared with control animals but by 180 min both groups had low activity scores. Following injection of naloxone hydrochloride, 4 mg kg^{-1} i.p., there was a marked increase in activity score for the morphine-treated animals but no change in the activity of the control animals. The mean score for locomotor activity for the 20 min period following naloxone injection was significantly greater for morphine-treated guinea-pigs than for controls ($0{\cdot}05 > P > 0{\cdot}01$, Student's t-test). There was also a significant increase in activity of morphine-treated animals during the first 20 min period following naloxone injection compared with that during each of the three 20 min periods before morphine injection ($0{\cdot}05 > P > 0{\cdot}01$).

Addition of naloxone, 1 μM, to the isolated ileum from guinea-pigs pretreated 2 h previously with morphine produced marked contracture (Fig. 2), whereas addition of naloxone to ileal segments from control animals produced little or no contracture. The mean height of contracture of ilea from morphine-treated guinea-pigs (50 ± 7% of the ACh maximum, mean ± s.e., n = 5) was significantly greater than that from controls (13 ± 8%, n = 5) ($0{\cdot}01 > P > 0{\cdot}001$). The responses to SP and ACh were similar on preparations from morphine-treated and control guinea-pigs. Atropine, 5 μM, added 5 min before naloxone, did not significantly affect the height of the naloxone-induced contracture of ilea from morphine-treated guinea-pigs (44 ± 10%, n = 8), but spantide, 10 μM, abolished the response (0 ± 0%, n = 5) (significantly different from mean response in absence of spantide, $P < 0{\cdot}001$) (Fig. 2). Responses to SP were also abolished by spantide.

Discussion

As predicted from previous experiments on isolated ileum (Chahl 1983), dependence on opiates occurred very rapidly. Two hours after guinea-pigs were pretreated with a single dose of morphine, naloxone precipitated an increase in locomotor activity and other signs of central nervous system withdrawal as well as contracture of isolated ileum indicative of enteric nervous system withdrawal. Rapidly induced dependence on morphine, as measured by withdrawal precipitated by naloxone, has been observed previously in mice (Kosersky et al 1974) and in dorsal horn neurons where naloxone-precipitated firing occurred within 10 min following contact with morphine (Johnson & Duggan 1981).

It has been proposed that SP or a related substance is the primary mediator of the opiate withdrawal response of guinea-pig ileum, not only of the atropine-resistant component as has been suggested (Gintzler 1980; Tsou et al 1985), but also of the atropine-sensitive component (Chahl 1983). Therefore spantide was used in the present experiments since it is a relatively potent antagonist of the direct muscle action of SP on the ileum while retaining antagonist potency against the indirect (atropine-sensitive) response (Chahl 1985). The pharmacology of the ileal withdrawal contracture precipitated by naloxone following morphine treatment of guinea-pigs in-vivo was similar to that previously observed for met^5-enkephalin in-vitro (Chahl 1983), since it was abolished by a SP antagonist and therefore presumably mediated by SP or another tachykinin. However, atropine did not significantly inhibit the morphine withdrawal contracture and this agreed with previous findings for met^5-enkephalin withdrawal which was atropine-sensitive following 2 min contact with met^5-enkephalin, but became atropine-resistant following 32 min contact (Chahl 1983).

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Lipid vehicles for intestinal lymphatic drug absorption

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The lipoprotein fractions in mesenteric lymph were monitored following intraduodenal administration of arachis oil and oleic, linoleic and linolenic fatty acids to rats. An increase in the chylomicron fraction, but not the VLDL or LDL fraction, was observed with each lipid. The greater the degree of unsaturation of the fatty acid, the more rapid the onset of chylomicron synthesis. The administration of linoleic acid and arachis oil produced the highest concentration of chylomicrons in the lymph. These results reflect differences in the rate of absorption and biochemical metabolism of the lipids and have implications for the selection of vehicles for the delivery of drugs by the lymphatic route.

The absorption of drugs into the lymphatic system following oral administration is of particular interest for compounds which are subject to first pass metabolism, as well as for anticancer agents directed to metastases within the lymphatics. Sieber et al (1974) and Noguchi et al (1985a) have suggested that the lymph concentration of chylomicrons (lipoprotein units produced in the enterocytes) is a major factor in controlling the uptake of lipophilic molecules into the lymphatic vessels. During the digestion of lipids containing long chain fatty acids, the synthesis of chylomicrons increases. These lipids can therefore be used to promote the intestinal absorption of drugs selectively absorbed via the lymphatic system (Palin & Wilson 1984). In the present study the chylomicron concentrations in rat mesenteric lymph were determined following intraduodenal administration of the triglyceride arachis oil and each of its constituent fatty acids; oleic, linoleic and linolenic acid. High performance size exclusion chromatography was employed to isolate the chylomicron fraction.

Materials and methods

The lipids used were oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) (Sigma Chemicals, Dorset) and arachis oil B.P. (Thornton and Ross, Huddersfield, Yorks).

Male Wistar rats were starved for 18 h, with free access to water, before anaesthesia with sodium pentobarbitone (Sagatal, 90 mg kg^{-1} i.p.). The superior mesenteric lymph duct was cannulated using a polyethylene cannula (0·8 mm i.d., 1.0 mm o.d.) as described by Noguchi et al (1985b). The inferior mesenteric lymph duct was cut and occluded using cyanoacrylate adhesive. An initial lymph sample was collected and then 0·3 mL phosphate buffered saline (pH 7·4), or lipid, was injected into the duodenum. The lymph output from the mesenteric duct was collected every 30 min for 240 min and stored at 4 °C before analysis.

Aliquots (20 μL) from each of the 30 min lymph samples were injected onto a TSK 6000PW aqueous exclusion column (Anachem, Luton, Beds) equilibrated in 0·9% w/v sodium chloride and eluted at 0·7 mL min^{-1}. The absorbance of the eluate at 290 nm was monitored; typical elution profiles are shown in Fig. 1. The area under the elution peaks was used to determine the proportion of the different lipoprotein fractions in the lymph samples. UV calibration curves were produced for serial dilutions of chylomicron peaks collected at different times during fat absorption. These followed Beer-Lambert behaviour and showed no significant differences following administration of the different vehicles. Alterations in size or chemical composition of the eluting lipoproteins were therefore assumed to have no effect on the extinction coefficient.

Results and discussion

The three major lipoprotein fractions in lymph are chylomicrons, very low density lipoproteins (VLDL) and low density lipoproteins (LDL). Analysis of the particle size in each eluate fraction by photon correlation spectroscopy (Malvern Instruments), gave 'z' average diameter values of 280 nm, 100 nm and <30 nm. These agree with previously quoted values for chylomicrons, VLDL and LDL, respectively (Counsell & Pohland 1982). The elution profiles obtained for the VLDL and LDL fractions were similar to those produced from plasma samples by previous workers, who found good correlation with other methods of lipoprotein separation, such as density gradient ultracentrifugation and soft agarose gel chromatography (Wehr et al 1982; Carroll & Rudel 1983). Repeated chromatography of the lipoprotein peaks, obtained after administration of arachis oil, indicated greater than 90% recovery of all three lipoprotein classes.

Throughout the experimental period, the composition of the lymph in the control group remained constant, with the relative percentages of each lipoprotein fraction being: chylomicrons 39 ± 3·2%, VLDL 56·7 ± 3·1%, and LDL 4·3 ± 0·5% (mean ± s.d.).

* Correspondence.

Following administration of each lipid, there was a significant increase in the percentage of the chylomicron fraction and a corresponding decrease in the VLDL and LDL percentages, compared with the control and the time zero samples. No significant differences were observed in the lymph flow rate. For each lipoprotein fraction in each sample, the ratio of the area under the elution curve to that of the sample taken before dosing was calculated. The ratios for both the VLDL and LDL fractions remained approximately one throughout the experiment for each lipid. The ratios for the chylomicron fraction showed significant, but differing increases with each lipid (Fig. 2). The ratio values were compared using a one way analysis of variance.

With each increase in the degree of fatty acid unsaturation, the lag-time to a significant increase in the chylomicron concentration was reduced compared with the control. This difference in the onset of chylomicron synthesis probably reflects a more rapid rate of absorption with increasing fatty acid unsaturation. The greater the degree of unsaturation of the fatty acid, the lower the melting point, the greater the fluidity at 37 °C and the less hydrophobic the molecule—all factors which will facilitate absorption. The longest lag time to increased chylomicron output was observed after administration of arachis oil. Unlike the fatty acids, arachis oil cannot be absorbed directly; it has to be hydrolysed by pancreatic lipase to the constituent fatty acids and 2-monoglycerides before uptake into the mucosal cells.

A steady state lymph chylomicron concentration was reached with each fatty acid, linoleic acid producing the highest concentration followed by linolenic acid and then oleic acid. These data probably reflect differences

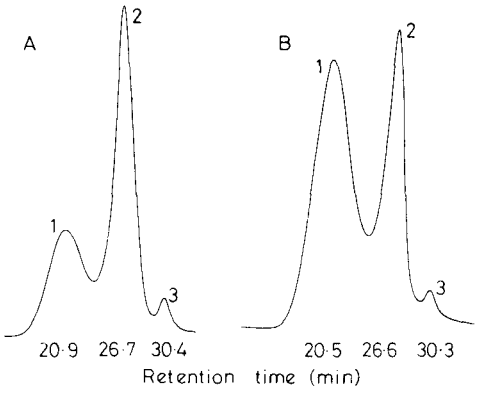

FIG. 1. The elution profile for (A) fasted rat and (B) rat dosed with arachis oil: 1, chylomicron peak; 2, VLDL peak; 3, LDL peak.

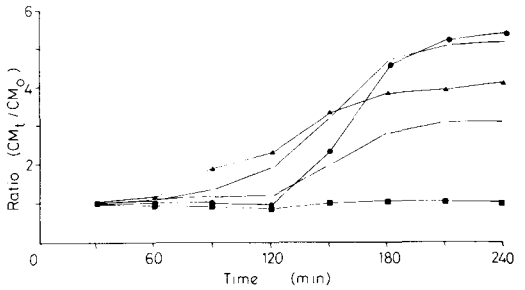

FIG. 2. The lymph chylomicron concentration (expressed as a ratio of the concentration at time t to that at time zero) following intraduodenal administration to rats of 0·3 ml volumes of ● arachis oil, □ oleic acid, ○ linoleic acid, ▲ linolenic acid, ■ phosphate buffered saline (pH 7·4), (mean, n = 6 per group).

in the biochemical pathways and rates of enzymatic reaction within the mucosal cell for the incorporation of the different fatty acids into chylomicrons (Brindley 1974). After a long lag time, by 240 min the lymph chylomicron concentration produced by administration of arachis oil was approaching a steady state equal to that of linoleic acid.

This study suggests that linoleic acid is the most suitable of the lipid vehicles investigated for the delivery of drugs to the lymphatic system since it rapidly stimulated high levels of chylomicrons in the lymph. Further investigations are required to assess the potential of fatty acids of different chain lengths and of mixed lipid systems.

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The topical anti-inflammatory effects of a topical preparation of meclofenamic acid on carrageenan-induced footpad swelling in mice

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A topical preparation of meclofenamic acid (Meclomen) was tested for anti-inflammatory activity in a murine model of carrageenan footpad oedema. The preparation significantly inhibited swelling when applied to the carrageenan-injected paw. Maximum inhibition was observed 4–5 h after carrageenan injection. The topical effects could not be attributed to systemic absorption because the preparation was more inhibitory when applied topically to the carrageenan-injected paw than to a distant site or orally.

Meclofenamic acid is a potent cyclooxygenase inhibitor which is used orally for a variety of inflammatory conditions (Wilkins 1978; Eberl & Dunky 1983; Petrick & Black 1983). Recent reports suggest that it may also be effective when administered topically in the treatment of psoriasis (Winthrop 1982; Ellis & Voorhees 1983). To investigate further the value of the drug, we prepared it as a topical preparation and tested it in murine carrageenan footpad oedema, a model of acute inflammation.

Materials and methods

Carrageenan paw oedema was induced in female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) as described by Levy (1969). Briefly, mice were injected intradermally in the left rear footpad with 50 μL of a 1% solution of carrageenan (FMC-Marine Colloids Division, Springfield, NJ) in 0·9% NaCl (saline), and in the contralateral footpad with 50 μL of saline. Meclofenamic acid (Meclomen, Warner-Lambert/Parke-Davis, Ann Arbor, MI) and indomethacin (Sigma, St Louis, MO) were dissolved in a vehicle containing 50% polyethylene glycol monolaurate, 10% H_2O, and 40% isopropanol. One hour after carrageenan injection, 50 μL of drug solution (or vehicle) was administered orally or topically by rubbing into the appropriate hindpaw for 15 s. Hindpaw swelling was assessed by a mercury plethysmograph (Buxco Electronics, Sharon, CN) at various times thereafter. Differences in volume between carrageenan- and saline-injected footpads were calculated for each experimental group (at least 8 mice per group). Each study was repeated at least once. Results are expressed as percent inhibition of swelling in drug-treated compared with vehicle-treated mice. Statistical differences between the experimental groups were determined by Student's *t*-test analysis.

* Correspondence.

Results and discussion

Topical administration of meclofenamic acid or indomethacin to the carrageenan-injected footpad induced a dose-dependent reduction in swelling (Fig. 1). For these experiments, paw volume was measured 5 h after carrageenan injection. The effects of the meclofenamic acid preparation were maximal at the 3% concentration (49·7% inhibition). Peak inhibition was also observed for indomethacin at this dose (39·0% inhibition), although similar effects were also observed for indomethacin at the 0·3 and 1% concentrations. The topical effects of indomethacin were comparable with published results for rat carrageenan paw oedema (Wada et al 1982).

The duration of action of the topical preparation was determined by studying the effects of the 3 and 5% solutions of the drug on swelling over 10 h during which the inflammatory response remained relatively constant in the vehicle-treated mice. A 3% solution of indome-

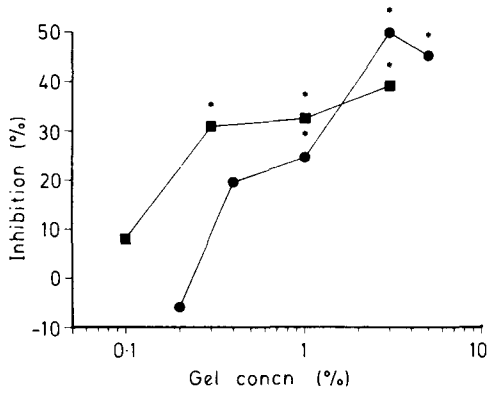

FIG. 1. Dose-response of topically-applied (●) meclofenamic acid and (■) indomethacin in mice with carrageenan footpad oedema. Results are expressed as the mean percent inhibition of oedema compared with vehicle-treated controls (* significant inhibition of oedema compared with vehicle-treated control mice, $P < 0·05$).

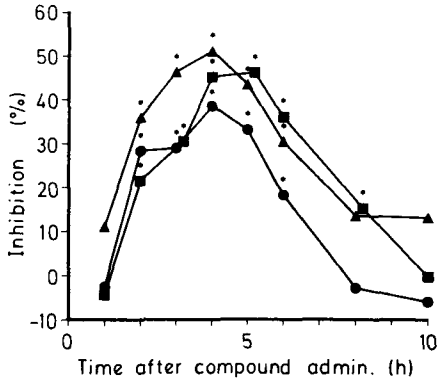

FIG. 2. Kinetic response of topically applied meclofenamic acid and indomethacin in mice with carrageenan footpad oedema. Results are expressed as the mean percent inhibition of oedema compared with vehicle-treated controls (*significant inhibition of oedema compared with vehicle-treated control mice, $P < 0.05$). Key: (●) 3% and (▲) 5% meclofenamic acid; (■) 3% indomethacin.

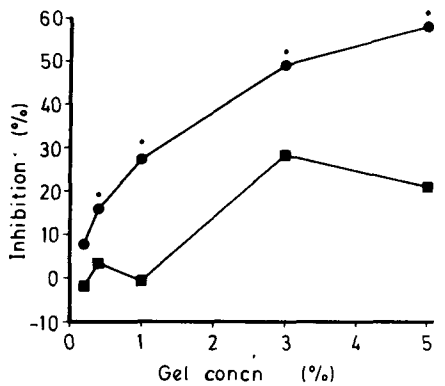

FIG. 4. A comparison of the anti-inflammatory effects of (■) orally and (●) topically applied meclofenamic acid to the carrageenan-injected paw. Results are expressed as the mean percent inhibition of oedema compared with vehicle-treated controls (*significantly greater inhibition when applied topically to the carrageenan-injected paw than orally at the same dose of the drug $P < 0.05$).

thacin was used for comparison. Both concentrations of meclofenamic acid inhibited swelling for 4–6 h beginning at 2 h (Fig. 2). A similar kinetic response was observed for indomethacin.

To determine whether the observed anti-inflammatory effect of the topical preparation was due to systemic absorption, two control studies were performed. In the first, the meclofenamic acid preparation was applied either to the carrageenan- or saline-injected paw and the effects on swelling were determined 5 h after carrageenan injection. Significantly greater inhibition was observed with the 1, 3, and 5% concentrations when these were applied to the carrageenan-injected compared with the saline-injected paw (Fig. 3), suggesting that the drug has significant local anti-inflammatory activity. To confirm these results, a second study was done in which the same protocol was used, except that the drug was administered either topically to the carrageenan-injected paw or orally. Inhibition of swelling in the topical treatment groups was significantly greater than that in the oral treatment groups at the 0·4, 1, 3, and 5% concentrations, which suggests that the topical effect cannot be explained entirely on the basis of oral absorption (as a result of preening) (Fig. 4). However, in several models of inflammation, including UV erythema and yeast pyresis, meclofenamic acid has been found to be extremely potent when administered orally 1 h before the inflammatory stimulus (Wax 1978). It is possible that prostaglandin-dependent events occur relatively early in the inflammatory response to carrageenan in mice. Therefore, if a cyclooxygenase inhibitor is given orally 1 h after carrageenan injection, prostaglandin-mediated inflammatory events may already have occurred. It is also conceivable, however, that the topical vehicle prevented optimal oral uptake.

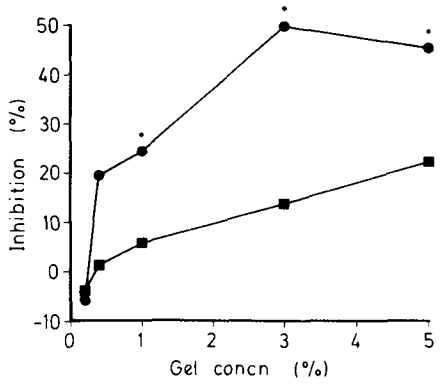

FIG. 3. A comparison of the topical anti-inflammatory effects of meclofenamic acid applied to the (●) carrageenan- or (■) saline-injected paw. Results are expressed as the percent inhibition of oedema compared with vehicle-treated controls (*significantly greater inhibition when applied to the carrageenan-injected than to the saline-injected paw at the same dose of drug, $P < 0.05$).

In conclusion, the results from the present study indicate that the topically applied meclofenamic acid has significant local anti-inflammatory activity. These effects cannot be explained on the basis of systemic absorption. Results from this study also indicate that murine carrageenan footpad oedema may be a useful model for development of novel topical anti-inflammatory agents.

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Effects of microiontophoretic pentobarbitone on conditioned inhibitions mediated by GABA-A receptors in the cuneate nucleus of the rat in-vivo

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We have studied the effects of microiontophoretic sodium pentobarbitone on the conditioned inhibition of the negative potential (N-wave) evoked in the cuneate nucleus of the rat by electrical stimulation (5 V, 0·2 ms, 0·5 Hz) of the ipsilateral forepaw. Five- or seven-barelled micropipettes were used, the tip being placed at a depth of 600–900 μm below the dorsal surface of the medulla oblongata. The conditioned inhibition was elicited by a previous identical stimulus. When the interval between the stimuli is shorter than about 40 ms (short duration) the inhibition is thought to be mediated by γ-aminobutyric acid (GABA), acting on GABA-A receptors. When it is longer (long duration conditioned inhibition) GABA-A receptors are not thought to be involved. Microiontophoretic sodium pentobarbitone potentiated both short (15 ms) and long (45 ms) duration conditioned inhibitions. The effect was current-dependent and appeared whether or not the first N-wave was depressed. Microiontophoretic application of (−)-bicuculline methiodide (a GABA-A antagonist) reduced the potentiation by pentobarbitone up to the basal inhibition when the interval between the stimuli was 45 ms or longer and to a greater extent when it was 30 ms or shorter. It seems likely that pentobarbitone prolongs the GABA-ergic mechanism which produces the short duration inhibition, making it visible with long stimulus intervals, superimposed upon the normal long duration conditioned inhibition which is not potentiated by local pentobarbitone.

Barbiturates potentiate inhibitions mediated by endogenous γ-aminobutyric acid (GABA) in the mammalian central nervous system, both in-vivo and in-vitro. Their mechanism of action appears to be the prolongation of the open time of the chloride channels associated with GABA-A receptors (Barker & Mathers 1981; Johnston & Willow 1982; Simmonds 1981). Ligand studies show that barbiturates inhibit the binding of [^{3}H]dihydropicrotoxinin, a non-competitive antagonist of GABA, to synaptic membranes (Olsen 1982; Ticku et al 1978). However, the actions of barbiturates in-vivo seem to be complex, judging from the variety of effects they cause (hypnotic, anaesthetic, anticonvulsant) (Enna 1981; Snodgrass 1983).

We describe here the effects of microiontophoretic sodium pentobarbitone, a hypnotic barbiturate, on a characterized and reproducible model of endogenous inhibition in-vivo: the inhibition by a previous stimulus (*conditioned* inhibition) of the negative potential (N-wave) evoked in the cuneate nucleus of the rat by electrical stimulation of the ipsilateral forepaw (Andersen et al 1970; Andres-Trelles et al 1976). When the interval between both stimuli is smaller than about 40 ms (*short duration* conditioned inhibition), the inhibition is due to GABA, since it is reduced by microiontophoretic GABA-A antagonists. When it is longer than 40 ms (*long duration*) the inhibition is resistant to GABA-A antagonists (Andres-Trelles et al 1976), as well as to antagonists of glycine, 5-hydroxytryptamine and histamine (unpublished observations). Nevertheless, the involvement of GABA in this long duration conditioned inhibition cannot be excluded, for it is now known that GABA receptors insensitive to (−)-bicuculline methiodide (GABA-B) are present in the cuneate nucleus of the rat (Orviz et al 1986).

Materials and methods

Male wistar rats (200–250 g) were anaesthetized with urethane (1·8 g kg^{-1} i.p.) or halothane (1–1·5% in 30% O_2 and 70% N_2O) and fixed in a stereotaxic frame. Two stainless steel electrodes placed in the centre pad of the paw and under the skin of the forelimb stimulated the forepaw at a rate of 0·5 Hz with supramaximal electric shocks (5 V), 0·2 ms wide. To study the conditioned

* Correspondence.

inhibitions we used standard stimulus intervals of 15 ms (short duration) and 45 ms (long duration).

The dorsal surface of the medulla oblongata was exposed and the potentials evoked in the ipsilateral cuneate nucleus were recorded via a silver–silver chloride electrode in one of the barrels, filled with 3 M NaCl, of a multibarrelled (five or seven compartments) borosilicate micropipette. Its DC impedance was lower than 4–6 MΩ. The tip of the micropipette (overall diameter 5–8 μm) was lowered into the nucleus to a depth of 600–900 μm, which, as previously reported, is the optimum position for recording the conditioned inhibition and the microiontophoretic effects of drugs (Andres-Trelles et al 1976; Orviz et al 1986). The indifferent electrode was placed under the skin of the head. The signal was led by a high impedance probe to a Grass P16 differential preamplifier from which the DC output was then led to a Neurolog System Digitimer amplifier and averager. Frequencies higher than 1 kHz were filtered out. The single sweeps and the averaged signal were displayed on a Tektronix oscilloscope. The displays were photographed either directly from the oscilloscope or after FM recording on a Hewlett-Packard 3964A recorder.

Solutions of sodium pentobarbitone (Abbott) 40 mM in distilled water, pH 9, and of (−)-bicuculline methiodide (Sigma) 5 mM in 150 mM NaCl, pH 3·5, were placed in the other barrels of the micropipette. These drugs were microiontophoretically applied into the cuneate nucleus by ejection currents lower than 100 nA, generated by a five channel Analog-Medical Systems Corp. microiontophoresis pump. Retaining currents of 25 nA were used to avoid passive diffusion. Balancing currents were automatically passed through one of the barrels, filled with 1 M NaCl.

Results

Microiontophoretic applications of sodium pentobarbitone significantly potentiated both short duration (15 ms stimulus interval) and long duration (45 ms interval) conditioned inhibitions (Figs 1, 2B). With higher ejection currents the effect was greater (Fig. 3). Overall, the amplitude of the first N-wave was not significantly affected with ejection currents up to 40 nA, but in particular experiments it was reduced and the reduction could be antagonized by (−)-bicuculline methiodide (Fig. 2A). In the experiments in which the amplitude of the first N-wave was not decreased (or was even slightly increased), the potentiation of the conditioned inhibitions by pentobarbitone was equally evident.

The GABA-A antagonist (−)-bicuculline methiodide, as expected, antagonized short but not long duration inhibition in control conditions (Fig. 1). However, the potentiation by pentobarbitone of both 15 and 45 ms inhibitions was diminished by (−)-bicuculline methiodide (Figs 1, 2B). After application of the antagonist in the presence of sodium pentobarbitone the degree of conditioned inhibition depended on the stimulus interval. When it was 45 ms or longer the amplitude of the second N-wave was comparable with the one recorded before the application of pentobarbi-

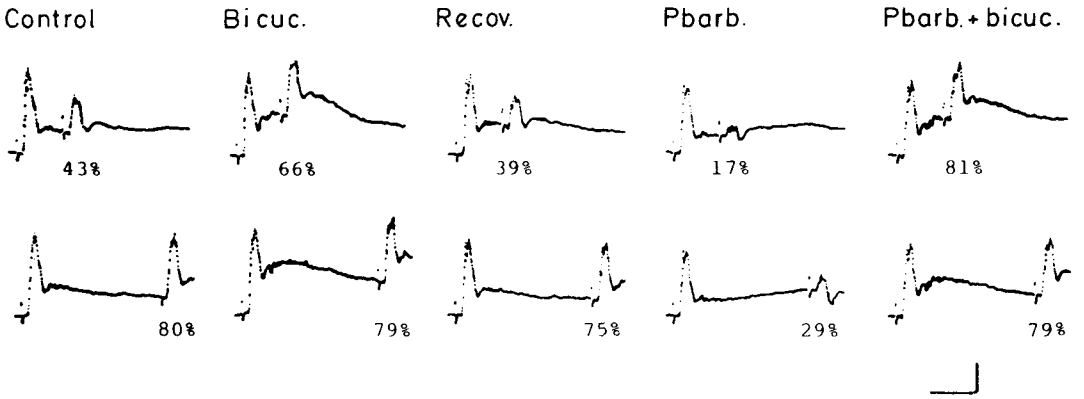

FIG. 1. Potentials evoked in the cuneate nucleus of an anaesthetized rat by two consecutive electric stimuli to the ipsilateral forepaw. Intervals between the stimuli are 15 ms (above) and 45 ms (below). The numbers show the amplitude of the second N-wave, expressed as a percentage of the first one, i.e. the smaller they are, the greater the conditioned inhibition is. Records are averages of eight consecutive single sweeps. Microiontophoretic (−)-bicuculline methiodide (Bicuc), 25 nA for 5 min, antagonizes the short but not the long duration conditioned inhibition. After recovering from bicuculline for 30 min, sodium pentobarbitone (Pbarb), 40 nA for 5 min, was microiontophoretically applied. It reduced the amplitude of the second N-wave. This potentiation of the conditioned inhibition was diminished by application of (−)-bicuculline methiodide (Pbarb + Bicuc), 25 nA for 5 min. Whereas the amplitude of the second N-wave was similar to the control when the interval was 45 ms, it was much greater when the interval was 15 ms. Vertical calibration bar = 250 μv negative upwards and horizontal bar = 15 ms.

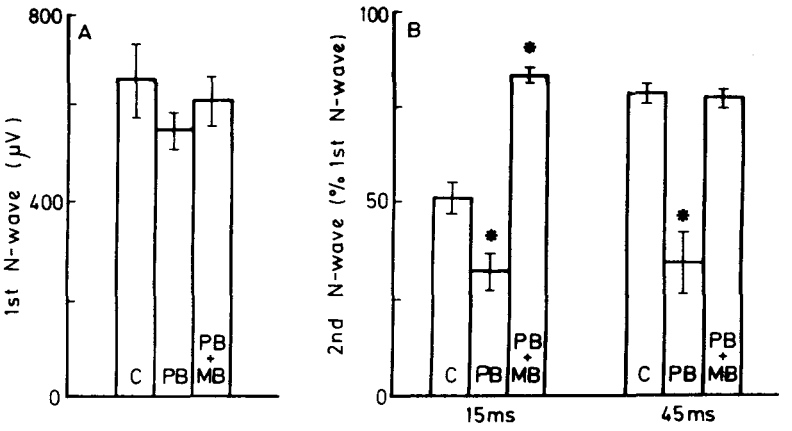

FIG. 2. A. Effect of microiontophoretic sodium pentobarbitone (PB), 40 nA for 5 min, on the amplitude of the first (i.e. non-inhibited) N-wave and influence on this effect of (−)-bicuculline methiodide (MB), 25 nA for 5 min. Vertical lines on each bar indicate the standard error of the mean (s.e.m.). C = Control. n ≥ 9. B. Effect of microiontophoretic sodium pentobarbitone (PB), 40 nA for 5 min, on the conditioned inhibition of the N-wave and antagonism by (−)-bicuculline methiodide (MB), 25 nA for 5 min. Intervals between the stimuli are 15 ms (left) and 45 ms (right). The histogram bars represent the amplitude of the second N-wave measured, as explained in Fig. 1, as a percentage of the first N-wave. As in A, vertical lines indicate s.e.m. C = control. * represents significant difference from the control ($P < 0{\cdot}05$, Student's *t*-test). n ≥ 9.

tone (control). On the contrary, when the interval between the stimuli was 30 ms or shorter the inhibition of the second N-wave was significantly smaller than the control (Figs 2B, 3).

Discussion

From our experiments it is clear that microiontophoretic sodium pentobarbitone potentiates the short duration conditioned inhibition of the N-wave, which seems to be mediated by activation of GABA-A receptors, as it is antagonized by (+)-bicuculline methochloride (Andres-Trelles et al 1976). The effect is dose-dependent and appears with doses (ejection currents) that do not affect the first N-wave. Hence, it does not seem to be due to direct depression of the evoked potential but to potentiation of an endogenous inhibitory mechanism. The same doses also potentiate the long duration conditioned inhibition. This is surprising, because in normal circumstances it is not sensitive to GABA-A antagonists. However, (−)-bicuculline methiodide also reduces this effect of pentobarbitone, as would be expected if it were caused by activated GABA-A receptors.

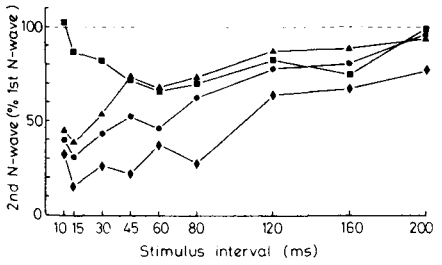

FIG. 3. Effect of different ejection currents of sodium pentobarbitone on the conditioned inhibition of the N-wave seen in an anaesthetized rat. The amplitude of the second N-wave is plotted, as a percentage of the first one, at each of several stimulus intervals (10 to 200 ms). ▲ Control. ● Sodium pentobarbitone, 20 nA for 5 min. ● Sodium pentobarbitone, 40 nA for 5 min. ■ (−)-Bicuculline methiodide, 25 nA for 5 min, in the presence of sodium pentobarbitone, 40 nA for 5 min.

A likely explanation of pentobarbitone potentiating the long duration inhibition seems to be that it prolongs the GABA-A-mediated mechanism which produces the short duration inhibition, making it visible with longer stimulus intervals. This is supported by the effect of (−)-bicuculline methiodide in the presence of sodium pentobarbitone at several stimulus intervals. When the interval is 15 ms, bicuculline antagonizes both the potentiation by pentobarbitone and the basal inhibition. When it is 45 ms or longer, bicuculline still antagonizes the effect of pentobarbitone but not the basal inhibition.

Our results show, therefore, the potentiation by local pentobarbitone of a GABA-A-mediated inhibition without obvious potentiation of other kinds of inhibition in the rat cuneate nucleus.

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Effects of dimethyl sulphoxide (DMSO) on aggregation of human blood platelets

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The effects were examined of the universal solvent dimethyl sulphoxide (DMSO) on human platelet aggregatory activity, in-vitro, of the endogenous mediators ADP, adrenaline, arachidonic acid, collagen and PAF-acether which are believed to play important roles in cardiovascular diseases in man. DMSO inhibited aggregation induced by all of the mediators in the order ADP > adrenaline = arachidonic acid = PAF-acether > collagen. Since DMSO is widely used as a solvent for drug substances, an awareness of its intrinsic activity in any such evaluations is essential.

Dimethyl sulphoxide (DMSO) was first synthesized over a century ago (Saytzeff 1867), though its useful solvent properties in relation to a variety of chemical substances have been recognized only within the last fifty years. It has, also, a wide spectrum of pharmacological activity of itself (David 1972; Haigler 1983; Brayton 1986) and has received renewed interest in the last few years as a therapeutic agent, particularly in North America. However, in view of reports linking DMSO to cataracts in laboratory animals (Rubin & Barnett 1967), and debate about its carcinogenic potential, some have cautioned its clinical use until adequate safety data become available (Savastano 1984).

Intravenously administered DMSO has been shown in-vivo to reduce the thrombotic response to surgical trauma (Dujovny et al 1983) in the rat and in a mouse model of pial arteriolar injury (Rosenblum & El-Sabban 1982) and to have anti-thrombotic effects when applied topically to rats (Gorog & Kovacs 1975). However, only few reports have appeared examining the effects of DMSO on platelet aggregation in-vitro, and of these there has been demonstrated an action against effects induced by ADP and adrenaline (Schiffer et al 1976) as well as thrombin (Holz & Davis 1972) and collagen (Shepherd et al 1984).

In view of the growing interest in discovering agents which beneficially modulate the roles of endogenous mediators in thrombosis and cardiovascular disease states, we have undertaken a systematic study of the effects of the universal solvent DMSO on aggregation of human platelets induced by ADP, adrenaline, arachidonic acid, collagen and PAF-acether.

Methods

Our studies of platelet aggregation were performed on citrated platelet-rich plasma (PRP). Blood was obtained via an antecubital vein from fasted healthy male adults who had not received any medication for at least one week before bleeding. PRP was prepared by centrifuging the citrated blood sample (9 volumes of blood to 1 volume of 3·8% aqueous sodium citrate) at 1500 rev min^{-1} for 15 min. The concentration of platelets in plasma was 300 000 ± 50 000 platelets μL^{-1}.

Aggregation experiments were conducted using a Chrono-log aggregometer model 550 (Coultronics), traces being recorded on an Omniscribe recorder. Aggregation was provoked by the following substances at the pre-determined concentrations indicated in parentheses: ADP (4 μM), adrenaline (1 μM), arachidonic acid (0·5 mM), collagen (10 μg mL^{-1}) and PAF-acether (1 μM). All the aggregating agents except PAF-acether, which were purchased from Sigma, were dissolved in physiological saline. PAF-acether (obtained from Bachem Corporation) was dissolved in 2·5% w/v aqueous bovine serum albumin (Fraction V).

Aggregation studies were conducted essentially as

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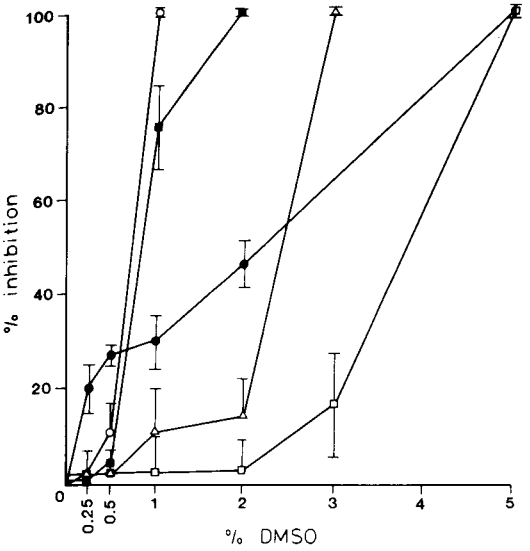

FIG. 1. Dose-response curves demonstrating the effect of dimethylsulphoxide (DMSO) on aggregation in human platelets induced by previously determined concentrations of ADP (●——●), adrenaline (○——○), arachidonic acid (△——△), collagen (□——□), and PAF-acether (■——■). Each curve is the mean ± s.e.m. from four experiments.

described by Born & Cross (1963). Estimates were made from the recordings of optical density changes and the percentage of inhibition of platelet aggregation (induced by each of the agents mentioned above) by the presence of each of the following concentrations of DMSO calculated: 0·25, 0·5, 1, 2, 3 and 5%. Four aggregations on different PRP were undertaken for each DMSO concentration.

Results and discussion

The results are indicated in Fig. 1. In essence, DMSO produced a significant inhibition of platelet aggregation provoked by all of the agents examined, being most potent (at a starting concentration of 0·25%) against aggregation induced by ADP, intermediately and equipotent (0·5% DMSO) against aggregation due to adrenaline, arachidonic acid and PAF-acether, and least potent (DMSO concentration 2%) in the case of collagen-induced aggregation.

The aggregating agents used in these studies are believed to play a role in cardiovascular disease in man. It is evident therefore that an awareness of these observed effects of the universal solvent DMSO is extremely important in the evaluation of any potential chemotherapeutic agent where DMSO may be the vehicle.

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