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Mechanism of inhibition of cyclo-oxygenase in human blood platelets by carbamate insecticides

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Carbamates are a widely used class of insecticides and herbicides. They were tested for their ability to affect human blood platelet aggregation and arachidonic acid metabolism in platelets. (1) The herbicides of the carbamate type have no, or only little, influence up to a concentration of 100 μM; the carbamate insecticides, however, inhibit both aggregation and arachidonic acid metabolism in a dose- and time-dependent manner. (2) Carbaryl, the most effective compound, inhibits platelet aggregation and cyclo-oxygenase activity completely at 10 μM. The liberation of arachidonic acid from phospholipids and the lipoxygenase pathway are not affected, whereas the products of the cyclo-oxygenase pathway are drastically decreased. (3) By using [^{14}C]carbaryl labelled in the carbamyl or in the ring moiety, it could be proved that the carbamyl residue binds covalently to platelet proteins. In contrast with acetylsalicylic acid, which acetylates only one protein, carbaryl carbamylates a multitude of platelet proteins. (4) One of the carbamylated proteins was found to be the platelet cyclo-oxygenase, indicating that carbaryl resembles in this respect acetylsalicylic acid, which is known to inhibit this enzyme specifically by acetylation.

INTRODUCTION

The blood represents one of the first compartments of the organism that is confronted with xenobiotics. Blood constituents, such as serum proteins, lipoproteins and blood cells, serve as carriers for such compounds (Skalsky & Guthrie, 1978; Maliwal & Guthrie, 1981*a*,*b*). In addition to the transport mechanisms within the blood, the direct influence of xenobiotics on these constituents is of great interest.Blood platelets have been shown to be a very sensitive target, not only for environmental chemicals, but also for drugs and other compounds. The ability of platelets to aggregate, to liberate arachidonic acid from their membrane phospholipids and the formation of eicosanoids are some of the important functions influenced *in vivo* and *in vitro* by drugs and other chemicals (Takeguchi & Sih, 1972; Vanderhoek & Lands, 1973; Flower, 1974; Lands & Rome, 1976; Lindgren *et al.*, 1977; Dewhirst, 1980; Tai *et al.*, 1980; Kangasaho & Vapaatalo, 1983), by xenobiotics such as heavy-metal compounds (Caprino & Togna, 1979; Caprino *et al.*, 1979, 1982; Macfarlane, 1981; Heyns *et al.*, 1985; Krug & Berndt, 1987) and by pesticides (Krug & Berndt, 1985).

One large group of chemicals in use today are the insecticides, and the carbamates are among the most important. It has been shown that carbaryl, a congener of carbamate insecticides, penetrates the cell membrane (Murakami & Fukami, 1979), exhibits its inhibitory activity by covalent binding to acetylcholinesterase (Metcalf, 1971; Corbett, 1974), carbamylates serine esterase (Aldridge, 1971; Pipy *et al.*, 1982), binds covalently to liver microsomal proteins (Miller *et al.*, 1979) and nuclear proteins of cultured human cells (Murakami & Fukami, 1980, 1982) and affects arachidonic acid metabolism (Krug & Berndt, 1985; Marousem *et al.*, 1985, 1986).

Our previous experiments have shown that several lipophilic carbamates inhibit the aggregation of platelets and thromboxane B_2 (TXB_2) formation in human blood platelets *in vitro* (Krug & Berndt, 1985). We report here the results of our experiments designed to gain more insight into the interaction of carbamates, in particular of carbaryl, with arachidonic acid metabolism and cyclo-oxygenase in human blood platelets.

MATERIALS AND METHODS

Chemicals

ADP, adrenaline (1-epinephrine) and Amberlite XAD-2 were obtained from Serva (Heidelberg, Germany); carbaryl was from Riedel de Haen (Hannover, Germany). Promecarb, bendiocarb and propoxur were generously given by Schering AG (Düsseldorf, Germany), AAgrunol-Stähler G.m.b.H. (Stade, Germany), and Bayer AG (Wuppertal, Germany) respectively. NCS solubilizer, l-naphthyl *N*-methyl-[^{14}C]carbamate ([*carbamyl*-^{14}C]carbaryl, 59.5 mCi/mmol), 1-[1-^{14}C]naphthyl *N*-methylcarbamate ([*ring*-^{14}C]carbaryl, 5.8 mCi/mmol), [*acetyl*-1-^{14}C]acetylsalicylic acid (26.2 mCi/mmol), and [^{3}H]arachidonic acid (151 Ci/mmol) were purchased from Amersham (Braunschweig, Germany). Unlabelled arachidonic acid and acetylsalicylic acid (ASA) were obtained from Sigma (München, Germany), thrombin from Calbio-

Abbreviations used: ASA, acetylsalicylic acid; TXB_2, thromboxane B_2; 12-HHT, 12-hydroxyheptadeca-5,8,10-trienoic acid; 12-HETE, 12-hydroxyeicosa-5,8,10,14-tetraenoic acid; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

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chem (Giessen, Germany), and collagen from Hormon Chemie (München, Germany). The silica-gel 60 plates were from Merck (Darmstadt, Germany). All other reagents and solvents used were of analytical grade.

Preparation and incubation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP)

PRP and PPP were prepared by centrifuging fresh human blood (Krug & Berndt, 1987). Before incubation of the platelets the carbamates were added as ethanolic solutions to PPP (final concns. 500 μM) and stirred for 30 min at 37 °C. These freshly prepared stock solutions with autologous PPP were used in all experiments. The platelet count was adjusted to 300000 platelets/μl by dilution with autologous PPP. After the indicated incubation periods, with stirring at 1000 rev./min, platelet aggregation was initiated with collagen (1.2 μg ml of PRP) or 2 μM-ADP or 5 μM-adrenaline or 1 mM-arachidonic acid, and light transmission during aggregation was monitored by use of an Elvi aggregometer at 37 °C. Control incubations contained the same ethanol concentrations, ranging from 0.34 to 17 mM.

Incubation of the PRP and extraction of arachidonic acid metabolites with Amberlite XAD-2 resin

Platelets in PRP were incubated with [^{3}H]arachidonic acid (1 μCi/ml) for 2 h at 35 °C with constant stirring. Subsequently they were incubated with or without the carbamates as described for the aggregation studies. After the appropriate preincubation times, arachidonic acid (1 mM final concn.) was added and platelets were incubated for further 10 min; then Amberlite XAD-2 resin (1 g/ml of PRP) was added to extract arachidonic acid and its metabolites (Keirse & Turnbull, 1973). In these experiments the liberation of arachidonic acid from phospholipids could not be measured because the [^{3}H]-arachidonic acid added for platelet labelling had not been removed before the incubation and extraction procedure, but the effects of the different compounds on aggregation and on the metabolism of arachidonic acid were determined simultaneously.

Incubation of suspensions of washed platelets and determination of arachidonic acid and its metabolites

Platelets were labelled with [^{3}H]arachidonic acid and washed with buffer as described by Krug & Berndt (1987). Samples of washed platelets (0.5 ml, containing 2.5×10^8 platelets) were incubated with 50 μl of autologous PPP containing the carbamate insecticides or ASA to give final concentrations of 10 μM, 20 μM or 100 μM. After preincubation times of 5–90 min, platelets were activated by the addition of thrombin (1.5 units/ml). Platelets were incubated for a further 10 or 15 min, and the reaction was terminated by transferring the samples to an ice-cold mixture of chloroform/methanol (1:2, v/v), and lipids were extracted as described (Siess *et al.*, 1982). Arachidonic acid and its metabolites were separated by t.l.c. (Lapetina & Cuatrecasas, 1979). Metabolites were counted for radioactivity and identified as described by Krug & Berndt (1987).

Determination of covalent binding of [^{14}C]acetylsalicylic acid or of [^{14}C]carbaryl to platelet microsomal proteins

Suspensions of washed platelets [(2–8) $\times 10^8$ platelets/ml] were sonicated three times for 10 s before incubation at 37 °C with [1-^{14}C]acetylsalicylic acid, [*carbamyl*-^{14}C]- or [*ring*-^{14}C]-carbaryl for 45 min. In experiments concerning the inhibition of protein acetylation by carbaryl or the inhibition of protein carbamylation by ASA, the platelet homogenates were preincubated for 45 min with the non-radioactive compounds. After treatment, the suspensions were centrifuged at 90000 *g* for 75 min at 4 °C. The platelet pellets were resuspended in buffer. To homogenize these cells, they were rapidly cooled in liquid N_2 and then rewarmed to 37 °C three times and then sonicated (Sonifier B-12; Branson Sonic Power Co.; MicroTip at 50W) three times for 10 s. Platelet homogenates were centrifuged at 90000 *g* for 75 min at 4 °C. The pellets were washed with 3 ml of 90% (v/v) acetone (Karlin *et al.*, 1976) and 3 ml of diethyl ether, and protein pellets were dried and dissolved in 500 μl of a solution consisting of 1 part of mercaptoethanol and 10 parts of a buffer containing 62.5 mM-Tris/HCl, pH 6.8, 10% (v/v) glycerol and 3% (w/v) SDS. The platelet proteins were separated by SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970), in 1-cm-diameter tubes, using a stacking gel of 3% (w/v) acrylamide and a 9%-(w/v)-acrylamide separation gel. The gels were fixed with 20% (w/v) trichloroacetic acid, stained with Coomassie Brilliant Blue and diffusion-destained (Weber & Osborn, 1969). Protein bands were cut out and the gel slices were treated with NCS/water solution (1:9, v/v) for 5 h at 60 °C with constant shaking. Radioactivity was measured in a liquid-scintillation counter after cooling the solution and adding 10 ml of scintillation cocktail. The M_r values for the platelet proteins were estimated by comparing their migration with that of six standard proteins of known M_r.

RESULTS

Inhibition of platelet aggregation by carbamate insecticides

Human blood platelets in PRP were stimulated to aggregate by the addition of different inducers. After preincubation of PRP with various carbamate insecticides, the induced aggregation was inhibited or diminished (Fig. 1). Arachidonic acid- and collagen-stimulated aggregation are very sensitive to the compounds used and were completely (Fig. 1*b*) or almost completely (Fig. 1*a*) inhibited.

When aggregation was induced with adrenaline or ADP, no complete inhibition, but an inhibition of the 'second wave' of aggregation, could be observed (Figs. 1*c* and 1*d*). The extent of inhibition varies partly according to the different carbamates used and partly according to which stimulatory agent was employed.

Carbaryl was found to be a very potent inhibitory compound, and its effect on platelet aggregation was investigated in more detail. The results are shown in Fig. 2. Total inhibition occurs when aggregation was induced by arachidonic acid after 10 min of incubation. Collagen-stimulated platelet aggregation could also be inhibited completely, but a higher carbaryl concentration and a longer preincubation period were necessary. ADP- and adrenaline-induced platelet aggregation were less sensitive to carbaryl.

Effect of carbamate insecticides on arachidonic acid metabolism in [^{3}H]arachidonic acid-labelled platelets

The induction of human platelet aggregation by

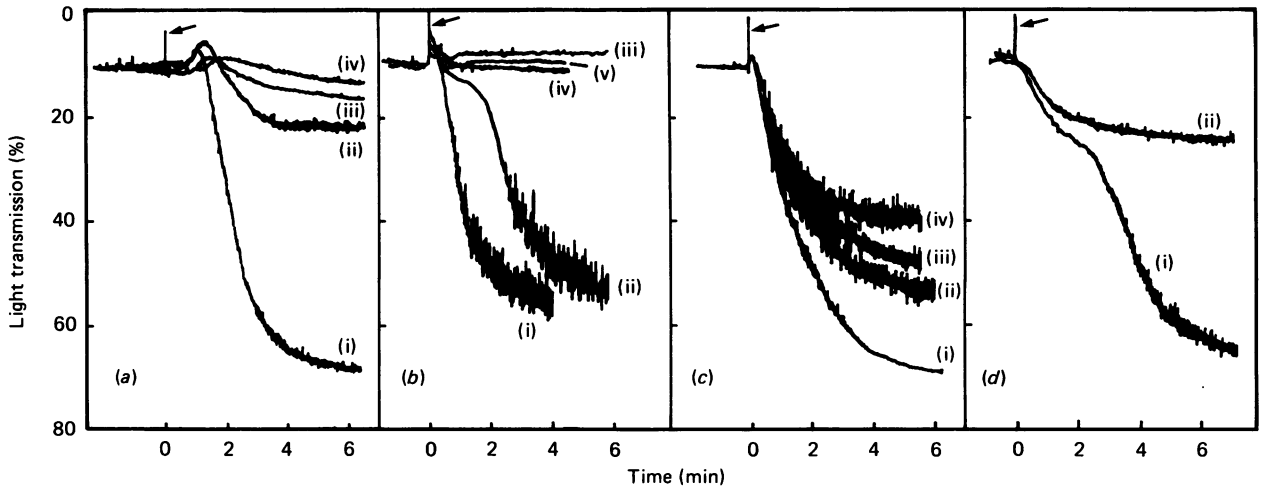

Fig. 1. Inhibition of human platelet aggregation by various carbamate insecticides

After the appropriate preincubation of PRP with or without the carbamates, aggregation was started by the addition of four different aggregating agents (*a–d*, arrows), and the light transmission was monitored for a further 6 min. (*a*) Induction of aggregation with 1.2 μg of collagen/ml of PRP (arrow) after 20 min of preincubation with (i) no insecticide (control), (ii) 100 μM-promecarb, (iii) 100 μM-bendiocarb and (iv) 100 μM-carbaryl; (*b*) induction of aggregation with 1 mM-arachidonic acid (arrow) after preincubation with (i) no insecticide (control), (ii) 10 μM-bendiocarb for 2 min and (iii) for 5 min, (iv) 10 μM-carbaryl for 2 min and (v) 100 μM-promecarb for 2 min; (*c*) induction of aggregation with 2 μM-ADP (arrow) after 20 min of preincubation with (i) no insecticide (control), (ii) 100 μM-bendiocarb, (iii) 100 μM-promecarb and (iv) 100 μM-carbaryl; (*d*) induction of aggregation with 5 μM-adrenaline (arrow) after preincubation with (i) no insecticide (control) and (ii) 20 μM-carbaryl.

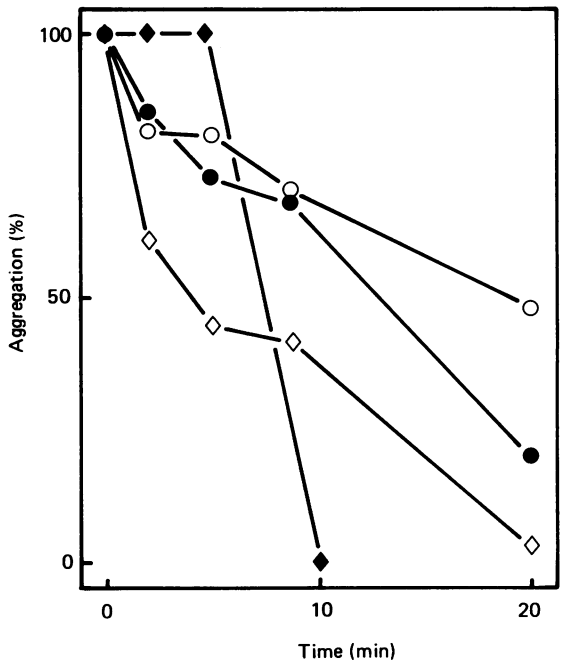

Fig. 2. Inhibition of platelet aggregation by the insecticide carbaryl

Platelets were preincubated with 10 μM- (◆) or 20 μM- (◇, ●, ○) carbaryl for the indicated times. Aggregation was induced by the addition of 1 mM-arachidonic acid (◆), 1.2 μg of collagen/ml of PRP (◇), 5 μM-adrenaline (●) and 2 μM-ADP (○). The ordinate shows the aggregation (% of control) after 6 min of incubation with the aggregating agents; the abscissa shows time of preincubation with carbaryl.

exogenous arachidonic acid depends strictly on cyclo-oxygenase activity (Siess *et al.*, 1983). When [^{3}H]-arachidonic acid-prelabelled platelets in PRP were incubated with the carbamate insecticides and stimulated after 10 min with exogenous arachidonic acid, the inhibition of the aggregation is paralleled by a decreased formation of TXB_2 during the stimulation (Fig. 3). The strongest effect on TXB_2 formation was found with carbaryl (10 μM); a comparable effect, but with a 10-fold higher concentration, was produced by ASA and bendiocarb; promecarb, however, exhibited an even smaller effect, and propoxur behaved similarly to the control (Fig. 3). ASA is known to inhibit cyclo-oxygenase by acetylation (Roth & Majerus, 1975; Roth *et al.*, 1975; Rome *et al.*, 1976). As can be seen from Fig. 3 and Table 1, ASA drastically inhibits the formation of TXB_2 as well as 12-HHT, and, as a consequence of this, the formation of the lipoxygenase product 12-HETE increases in comparison with the control. Carbaryl resembles ASA in this respect: in carbaryl-treated platelets the same shift from the cyclo-oxygenase pathway (TXB_2 and 12-HHT formation) to the lipoxygenase pathway (12-HETE formation) could be observed. Bendiocarb and promecarb exhibited a similar effect.

Covalent binding of acetylsalicylic acid and of carbaryl to platelet proteins

The data summarized in Table 1 suggest that carbaryl may act on cyclo-oxygenase via a way similar to, or even identical with, that of ASA, i.e. carbamylation of this enzyme. This was investigated in more detail. Initially, the acetylation of cyclo-oxygenase by [^{14}C]ASA was performed as described by Roth & Majerus (1975) and Roth *et al.* (1975): incubation of sonicated suspensions of washed platelets with [^{14}C]ASA for 45 min, washing of

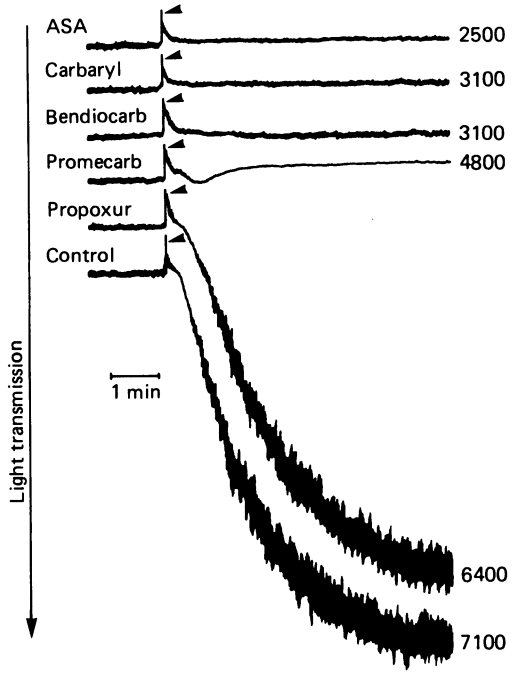

Fig. 3. Inhibition of arachidonic acid-induced platelet aggregation and TXB_2 formation by carbamate insecticides and ASA

[^{3}H]Arachidonate-prelabelled platelets were treated with 10 μM-carbaryl, with 100 μM concentrations of the other compounds, or without inhibitor (control). After a preincubation period of 10 min, platelets were stimulated by the addition of 1 mM-arachidonic acid (arrowheads). Light transmission was monitored for 6 min and then TXB_2 was extracted with Amberlite XAD-2 resin. The levels of radioactively labelled TXB_2 (c.p.m.) formed during aggregation are given on the right.

the platelet proteins and subsequent SDS/polyacrylamide-gel electrophoresis resulted in incorporation of the radioactive acetyl moiety of ASA into a single protein peak, with an apparent M_r of 80000 (Fig. 4), corresponding to cyclo-oxygenase (for a review, see Marcus, 1978). When platelets were incubated with [*carbamyl*-^{14}C]carbaryl, incorporation of radioactivity into many proteins was found. On the other hand, when [*ring*-^{14}C]carbaryl was used, binding to proteins was substantially lower, the radioactivity was evenly distributed throughout the chromatogram and no discrete labelled protein peak could be seen (Fig. 4). The labelling of proteins in the M_r region 77000–82000 is quite different for the three radioactive compounds (stippled area in Fig. 4): (1) the acetyl moiety from ASA binds specifically, with >90% (350 c.p.m.) of the total radioactivity in one peak; (2) the carbamyl moiety of carbaryl binds non-specifically to many proteins; 8% (3000 c.p.m.) of the total radioactivity binds to proteins in the 80000-M_r area; (3) the ring moiety of carbaryl scarcely binds to proteins, and there is no specific binding in the 80000-M_r area. From these results it can be concluded that the acetyl moiety of ASA binds to one protein, shown by others to be cyclo-oxygenase, whereas the carbamyl moiety of carbaryl binds to many proteins, one of which is probably cyclo-oxygenase, as indicated by radioactive labelling in the 80000-M_r area.

Inhibition of ASA-mediated protein labelling by carbaryl and vice versa

The binding of the acetyl moiety of ASA and of the carbamyl moiety of carbaryl to the same site of the cyclo-oxygenase is further corroborated by the fact that carbaryl inhibits the acetylation of cyclo-oxygenase by ASA. After preincubation of sonicated platelet suspensions with non-radioactive carbaryl, the uptake of the [^{14}C]acetyl residue from ASA is blocked (Table 2). The reverse experiments, using non-radioactive ASA, followed by an incubation with [^{14}C]carbaryl showed a 30% decrease in labelled protein in the 77000–82000-M_r region when [*carbamyl*-^{14}C]carbaryl was used; there was no effect with [*ring*-^{14}C]carbaryl (Table 2). Moreover, the extent of the inhibition of the acetylation by non-radioactive carbaryl depends on the molar ratio of unlabelled to labelled compound (Fig. 5).

The irreversible covalent inhibition of the cyclo-oxygenase was first tested by washing the sonicated

Table 1. Effect of carbamates and ASA on arachidonic acid metabolism in human platelets

Suspensions of washed platelets, prelabelled with [^{3}H]arachidonic acid, were preincubated with the different compounds for the times indicated and then stimulated by the addition of thrombin (1.5 units/ml) for further 10 or 15 min at 37 °C. Lipids were extracted, separated and counted for radioactivity as described in the Experimental section. Abbreviation used: AA, arachidonic acid.

Treatment	Incubation time (min)	Radioactivity of total liberated AA (c.p.m.)	Metabolites formed (% of total radioactivity)			
			TXB_2	12-HHT	12-HETE	Free AA
Control	5	12180	36	43	18	3
ASA (100 μM)	5	12570	1	4	44	51
Bendiocarb (100 μM)	5	12190	17	24	25	34
Promecarb (100 μM)	5	14800	24	29	18	29
Carbaryl (10 μM)	5	12130	13	18	26	43
Carbaryl (20 μM)	10*	12080	7	19	71	3
Carbaryl (20 μM)	90*	10160	3	16	79	2

* 15 min stimulation with thrombin; all others stimulated for 10 min (demonstrating the total conversion of liberated AA into 12-HETE when stimulated for a longer period).

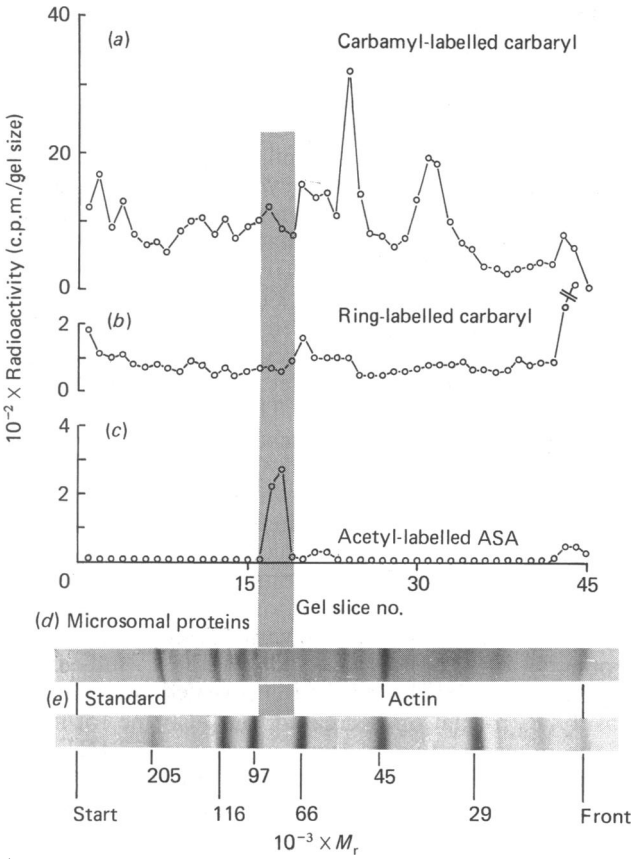

Fig. 4. Incorporation of radioactivity from [^{14}C]carbaryl or [^{14}C]ASA into microsomal proteins from human blood platelets

Sonicated suspensions of washed platelets were incubated with (*a*) 1 μCi of (7 μM-) carbamyl-labelled carbaryl, (*b*) 5 μCi of (340 μM-) aromatic-ring-labelled carbaryl or (*c*) 5 μCi (76 μM-) acetyl-labelled ASA for 60 min at 37 °C. After incubation the proteins were collected and subjected to SDS/polyacrylamide-gel electrophoresis. The protein bands were stained with Coomassie Brillant Blue [see the electrophoretograms of platelet microsomal proteins (*d*) and molecular marker proteins (*e*) at the bottom of the Figure], cut out, dissolved in NCS solubilizer and measured for radioactivity in a liquid-scintillation counter. The stippled area ($\sim 80000\ M_r$) corresponds to cyclo-oxygenase.

platelets with buffer twice after preincubation with the non-radioactive compound. Subsequently the platelets were incubated with labelled ASA or carbaryl. These experiments revealed the same results (results not shown) as those without the washing step between the two treatments (Table 2). Consequently this time-consuming step was then omitted.

DISCUSSION

Out of a group of 18 insecticides and herbicides of the carbamate type tested, only the lipophilic *N*-methyl-carbamates (Fig. 6) had the effect described here, whereas none of the hydrophilic *N*-methylcarbamates (seven insecticides) or the lipophilic *N*-phenylcarbamates (seven herbicides) had any inhibitory activity on human platelet aggregation or arachidonic acid metabolism (results not shown).

We compared the inhibitory activity of the four carbamates and ASA with regard to the concentrations used and the effect on aggregation and TXB_2 formation (Fig. 3), and the following sequence of inhibitory potency was found:

Carbaryl > ASA > bendiocarb > promecarb ≫ propoxur

This order of inhibition is possibly explicable by increasing steric blocking and chemical differences among ring substituents from left to right (Fig. 6).

Siess *et al.* (1981) have shown that the potential for TXB_2 formation of platelets increased in the following order:

ADP < adrenaline < collagen < arachidonic acid

Furthermore, they describe a correlation between TXB_2 formation and the 'second wave' of platelet aggregation, in particular, after stimulation with adrenaline. In our experiments the inhibition of platelet activity by the carbamates (Figs. 1 and 2) follows the same order: weak inhibition of ADP- to total inhibition of arachidonic acid-induced aggregation and inhibition of the 'second wave' of aggregation induced by adrenaline (Fig. 1*d*). Taken together with our previous results (Krug & Berndt, 1985), these data point to an inhibitory activity of the carbamates within the arachidonic acid cascade.

The insecticides of the carbamate type exert their function by carbamylation of the enzyme acetyl-

Table 2. Inhibition of ASA-mediated protein acetylation by carbaryl and of carbaryl-mediated protein carbamoylation by ASA

Sonicated suspensions of washed platelets (corresponding to 5 mg of protein) were preincubated for 45 min with non-radioactive carbaryl or ASA. Controls were preincubated without any addition. Incubation was continued for a further 45 min in the presence of radioactively labelled ASA or carbaryl. Platelet proteins were separated by SDS/polyacrylamide-gel electrophoresis and the radioactivity in the 80000-M_r region was measured (gel slices 16–18). Values represent means ± S.E.M. for four or five experiments with the blood of different donors. Statistical significance: $*P < 0.001$ and $**P < 0.05$, paired *t*-test as compared with control results (line above).

Radioactive treatment	Non-radioactive pretreatment	Radioactivity (c.p.m. in gel slices 16–18)
[*acetyl*-^{14}C]ASA (5 μCi)	None	375 ± 25
	Carbaryl (1 mM)	55 ± 19*
[*ring*-^{14}C]Carbaryl (5 μCi)	None	182 ± 42
	ASA (1 mM)	154 ± 25
[*carbamyl*-^{14}C]Carbaryl (5 μCi)	None	4740 ± 267
	ASA (1 mM)	3280 ± 630**

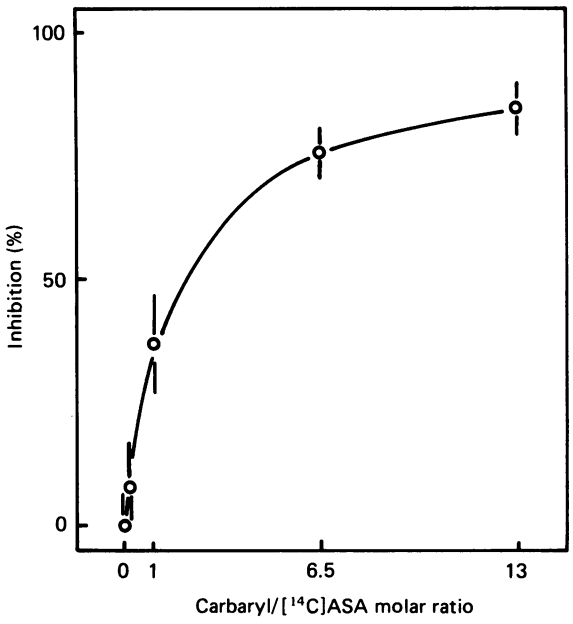

Fig. 5. Inhibition of ASA-mediated protein acetylation by carbaryl

Sonicated suspensions of washed platelets (corresponding to 5 mg of protein) were preincubated for 45 min with four different concentrations of non-radioactive carbaryl to give the molar ratios of 0.1, 1, 6.5 and 13 as indicated on the abscissa. Control was incubated without carbaryl. The ordinate shows inhibition of [^{14}C]ASA-mediated acetylation of cyclo-oxygenase within the gel slices 16–18 (cf. Fig. 4, stippled area). The vertical lines represent the S.E.M. values for four experiments with different blood donors. Other details are as in Table 2.

cholinesterase in insects, mimicking its natural substrate, acetylcholine (Aldridge, 1971; Metcalf, 1971; Corbett, 1974). The acetylated acetylcholinesterase is hydrolysed immediately and re-activated; the carbamylated enzyme, however, resists hydrolysis, and the enzyme is blocked (Corbett, 1974). A similar mechanism occurs in human blood platelets when cyclo-oxygenase is acetylated by ASA. It has been shown (and we have confirmed it; Fig. 4) that, after incubation of platelets with radioactively labelled ASA, (i) this enzyme becomes labelled by the acetyl residue, but not by the aromatic-ring moiety (Marcus, 1978; Roth *et al.*, 1975, (ii) covalent binding to only one protein occurs (Fig. 4) and (iii) this protein is known to be platelet cyclo-oxygenase (for a review, see Marcus, 1978). The similarity of these reactions to effects of the carbamates on aggregation and TXB_2 formation (Figs. 1–3) suggests that cyclo-oxygenase may be blocked by these xenobiotics.

The decline in TXB_2 formation, however, is not necessarily a consequence of decreased cyclo-oxygenase activity; it may be also the result of phospholipase or thromboxane synthase inhibition. An investigation of arachidonic acid metabolism with respect to the major products of lipoxygenase (12-HETE) and cyclo-oxygenase (12-HHT and TXB_2) revealed that not only was TXB_2 formation decreased, but also the formation of the other product of cyclo-oxygenase, 12-HHT was diminished. Prolonged incubation of platelets with carbaryl produced an almost complete conversion of arachidonic acid, liberated from platelet phospholipids by phospholipase, into 12-HETE, whereas TXB_2 formation was negligible and 12-HHT formation was low (line 7 in Table 1). Both ASA and carbaryl, a congener of the *N*-methylcarbamates, cause a shift from the cyclo-

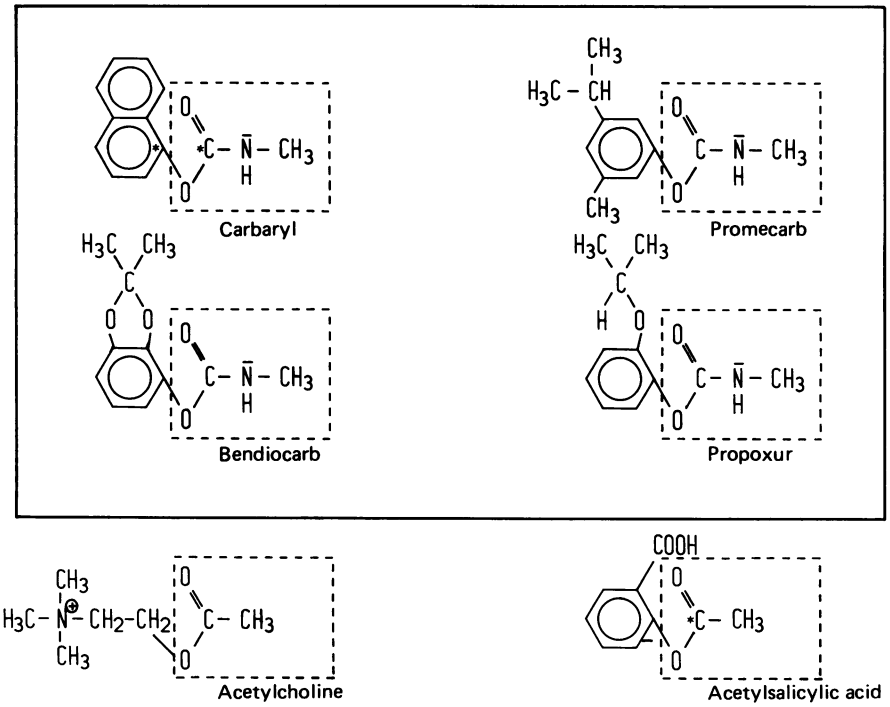

Fig. 6. Structures of four lipophilic *N*-methylcarbamates (insecticides) described here as cyclo-oxygenase inhibitors working via carbarmylation of the enzyme

In comparison with the carbamates, the structures of the natural substrate, acetylcholinesterase, and the covalent cyclo-oxygenase inhibitor, ASA, are shown. All compounds were able to transfer carbamyl or acetyl residues (shown in broken rectangles) to enzymes by carbamylation or acetylation respectively. Asterisks (*) indicate ^{14}C-labelled carbon atoms.

oxygenase pathway towards the lipoxygenase pathway without affecting the liberation of arachidonic acid by phospholipases. Carbaryl (and the other carbamates tested) resembles ASA with respect to its (their) effect on arachidonic acid metabolism.

We show here a substantial covalent labelling of platelet proteins by the [^{14}C]carbamyl moiety of carbaryl, whereas aromatic-ring-labelled carbaryl has only a very small labelling efficacy (Fig. 4). In contrast with the experiments with [^{14}C]ASA, many proteins have been labelled by carbamylation (Fig. 4), indicating a rather non-specific covalent binding of the carbamyl moiety to platelet proteins compared with acetylation of cyclo-oxygenase by ASA. Nevertheless, incubation of platelets with unlabelled ASA before incubation with labelled carbaryl resulted in a (30%) decrease in the incorporation of label into the 77000–82000-M_r fractions (Table 2). The reverse test, namely the inhibition of acetylation by non-radioactive carbaryl, however, demonstrated that both compounds bind to the same protein and, if there is no conformational change, compete for the same binding site.

During carbamylation of the proteins by carbaryl and other carbamates, phenolic metabolites from these compounds are formed (i.e. α-naphthol). These phenols are known to inhibit cyclo-oxygenase, owing to their antioxidative properties (Takeguchi & Sih, 1972; Vanderhoek & Lands, 1973; Dewhirst, 1980; Lands & Rome, 1976; Lindgren *et al.*, 1977). In the experiments described here, α-naphthol had an inhibitory effect, comparable with that of carbaryl, on platelet aggregation and also on eicosanoid formation (results not shown). As was reported by others (Lands & Rome, 1976; Lindgren *et al.*, 1977; Vanderhoek & Lands, 1973), the inhibitory effect of naphthol was found to be reversible, a finding positively consistent with the fact that this part of the carbamate molecule does not bind to proteins, especially not to cyclo-oxygenase. With respect to the effect of phenols on platelet aggregation and arachidonic acid metabolism, we could thus confirm the findings of others.

The irreversible inhibition of cyclo-oxygenase by ASA as well as by carbaryl is directly associated with their ability to acetylate or carbamylate this enzyme. Once the protein modification has occurred, it could be neither reversed or undone by washing the platelet homogenates with buffer between pretreatment with the non-radioactive, and incubation with the radioactive, compounds (results not shown), nor by extraction of the platelet proteins with 90% (v/v) acetone and diethyl ether, nor by dilution of the proteins in buffer containing SDS and subsequent gel electrophoresis. This clearly demonstrates that, at least in the case of carbaryl and presumably also for the other carbamates used, an aspirin-like covalent binding to the active site of cyclo-oxygenase is the irreversible inhibitory mechanism within the arachidonic acid cascade. On the other hand, however, this enzyme is not the only one carbamylated by carbaryl; ASA, however, acetylates solely this protein.

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