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STUDIES ON THE ARACHIDONIC ACID CASCADE—I

INHIBITION OF PHOSPHOLIPASE A_2 *IN VITRO* AND *IN VIVO* BY SEVERAL NOVEL SERIES OF INHIBITOR COMPOUNDS

DONALD P. WALLACH* and VIVIAN J. R. BROWN
Department of Experimental Biology, The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

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Abstract—A series of compounds have been discovered which are potent inhibitors *in vitro* of hog pancreas, cobra venom, and bee venom phospholipase A_2. Collagen-induced aggregation of human platelets was prevented by representatives of this series. The inhibition was reversed by aggregatory concentrations of arachidonate, indicating the hydrolysis of esterified arachidonate had been prevented. In the isolated perfused guinea pig lung sensitized to ovalbumin, the release of prostanoids, resulting from a challenge dose of the antigen, was prevented by exemplars of these compounds. Subsequent administration of arachidonate in the presence of the inhibitor resulted in full prostanoid synthesis and secretion, again indicating that the block was at the phospholipase level. Administration of some of these compounds to guinea pigs by subcutaneous or intraperitoneal routes delayed the onset, and decreased the severity, of erythema induced in depilitated skin by the controlled application of ultraviolet light. This result was, also, consistent with phospholipase A_2 inhibition. Kinetic experiments with hog pancreas phospholipase A_2 demonstrated that, with representatives of this series, the inhibition induced was noncompetitive, and appropriate dissociation constants have been calculated.

Recently, Vogt [1] and Isakson *et al.* [2] reviewed the role of phospholipase A_2 in the arachidonic acid cascade, the series of enzymically catalyzed oxidative transformations of arachidonic acid leading to the formation of prostaglandins, prostacyclins, thromboxanes, and other hydroxylated derivatives. It is now generally accepted that a wide variety of stimuli can cause activation of phospholipase A_2 [1, 3, 4], resulting in hydrolysis of arachidonate rich phospholipids and, thereby, providing substrate to the enzymes of the cascade.

Despite the central importance of phospholipase A_2 in initiating the cascade, there has been little work directed toward the discovery of specific, physiologically acceptable inhibitors of this enzyme. Rather, the actions of known drugs have been examined with a view toward explaining the mode of action of phospolipose A_2. The paradigm for this mode has been the action of the anti-inflammatory steroids, which may be looked upon not as inhibitors of the phospholipase, but as compounds that effectively mimic such action *in vivo* by, in some way, depriving the enzymes of the cascade of substrate [2, 3, 5–7]. Apparently, they do not work directly on the phospholipase to induce inhibition. This is also true for a pyrophtalone derivative [8].

In a recent and important contribution, Flower and Blackwell [9] presented evidence that the steroids induce synthesis of a factor, presumed to be a peptide, that has anti-phospholipase A_2 activity. This could explain the frequently observed discrepancy between the *in vitro* and *in vivo* actions of steroids on the phospholipase.

On the other hand, some true inhibitors are known. These comprise indomethacin [10], chlorpromazine [3], mepacrine [2–4, 11], local anesthetics [1, 12] that work by competing with calcium (a requirement for phospholipase A_2 activity), and bromphenacyl bromide [11]. This last compound acylates a histidine residue at the active site [13, 14].

Phospholipase A_2 has been studied in detail, and its structure and amino acid sequence have been elucidated [15, 16]. Drenth *et al.* [16] have stated that, irrespective of source, the amino acid sequences of these enzymes are similar, resulting in similar three-dimensional structures. They also postulated that hog pancreatic phospholipase A_2 represents an exemplar for this class of enzymes.

By using this enzyme and soybean lipoxidase in a coupled system and monitoring the release of unsaturated fatty acids from a semi-synthetic phosphatidyl choline substrate by oxygen consumption measurements, a screening program was initiated which was aimed at discovering specific and potent inhibitors of this phospholipase that would be effective *in vivo*. This report describes results of this work.

MATERIALS AND METHODS

The phosphatidyl choline substrate was made by Dr. W. E. Heyd of these laboratories. The fatty acid composition, upon saponification, was 2% of 16:0, 1% of 18:0, 3% of 18:1, 82% of 18:2, and 12% of 18:3 fatty acids; the largest fraction was linoleic acid. The estimated mol. wt was 780. Hog pancreatic, snake venom and bee venom phospholipases A_2, and soybean lipoxygenase, were obtained from the Sigma Chemical Co., St. Louis, MO, as was deoxycholic acid, Tris hydroxymethylaminomethane buffer, indomethacin, propranolol hydrochloride, (−)-sco-

* Author to whom all correspondence should be addressed.

polamine hydrobromide, and pyrilamine maleate. Methysergide maleate was purchased from Sandoz, East Hanover, NJ, and phenoxybenzamine hydrochloride from Smith, Kline & French Laboratories, Philadelphia, PA. Prostaglandin cyclodioxygenase was prepared in these laboratories [15]. 2-Amino-2-methyl-1,3-propanediol (Ammediol) (practical grade) buffer was purchased from Eastman Organic Chemicals, Rochester, NY. Organic solvents were purchased from Burdick & Jackson Laboratories, Muskegon, MI. Components of Krebs–Ringer bicarbonate solution were purchased from Mallinckrodt, Inc., Paris, KY. Arachidonic acid, 99%+ pure, was purchased from NuChek Laboratories, Inc., Elysian, MN. Guinea pigs of the Hartley strain were obtained from the Kuiper Rabbit Ranch, Gary, IN. Female rats, 250–300 g, were obtained from The Upjohn Animal Rearing Facility, Portage, MI. All compounds reported in this paper were made in these laboratories by Dr. Dan Lednicer and Dr. Gilbert A. Youngdale, with the exception of propranolol and practolol, which were obtained from Ayerst.

For measurements of oxygen consumption, a Yellow Springs Instrument Company "oxygraph", coupled to a Sargent-Welch SRG recorder through a scale expansion and switching device made by Mr. Jim Jameson of these laboratories, was used.

A Kromayer No. 10 ultraviolet lamp made by Hanovia Ltd., Slough, England, was used for the induction of erythema on the skin of guinea pigs. A Branson sonifier was used to make drug and substrate suspensions.

The instrument used to record the contractions of the smooth muscle strips was a Grass model 79D polygraph, equipped with three recording channels. The instrument was calibrated each day just before each run. The results were graphically recorded in the isometric mode.

Phospholipase assay system. Micellar solutions of phosphatidyl choline were made from 78 mg of this substrate in a 25 ml beaker, to which 7–8 ml of 0.1 M ammediol–HCl buffer (pH 8.0) containing 1% deoxycholate was added. The material was sonicated with a Branson sonifier, tuned to maximal output for 1 min. The sonicated material was allowed to stand until the foam subsided and, then, was made up to 10 ml in a volumetric flask. The substrate could be stored at 0–3° for 3–4 days and used with consistent results.

The hog pancreas phospholipase A_2 (EC 3.1.1.4) preparation routinely contained 6000–7000 units/ml. This was diluted 1:40, and approximately 9 units (0.05 ml) was added to each of four oxygraph cells that were equipped with magnetic stirring bars and contained 2.5 ml of ammediol–HCl buffer (pH 8.49) with $1\ 10^{-4}$ M calcium and 0.15 mg of soybean lipoxygenase (EC 1.13.1.13). When snake venom (*Naja naja*) was used, it was dissolved in water at a concentration of 2 mg/ml, and 0.02 ml, equivalent to 28 units, was added to each cell.

When bee venom was employed, 7.5 units was added to each oxygraph cell with all other components remaining the same.

When an inhibitor was added to the incubation mixture, it was dissolved in water, methanol, or acetone, at a concentration of 0.01 M, and 0.1 ml of one of the solutions was then added with mechanical stirring to ensure thorough mixing. The final concentration of each inhibitor was $3.8\ 10^{-4}$ M. (At this concentration in the final mixture, the organic solvents had no discernible effects on the reaction.) The oxygen probes of a Yellow Springs "oxygraph" were then inserted in the cells, care being taken to exclude all air bubbles. The instrument settings were 5 mV full scale; the "air" setting was used, with a medium chart speed of 10 abscissa units/min. A 3-min preincubation period with stirring was used to ensure contact of inhibitor with enzyme and, also, to allow for temperature equilibration at 37°. Then 0.05 ml of 0.01 M phosphatidyl choline was added to the first cell to initiate the reaction, which was monitored continuously thereafter for oxygen consumption.

Once the tracing of oxygen consumption was sufficient, the process was repeated with the remaining three cells. The slopes of initial rates of oxygen consumption could then be calculated.

If inhibition of the reaction was observed, 0.03 ml of 0.01 M potassium arachidonate was added. If no inhibitory effect on the lipoxygenase was discerned then, the inhibitor obviously had [illegible] the phospholipase.

Appropriate dilutions of inhibitor were run to obtain partial inhibition at one or more concentrations. The degree of inhibition could be calculated from the diminution in slopes when compared with noninhibited controls. The I_{50} values could then be calculated.

Enzyme kinetics. When kinetic experiments were run, all inhibitor solutions were made up at 0.01 M concentrations in acetone for stock solutions, and appropriate dilutions were made in acetone so that in all cases a constant volume of 0.01 ml of solution was added to the oxygraph cells. The conditions remained as outlined for the testing of inhibitors. Substrate dilutions were made in buffer so that constant volume of substrate was also added. At least four samples were run at each substrate concentration with a given inhibitor concentration. The reciprocal of the mean slope of the four samples was then calculated, as were those for other substrate concentrations with a given inhibitor concentration. The slopes of $1/V$ (ordinate) vs $1/S$ (abscissa) were then calculated for the samples, both with and without inhibitor. The K_m for the uninhibited enzyme was then calculated, and the K_i was determined for the inhibited case. The intercepts were compared statistically using a program developed by Ms. P. L. Kemp and Dr. P. I. Good of these laboratories (unpublished observations).

Sheep seminal vesicular cyclodioxygenase assay. When inhibitors of phospholipase A_2 were tested with the sheep seminal vesicular cyclodioxygenase, microsomal acetone-pentane powders, made by a method published previously [17], were used. Finely ground powder was suspended in a mixture of 0.25 M Tris–HCl buffer (pH 8.25) containing $7\ 10^{-4}$ phenol, with the aid of a Dounce homogenizer. The suspension was held at room temperature for 10 min to "activate" the cyclodioxygenase, after which the enzyme was chilled and kept in ice.

To four oxygraph cells was added 2.5 ml of

Tris–HCl buffer with phenol (as above), a magnetic stirrer, and an inhibitor at a 3.8 10^{-4} M final concentration. Three minutes were allowed for preincubation (as before), and then the cyclodioxygenase reaction was initiated by addition of 0.03 ml of 0.01 M potassium arachidonate. With the recorder set at 5 mV full scale, the initial slopes of oxygen consumption were calculated as before and comparisons made in the presence and absence of inhibitors.

Guinea pig skin erythema assay. When guinea pigs were used to evaluate the anti-erythema effects of phospholipase inhibitors *in vivo*, the hair of animals weighing between 300 and 400 g was clipped on either the back or the abdomen with coarse and fine clippers to remove as much as possible. The clipped area was then coated with "Nair", the commercial depilatory, and left for a period of 8 min. The animals were then washed with warm water and gently dried with towels.

A suspension of an inhibitor compound in an aqueous vehicle consisting of 0.5% carboxymethylcellulose, 0.4% polysorbate 80, 0.9% sodium chloride, and 0.9% benzyl alcohol was then administered i.p., s.c. over the back, or orally to each animal, initially at a dose of 0.3 ml of 0.02 M compound/100 g of body weight. The suspensions were made by weighing out the requisite amount of compound into a 20 ml beaker, adding vehicle, and sonicating the mixture at maximal output for 30 sec to 1 min as required. Stable uniform suspensions were produced by this method. At least three animals per group were used.

The guinea pigs, following compound administration, were held for 30 min to permit absorption of the compound. Then they were exposed to ultraviolet irradiation for 30 sec at four different locations on the abdomen with a Kromayer lamp with u.v. emissions at 296.7 n M, known to cause erythema [18].

The size of the lamp opening was 20 mm in diameter and the distance from the Quartz lens to the skin was 1.5 cm. At hourly intervals after exposure, the intensities of the spots were scored in direct comparison with the vehicle-treated controls. In a modification of the method of Adams and Cobb [19], a scoring system, based on a scale of 0—5, was used as follows:

0 = no apparent reddening of the skin;
0.5 = some slight reddening without a definite border;
1.0 = a discreet circle of reddening with a definite border;
2.0 = a discreet circle of reddening with a definite border, more intense than 1.0;
3.0 = the same circle with still greater intensity;
4.0 = a maximum erythema response but without any elevation of the irradiated area above the level of the surrounding skin;
5.0 = a raised edematous circle with redness of maximum intensity.

The animals were scored after treatment at hourly intervals for the first 4–5 hr and again at 24 hr, the scores were averaged for presentation of data.

Platelet aggregation assay. Platelet-rich plasma was prepared from freshly drawn human blood obtained from donors, fasted overnight, who had taken no medication for the previous 5 days. The blood was diluted with 3.8% sodium citrate (9 parts of blood to 1 of citrate) and centrifuged at 200 *g* for 10 min at 25°.

Platelet aggregation was monitored at 37° in a Payton aggregometer with constant stirring at 1100 rpm. Platelets were incubated for 2 min at 37°, and then aggregation was initiated by addition of 400 μg/ml of arachidonic acid. If aggregation was not seen following this treatment, the platelets were discarded. Collagen was then added until the concentration was reached that produced irreversible aggregation (60–80 per cent transmission) that was blocked by the addition of 10 μg/ml of indomethacin.

With these preconditions established, the compounds, dissolved in organic solvents, were then added to the aggregometer cuvettes and evaporated to dryness. Dispersal of the compound in the platelet-rich plasma was assumed to take place during the 2-min preincubation period.

Experiments with the isolated guinea pig lung sensitized to ovalbumen, in the tissue cascade technique. Guinea pigs were sensitized to ovalbumen by the method of Fleisch *et al.* [20] and Hemker and Aiken [21]. The guinea pigs were ready to use as early as 12 days after the first sensitization injection. Maximum responses were usually seen from day 15 to 20, the lungs responding to 1–10 ng of ovalbumen. Thereafter, larger challenge doses of ovalbumen were required.

The animals were killed by cervical dislocation. The thoracic cavity was rapidly opened, the pericardium was incised, and 1 ml of Krebs–Ringer bicarbonate containing 100 units of heparin was injected intracardially. The pulmonary artery was cannulated via an incision in the wall of the right atrium. The cardiac ventricles were removed along with the left atrium, and the lung was flushed with 10–20 ml of Krebs–Ringer solution containing 10 units heparin/ml. With removal of blood the lungs turned white. The trachea was then cannulated, and the lungs were expeditiously removed from the animal, inserted into the first chamber of the "Cascade" superfusion apparatus described by Vane [22], and attached to the perfusion apparatus by the pulmonary artery cannula. Krebs–Ringer bicarbonate, gassed continuously with 95% O_2 and 5% CO_2 and warmed to 37°, was then pumped through the lungs at the rate of 10 ml/min. Via the tracheal cannula the lungs were then "puffed", by the addition of 10–20 ml of air, to fully extend the alveoli. The tracheal cannula was then clamped.

To the effluent from the lungs was added a mixture of antagonists described by Aiken and Vane [23] and a 1 mg/ml solution of indomethacin, both added at the rate of 0.1 ml/min.

The effluent was conducted first over an aortic strip removed from a rat exposed to a lethal concentration of carbon dioxide. The strip was cut spirally, at a 45° angle, as described by Furchgott [24], to assay the content of thromboxane A_2 in the effluent from the lungs. In a second chamber, the effluent from the lungs was conducted over a stomach fundus strip from the same animal. This strip was cut longitudinally along the curve of the stomach.

Table 1. Structures of phospholipase A_2 inhibitors and values of I_{50} for phospholipases A_2 from hog pancreas, cobra (*N. naja*) venom, and bee (*A. mellifera*) venom

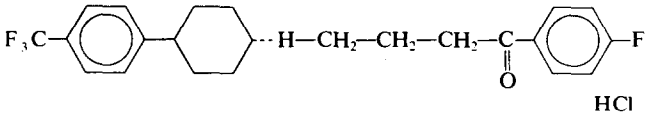
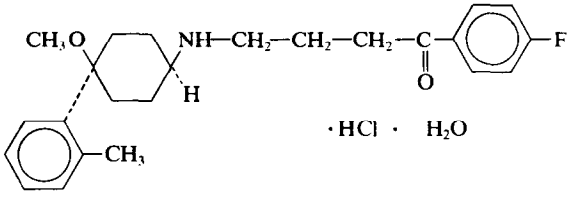
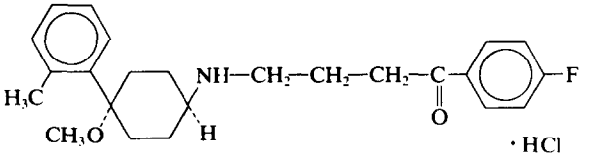
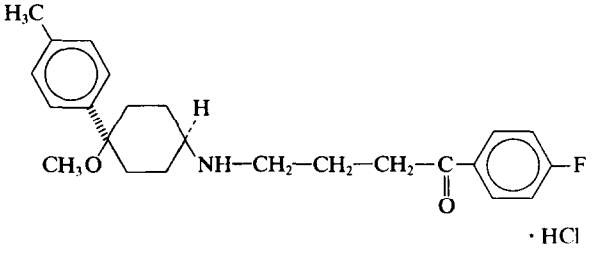
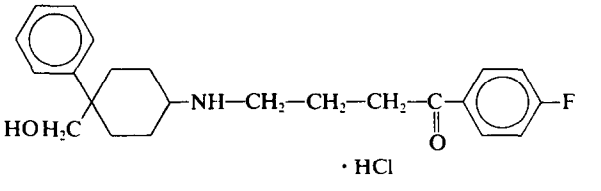
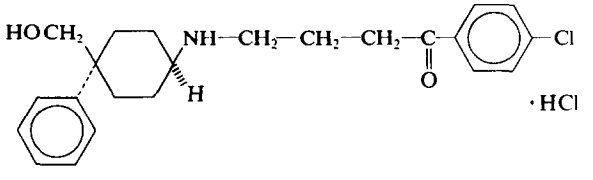
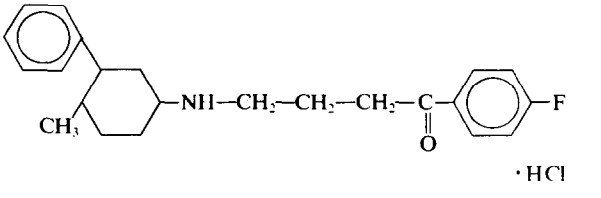
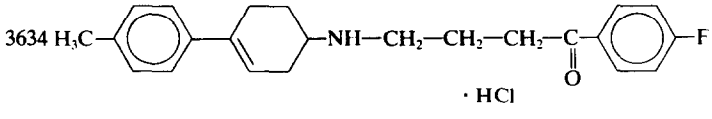
Compound	Structure	I_{50} (M) Hog pancreas	Cobra venom	Bee venom
3585		5.1×10^{-6}	3.2×10^{-4}	1.4×10^{-3}
3778		1.1×10^{-5}		
3776		1.2×10^{-5}	3.0×10^{-4}	4.0×10^{-4}
3829		1.5×10^{-5}	3.2×10^{-4}	1.8×10^{-3}
3976		2.4×10^{-5}	2.2×10^{-3}	1.2×10^{-3}
4087		3.0×10^{-5}		
4077		3.1×10^{-5}		
3634		3.5×10^{-5}	3.3×10^{-4}	5.9×10^{-4}

Table 1 (cont.)

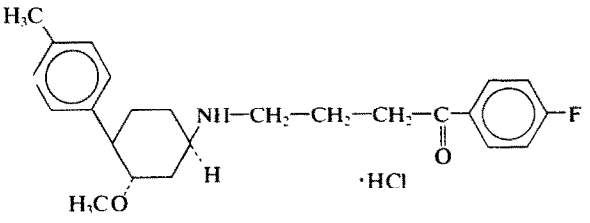
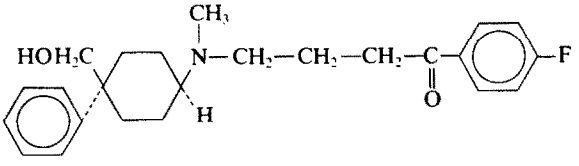
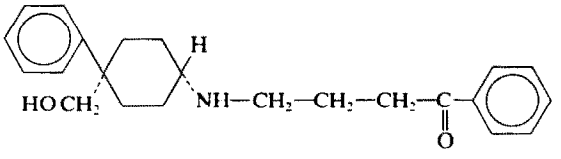
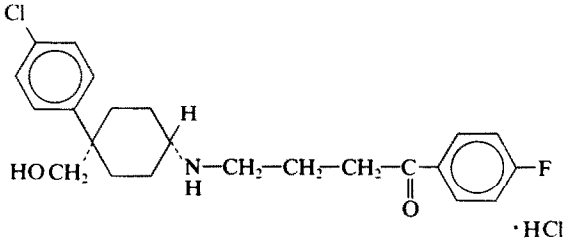
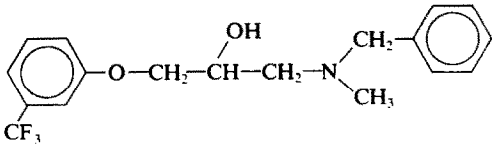
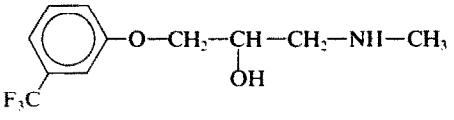
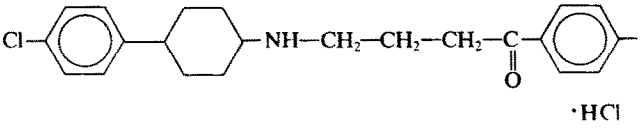
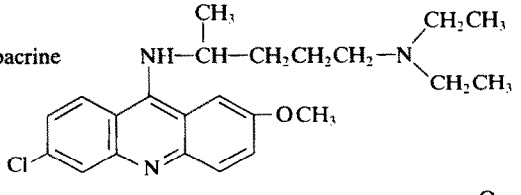
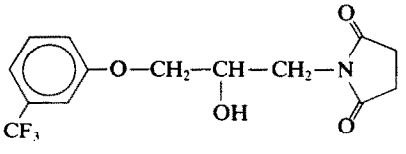
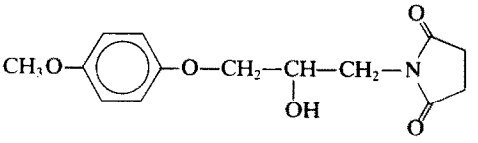
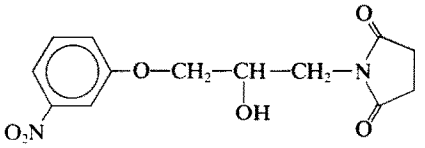
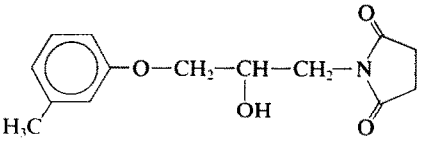
Compound	Structure	I_{50} (M) Hog pancreas	Cobra venom	Bee venom
3557		4.0×10^{-5}	3.2×10^{-4}	1.2×10^{-3}
4078		4.3×10^{-5}		
4045		4.7×10^{-5}		
4046		5.5×10^{-5}	2×10^{-4}	2.5×10^{-3}
1002		1.6×10^{-4}	4.1×10^{-4}	4.9×10^{-4}
2021		1.6×10^{-4}	5.2×10^{-4}	7.4×10^{-4}
3674		2.4×10^{-4}	1.8×10^{-4}	1.8×10^{-4}
Mepacrine		2.4×10^{-4}		
2193		3.5×10^{-4}	5.1×10^{-4}	5.2×10^{-4}

Table 1 (cont.)

Compound	Structure	I_{50} (M) Hog pancreas	Cobra venom	Bee venom
1146		3.8×10^{-4}	7.2×10^{-4}	5.8×10^{-4}
1227		4.9×10^{-4}	5.9×10^{-4}	
1325		5.9×10^{-4}	5.0×10^{-4}	7.9×10^{-4}

RESULTS

In Table 1 are shown the I_{50} results for a variety of compounds, including mepacrine for comparative purposes, tested under the conditions indicated for the individual phospholipases. The compounds are listed in order of decreasing potency. The most potent compound was no. 3585, and the least potent compound was no. 1325. In general, the enzyme most sensitive to these inhibitors was the hog pancreas enzyme, whereas the least sensitive was the bee venom enzyme. The cobra venom phospholipase A_2 fell midway between these two.

All the compounds were tested for their inhibitor activities *in vitro* toward both the soybean lipoxygenase and the sheep seminal vesicular cyclodioxygenase; none showed appreciable activity.

As there was no effect on the lipoxygenase, the coupled system was a valid model for kinetic studies with prototypes of these compounds.

Table 2 presents the results of kinetic studies with one of the phospholipase inhibitors, no. 3585, with appropriate confirmatory runs. The correlation coefficients of the calculated slopes were 95 per cent or better. The Michaelis constants were in reasonable agreement, as were the K_i values. When statistically evaluated, the intercepts of the inhibited slopes were significantly ($P < 0.001$) different from the control slopes. The inhibited slopes compared to the control slopes were not significantly different statistically. The mechanism of inhibition for this compound was therefore consistent with the "non-competitive" model.

In vivo *evaluation.* For almost thirty years the ameliorative effects of anti-rheumatoid arthritis drugs on dermal erythema induced by ultraviolet light have been known [25–27]. More recent evidence indicates that the erythema is caused by release of prostaglandins [28–30] and that it can be delayed or inhibited by both steroid and nonsteroidal anti-inflammatory drugs [19, 30–36].

Despite reports [29, 36] that an erythema can be induced in rat skin by exposure to ultraviolet light, preliminary trials with rats resulted in the production

Table 2. Phospholipase A_2 kinetics with compound 3585

Concn of 3585 (M)	Substrate concn range in medium (M)	Number of substrate concentrations run	Correlation coefficients (%)	Slope M × (rate of O_2 uptake)$^{-1}$	Intercept (rate of O_2 uptake)$^{-1}$	K_m (M)	K_i
0	1.92×10^{-4} to 1.92×10^{-5}	5	99	2.1×10^{-6}	9.25×10^{-2}	2.27×10^{-5}	
1.92×10^{-5}	1.92×10^{-4} to 1.92×10^{-5}	5	99	1.19×10^{-5}	2.97×10^{-1}		4.04×10^{-6}
3.8×10^{-5}	1.92×10^{-4} to 1.92×10^{-5}	5	95	3.22×10^{-5}	9.1×10^{-1}		2.63×10^{-5}
3.8×10^{-5}	1.92×10^{-4} to 1.92×10^{-5}	5	95–99	3.84×10^{-5}	5.07×10^{-1}		2.18×10^{-6}

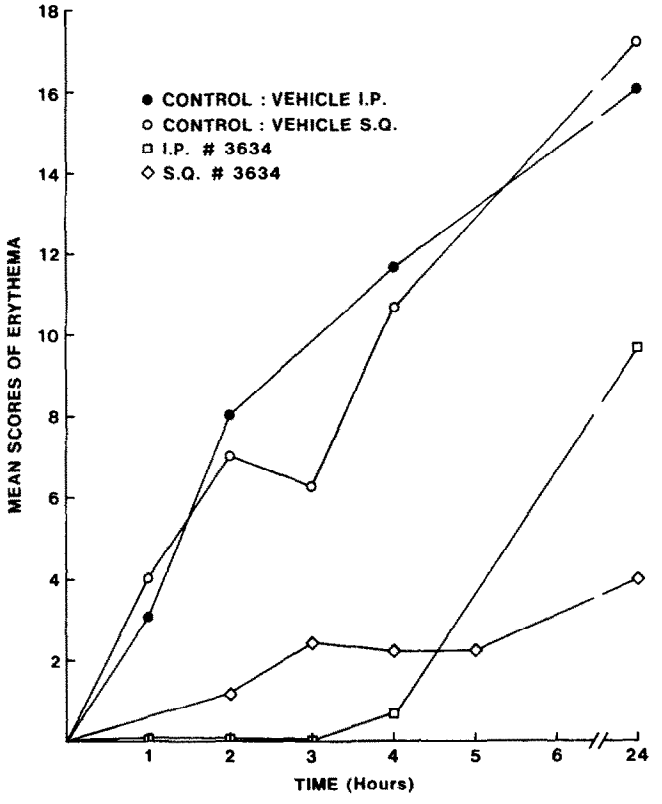


Fig. 1. Anti-erythemic effects of compound 3634 administered i.p. and s.c. compared to vehicle-treated controls.

of edematous weals on the skin which were not erythematous. We, therefore, resorted to the guinea pig, for which there is ample precedent [19, 31–33, 35, 37].

Using the scoring system and methods outlined, it was determined that, in otherwise untreated animals, after 30 sec exposure to u.v. light, the erythema reached a maximum of 4–5 hr, a result consistent with the experience of Gupta and Levy [31]. The major difference between the results at 4–5 hr, and subsequently, was the production of edema, indicated by a raising of the erythematous area above the surface of the surrounding skin.

Parenteral routes of administration of the phospholipase A_2 inhibitors were chosen after some untoward experiences with topical application. Figure 1 shows a comparison of the results obtained with no. 3634 by the intraperitoneal and subcutaneous routes. It is apparent that the results by the intraperitoneal route were not the result of "counter-irritant" hyperemic effects on the gut, as the s.c. results also indicated the anti-erythemic effect of the compounds. The results with somewhat less active compounds are shown in Table 3.

In Figure 2 are shown the mean erythema scores (three animals at each point) with three different dose levels at 1, 2 and 3 hr. It is apparent that the degree of anti-erythemic effect was related to the dose at all three time periods. The effects were maximum at 2 and 3 hr with the two highest doses; i.e. there was no increased increment of anti-erythemic effect at 2 and 3 hr with a dose higher than 0.3 ml of 0.01 M no. 3634.

There were other effects of these and other compounds of this series. At the highest dose of no. 3634

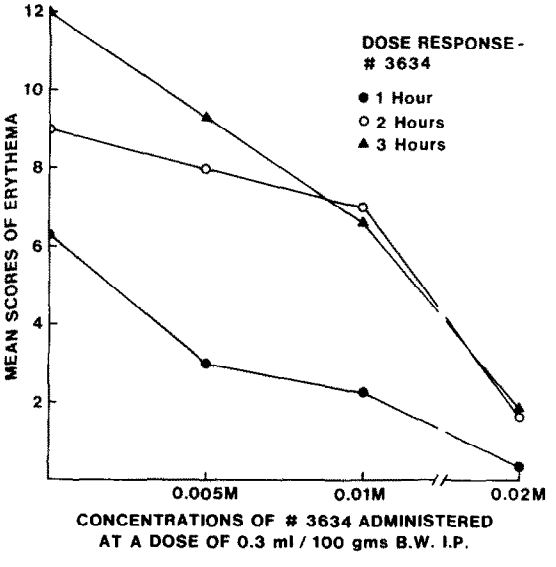


Fig. 2. Dose–response curves at 1, 2, and 3 hr after exposure to u.v. light for 30 sec.

Table 3. Mean erythema scores with additional phospholipase inhibitors*

Compound	1 hr		2 hr		3 hr		4 hr		24 hr	
	i.p.	s.c.	i.p.	s.c.	i.p.	s.c.	i.p.	s.c.	i.p.	s.c.
Vehicle control	4.0	6.2	6.4	6.3	6.8	8.3	8.8	8.3	15.0	18.4
3778	1.0	—	1.5	2.0	1.7	2.0	0.6	2.6	12.0	16.0
3557	1.33	—	6.0	2.2	8.0	4.6	5.3	4.0	18.0	16.0
3776	2.6	3.2	4.3	3.5	4.0	3.0	4.0	3.3	9.0	13.3

* Three guinea pigs/group compounds at a dose of 0.3 ml of 0.02 M compound/100 g body wt.

administered (0.3 ml of 0.02 M/100 g body wt), the animals appeared cyanotic, were cold to the touch, and appeared to be tranquilized. Some deaths occurred with this dose which did not happen at lower doses. Tranquilization was common with these compounds at effective anti-erythemic doses.

In general, with respect to the anti-erythemic effects of these compounds, it is consistent with the data to state that they delayed the onset of the erythema and decreased its severity. There was little or no effect on the edema.

Platelet aggregation. Figure 3 shows the results with a phospholipase inhibitor of this series, no. 4046, and its anti-aggregatory effects on human blood platelets in the presence of an aggregating concentration of collagen. It is apparent that there was a gradation of response in the range of 10–100 μg/ml, with complete inhibition of aggregation at 100 μg/ml. When arachidonic acid was added to the platelets in the presence of this concentration of no. 4046, aggregation again occurred, demonstrating that the inhibition must have been at the arachidonate releasing step. With the higher concentration of 150 μg/ml of no. 4046, there was no effect on platelet aggregation stimulated by epinephrine, or by ADP.

Results in the isolated perfused guinea pig lungs sensitized to ovalbumen. Figure 4 shows the results with one of these inhibitors, 1002, in an isolated perfused guinea pig lung derived from an animal that received the first sensitized dose of ovalbumen 12 days beforehand. Segments 1 and 2 show the responses of the rat aorta and fundus when 30 μg of arachidonate and 500 ng of ovalbumen were added to the perfusate respectively. Compound 1002 was then added to the perfusate at a concentration of 4×10^{-7} M for 10 min, and this was followed 15 min later by a challenge dose of ovalbumen. The result (segment 3) was a diminished response of both the aorta and fundus to thromboxane and prostaglandin E_2 (PGE_2) respectively. The concentration of no. 1002 was increased to 1×10^{-6} M in the perfusate for 10 min, and another dose of ovalbumen was again administered (segment 4). The response of the aorta to thromboxane A_2 was abolished, and the PGE_2 response of 90 per cent was inhibited as judged by the height of the contraction compared with the control. Segment 5 shows that in the presence of the same concentration of inhibitor the responses of the strips were undiminished when 30 μg of arachidonate was added to the perfusate entering the lungs. This demonstrates that neither cyclodioxygenase leading to the synthesis of PGH_2 and to successive steps in prostanoid synthesis nor the ability of the strips to contract was affected by the compound. It is consistent with the evidence to aver that the block is at the phospholipase level. The infusion of no. 1002 was then stopped, and 30 min later a challenge dose of ovalbumen to the lung evoked the responses shown in segment 6. Although not equivalent to the controls, it is obvious that the prostanoid response as a result of antigenic challenge was returning.

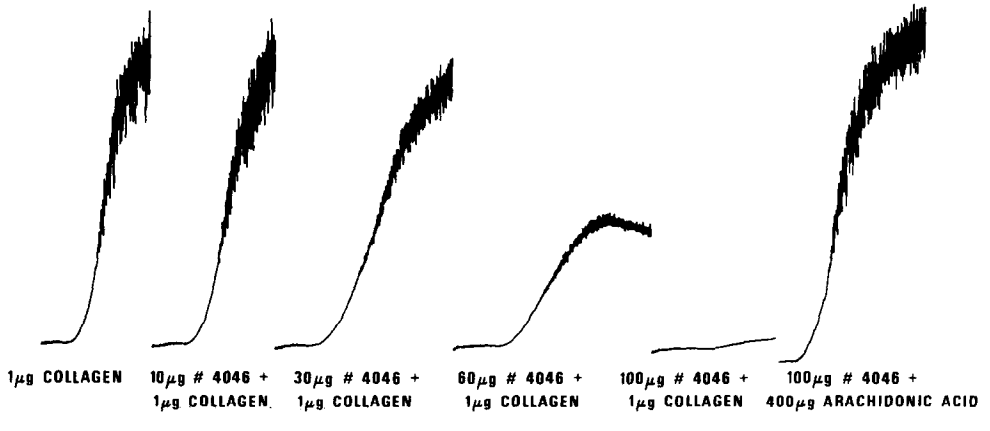


Fig. 3. Effect of a phospholipase inhibitor, no. 4046, on collagen-induced platelet aggregation and the reversal of this effect by arachidonic acid.

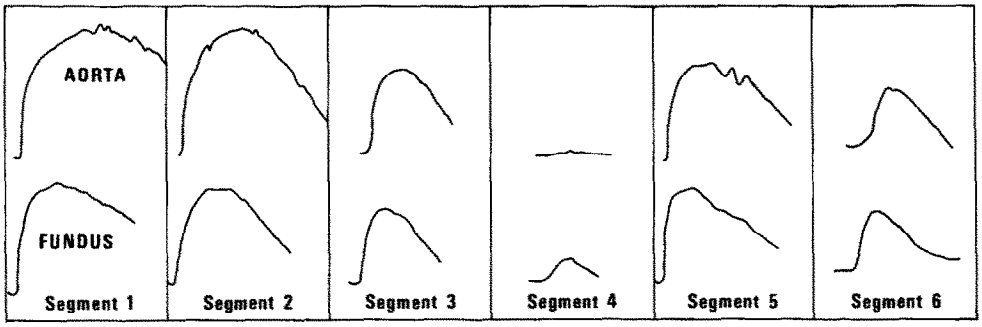


Fig. 4. Inhibition by compound 1002 of prostanoid elaboration in the perfused, ovalbumen sensitized, guinea pig lung. Substances were administered by bolus injection into the perfusate entering the lungs. Treatment of segments was as follows: segment 1: 30 μg of arachidonate; segment 2: 500 ng of ovalbumen; segment 3: infusion of compound 1002 was begun with a concentration in the perfusate of 4×10^{-7} M for 15 min; 500 ng of ovalbumen was then administered; segment 4: the concentration of compound 1002 was increased to 1×10^{-6} M and continued for 10 min, followed by another challenge of 500 ng of ovalbumen; segment 5: the perfusion of compound 1002 continued for 10 min longer, followed by administration of 30 μg of arachidonate; and segment 6: infusion of compound 1002 was discontinued for 30 min; 500 ng of ovalbumen was then administered.

DISCUSSION

There are three commonly accepted criteria that must be fulfilled before a compound inhibiting an enzyme *in vitro* can be said to be doing the same *in vivo*: (1) there must be evidence that the same enzyme is inhibited *in vivo*, (2) the enzyme must be inhibited at approximately the same concentrations as required *in vitro*, and (3) a dose–response relationship must exist.

In fulfillment of these criteria, it must be conceded at the outset that we have no direct measure *in vivo* of phospholipase A_2 inhibition induced by these compounds. Evidence has been presented, however, that three different phospholipase A_2 enzymes, derived from hog pancreas, cobra venom and bee venom, are all inhibited *in vitro*. Furthermore, selected members of this series of inhibitors block the "second-stage" of collagen-induced platelet aggregation. This stage is attributable to the hydrolysis of glycerol phosphatides resulting in the release of arachidonate for the synthesis of thromboxane A_2. These compounds block this lipolysis, preventing platelet aggregation, and this inhibition is reversible by added arachidonate. Thus, four different phospholipases A_2 are inhibited by these compounds *in vitro*, and potentially, they could be expected to have the same effects *in vivo*.

The experiments with the isolated perfused guinea pig lung sensitized to ovalbumen are one more link in the chain of evidence showing that the compounds presented in this report do possess phospholipase A_2 inhibitory activity. Evidence is presented that no. 1002, at 4×10^{-7} M in the perfusate, caused apparent inhibition. When the concentration was increased to 1×10^{-6} M, thromboxane elaboration was abolished, and PGE_2 secretion was diminished approximately 90 per cent. In the presence of the inhibitor, arachidonate was still able to evoke prostanoid synthesis, indicating that the compound had no adverse effect on the assay strips, nor did it affect other enzymes of the arachidonic acid cascade. This result is also consistent with phospholipase A_2 inhibition.

Flower and Blackwell [9] have demonstrated very recently that the mechanism of action of three anti-inflammatory steroids, dexamethasone, triamcinolone, and hydrocortisone, is to induce synthesis of a factor, as yet uncharacterized, that has "anti-phospholipase" activity. The effect of steroids on ultraviolet light induced erythema of the skin, like that of the phospholipase inhibitors of this report, is to delay the onset and reduce the severity of the reaction [32, 34, 36], although this effect in guinea pigs seems to be dependent upon the intensity of the u.v. light. Agreement on this point is not unanimous [31, 32, 37].

The compounds of this report are not cyclooxygenase inhibitors of the non-steroidal anti-inflammatory type, nor do they owe their activity to ability to filter out erythema causing wavelengths of u.v. light. Despite treatment with these compounds, some erythema always developed, and compounds closely related to the active ones, which would presumably filter u.v. light equally well, did not prevent the development of erythema. It seems reasonable to conclude therefore that the anti-erythemic effect of these compounds was due to their effects as inhibitors *in vivo* of phospholipase A_2.

The second link in the chain of evidence, that of the similarity of concentrations required to inhibit the enzyme *in vitro* and *in vivo*, must also be deduced by inference. In the case of one inhibitor, no. 3634, the I_{50} concentration *in vitro* was found to be 3.5×10^{-5} M. The dose of this compound required to block development of erythema in guinea pigs for at least 3 hr after exposure to u.v. light (see Fig. 1) was 0.3 ml of 0.2 M compound/100 g body wt. This is equivalent to a concentration, assuming equal distribution of the compound throughout the organism, of 6.5×10^{-5} M. Even at half this dose, as shown in the dose–response curve (Fig. 2), substantial inhibition of the development of erythema resulted. Therefore, it is reasonable to conclude that a close relationship does exist between the concentrations required *in vitro* and *in vivo* to inhibit phospholipase A_2 in the erythema model.

With guinea pig lung, a discrepancy exists. Compound 1002 exhibited I_{50} values with all three test enzymes *in vitro* in the 10^{-4} M range. As shown in Fig. 4, prostanoid synthesis and elaboration by the guinea pig lung were virtually completely abolished at a concentration of compound 1002 in the perfusate of 1×10^{-6} M. The compound was perfused through the lungs for 10 min and, during this time, there could have been some accumulation of the compound at the phospholipase site.

The third component, that of a dose–response relationship, is shown by the data in Fig. 2 and also in the *in vitro–in vivo* system of blood platelets shown in Fig. 3. It is apparent, in both these instances, that a dose–response relationship did, in fact, exist.

With regard to the effects of these compounds on platelets, there is a paradox that deserves mention. Rittenhouse-Simmons [38] and Bell *et al.* [39, 40] have presented evidence that the phosphatide supplying arachidonate for thomboxane formation in platelets is phosphatidyl inositol. Bell *et al.* [39, 40] have also defined the lipolytic pathway as due first to the action of a phospholipase C, converting phosphatidyl inositol to a diglyceride with arachidonate in the 2 position. The diglyceride then would become a substrate for a 1,2-diglyceride lipase that is the enzyme responsible for releasing arachidonate. If this is true, it would mean that either one or both of these enzymes is sensitive to some of the inhibitors presented in this report. It could also mean, despite the evidence for this pathway, that a major pathway is via a phospholipase A_2, which is inhibited by these compounds. This important point awaits resolution.*

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* Dr. Susan Rittenhouse-Simmons of the Boston Veterans Administration Medical Center, has tested compound 4046 in a phospholipase C preparation derived from human platelets. She found that, at a concentration of 20 μM in the medium, there was no significant inhibition of the enzyme. If the diglyceride lipase was unaffected by the inhibitor, phospholipase A_2 would be the important pathway.