or rhythmic activity induced by pentazocine. In contrast, the excitatory effect of pentazocine was antagonized by isoprenaline $(3 \times 10^{-6} \text{ M})$ or prostaglandin E_1 $(1 \times 10^{-5} \text{ M})$. Pharmacological actions of opiates can be blocked by $(-)$-naloxone (Snyder & Goodman 1980), although higher doses are required to block κ than μ effects. As shown in Fig. 1c, $(-)$-naloxone $(1 \cdot 4 \times 10^{-5} \text{ M})$ slightly reduced the excitatory effect of pentazocine.

The action of pentazocine does not seem to be mediated through acetylcholine or 5-HT, or through an adrenergic pathway. Moreover, a nervous pathway does not seem to be involved, as indicated by the lack of effect of tetrodotoxin. The partial antagonism of pentazocine by high concentrations of naloxone suggests that pentazocine does not act entirely on κ receptors.

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# Effect of cyclo-alkyl lactamimides upon amylase, lipase, trypsin and chymotrypsin

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Recent studies of certain cyclo-alkyl lactamimides have indicated a potential therapeutic usefulness arising from their ability to reduce gastric hydrogen ion secretion (Cheng et al 1976), inhibit the activation of pepsinogen to pepsin (Roberts & Taylor 1978) and inhibit adenylate cyclase activity (Siegel & Wiech 1976).

We now report further in vitro observations of the effect of various lactamimides upon the activity of the pancreatic enzymes amylase, lipase, chymotrypsin and trypsin. Of particular interest was whether there were any inhibition characteristics relating the pancreatic enzymes to pepsinogens and pepsins, as was noted with various triterpenoid derivatives (Waft et al 1974).

The general formula for the lactamimides studied can be written (Grisar et al 1973) as
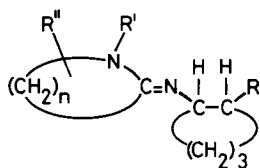
where, for the compounds studied so far, $R = C_6H_5$ and usually $R' = H$, $R'' = H$, and n, which determines the size of the lactam ring, was varied as shown in Table 1.

Materials and methods

Human pancreatic juice, from a patient with a pancreatic cyst and fistula, was used as the source of α-amylase (E.C. 3.2.1.1.). Purified bovine pancreatic chymotrypsin (E.C.

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3.4.21.1.) and trypsin (E.C. 3.4.21.4) and semipurified porcine pancreatic lipase (E.C. 3.1.1.3.) were obtained from the Sigma Chemical Co Ltd, St Louis, U.S.A. The cyclo-alkyl lactamimides, as the hydrochloride, were kindly donated by Dr N. L. Wiech of Merrell-National Laboratories, Cincinnati, Ohio, U.S.A. Measurements of pH were carried out with the Vibron pH meter (model 39A, Electronic Instruments Ltd, Richmond, Surrey, U.K.). Bovine haemoglobin substrate powder was obtained from Armour Laboratories, Eastbourne, Sussex, U.K.

The reagents for amylase and lipase measurement were obtained from American Hospital Supply (U.K.) Ltd, Didcot, Oxfordshire, U.K. Amylase substrate was a stable suspension of $0 \cdot 8\%$ (w/v) amylopectin in $0 \cdot 1$ M Tris HCl buffer of pH $7 \cdot 2$ prepared as described by the manufacturers (Amylase test reagent kit D-674, Perkin-Elmer, Coleman Instruments Division, Oakbrook, Illinois, U.S.A.). Lipase substrate (kit D-675 of the same manufacturers) was a stable emulsion of $0 \cdot 076\%$ (w/v) glycerol trioleate, purified from olive oil, in $0 \cdot 01$ M sodium deoxycholate, $0 \cdot 001$ M ascorbic acid and $0 \cdot 04$ M Tris HCl buffer at pH $9 \cdot 0$. When prepared according to the manufacturer's instructions, the triolein emulsion was stable at 4 °C for a period of at least seven days. Pancreatic lipase has a pH-optimum of $9 \cdot 0$ (Vogel & Zieve 1963); other lipases in plasma show relatively little activity at pH $9 \cdot 0$.

Amylase and lipase activity were measured with the Hyland laser nephelometer (Hyland, Costa Mesa, California 92626, U.S.A.) using the procedures set out by the manufacturers. Basically the method consisted in taking 1 ml of the appropriate substrate suspension and incubating

Table 1. Activity of various pancreatic enzymes with two concentrations of each lactamimide. The concentration of the potential inhibitor is that in the pre-incubation mixture. Each value is expressed as a mean percentage of the control value. R', R" and n refer to the formula given in the introduction. 100% activity was for amylase 40 Coleman units, lipase 2·6 Coleman units, chymotrypsin 5 μg pure solid, trypsin 5 μg pure solid.

| | | | | | | Activity of pancreatic hydrolases | | | |
Compound	n	R'	R"	isomer	Concn. of lactamimide mmol litre⁻¹	Amylase	Lipase	Chymo-trypsin	Trypsin
1	3	H	H	cis	0·25	105	97	100	92
					3·0	110	75	100	100
2	4	H	H	cis	0·25	104	86	102	105
					3·0	110	56	101	95
3	5	H	H	cis	0·25	113	85	105	91
					3·0	113	46	100	93
4	5	H	H	trans	0·25	98	86	105	90
					3·0	105	56	108	88
5	7	H	H	cis	0·25	95	88	95	98
					3·0	110	40	100	96
6	11	H	H	cis	0·25	102	62	103	95
					3·0	65	30	90	98
7	5	H	5 tert-butyl	cis	0·25	110	58	99	91
					3·0	115	2	98	92
8	3	CH₂C₆H₅	H	cis	0·25	98	60	92	102
					3·0	106	13	96	104

One Coleman unit of amylase is approximately equivalent to one Somogyi unit; one Coleman unit of lipase is the quantity of enzyme required to release 0·14 μmol of fatty acid from triglyceride in 1 h at 37 °C. Both enzymic activities are determined as the rate of change of formazin turbidity min⁻¹ ml⁻¹ of serum (Zinterhofer et al 1973a,b).

with 100 μl of the enzyme solution at 37 °C for 20 min. The decrease in light scattering of the test solution as compared with the control (i.e. no enzyme added) was taken as the measure of substrate hydrolysis and hence of enzymic activity. Activity was thus reported as a change in relative light scatter units (Δ RLS). Both enzyme assays showed a direct linear relationship of enzymic activity to increasing enzyme concentration.

The proteolytic activity of chymotrypsin and trypsin was measured at pH 7·4 in 0·1 M phosphate buffer containing 3·3 g litre⁻¹ bovine haemoglobin substrate, after 30 min incubation at 37°C as described by Roberts & Taylor (1973).

In determining the effect of the cyclo-alkyl lactamimides on each enzyme 50 μl of the enzyme solution was pre-incubated with 50 μl of the potential inhibitor for 30 min at 37°C at pH 7·4. Control tubes were set up with 50 μl of enzyme and 50 μl of buffer without inhibitor. The subsequent hydrolytic activity was then measured as already described. The inhibitors had no direct effect on substrate turbidity. Similar experiments were also carried out without pre-incubation, the enzyme being added to start the reaction. Pre-incubation of each enzyme with the lactamimides was carried out to test directly the effect of the potential inhibitor on the enzyme and to avoid any non-specific binding with the substate. The 30 min period of pre-incubation was carried out in accordance with earlier established practice (Roberts & Taylor 1973, 1978).

The cyclo-alkyl lactamimides and lipase were each dissolved in 0·01 M phosphate buffer, pH 7·4, which was also used for the dilution of pancreatic juice for amylase determination.

The effect of temperature upon inhibition was studied by pre-incubation of the enzyme and inhibitor and subsequent assay at the temperature recorded in the text.

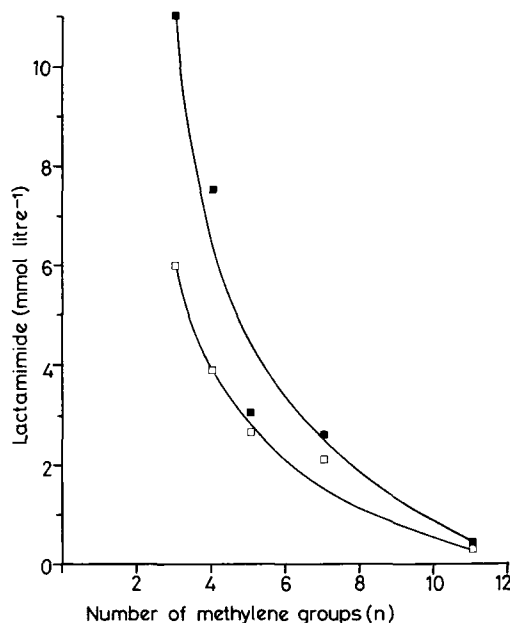All determinations were carried out in duplicate or

Fig. 1. Relationship of the lactamimide concentration resulting in 50% inhibition (I50) of lipase activity to the number of methylene groups (n), where R' = H and R" = H. ■ without pre-incubation; □ with pre-incubation.

Table 2. Effect of the lactamimides on the serum lipase activity of a patient being investigated for acute pancreatitis. An aliquot of serum (50 µl) was pre-incubated with 50 µl of a solution of each lactamimide at 0·25 and 3·0 mmol litre⁻¹ final concentration. Each value is expressed as a mean percentage of the control value. 100% activity corresponded to 2·0 Coleman units, or 92 µmol free fatty acid min⁻¹ litre⁻¹. Compounds 1–8 are as reported in Table 1.

Compound	Serum lipase activity as percentage of control value							
	1	2	3	4	5	6	7	8
Concn mmol litre⁻¹								
0·25	105	120	110	82	110	84	96	97
3·0	93	105	100	75	90	42	26	55

triplicate and in accordance with previous practice (Roberts & Taylor 1978) differences between mean control value and mean test value of 5% or less were considered to be not significant; those between 5 and 15% were considered arbitrarily to show marginal inhibition.

Results

Table 1 shows the effect of the lactamimides after pre-incubation at two different concentrations on the hydrolytic activity of various pancreatic enzymes. Inhibition of more than 20% of the control activity was not found for chymotrypsin or trypsin. Compound 6 partially inhibited amylase at 3·00 mmol litre⁻¹, by 35%. All the lactamimides inhibited lipase activity to some extent with compound 7 resulting in loss of 98% of activity.

A series of concentration-dependent inhibition curves for lipase, with and without pre-incubation of the inhibitor with the enzyme, were set up for compounds, 1, 2, 3, 5 and 6, the unsubstituted derivatives in the cis-configuration. The concentration of each lactamimide (mmol litre⁻¹) resulting in 50% inhibition of lipase activity (I 50) was thus calculated, and plotted against the number of methylene groups (n) present in the lactamimide molecule. Fig. 1 shows that the I 50 values for each lactamimide were inversely related to the number of methylene groups. The effect of pre-incubation on the I 50 values was slight with compounds containing 11, 7 and 5 methylene groups compared with those containing 3 and 4 methylene groups, where pre-incubation reduced the I 50 concentrations by approximately half.

For compound 7 which has 5 methylene groups the I 50 values with and without pre-incubation were, respectively, 0·40 and 0·53 mmol litre⁻¹; for compound 8, with 3 methylene groups, the I 50 values with and without preincubation were, respectively, 0·55 and 0·88 mmol litre⁻¹. Thus the effects of reduction in the number of methylene groups was compensated for by introduction of hydrocarbon residues, i.e. 5-tertiary butyl at R' and CH₂C₆H₅ at R".

The effect of temperature on the inhibition of lipase was studied for compounds 5 and 7. Fig. 2 shows that with compound 7 inhibition reaches a maximum at 37°C and decreases as the temperature is raised or lowered. At the lower concentration lipase is activated at 20° and 50°C.
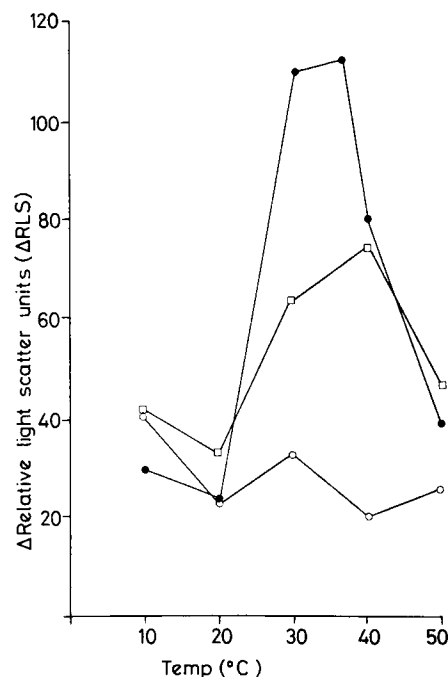

Fig. 2. The effect of temperature on the inactivation of lipase by the lactamimide no. 7. The values plotted represent the activity of the enzyme, expressed as a change in the relative light scatter units (107 ΔRLS = 2·6 Coleman units at 37 °C). ● Enzyme alone. □ Lactamimide concentration 0·16 mmol litre⁻¹. (0·05 mg ml⁻¹). ○ Lactamimide concentration 3·3 mmol litre⁻¹ (1·0 mg ml⁻¹).

Similar results were obtained for compound 5.

Table 2 shows the effect of each lactamimide, after pre-incubation, on the lipase activity present in serum. Only compounds 4, 6, 7 and 8 produced significant inhibition at a concentration of 3·0 mmol litre⁻¹ of the lactamimide, with compounds 1, 2, 3 and 5 showing either no inhibition or marginal inhibition.

Discussion

Roberts & Taylor (1978) have previously described inhibition by a series of cyclo-alkyl lactamimides of the conversion of pepsinogens to pepsins and have shown that the degree of inhibition was related to the number of methylene groups (n) present in the lactamimides. They postulated that surface carboxyl groups on the pepsinogen molecule were binding with the amidino-groups of one or several molecules of the lactamimides in a manner which prevented release of inhibitory peptides to form active pepsins. In this further study of the pancreatic enzymes only lipase showed a similar relationship of inhibition and lactamimide structure. The enzymes amylase, chymotrypsin and trypsin showed little or no inhibition.

It is likely that similar interactions would take place between the amidino group and the surface carboxyl groups

of lipase, amylase, trypsin and chymotrypsin. If they do, access of substrate to the active site is not prevented, except with lipase. An alternative explanation of the structural-inhibitory relationship of the lactamimides would be that by increasing the number of methylene groups, the hydrophobic nature of the lactamimide would become greater. As a result increased hydrogen bonding between enzyme and lactamimide would occur with hindered substrate-access to the active site in the case of the lipases and hindered activation of the pepsinogens. The temperature effects described above are difficult to explain but would on the whole favour this latter explanation. Thus hydrogen bonding would diminish as the temperature rose above 37° and substrate access to the active site would increase; this effect would be concentration-dependent and super-imposed upon the inactivation of the enzyme itself with increasing temperature.

In acute pancreatitis there is release of pancreatic enzymes into the abdominal cavity and, as is well-known, the released lipase hydrolyses omental fat. The serum lipase (glycerol trioleate esterase activity at pH 9·0) also rises, indicating a release of pancreatic lipase into the blood stream. A suitable lipase inhibitor that could reach the pancreas or the peritoneal cavity may possibly have a role in preventing destruction of omental fat, although it is a matter for conjecture as to whether the prevention of lipolysis would materially influence the course of the disease. The ability of compounds 4, 6, 7 and 8 to inhibit the glycerol trioleate esterase activity of human serum is nevertheless of interest. The lack of activity of compounds 2, 3 and 5 probably arises from high binding to serum proteins, since these compounds inhibited porcine pancreatic lipase in the absence of serum proteins. Compounds 4, 6, 7 and 8 ought therefore to be active inhibitors in the abdominal cavity, where the protein content of any free fluid is always lower, and often very much lower, than in serum. The compounds studied in this paper unfortunately only give effective inhibition in relatively high concentrations so that a compound which would achieve inhibition at lower concentrations is desirable. Our results show that substitution at R′ and R″ (compounds 8 and 7 respectively) increases the inhibitory action of compounds 1 and 3, so that similar substitutions in compound 6, the most potent unsubstituted inhibitor studied, may give a more potent lipase inhibitor. It would be important to ensure that such a compound does not activate other enzymes, such as trypsin and chymotrypsin, which are released in acute pancreatitis. It would be important also to know that such a compound does not exert hypoglycaemic activity; on the present evidence this is unlikely as compound 6 was found not to be hypoglycaemic in rats nor was compound 7, although compounds 2, 4, 5 and 8 were hypoglycaemic as was compound 3, the unsubstituted precursor of compound 7 (Grisar et al 1973).

In conclusion we would draw attention to the similarity between the pepsinogens and lipases to inhibition by the lactamimides studied. Some similarity of three-dimensional form of these enzymes is implied, as was noted among the pepsins, among the pepsinogens and between the pepsins and chymotrypsin during a study of triterpenoid inhibitors (Waft et al 1974).

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