grating spectrophotometer, i.e. at 610–620 cm^{-1} for each of the different forms. These differences have since been confirmed in a personal communication from Drs Axon & Mitchell.

Our results suggest that at least two polymorphic forms of methisazone exist. The unground and micronized materials both give evidence of a phase change in the shape of their endothermic peaks using differential analysis. The milled sample appears to give results somewhere between the unground and micronized samples for both X-ray and differential thermal analysis as might be expected from their previous history.

The unground material is probably prepared by precipitation as shown by the similarity of the two thermograms (Figs 2A, D), whereas a single crystalline variety is produced by seeding or slow recrystallization (Figs 2E, F).

The unground methisazone may change crystal structure at elevated temperature as evidenced by its thermogram (Fig. 2A). Grinding of this material (Figs 2C, B) leads to the formation of a more active polymorph, which will revert more rapidly to a more stable polymorph as evidenced from the work of Deavin & Mitchell (1965). This would also account for the reported (Axon, 1972) increased solubility, biological activity and instability obtained with use of the micronized form in pharmaceutical formulations.

November 23, 1976

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Comparison of retention of activity of soluble and insolubilized forms of trypsin in a simulated gastric medium

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In pancreatic insufficiency, or after pancreatectomy, pancreatic extracts from animals (pancreatin) are administered orally to patients to supplement, or substitute, the secretion of proteolytic, lipolytic and amylolytic enzymes, normally secreted into the duodenum (Soergel, 1973; Warren & Jefferson, 1973; Anderson & Goodchild, 1976). Frequent incomplete response to even very high doses of pancreatin can be blamed on several factors:

(i) gastric inactivation of trypsin and lipase have been well documented both *in vivo* and *in vitro* by Heizer, Cleaveland & Iber (1965); whereas trypsin is denatured by the combination of pepsin and low pH, lipase is inactivated by acidic pH alone;

(ii) reduced pH in the duodenum due to poor bicarbonate secretion often accompanies pancreatic insufficiency (Hadorn, Zoppi & others, 1968); this is significant in view of the pH-dependence of enzymatic activity. pH optima for trypsin, lipase and amylase are 7 to 8, 7 to 9 and 6·9, respectively (Diem & Lentner, 1970);

(iii) inadequate mixing of the enzymes with food;

(iv) 'autodigestion' of trypsin, lipase and amylase by trypsin in pancreatin.

Attempts to overcome these problems by, e.g. sprinkling the enzymes directly on food, co-administration of sodium bicarbonate, or enteric coating are either unsuitable for chronic treatment, or they do not always yield the expected results (Anderson & Goodchild, 1976).

It is now recognized that insolubilization of enzymes by physical or chemical attachment to macromolecular carriers is a versatile method for modification of the enzymic properties (Chang, 1975). Thus, it may be possible to formulate enzyme preparations with enhanced storage stability or pH-profiles optimized for a particular disease state. Our preliminary investigations (Wiseman & Gonda, 1976) indicated that trypsin immobilized on a variety of inert carriers exhibited a much greater resistance towards inactivation by simulated gastric fluid than the soluble form of trypsin. The experimental procedure reported in the present paper is a simplified version of the method described in the previous communication. The results demonstrate again the superior resistance of the insoluble forms of trypsin, although some quantitative differencies from the results obtained in the previous tests can be observed.

Materials. A stock solution of trypsin (3150 NF, crystallized, Armour Pharmaceutical Company Ltd.) was prepared by dissolving approximately 25 mg of the enzyme in 10 ml 0·001 N HCl. This solution was

diluted 1:10 in 0·0015 M borate buffer containing 2·93 g $CaCl_2.2H_2O$ litre^{-1} pH adjusted to 7·8 with 1 N HCl, to prepare Standard Trypsin Solution used in the tests described below.

A solution of pancreatin was prepared by dissolving approximately 0·160 g of pancreatin (3 NF, Armour Pharmaceutical Company Ltd.) in 25 ml of the same borate buffer as described above, and used in this form for testing.

ENZITE-trypsin (3 × crystallized bovine trypsin attached by the Curtius azide method on carboxymethyl cellulose, Miles Laboratories Ltd.), and insoluble trypsin on polyacrylamide (bovine trypsin attached to polyacrylamide, Sigma Chemical Company Ltd.) were used directly as powders. ENZITE-agarose-trypsin (3 × crystallized bovine trypsin attached by the triazine method to agarose, 25% suspension in 20% $(NH_4)_2SO_4$, 0·01 M acetate buffer pH 5·0 and 0·01% NaN_3, Miles Laboratories Ltd.) was also used directly in the form supplied by the manufacturers.

0·25 M solution of α-*N*-benzoyl-L-arginine ethyl ester HCl (BAEE) was prepared by dissolving an appropriate amount of BAEE (BDH Chemicals Ltd.) in de-ionized water.

0·02 N aqueous NaOH was used as the titrant.

Simulated gastric fluid (GF) was prepared from 0·160 g pepsin (2 × crystallized and lyophilized, 2710 units mg^{-1} using haemoglobin as substrate, Sigma Chemical Company Ltd.) dissolved in 50 ml 0·05 N HCl containing 0·25 mol litre^{-1} $CaCl_2$.

Apparatus. Radiometer (Copenhagen) consisting of a Titrator TTT2, Titrigraph Module PHA 943, Titrigraph Pen Drive REA 300, Titrigraph SBR 3, Autoburette ABU 12 fitted with a 25 ml burette, and a 100 ml jacketed titration vessel, was used in the pH-stat mode.

The temperature in the titration vessel was kept constant with a Churchill (U.K.) Thermocirculator LTC.

Method. The proteolytic activity of the enzymes against the model substrate BAEE was assayed at pH = 8, 37°.

Test 1: The activity before exposure to GF was measured as follows: 5 ml GF and 1 ml 0·25 N NaOH were placed in the titration vessel and kept under an atmosphere of nitrogen. 4 ml 0·25 M BAEE was then added, and the pH of the mixture was adjusted rapidly to 8 by the automatic titration assembly. After addition of approximately 20 units* of one of the trypsin preparations, the automatic titration was restarted with 0·02 N NaOH. The contents of the titration vessel were stirred vigorously during this process with a mechanical stirrer. Activity of the enzymes was calculated from the recorded curves of the titrant volume *vs* time. It was found that under these conditions a steady state was reached in about 1 min. Consequently, the enzyme activity was calculated from the slope of the terminal straight line portions of the curves. The excess of BAEE used in the assays was sufficient for the steady state to persist for prolonged periods of time, as one would expect on the basis of Michaelis–Menten kinetics.

Test 2: Approximately 20 units of each enzyme were mixed with GF, and stirred for 15 min at 37° under an atmosphere of nitrogen in the titration vessel. The resultant pH was 1·45 ± 0·15. 1 ml 0·25 N NaOH and 4 ml 0·25 M BAEE were then added to the mixture, and the pH was adjusted rapidly to 8. The enzymatic activity was assayed as in Test 1.

Test 3: 5 ml GF, 1 ml 0·25 N NaOH and 4 ml 0·25 M BAEE, or 5 ml GF, 1 ml 0·25 N NaOH and approximately 20 units of trypsin, were placed in the titration vessel at 37° under an atmosphere of nitrogen. After rapid adjustment of pH to 8, these mixtures were also titrated automatically.

Results and discussion. Activities of the enzymes tested in these experiments before (Test 1) and after (Test 2) exposure to GF are given in Table 1. The values of retention of activity (RA) were calculated as

$$\%RA = \frac{\text{activity in Test 2}}{\text{activity in Test 1}} \times 100$$

The proteolytic activities of trypsin and pancreatin are virtually destroyed in Test 2. The retained activity is debatable as it is of the same order of magnitude as the background drift observed in Test 3, caused probably by spontaneous hydrolysis of BAEE in one case, or a slow attack of trypsin on pepsin in the other case. Results of Test 3 were equivalent to less than 0·4 units/assay. Obviously, such a low background drift is insignificant in the calculation of the high RA values for the insoluble forms of trypsin.

No recovery of activity was observed once the steady state has been reached in Test 2. Thus, the process of inactivation quantified by the RA values in Table 1 can be regarded as essentially irreversible.

In preliminary experiments, bubbling of nitrogen gas through the solutions led to extensive foaming; the results reported here were obtained by keeping the solutions in the titration vessel under nitrogen atmosphere only. In spite of this, repeated measurements were highly reproducible.

Manufacturers of insolubilized enzymes often advise the reconstitution of powdered formulations by repeated washing, filtering, and finally suspending in a suitable medium. We have found that using accurately weighed out quantities of powders directly in the tests leads to much more reproducible results than pipetting reconstituted suspensions. In fact, ENZITE-trypsin-agarose supplied as a suspension had to be shaken vigorously immediately before every assay to achieve

* 1 unit = the enzyme activity which under the conditions specified above hydrolyses 1 μmol BAEE min^{-1}.

Table 1. *Esterase activities of soluble and insolubilized proteolytic enzymes before* (A_1)* *and after* (A_2)* *exposure to simulated gastric fluid.*

Substance	Nominal activity	A_1*	A_2*	$RA\,(\%) = \frac{A_2}{A_1} \times 100$*
Trypsin (crystallized)	3150 NF units†	(66·0 ± 0·3) units mg^{-1}	≤1·8 units mg^{-1}	≤3
Pancreatin	3 NF units††	(1·05 ± 0·04) units mg^{-1}	≤0·02 units mg^{-1}	≤2
ENZITE-trypsin	0·53 units mg^{-1} at pH = 8·0, 25°	(0·58 ± 0·03) units mg^{-1}	(0·24 ± 0·01) units mg^{-1}	41 ± 4
ENZITE-agarose trypsin	12·6 units ml^{-1} at pH = 8·0, 25°	(69 ± 2) units ml^{-1}	(53 ± 1) units ml^{-1}	77 ± 4
Insoluble trypsin on polyacrylamide	0·52 units mg^{-1} at pH = 8·0, 30°	(0·75 ± 0·03) units mg^{-1}	(0·69 ± 0·05) units mg^{-1}	92 ± 10

* Error margins indicate reproducibility from duplicates or triplicates.

† One NF unit is the amount of trypsin causing a change in absorbance at 253 nm of 0·03 min^{-1} under conditions given in the National Formulary XIV, 'Trypsin', using BAEE as the substrate.

†† One NF unit of protease activity is contained in the amount of pancreatin that digests 1·0 mg of casein under the conditions specified in the National Formulary XIV, 'Pancreatin'.

good reproducibility. It is important, however, to titrate for a sufficient period of time to reach a true steady state, manifested by a straight line plot of the volume of titrant *vs* time. In this way, any swelling or disintegration necessary for the full expression of the activity of insolubilized enzymes is achieved.

In contrast to the experiments reported before (Wiseman & Gonda, 1976), the original activity of the enzymes (Test 1) was measured in neutralized GF. Preliminary observations suggest that there may be a slight interference of pepsin with the assay of proteolytic activity using BAEE; it was decided therefore, to do both Test 1 and Test 2 under identical conditions.

The previous communication (Wiseman & Gonda, 1976) is in qualitative agreement with the results presented here. The differencies between the two sets of values for the insolubilized enzymes are likely to be due to the modification of the testing procedure, some batch-to-batch variations, and possibly also due to ageing of the preparations: ENZITE-trypsin and insoluble trypsin on polyacrylamide were from different batches than those used in the previous series of experiments; the ENZITE-agarose-trypsin was almost two years old.

Although the three insolubilized forms of trypsin used in our investigations have not been designed for therapeutic use, the results illustrate that immobilization of pancreatic enzymes may lead to a great improvement in the efficacy of these substances. It remains to be seen whether the same dramatic reduction of susceptibility to gastric fluid can be achieved by insolubilization of the pancreatic lipase and amylase.

It is not possible as yet to extrapolate our observations to the situation *in vivo*. Often, one of the consequences of insolubilization is that the activity towards macromolecular substrates is diminished by steric or diffusional hindrance. Although we have not tested the proteolytic activity of the three insolubilized enzymes used here against casein, immobilized trypsin with high activity towards this macromolecular substrate has been prepared (Levin, Pecht & others, 1965).

I should like to thank Mrs Christine Preston and Mr G. Elson for technical assistance. The samples of trypsin and pancreatin were supplied generously by Armour Pharmaceutical Company Limited.

October 11, 1976

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