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Further Purification of Cholecystokinin and Pancreozymin

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In 1961 Jorpes and Mutt[1] described the preparation of cholecystokinin-pancreozymin material with about 100 Ivy cholecystokinin and 600 Crick, Harper and Raper units of pancreozymin per mg. In all the purification steps leading to this preparation from the crude starting material[2] the activities of cholecystokinin and of pancreozymin did not separate. Subsequently Dhariwal *et al.*[3] succeeded in pyrifying pancreozymin to an activity of 3750 pancreozymin units per mg.

By minor modifications of the technique described in 1961 we have routinely obtained preparations with 250 cholecystokinin and 1500 pancreozymin units per mg. This has been our starting material for further work. First we attempted purification by counter current distribution. Of the systems tried an aqueous acetic acid-sodium chloride-butanol system[4] and especially, an aqueous acetic acid-pyridine-butanol system[5,6] gave encouraging results. Again both cholecystokinin and pancreozymin were purified to the same extent. However, because there were disconcerting losses in activity during recovery of the material from the solvents after distribution and also because the distribution patterns showed that the starting material was still quite impure simpler methods for purification were tried. Dhariwal *et al.*[3] had used filtration through Sephadex, G-50 in 0.2 M AcOH for the purification of pancreozymin. We tried this technique on our starting material with some success. However, distinctly better separations were obtained on filtration at higher pH values. Filtration in 0.25 M Na_2HPO_4 adjusted to pH about 8 was uniformly successful. After recovery in lyophilized form from the buffer solution the material from the active fractions was found to be about six times purer on a weight basis with respect to both cholecystokinin and pancreozymin. This material was further purified by a factor of about 2 by chromatography on the ion exchange resin Amberlite XE–64 (Rohm and Haas Co.), again with respect to both cholecystokinin and pancreozymin. This material consequently assayed at 3000 cholecystokinin and 18 000 pancreozymin units per mg.

In acid hydrolysates of the most purified material the following amino acids were identified by the two dimensional system of Redfield:[7] alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, lysine, proline, leucine, methionine, phenylalanine, serine, tyrosine and valine. Only traces of threonine were present. After performic acid oxidation no cysteic acid could be demonstrated.[8] Analysis for tryptophan by the Voisenet-Rhode *p*-dimethylaminobenzaldehyde method[9] showed that this amino acid was present.

Experimental. The starting material was prepared as described by Jorpes and Mutt.[1] It was recovered from the eluate from the TEAE-cellulose column by adsorption on alginic acid at pH 3, elution with 0.2 M HCl, exchange of the chloride for acetate on a column of DEAE Sephadex and lyophilization from dilute aqueous acetic acid. The lyophilized material had an activity of 250 Ivy dog units of cholecystokinin and 1500 Crick, Harper and Raper units of pancreozymin per mg.

First purification step. 100 mg of the starting material was dissolved in 1 ml of 0.25 M Na_2HPO_4, which had been preadjusted to pH 8.0 ± 0.1 with M H_3PO_4 and allowed to sink into a column, 1.5 × 90 cm of Sephadex, G-50, fine, bead form (Pharmacia, Uppsala). The column had been equilibrated with the same buffer and the chromatogram was also developed with it. Fractions of 5 ml each were collected. The flow rate was approximately 8 min per fraction. The polypeptide content of the fraction was estimated by measuring the OD at 280 mμ. The cholecystokinin activity was determined on appropriate dilutions by the technique of Ljungberg,[10] the pancreozymin content by determining the capacity of the fractions to effect protein expulsion from the pancreas of cats operated as for the assay of secretin,[11] and secreting a juice of low protein content under the influence of secretin; Fig. 1 summarizes a typical chromatogram. The active fractions were combined, diluted with 20 volumes of distilled water and the pH was brought to 3 ± 0.1. 250 mg of alginic acid, prewashed with 0.1 M HCl, and then water was stirred with the solution. The alginic acid carrying the adsorbed hormone material was filtered off, washed on the filter with 0.001 M cold HCl and then eluted with 15 ml of ice-cold 0.2 M HCl. The eluate was allowed to sink into a column of DEAE-Sephadex, coarse, (Pharmacia, Uppsala) prewashed with 0.2 M AcOH and the polypeptide material washed out of the column with the dilute acetic acid. After lyophilization the material was assayed and found to be approximately 6 times purer on a weight basis than the starting material. This with respect to both cholecystokinin and pancreozymin. Yield in four similar experiments 8–9 mg.

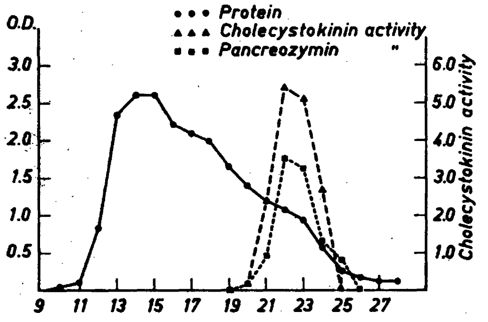

Fig. 1. Column: Sephadex G-50, fine, 1.5 × 90 cm. *Buffer:* Sodium orthophosphate, 0.25 M, pH 8 ± 0.1. *Chromatographed material:* 100 mg PC–TEAE–C. *Fraction volume:* 5 ml. *Flow rate: ca.* 8 min per fraction. ●, Optical density of fractions at 280 mμ. ▲, Cholecystokinin activity of fractions; Ljungberg[10] method (relative values). ■, Colour (Lowry[12]) at 660 mμ of the pancreatic juice, diluted to 25 ml, of a cat after injection of equal aliquots of the fractions.

Second purification step. A column of the cation exchange resin Amberlite XE-64 (Rohm and Haas Co.) 40 × 1 cm was equilibrated with a Na_2HPO_4/NaH_2PO_4 buffer of pH 7.5, 0.05 M in phosphate, 12 mg of the Sephadex purified material was dissolved in one ml of buffer and allowed to sink into the column. The chromatogram was developed with the same buffer. Fractions of 3 ml each were collected. The flow rate was one fraction per 14 min. The fractions were analyzed as described for the Sephadex column. Fig. 2 summarizes the results. The active fractions were combined and treated as described above for those from the Sephadex-G-50 column.

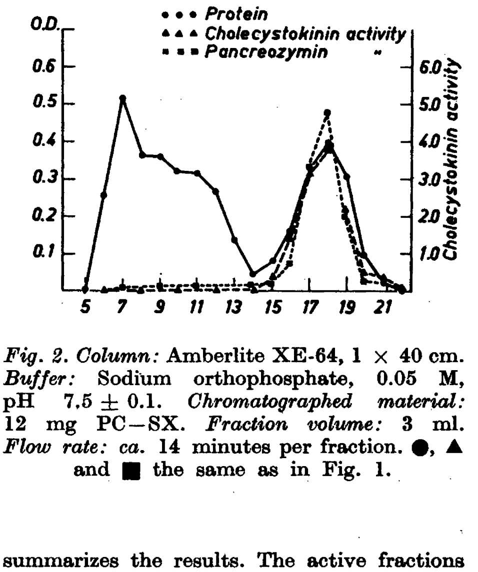

Fig. 2. Column: Amberlite XE-64, 1 × 40 cm. *Buffer:* Sodium orthophosphate, 0.05 M, pH 7.5 ± 0.1. *Chromatographed material:* 12 mg PC–SX. *Fraction volume:* 3 ml. *Flow rate: ca.* 14 minutes per fraction. ●, ▲ and ■ the same as in Fig. 1.

The lyophilized product weighed 2.4 mg and had an activity of *ca.* 3000 cholecystokinin and 18 000 parcreozymin units per mg.

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The Effect of Sodium Fluoride on Sarin Inhibited Blood Cholinesterases

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During studies on the mechanism of ageing of phosphorylated cholinesterases some substances were tested for their ability to block ageing of Sarin (methyl-isopropoxy-phosphoryl fluoride) inhibited human plasma cholinesterase. The method used was the one described earlier for *in vitro* reactivation and ageing studies on Tabun inhibited blood cholinesterases.[1] Thus after inhibition at 0°C all samples were dialysed against cold saline for two days in order to remove excess of Sarin. The compound to be tested for its effect upon ageing was added to part of the control and part of the inhibited enzyme immediately before the samples were incubated at pH 7.4 and 37°C. Compounds lowering cholinesterase activity were used in concentrations which gave up to 50 % inhibition of the original enzyme activity.

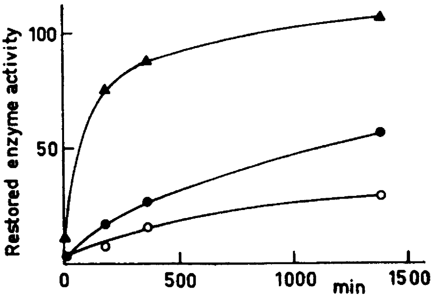

Fig. 1. Return of enzyme activity at pH 7.4, 37°C from methyl-isopropoxy-phosphorylated cholinesterase of human plasma. O = veronal buffer only, ● = veronal bufter and 10^{-4} M sodium fluoride, ▲ = veronal buffer and 10^{-3} M sodium fluoride.

The ability to reactivate the Sarin inhibited enzyme was tested with 10^{-2} M P2S (N-methylpyridinium-2-aldoxime methane sulphonate).

Under the conditions used (veronal buffer) it was observed that 10^{-1} M and 10^{-2} M sodium fluoride prevented ageing; also the compound itself inhibited plasma cholinesterase at this concentrations. Repeated experiments with 10^{-3} M and 10^{-4} M sodium fluoride revealed that the reason for the prevented ageing was to be found in a reversal of cholinesterase activity after addition of sodium fluoride, meaning that these concentrations of sodium fluoride were able to restore enzyme activity before any ageing of the inhibited sample had occurred. As seen in Fig. 1, 10^{-3} M sodium fluoride was able to restore the enzyme activity completely, while 10^{-4} M sodium fluoride only restored part of the activity. Ageing of the still inhibited enzyme was not prevented by 10^{-4} M sodium fluoride. Enzyme activity of a previously aged Sarin inhibited plasma cholinesterase preparation was not restored upon addition of either 10^{-3} M sodium fluoride alone or together with 10^{-2} M P2S.

Experiments also showed that Sarin inhibited human erythrocyte cholinesterase regains enzyme activity upon addition of sodium fluoride. Further experiments are in progress.

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