a decrease is seen of the content of ATP and G-6-P, and an increase of the ADP and AMP content in the cardiac muscle. These results are consistent with an increasing phosphofructokinase activity in the cell.

Table II shows a serie of experiments in which all the hearts were perfused for 10 min. The zero time represents the controls in which the hearts were perfused for 10 min with the DNP-free buffer. The periods of $1^1/_2$ and 5.0 min represent determinations in which the hearts were preliminary perfused for $8^1/_2$ and 5.0 min respectively with DNP-free buffer, after which they were rapidly switched to a buffer containing DNP. Time 10 min represents determinations in which the hearts were perfused for 10 min with a DNP-containing buffer. As can be seen, after $1^1/_2$ min of perfusion with DNP the content of G-6-P in the muscle was found to be lower, and at the same time only a small increase in the content of phosphorylase *a* could be noticed.

From these results, it was possible to assume that the glycogenolytic effect of DNP is not explained by the 'push mechanism', where the G-6-P level is found to be higher than in the case of catecholamines[4]. The G-6-P and nucleotides levels found after a short period of exposition of the hearts to the drug ($1^1/_2$ min), suggest that the DNP may cause primary activation of the phosphofructokinase, and in consequence a higher rate of glycogenolysis. On the other hand, considering that G-6-P is an inhibitor of phosphorylase *b* kinase activity[12], the low levels of this metabolite would be responsible for the higher activity of the phosphorylase *b* kinase found in the hearts perfused with DNP.

Studies to be described elsewhere show that the G-6-P concentration observed into the cardiac cell in normal conditions of perfused hearts causes significant inhibition of the phosphorylase *b* kinase.

Summary. To explain the mechanism of DNP action the contents of ATP, ADP, AMP and G-6-P were determined in perfused rat hearts in a period of time in which the rate of the glycogenolysis was increased. The levels of these metabolytes in the extract were consistent with an increase of the phosphofructokinase activity. On the other hand, the finding of higher activity of phosphorylase *b* kinase in DNP-poisoned animals could be explained as due to the low content of G-6-P in the perfused hearts subjected to the action of the drug.

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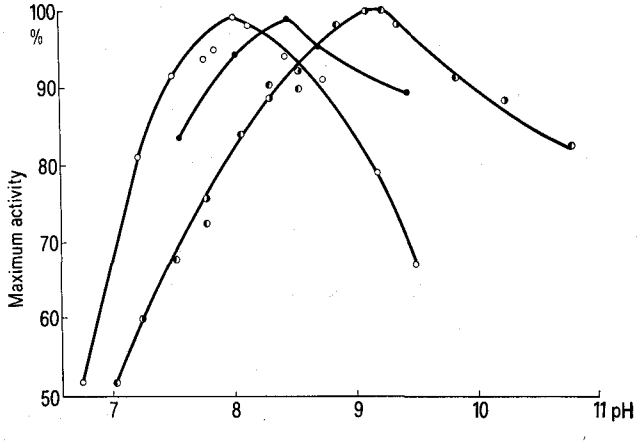
Preparation and Properties of a Soluble Trypsin-Dextran Conjugate

The properties of insoluble, immobilized enzymes have been extensively investigated and documented[1]. By comparison, however, data available for enzymes immobilized in solution are limited, although early investigations by MITZ and SUMMARIA[2] indicated that soluble chymotrypsin-cellulose conjugates exhibited higher catalytic activities than their insoluble counterparts. More recently several chymotrypsin-dextran graft co-polymers have been shown to possess increased stability with unaltered catalytic properties[3,4].

Trypsin (EC 3.4.31.4) covalently attached to neutral polyaldehyde dextran is also more stable than the free enzyme but its activity towards polymeric substrates and its pH-rate profile have both been altered by immobilization.



pH-activity profiles for trypsin, I = 0,17 (○); acetaldehydetrypsin, I = 0.17 (●); PADT, I = 0.17 (◐); PADT, I = 1.17 (◑). All reaction mixtures contained 0.1 mg/ml protein and 1.27×10^{-3} *M* BAPNA.

Dextran T70 (Pharmacia, G.B., Ltd) was oxidized by an acidic solution of sodium periodate for 16 h, 25 °C in the dark. The polyaldehyde dextran (PAD) so formed was recovered by dialysis and lyophilization. Trypsin (Boeringer Corporation, London, Ltd.) was stirred with an excess of PAD for 24 h at 5 °C in 0.1 *M* citrate buffer pH 7.0. No insoluble material was formed during conjugation and the soluble polyaldehyde dextran-trypsin conjugate (PADT) was isolated by gel chromatography. Rechromatography of the isolated complex at high ionic strenght had no effect on the protein concentration in the complex. A typical preparation of the PADT complex contained 0.41% nitrogen equivalent to approximately 3% trypsin (w/w).

Tryptic activity was assayed spectrophotometrically at 30° with benzoyl-D,L-arginine *p*-nitranilide hydrochloride (BAPNA) (Boeringer Corporation, London, Ltd.) Apparent Michaelis-Menten parameters for BAPNA were estimated from Lineweaver-Burk plots and were refined using a procedure similar to that of WILKINSON[5]. The proteolytic activity of trypsin was estimated by its catalyzed release of TCA soluble products from 0.5% casein solution[6].

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The single stage coupling of trypsin to the pre-activated dextran matrix is complete within 24 h at 5 °C, as shown by the absence of free enzyme on gel chromatography. The resultant graft is stable at room temperature in aqueous solutions of neutral pH.

The Figure shows pH-rate profiles for the hydrolysis of BAPNA catalyzed by free and bound trypsin at ionic strengths of 0.17 and 1.17. The pH optimum is displaced by approximately 1 pH unit towards more alkaline values by conjugation. A similar shift towards higher pH values has been observed previously for trypsin-polyelectrolyte derivatives and in that case was attributed to the neighbouring carrier bound charged groups[7]. For this system such an explanation is inadequate. Oxidation of polysaccarides by sodium metaperiodate, under the above conditions terminates at the aldehyde level of oxidation, and the pH shift is not reduced by the large change in ionic strength[8].

The resultant pH-activity profile after treatment of trypsin with excess acetaldehyde was determined in order to separate the effects due to the matrix from those due to chemical modification of the protein molecule (Figure). In agreement with LABOUESSE and GERVAIS[9] a small alkaline shift was observed, but smaller than that caused by the polyaldehyde dextran.

The matrix had no effect, within experimental error, on the substrate dependant kinetics of trypsin. Both free and attached enzyme obeyed the Michaelis-Menten equation for the hydrolysis of BAPNA over the range $0.1–1.27 \times 10^{-3}$ *M*; the parameters determined at each pH optimum are shown in Table I.

Immobilization imparts increased resistance to autolysis (Table II). Trypsin loses 98% of its activy within 72 h at 30 °C pH 7.0, but somewhat less (86%) in the presence of 0.2 *M* calcium chloride. By contrast the conjugate retains 42% of its initial activity even after 34 days under the same conditions; calcium ions also have a small stabilizing effect on the conjugate, Table I (care was taken to avoid bacterial contamination).

This reduced autolytic capability of the carrier bound enzyme is the result of two main factors. By analogy with the usual reaction products of aldehydes with proteins, the expected sites of reaction would be to the E-amino groups of lysine so preventing the latter from binding to the active sites of other trypsin molecules. In addition, the presence of the attached dextran will sterically hinder the mutual approach of trypsin molecules. This effect is probably also responsible for the decreased degradation of casein by the conjugate which is only 4% of that of the native enzyme.

As a requisite to further elucidation of the observed kinetic properties of the conjugate, its structure is under investigation.

Table I. Apparent Michaelis constants for trypsin catalyzed hydrolysis of BAPNA in 0.1 *M* veronal buffers, plus 0.02 *M* calcium chloride, 30 °C

	$K_{(app)}$ ($\times 10^4$ *M*) pH 8.0	pH 8.85
Trypsin	7.40	5.80
PADT	5.60	5.95

Table II. Percentage retention of tryptic activity towards BAPNA, at 30 °C in 0.1 *M* citrate buffer pH 7.0 (protein concentration, 1 mg/ml).

	3 days	34 days
Trypsin	2	0
Trypsin plus 0.02 *M* $CaCl_2$	14	0
PADT	46	42
PADT plus 0.02 *M* $CaCl_2$	53	55

Zusammenfassung. Durch kovalente Bindung an Polyaldehyd-dextran wird Trypsin immobilisiert. Die polymerische Matrix stabilisiert das Enzym und bewirkt eine pH-Erhöhung der maximalen Aktivität, beeinflusst aber den Michaelis-Menten Parameter für die Hydrolyse des Substrates BAPNA nicht.

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Alteration of Erythrocyte ($Na^+ + K^+$)-ATPase by Replacement of Cholesterol by Desmosterol in the Membrane

It was shown by us that treatment of rats with an inhibitor of desmosterol reductase, i.e. 20.25-diazacholesterol, results in an increased specific ($Na^+ + K^+$)-ATPase activity in membranes of different tissues. Such an effect was shown for the sarcolemma of skeletal and cardiac muscle[1,2] and erythrocyte ghosts[3,4]. Preliminary studies[5] suggest that the alteration in ($Na^+ + K^+$)-ATPase activity is due to the replacement of cholesterol by demosterol in the membranes of animals treated with this substance.

Male Wistar rats were daily treated with 10 mg 20.25-diazacholesterol dihydrochloride in 0.2 ml of water given by an oesophageal cannula for a period of 8 weeks. Control animals were subjected to the same procedure using 0.2 ml of water only. Blood was drawn from one treated and one control animal into heparinized syringes by aortic puncture of the anesthetized animal. Plasma and red

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