On the Mechanism of the Glycogenolytic Effect of Dinitrophenol. Activity of Phosphofructokinase in Perfused Hearts

The relationship between the glycogenolytic effect of the 2,4-dinitrophenol (DNP), either in vivo or in perfused hearts, and the activity of the phosphorylase *b* kinase, was recently shown in studies from this laboratory[1,2]. However, attempts to demonstrate the effect of the drug directly on the activity of partially purified preparation of the enzyme, or on the adenosine-3′,5′-phosphate cyclic level in the muscle, were unsuccessful[3]. These facts suggested that DNP could stimulate glycogenolysis by a mechanism other than that described for catecholamines, i.e. the so-called 'push mechanism' according to CORY's[4] terminology. Thus the 'pull mechanism' in which the phosphofructokinase represents an important regulatory enzyme, could be invoked to explain the glycogenolytic effect of the DNP.

The DNP, among other properties, presents a well-known ATPasic action[5]. Since the activity of phosphofructokinase is highly dependent on the ATP level of the cell, experiments have been carried out to accumulate evidence on the activity of this enzyme in perfused hearts in conditions under which the glycogenolysis was stimulated. The results also helped to suggest an explanation for the high activity of the phosphorylase *b* kinase found in animals poisoned by the DNP.

Thus, heparinized adult rats were anesthetized with ether and the hearts were rapidly removed and perfused with a Krebs-Henseleit bicarbonate buffer, gassed with a mixture of O_2:CO_2 (95:5) at 37 °C for 10 min, in order to adapt the hearts to the new physiological conditions according to MORGAN et al.[6], and then for a further period of 10 min either in the presence or absence of 10^{-5} *M* DNP. After this treatment, the hearts were frozen by imersion in liquid nitrogen and a sample was weighed and ground in ice-cold solution containing 0.001 *M* EDTA 0.02 *M* sodium fluoride, pH 6.8 in the proportion of 25 ml/g of muscle. After centrifugation, the phosphorylase activities were determined as previously described[1] and portions of the remaining muscle were dumped onto 10% percloric acid and ground. After centrifugation, aliquots were taken from this extract for determination of glucose-6-phosphate, ATP, ADP and AMP.

The levels of nucleotides were estimated by the PETERSEN and KALCKAR method[7], using a cuvette containing 2.5 ml of 0.3 *M* succinate buffer, pH 6.1; 20 µl 0.1 *M* $MgCl_2$; 500 µl of neutralized tissue extract and 10 µl (10 µg/ml) solution of 5-adenylic acid deaminase[8]. After no change in absorbance at 265 nm, 20 µl (20 µg/ml) of myokinase solution[9] and 50 µl of (10 µg/ml) solution of potato apyrase[10] were added and the contents of ADP and ATP were determined by the decrease in absorbance at 265 nm of the reaction mixture. Glucose-6-phosphate (G-6-P) was determined by the increase in absorbance at 340 nm[11] after the addition 10 µl (1 mg/ml) of glucose-6-P dehydrogenase solution to a cuvette containing 0.5 ml of tissue extract and 0.5 ml of 0.20 *M* *Tris*-Cl solution, 0.01 *M* $MgCl_2$ and 0.1 m*M* NADP at pH 7.5.

Table I shows that, in 10 min of perfusion with DNP, the rate of glycogenolysis is increased as determined by the activity of the phosphorylases system. In the same table,

Table I. The phosphorylase activity and the levels of G-6-P, ATP, ADP and AMP in hearts perfused 10 min with 10^{-5} *M* DNP

Determinations	Control	DNP
Phosp *a*/total × 100	10.70 ± 1.8[a]	37.5 ± 7.4
G-6-P (µmoles)	0.69 ± 0.03	0.48 ± 0.04
ATP (µmoles)	17.4 ± 1.1	14.4 ± 0.8
ADP (µmoles)	1.6 ± 0.2	3.63 ± 0.4
AMP (µmoles)	0.37 ± 0.08	0.69 ± 0.1
ATP/ADP	10.8 ± 1.8	4.07 ± 1.2

The results are expressed per g of dry hearts.
[a] S.E.M.

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Table II. Level of G-6-P and ratio of phosphorylase *a*/total phosphorylase in hearts perfused in either the presence or not of 10^{-5} *M* DNP in the perfusate

Perfusion	Time of DNP action (min)	G-6-P (µmoles/g)	Phosp $\frac{a}{\text{total}}$ × 100
10 min KH (14)[a]	0	0.69 ± 0.03[b]	10.70 ± 1.8
$8^1/_2$ min KH + $1^1/_2$ min DNP 10^{-5} *M* (8)	$1^1/_2$	0.35 ± 0.02	15.5 ± 1.64
5 min KH + 5 min DNP (5)	5	0.48 ± 0.04	40.5 ± 5.52
10 min DNP (7)	10	0.49 ± 0.03	37.50 ± 7.4

The DNP was dissolved in the same Krebs-Henseleit buffer (KH) (see text).
[a] Represents the number of rat hearts used. [b] S.E.M.

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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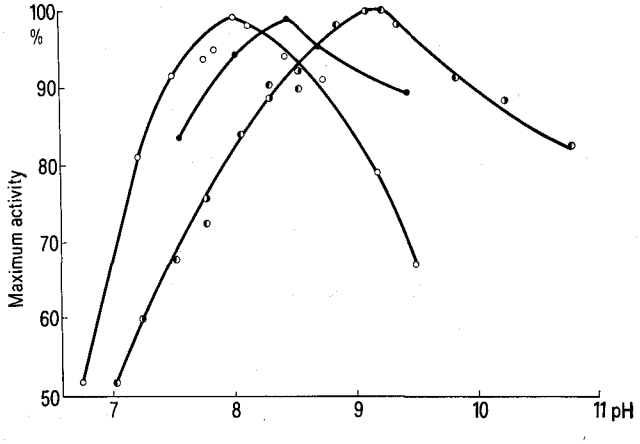
Preparation and Properties of a Soluble Trypsin-Dextran Conjugate

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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