Histochemically Demonstrable Phosphorylase as an Early Index of Anoxic Myocardial Damage

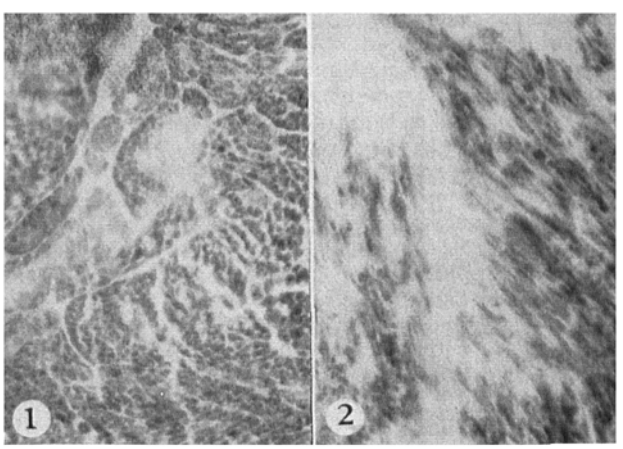
Morphologic differential diagnosis between primary (i.e. non-occlusive or metabolic) and secondary (occlusive or anoxic) necrotizing cardiomyopathies is one of the basic, yet still unsolved, aspects of cardiovascular pathology[1]. This problem becomes more to the fore as it is increasingly recognized that some forms of human cardiac necroses and interstitial myocarditis represent reactions to direct metabolic injury in hearts with a normal blood supply[2-4]. Our earlier histochemical studies on the alterations in glycogen metabolism and on the enzymatic changes that occur during the devlopment of various experimentally induced cardiomyopathies indicated that myocardial phosphorylase behaves differently from all other enzymes studied so far[5,6]. Consequently, in the present investigations the question was broached whether changes in the activity of phosphorylase and in the amount of stainable glycogen reserve possess any value from a differential-diagnostic point of view.

Three hundred and sixty female rats of the Sprague-Dawley strain, with an average body weight of 112 g, were used. Six different (three primary and three secondary) cardiomyopathies were analysed comparatively throughout their entire development. Primary myocardial lesions were induced by: (a) plasmocid (a metabolic inhibitor); (b) dihydrotachysterol, or DHT (a steroid of the vitamin D group); and (c) a K-deficient diet. Secondary myocardial lesions were produced by; (a) ligature of the left main coronary artery; (b) methoxamine (a synthetic vasopressor amine); and (c) metaraminol (another potent vasopressor compound). The details of methods used for eliciting these necrotizing cardiomyopathies are described elsewhere[1]. Following surgical occlusion of the coronary artery or a single injection of the cardiotoxic compounds, the animals (always four rats at a time) were killed by decapitation at different intervals ranging from 1 min to 96 h. In the case of the more slowly developing cardiac lesions induced by the K-deficient diet, the rats were sacrificed at 24 h intervals, from the 1st until the 16th day. At autopsy, transventricular ablation was rapidly performed (after which, cardiac contraction ceases instantaneously) and the remaining lower two-thirds of the ventricles was frozen with liquid air. Serial sections (thickness 10 μ) were cut from each specimen in a Pearse cryostat. The activity of phosphorylase was demonstrated after TAKEUCHI et al.[7,8] (incubation time 120 min), while glycogen was identified by its staining characteristics with the periodic acid-Schiff technique (on frozen sections) following fixation with Rossman's fluid, and by its digestibility with diastase. For routine morphologic studies, the frozen sections were post-fixed with formalin and stained with hematoxylin-phloxine-saffron.

In the primary cardiomyopathies (induced by plasmocid and DHT), both a spotty decrease and an increase in the activity of phosphorylase occurred equally in the same hearts during early stages of involvement; nevertheless, these changes always progressed slowly, so that they usually did not become obvious until 1 to 2 h after injection of plasmocid, or 24 h after administration of DHT. Moreover, complete loss of the enzyme reaction did not prove to be a prenecrotic alteration; in fact, phosphorylase activity could often be detected in fibres already in various stages of disintegration. Furthermore, the gradients for phosphorylase activity constantly followed the opposite direction from those for glycogen reserves; sometimes, in fibres completely devoid of glycogen, the reaction for phosphorylase was obviously increased. In the hearts of rats maintained on the K-deficient diet, the disturbance in phosphorylase activity was similar to that seen in the other two metabolic cardiomyopathies. However, here, the glycogen content of myofibres gradually decreased; it completely disappeared several days before the first morphologic signs of degeneration became obvious.

In the secondary cardiomyopathies (produced by coronary artery ligature and injection of vasopressor substances) the behaviour of phosphorylase and glycogen proved to be quite different from that observed during the development of primary lesions. A decline or even complete loss of phosphorylase – with a parallel disappearance of the stainable glycogen – could clearly be detected as soon as 1 to 5 min after coronary ligature, within the areas of the anticipated infarction. During later stages of involvement these changes remained constant, independently of the duration of anoxia and of the degree of degeneration. A similar parallel decrease of phosphorylase and glycogen began as early as 15 to 30 min after the injection of the vasopressor amines; nevertheless, here the foci showing losses in these two substances were rather spotty and mainly located in the subendocardial and apical areas, where the necrotic lesions usually developed.

These comparative histochemical studies suggest that (a) the early alterations in glycogen metabolism of primary and secondary cardiomyopathies are basically different; (b) a decrease or loss in the histochemically demonstrable activity of phosphorylase is a sensitive and



Behaviour of myocardial phosphorylase during early stages of anoxic heart damage. Fig. 1. Spotty decline of phosphorylase activity within subendocardial areas 15 min after injection of methoxamine. Fig. 2. Rather confluent loss of enzyme reaction in the left ventricular wall 3 min after coronary artery occlusion.

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early index of anoxic myocardial damage; and (c) a comparative evaluation of the changes in the reaction for phosphorylase and in variations in the amount of the stainable, labile fraction of glycogen is of value concerning pathologic differential diagnosis, since it provides a basis for etiologic considerations.

Résumé. Il fut démontré que les changements précoces d'activité de la phosphorylase au niveau du myocarde sont essentiellement différents selon qu'il s'agit d'une cardiomyopathie primaire (non-occlusive) ou secondaire (occlusive).

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Solubilisation de la monoamineoxydase des mitochondries de foie de rat

Depuis la découverte par HARE[1] en 1928 de l'enzyme capable de catalyser l'oxydation de la tyramine, enzyme connu depuis, sous le nom d'amineoxydase, les études concernant sa structure et son mode d'action ont relativement peu progressé. Cela est dû en particulier au fait que cet enzyme qui est essentiellement localisé sur les mitochondries[2] est très difficile à extraire et à solubiliser. Divers auteurs ont essayé l'action d'agents solubilisants tels que le désoxycholate de sodium associé à l'action des ultrasons[3], l'isooctylphénoxypolyphénoxyéthanol[4]. Pour obtenir une solubilisation avec un meilleur rendement, nous avons étudié l'action de quatre produits réputés tensioactifs: la digitonine, une saponine, le tween 20 et le laurylsulfate de sodium.

Matériel et méthodes. Nous avons choisi comme source enzymatique la suspension de mitochondries de foie de rat, préparées selon WEINBACH[5]. Ces suspensions ont un degré de pureté de 95%.

Les protéines sont dosées par la méthode du biuret[6].

L'activité monoamineoxydasique est évaluée:

Soit manométriquement, par la consommation d'oxygène mesurée au respiromètre de Warburg, en présence de tampon phosphate (pH 7; 0,08 *M*) et à 38°C, selon CREASY[7]. Le substrat est la sérotonine, sous forme de sulfate double de sérotonine et de créatinine à la concentration de $3{,}28 \cdot 10^{-3} M$.

Soit en dosant l'ammoniac dégagé suivant la méthode de Conway.

Action de la digitonine. Nous avons utilisé la digitonine en nous inspirant de la technique de LEHNINGER[8], pour l'obtention de fragments mitochondriaux. Les mitochondries fraîchement préparées, sont traitées par la digitonine à 0,8%, à pH 7 pendant 30 min à +1°C, puis centrifugées 30 min à 50000 *g*. Le surnageant est alors centrifugé à nouveau 30 min à 100000 *g*.

Le surnageant final possède une activité spécifique sensiblement égale à celle de la suspension initiale, mais son activité totale ne représente que 20% au maximum de l'activité initiale.

Le premier culot de centrifugation a une activité spécifique légèrement supérieure et le deuxième une activité spécifique 4 à 5 fois supérieure à celle des mitochondries de départ.

Observant donc que l'activité monoamineoxydasique spécifique augmente au fur et à mesure de la rupture des mitochondries nous avons vérifié ce phénomène en soumettant une suspension de mitochondries à l'action d'un broyeur Ultraturax et en la centrifugeant successivement à 20000, 40000, 80000 *g*: on obtient un accroissement de l'activité parallèle à la diminution de taille des particules: avant tout traitement nous mesurons une consommation d'oxygène de 6,9 ml de CO_2 par mg de protéine pendant 40 min; après broyage au turax, cette consommation passe à 7,8 pour une accélération centrifuge de 20000 *g* à 15 pour 40000 *g*, à 28 pour 80000 *g*.

	Activité[a] globale	Activité spécifique
Suspension de mitochondries de départ	6900	6,7
C_1[b]	3900	11
S_1	2000	2,6
C_2	2100	15,7
S_2	1000	3,6
C_3	900	16,2
S_3	0	0

[a] Activité globale: $\mu l O_2$ consommée/40 mn/1 ml; spécifique: $\mu l O_2$ consommée/40 mn/mg protéine.

[b] Centrifugation de la suspension de mitochondries de départ, traitée par le laurylsulfate de Na à 0,2%.

→ Surnageant S_1

Culot C_1 (remis en suspension dans du saccharose 0,25 *M*) même traitement que la suspension de départ

→ S_2

C_2 (traitement identique à celui de C_1)

→ S_3

C_3

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