THE EVALUATION OF THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE UNDER DIFFERENT PATHO-PHYSIOLOGICAL CONDITIONS: A COMBINED ENZYME HISTOCHEMICAL AND BIOCHEMICAL APPROACH.

DE EVALUATIE VAN DE ACTIVITEIT VAN GLUCOSE-6-FOSFAAT DEHYDROGENASE EN 6-FOSFOGLUCONAAT DEHYDROGENASE ONDER VERSCHILLENDE PATHO-FYSIOLOGISCHE OMSTANDIGHEDEN: EEN GECOMBINEERDE ENZYM-HISTOCHEMISCHE EN BIOCHEMISCHE BENADERING.

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. A.H.G. RINNOOY KAN
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
DONDERDAG 17 MAART 1988 DES NAMIDDAGS
TE 13.30 UUR.

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EZZAT ADLY ELIAS
geboren te Zawiet El-masloub,
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drukwerkverzorging OPTIMAX
Wijk bij Duurstede

Promotiecommissie

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The investigations necessary for this dissertation and allied publications were performed under the supervision of Prof. Dr. A.E.F.H.Meijer at the Department of Pathology, Histochemical and Biochemical Section, Academic Hospital Wilhelmina Gasthuis, University of Amsterdam and the Central Laboratory for Public Health in Friesland, Leeuwarden, The Netherlands.

I am an open book
written in a foreign language.

.... pour toi

et pour toujours....

W O O R D E N V O O R A F :

Een dankwoord aan allen die met een proefschrift te maken hebben of hadden is zeker op zijn plaats. Desondanks schiet een dergelijke verwoording altijd te kort. In de haast vergeet je velen te noemen die steeds een warme plek in je hart hebben. Kort en krachtige **be-voor-woording** is een doel dat door te veel willen vaak niet wordt bereikt. Alhoewel 5000 jaar bekende medische geschiedenis een lange tijd lijkt, is het nog steeds niet duidelijk of we met **geneeskunde** of met **geneeskunst** te maken hebben. Amn-Hotep, van beroep medicus, werd indertijd verheven, als dat woord gebruikt mag worden, van een volksjongen tot een goddelijkheid door zijn inzicht in de mens en de **kunde** waarmee hij zijn **kunst** uitoefende. Terwijl Augustinus **God** ontdekte, simpelweg door zijn hersenen te gebruiken, zocht de nuchtere Erasmus de verbetering van binnenuit. Weliswaar kerkelijk bedoeld, maar wat is het verschil tussen de kerk als geheel en de mens als individu daarin ? Zoeken naar begrip is oermenselijk, doch zelfs onverstane of onverstaanbare woorden hebben iets te verbergen terwijl onze onzekerheid in zich een zekerheid is. Taal is **slechts** een communicatie middel dat door ons gemakshalve ontwikkeld is.
Een nuchtere letterkundige vertaling van alles wat je mee maakt, je ervaringen en belevenissen van 20 jaar actief en 10 jaar passief in het leerproces, is haast een onmogelijke opgave; te meer daar het verschil tussen actief en passief in deze context onbeduidend is. Je kunt bij wijze van spreken het alfabet citeren en de zorg voor de volgorde, combinaties en de interpretaties daarvan aan de anderen laten. Wij zien **elkaar** toch niet zoals **wij** c.q. **zij** zijn maar zoals **wij** dat willen zien. Mijn groot leerboek: **"de patient"** is grenzeloos, eindeloos en onuitputtelijk.

Vele illusies moet je niet hebben. Zelfs de wetenschap is ten minste ten dele een slachtoffer van de, op een gegeven moment, heersende modeverschijnselen; blijkbaar zal dit voor altijd zo blijven. Kijk maar naar de actuele zg **monoclonal revolution.** Ten grieve of tot verdriet van vele waarnemers, wordt de ware waarde van iedere inbreng niet bepaald door mate van belangstelling maarwel door de test der tijd. In de schaduw werken heeft ook zijn voordeel als je maar niet voortdurend gestoord wordt door aasgieren die een graantje mee willen pikken van iedere aangeklede tafel of proberen je te hinderen in het doorgaan, gedreven door niet nader te beschrijven motivaties. Dat je van menige publicatie meer dan 1500 afdruk-aanvragen krijgt en op enkele andere publicaties slechts circa 25 reacties ontvangt, is geen bewijs voor de kwaliteit van hetgeen je schrijft. Nuchter gezien is dit slechts een onkosten factor. Het kan ook een parameter zijn voor de toevallige smaak van de lezers op **dat** moment. Eveneens betekent het feit dat je beste vrienden je voornaam na 20 jaar nog steeds verkeerd hanteren niet, dat de vriendschap hierdoor minder wordt of eronder mag lijden.
Als ons bestaan een voortbestaan van onze ouders is en als de genetica een materie van informatie dragen is, zouden **ze** waarvan ik iets geleerd heb een blijvend stukje van mijn eigen ik vormen. Dit is mijn enige troost jegens de namen die ik vergeten te melden heb:
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Ik heb veel waarvoor ik blijf danken.

Ezzat,
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CONTENTS:

CHAPTER 1

GENERAL INTRODUCTION.

1.1 General and historical considerations

The heritage of knowledge available in the microscopic anatomy resulted from a long history of experience evolved gradually since the early days of light microscopy. Our information is primarily derived by the examination of tissue sections from normal and pathological specimens after an array of histological manipulation techniques. These are essentially based on appropriate methods for collection, preservation, dehydration, clearance, impregnation, embedding, cutting and staining representative material. The classic way of fixation is the simple use of aqueous solution of formaldehyde or more sophisticated fixatives. For embedding paraffin has been very popular for a long time, replaced today gradually by synthetic polymers. The examination of light transmitting tissue sections is often performed after a classic staining of deparaffinized sections with haematoxylin & eosin stain or one of a long list of other special histological or histochemical stains.

This approach has been beneficial for morphological studies. However the value of such techniques is limited. The effect of fixation, dehydration and embedding, results in denaturation of tissue proteins and a definite shrinkage of the cells and tissues which may amount to a 30% loss of the original size according to Baker (1958). It is obvious that fixatives have an effect on the subcellular components and especially on the enzymes resulting often in a partial or complete inactivation. Such specimens do not reproduce reliable metabolic studies, such as those dealing with problems of energy metabolism.

On the other hand, the application of enzyme histochemical techniques, provided that they are performed under optimal conditions, may provide a better representation of the presence, distribution and activity of the enzymes involved and the metabolic status of the tissue cells investigated either in light microscopy or to a lesser degree in electron microscopy.

Therefore it is easy to assume that enzyme histochemistry can be a bridge between biochemistry and morphology, forming a logical step in the development of both.

The inter-connections between morphological studies and histochemical technology have long been well established. It is to the credit of morphologists that they have introduced many histochemical techniques suitable for investigating the activity of a large series of enzymes, (Gomori, 1939; Takamatsu, 1939; Menten et al., 1944; Danielli, 1946; Glick, 1949a & b; Pearse, 1953; Lillie, 1954; Graumann & Neumann, 1958:1964; Gurr, 1958; Barka, 1959; Casselman, 1959; Davenport, 1960).

This is understandable assuming that the morphologists are interested in correlating form, site, size and distribution with the aesthetic features of subjects investigated. Indeed the enzyme histochemistry can be described as a branch which has evolved from histology on the base of biochemical knowledge.

The actual sharp demarcation between morphology and pure biochemical research results in a widening gap between workers in both fields. Today only a few biochemists are demonstrably interested in morphological studies or in microscopical anatomy as a whole. On the other hand, the modern morphologists suffer from a shortage in knowledge because of the rapid advancements in biochemistry. Interpretation of the significance of enzyme histochemical observations has become more and more complicated to the pathologists in particular.

It is inevitable that the future will recessitate the pathologist to attempt answering more questions in the metabolic field as a result of the growing interest and insight into the biological backgrounds of the pathogenesis of the diseased cell and the therapeutic consequences based on such information. One of the fields in which such questions

are already being felt is muscle pathology, primarily because identification and further classification of certain muscle diseases has become only possible on the base of the metabolic parameters (Salter, 1968; Bonilla & Schotland, 1970; DiMauro et al., 1970; Lake, 1970; Busch et al., 1981; Johnson & Walton, 1981). Biochemical investigations are, in general, tissue consuming and have as a major drawback a lack of discriminative precision when done on tissue homogenates. The origin and location of enzymes investigated is indeterminable when the homogenates contain several contaminants other than the cells concerned. Enzyme histochemical investigations, on the other hand, are tissue saving and provide a positive correlation between the morphology, distribution and activity of enzymes investigated although they often lack the quantitative accuracy of the biochemical assays.

Therefore, it is a practical advantage to use enzyme histochemical techniques as a reliable method for the study of metabolic processes.

Skeletal muscle biopsies of patients investigated for acquired or inherited neuromuscular diseases can be processed in almost every laboratory familiar with histochemical technology (Dubowitz & Brooke, 1973; Dubowitz, 1978). This is true for both laboratories at the University of Amsterdam and The Central Laboratory for Public Health in Friesland where most of the investigations necessary for this dissertation were performed. Programs of combined histological, histochemical and when necessary, biochemical techniques were set up and performed to allow a better understanding of the conditions concerned. As will be seen in the publications and other references of applied techniques, the most reliable technology available in the field of enzyme histochemistry was utilized. New techniques were often developed and tested specifically for the data investigated. The results of other studies proved that many histochemical techniques available need modification in light of the conditions and tissues investigated in order to provide greater accuracy in observation and interpretation (Meijer,1978).

The shortages of many conventional enzyme histochemical techniques emerged as a result of the investigation of non-structurally bound or weakly bound enzymes which can diffuse into the incubation medium (Kalina & Gahan, 1965;

Altman & Chayen, 1966; Arnold et al., 1968; Meijer, 1972,1973,1980; Meijer & de Vries, 1974,1975; Meijer & Vloedman, 1973; Wachsmuth et al., 1975). Other difficulties arose in the determination of activity of enzymes dependent on the presence of auxiliary enzymes. this rendered the demonstration of the former only possible in relation to the site and equivalent activity of the auxiliary enzyme. This phenomenon may result in a "false localization" leading to a false interpretation. A "false localization" can be defined as a positive reaction due to enzymatic content appearing at sites other than the primary location. This may also be a result of enzymatic diffusion on subcellular level from the original site of action and adsorption at another (Pearse, 1968; Meijer & de Jong, 1988). A second possibility is the diffusion of the reaction products of the enzymatic hydrolysis from their site and subsequent adsorption or deposition at other sites (Cornelisse & van Duijn, 1974; Raap, 1983).

Reasonable advances have been achieved through, the development of a poly-acrylamide gel technique (Ruitenbeek & Scholte, 1976) and the poly-acrylic membrane techniques for cytology material (van Noorden et al, 1982; Elias et al., 1984a & b).

The application of macromolecular substances in the incubating media such as gelatine, dextran, polyvinylpyrrolidone, agarose and agar may facilitate prevention of leakage of soluble or diffusable enzymes (Ritter et al., 1971; Meijer, 1972,1973,1980; Meijer & deVries, 1974,1975; Meijer & Vloedman, 1973; Wachsmuth et al., 1975; Ruitenbeek & Scholte, 1976). Still, the macromolecular substances may inhibit the enzyme activities (Dahl & From, 1971; Lisý et al., 1971; Meijer, 1972, 1973). The practice of applying fixation techniques to diminish enzyme diffusion is not always the method of choice because it results in deterioration of many enzyme activities. Furthermore, the hydrous fixatives cause similar partial to complete leakage of enzymes into the fixation medium.

Such errors of leakage, diffusion and unreliable determination of the actual site of activity of enzymes under investigation were greatly diminished by the use of the semipermeable membrane techniques (Meijer, 1980), which were also more sensitive. The interposition of a semipermeable membrane

between the tissue sections and a gel-form incubation medium which contains appropriate enzyme inhibitors to abolish the function of cytochrome c oxidase and sufficient electron carriers to carry on the electron transfer have, from experience, shown to be of a great value in improving and creating many enzyme histochemical staining reactions. The techniques which were developed include reactions for the demonstration of activity of some hydrolases, oxidoreductases, transferases and isomerases.

1. 2. Scope and outlines:

The studies presented in this dissertation as well as in many other related publications evolved gradually through the continuous association with Prof. Dr. A. E. F. H. Meijer within the work group Histochemistry, of the Department of Pathology, Academic Hospital Wilhelmina Gasthuis, University of Amsterdam. The first contacts were in the early seventies and continued after my departure to work as a consultant pathologist at the Laboratory for Public Health in Friesland.

The available enzyme histochemical technology was under thorough revision at that time within the work group. The newly introduced semipermeable techniques proved promising. The demonstration of the activity of many enzymes was made possible and adaptation or improvement of techniques for the demonstration of other enzymes was under investigation. The challenge of a possible increase in the activity of the pentose phosphate pathway enzymes under certain conditions and the metabolic significance of such change has intrigued me since my early days as a trainee pathologist interested in the diagnostic implications and biological backgrounds of diseases.

The cooperation between a biochemist-histochemist and a pathologist proved fruitful in the following years. A series of several publications and scientific presentations, intended to illustrate the value of enzyme histochemical investigations as a diagnostic measure and a means to study the patho-physiology of conditions concerned, could be realized. The studies were completed when necessary by adjuvant biochemical assays on tissue homogenates for confirmation of the morphological observations and to obtain more quantitative data. Experience showed that a combined histochemical and biochemical approach conducted on the same tissue material is the most effective procedure for an accurate interpretation and evaluation of the observations.

Since the beginning the attention was directed to muscle diseases because of availability of material, the possibility of good correlation with clinical data, and a familiarity with the subject. Apparently healthy and diseased human muscle

specimens were studied and completed with further investigations on experimental animals.

The results encouraged us to extend the studies to the human heart and its conducting system as well as to other tissue cells under different conditions. In this way, different metabolic activities with special reference to early and late ischemic changes on the working myocardial fibres and the Purkinje fibres were investigated.

The enzymatic fluctuations in the human endometrium under different hormonal controlled conditions and in malignancy were a link to further study of metabolic changes in cancer and other proliferative diseases in a variety of organs.

The logic of capita selecta incorporated in this thesis emphasizes several approaches to the subject of metabolic deviations of the activity of glucose-6-phosphate dehydrogenase (GPDH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (PGDH, EC 1.1.1.44). The site and degree of activity of both enzymes and the metabolic significance of any change under conditions described was considered the linking factor throughout the actual presentation. It also may form the base for experiments planned for the future. In several studies the investigations were expanded to the exmination for the activity of about 20 enzymes which play a main role in the major aerobic and anaerobic pathways of energy metabolism and other enzymes of a special importance, in combination with the examination of presence and distribution of glycogen and lipid contents. In the meanwhile the correlation between the morphology, the histochemical observations and relevant clinical data has been considered necessary for evaluating the patho-physiological status of the conditions and specimens under investigation.

Only through the cooperation, hospitality and critical advices of Prof. Dr. R. O. van der Heul as a promotor, the presentation of this thesis in its actual form at the Erasmus University Rotterdam has been made possible.

1. 3. The Pentose Phosphate Pathway

The pentose phosphate pathway, also designated pentose phosphate shunt, hexose monophosphate pathway or phosphogluconate oxidative pathway is generally known as a non glycolytic pathway of glucose metabolism. It has been considered of subsidiary importance and not as one of the major metabolic pathways of energy metabolism. It is a multifunctional pathway which produces NADPH and/or ribose-5-phosphate depending on the needs and metabolic state of the cell.

The combined efforts of two American research teams of Horecker and Racker provided a great deal of the descriptive data about this pathway in the form depicted today (Horecker, 1968). Their conclusions were partially based on knowledge obtained from previous work of Lipmann (1936), Warburg & Christian (1937a & b), Dickens (1938a & b) and Racker (1961).

This extramitochondrial (cytosolic) pathway has two branches illustrated in figures 1 & 2: an oxidative part which converts glucose-6-phosphate into ribulose-5-phosphate and a nonoxidative part. The latter converts ribulose-5-phosphate into phosphates of octulose, heptoses, hexoses, pentoses, tetroses and trioses.

Apparently, glucose-6-phosphate dehydrogenase (GPDH) and transketolase (TK) are the two principal enzymes controlling the two branches of the pathway, with some indications that there is a possible additional control at the level of 6-phosphogluconate dehydrogenase (PGDH) and transaldolase (TA). It is generally agreed that the oxidative branch is controlled by the metabolic need for NADPH. The control mechanism of the non oxidative branch at the step of TK is less well established.

In plants the pentose phosphate pathway participates in a reverse direction in the formation of hexoses from CO_2 in the process of photosynthesis.

Several types of this pathway are described in literature. The classical F-type formulated by Horecker & Mehler (1955) is

presented in figure 1. It occurs primarily in fatty cells and shows interconnections with the Embden-Meyerhof pathway. This formulation has been called into question by Williams (1980).

The L-type of this pathway, figure 2, is described by Williams (1981) to occur primarily in liver cells. It distinguishes itself from the F-type by 4 additional intermediates and three additional enzymes.

The new intermediates are: D-arabinose-5-phosphate, D-glycero-D-ido-octulose-1,8-bisphosphate, D-altro-heptulose-1,7-bisphosphate and D-glycero-D-ido-octulose-8-phosphate.

This L-type invokes the additional action of: aldolase, D-arabinose-5-phosphate-2-epimerase and a phosphotransferase to catalyse the transfer of a phosphate group between heptulose and octulose bisphosphates. Currently, evidence of the existence of the L-type appears somewhat tenuous (Katz, 1981; Landau, 1981; Wood, 1981). Other alternative formulations of the pentose phosphate pathway were described by Wood (1975a, b & c, 1985) and by Severin & Stepanova (1980).

The exact formulation is not yet defined with certainty and is currently a subject of further investigation. Any how, the metabolic role of the pathway in any formulation is exactly the same. Therefore we used the classical F-type as a model for the discussion of the metabolic activities of the pathway.

The total reaction of the pathway can be summarized according to Mayes (1985) as follows:

$$3\ \text{glucose-6-phosphate} + 6\ NADP^{+} \longrightarrow 3\ CO_2 + 2\ \text{glucose-6-phosphate} + \text{glyceraldehyde-3-phosphate} + 6\ NADPH + 6\ H^{+}$$

1. Functions of the oxidative branch of the pentose phosphate pathway:

The enzymatic reactions of this branch run in sequence catalyzed by GPDH, a lactonase and PGDH. The total result can be equated as:

$$\text{Glucose-6-phosphate}^{-2} + 2\ \text{NADP}^{+} + \text{OH}^{-} \longrightarrow$$
$$\text{Ribulose-5-phosphate}^{-2} + 2\ \text{NADPH} + 2\ \text{H}^{+} + \text{HCO}_3^{-}$$

The produced NADPH is essential for several reductive bio-synthetic reactions (Danielsson & Tchen, 1968; Green & Allmann, 1968; Horecker, 1968). This reductive function of NADPH is manifest in the reductive bio-synthesis of fatty acids and steroids from acetyl-CoA in lipid synthesizing tissues as liver, mammary glands and adrenal cortex.

Another function of this reduced coferment is the reduction of the oxidized form of the tripeptide glutathione. Reduced glutathione is a protector against lipid auto-oxidation, as seen in the following equations.

$$O_2 + e \longrightarrow O_2^{\bar{\cdot}}$$

$$2\ O_2^{\bar{\cdot}} + 2\ H^{+} \longrightarrow H_2O_2 + O_2$$

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + 2\ H_2O$$

$$H^{+} + NADPH + GSSG \longrightarrow 2\ GSH + NADP^{+}$$

(The presence of the unpaired electron is represented by a superscript bold dot).

Consequently NADPH plays a role in elimination of harmful peroxides and free radicals (Autor, 1982; Freeman & Carpo, 1982). Also the NADPH plays a role in the elimination of singlet oxygen (Bodaness, 1982) as illustrated below:

$$O_2 + h\nu \longrightarrow {}^{1}O_2$$

$${}^{1}O_2 + H^{+} + NADPH \longrightarrow H_2O_2 + NADP^{+}$$

Lastly NADPH is necessary for those enzymes, as cytochrome P-450 reductase, which need for their action the reduced coferment.

The oxidative branch is, as mentioned, essentially irreversibe in animal tissues and functions as a rate limiting control site for the whole pathway.

2. Functions of the non-oxidative branch of the pentose phosphate pathway:

The enzymatic reactions of the non-oxidative branch are catalyzed by pentose-5-phosphate isomerase, pentose-5-phosphate-3-epimerase, transketolase (TK) and transaldolase (TA), while TK and TA link it with glycolysis.

It is not figurative to reproduce an equation to illustrate the total reactions of the branch because the direction of the different reversible enzymatic reactions depends on the availability of and demands for the metabolites produced, such as NADPH and ribose-5-phosphate which serve for the synthesis of important bio-molecules such as ATP, CoA, NAD^+, $NADP^+$, FAD, RNA and DNA. Thus, when the need for ribose-5-phosphate is greater than that of NADPH, the glycolytic pathway converts most of glucose-6-phosphate into fructose-6-phosphate and glyceraldehyde-3-phosphate. TK and TA then convert two molecules of fructose-6-phosphate and one molecule of glyceraldehyde-3-phosphate into three molecules of ribose-5-phosphate.

If the demand for NADPH and ribose-5-phosphate is balanced the predominant reaction of the pathway is the formation of two molecules of NADPH and one molecule of ribose-5-phosphate from glucose-6-phosphate, through the oxidative branch of the pathway. Conversely, when the need for NADPH is greater than that of ribose-5-phosphate, glucose-6-phosphate is completely oxidized into CO_2. Three groups of reactions are involved in this situation. First, two NADPH and one ribulose-5-phosphate are formed by the oxidative branch of the pentose phosphate pathway. Then, ribulose-5-phosphate is converted into fructose-6-phosphate and glyceraldehyde-3-phosphate by TK, TA and the isomerase of the non-oxidative branch. Finally, glucose-6-phosphate is resynthesized from the fructose-6-phosphate and glyceraldehyde-3-phosphate by the gluconeogenetic pathway. Alternatively, ribose-5-phosphate formed by the oxidative branch of the pathway can be converted

into pyruvate. Fructose-6-phosphate and glyceraldehyde-3-phosphate derived from ribose-5-phosphate proceed down the glycolytic pathway rather than revert to glucose-6-phosphate. In this way ATP and NADPH are generated. Pyruvate formed by these reactions is oxidized to generate ATP and CO_2.

The above given data show that the pentose phosphate pathway, through the production of triose phosphate or dihydroxyacetone phosphate as seen in figure 1, functions to bypass the rate limiting step of glycolysis: the reaction of phosphofructokinase (Kapucinski & Williams, 1981).

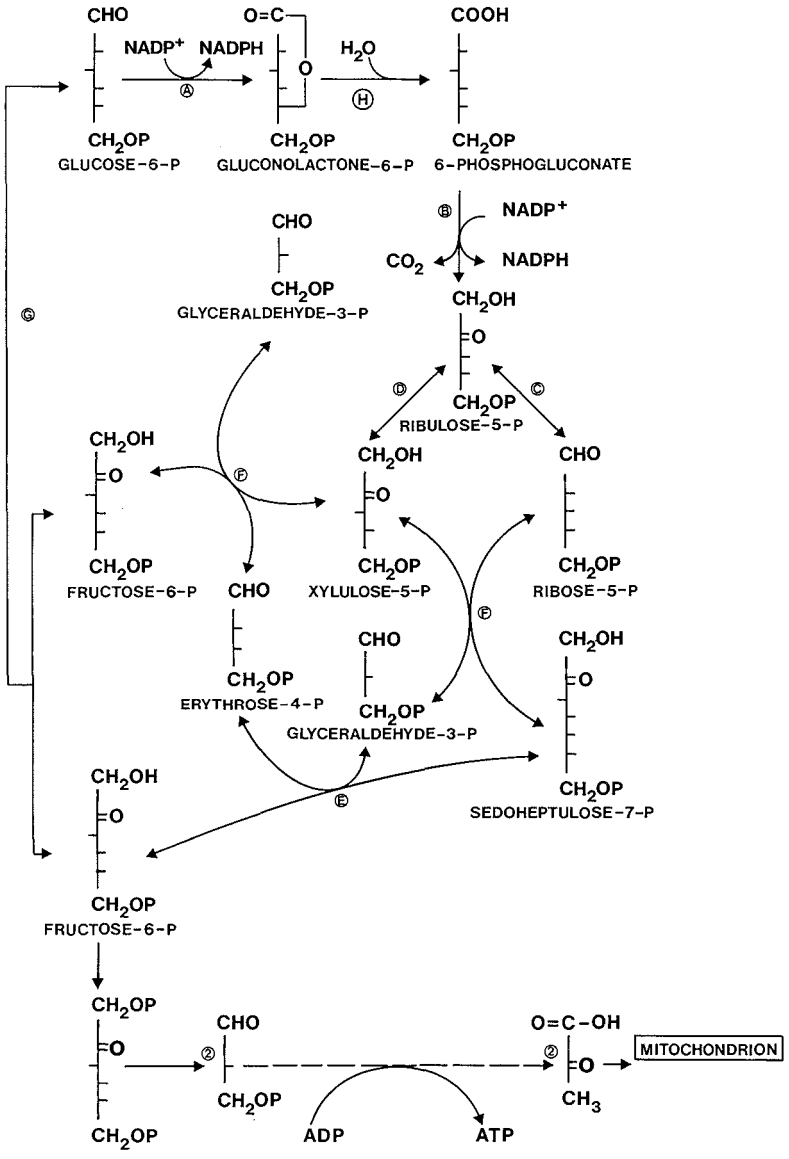

Figure 1:

The classical formulation of the pentose phosphate pathway according to Horecker and Mehler (1955). A: Glucose-6-phosphate dehydrogense, B: 6-phosphogluconate dehydrogenase, C: pentose-5-phosphate isomerase, D: pentose-5-phosphate-3-epimerase, E: transaldolase, F: transketolase, G: phosphohexose isomerase & H: phosphogluconolactone.

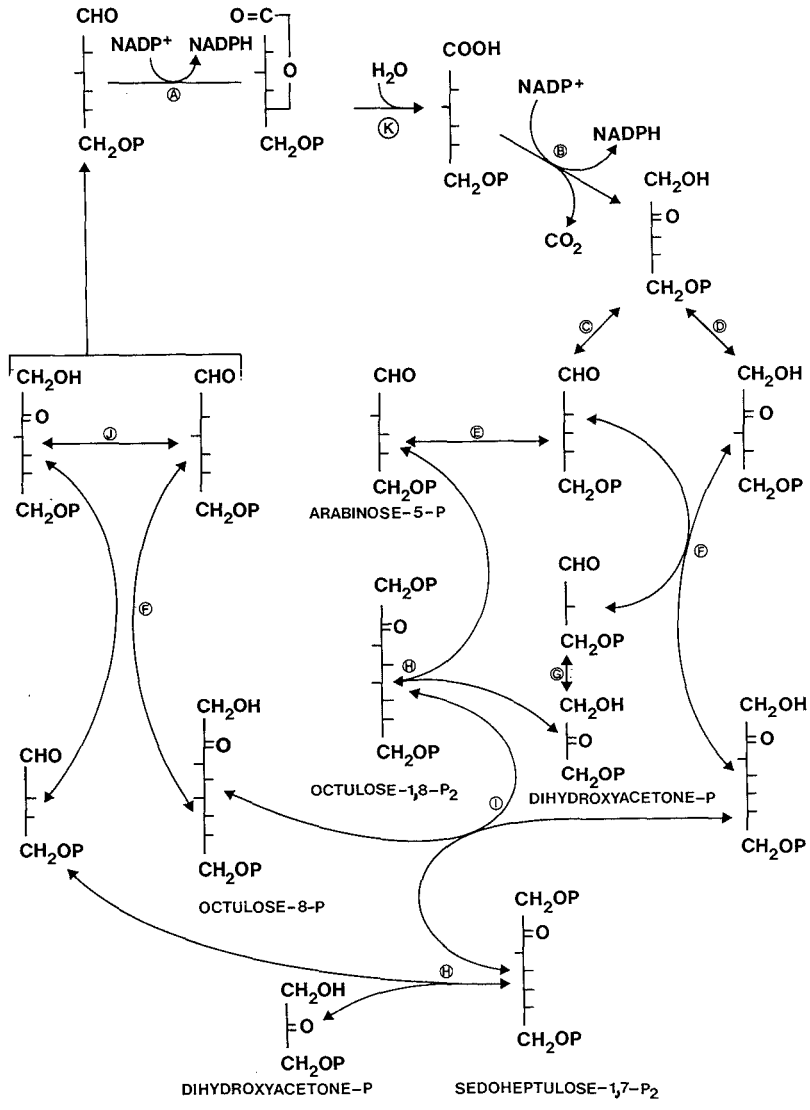

Figure 2:

The L-type of the pentose phosphate pathway according to Williams (1981). A,B,C,D & F: signify the same as in Figure 1., E: pentose-5-phosphate-2--epimerase, G: triosephosphate isomerase, H: aldolase, I: phospho-transferase, J: phosphohexose isomerase & K: 6-phosphogluconolactonase.

CHAPTER 2

MATERIALS AND METHODS

As seen in the publications and in other chapters of the actual presentation different types of tissues and cells were used in the course of this project.

The methodology including sampling techniques, collecting and preparing the specimens, freezing procedures, eventual use of fixatives, cutting and staining methods were given in details in each paper.

The backgrounds of the techniques used for treating our material might be considered similar but minor adaptative variations were inevitable depending on the nature and type of tissues or cells investigated. Experience showed that immediate freezing in pre-cooled isopentane had an absolute preferability when compared with other freezing techniques.

The lists of enzymes histochemically investigated for each study were given in every publication separately and summarized in Table 1 of this chapter. The biochemical enzymatic assays on tissue homogenates were also referred in each publication together with the methods for preparing the cytosolic extracts. A list of the biochemical methods is given in Table 2.

Additional non-enzymatic histochemical and/or histological stains were applied in addition to the classical Haematoxylin and Eosin stain. These were listed together with all appropriate references in the publications and/or general references. The details of these stains when not mentioned, modified or referred were performed as listed by Pearse (1968).

TABLE1: ENZYME HISTOCHEMICAL TECHNIQUES USED

ENZYMES WITH ABBREVIATIONS	E.C. NUMBERS	REFERENCES
Lactate:NAD^+ oxidoreductase (LDH)	1.1.1.27	Meijer (1973)
3-Hydroxybutyrate:NAD^+ oxidoreductase (HBDH)	1.1.1.30	Barke & Anderson (1963)
Malate:NAD^+ oxidoreductase (MDH:NAD^+)	1.1.1.37	Barka & Anderson(1963)
Malate:$NADP^+$ oxidoreductase (MDH:$NADP^+$)	1.1.1.40	Meijer & deVries(1975)
Isocitrate:NAD^+ oxidoreductase (ICDH:NAD^+)	1.1.1.41	Barka & Anderson(1963)
Isocitrate:$NADP^+$ oxidoreductase (ICDH:$NADP^+$)	1.1.1.42	Meijer & deVries(1975)
Phosphogluconate:$NADP^+$ oxidoreductase (PGDH)	1.1.1.44	Meijer & deVries(1974)
Glucose-6-phosphate:$NADP^+$ oxidoreductase (GPDH)	1.1.1.49	Meijer & deVries(1974)
Glycerol-3-phosphate: menadione oxidoreductase (GPOX)	1.1.99.5	Lojda et al.(1979); Elias et al.(1984b)[6]
Glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (GADH)	1.2.1.12	deVries et al.(1980)
Succinate:PMSoxidoreductase (SDH)	1.3.99.1	Nachlas et al.(1957)[1]
Amine:O_2 oxidoreductase (MAO)	1.4.3.4	Glenner et al.(1957)2
NADPH:Nitro BT oxidoreductase (NADPH)	1.6.99.1	Burstone (1962)[4]; Elias et al (1984b)[6]
NADH:Nitro BT oxidoreductase (NADH)	1.6.99.3	Burstone (1962)[4]; Elias et al.(1984b)[6]
Cytochrome c oxidase (CO)	1.9.3.1	Burstone (1962)[3]
Peroxidase (PO)	1.11.1.7	Christie & Stoward(1978)
a-Glucan phosphorylase (GP)	2.4.1.1	Meijer (1968)
Phosphofructokinase (PFK)	2.7.1.11	Meijer & Stegehuis(1980)
Phosphoglucomutase (PGM)	2.7.5.1	deVries & Meijer(1976)
Arylesterase (NE)	3.1.1.2	Meijer & Vloedman(1973) Elias et al.(1984b)[6]
Acetylcholine esterase (ACE)	3.1.1.7	Karnovsky & Roots(1964)
Alkaline phosphatase (ALP)	3.1.3.1	Pearse (1972); [5] Elias et al (1984a)[6]
Acid phosphatase (AP)	3.1.3.2	Meijer (1972); Elias et al (1984b)[6]
5-Nucleotidase (AMP)	3.1.3.5	Pearse (1972)
Aryl sulphatase (AS)	3.1.6.1	Koudstaal (1975)
Mitochondrial ATPase (Mg^{++}activated)(ATP-Mg^{++})	3.6.1.3	Meijer & Vloedman(1980)
Myosin ATPase (ATP-Ca^{++})	3.6.1.3	Meijer (1970)
B-glucuronidase (Gluc)	3.2.1.31	Meijer & Vloedman(1973); Elias et al (1984b)[6]
Glucose-6-phosphate isomerase (GPI)	5.3.1.9	deVries & Meijer(1976)

NOTES:

1. **Phenazine methosulphate** was added to the incubation medium at a final concentration of 50 μg/ml.
2. **Epinephrine** was used as substrate.
3. **P-Aminodiphenylamine** and **p-methoxy-p-aminodiphenylamine** were used as substrates. The reactions were performed with and without **cytochrome c** added to the incubation medium at a final concentration of 0.5 mg/ml.
4. **NADH** or **NADPH** was used as substrate.
5. **Naphthol AS-MX phosphate** was used as substrate and **fast blue BB** as diazonium coupler.
6. Exclusively for cytologic material.

TABLE 2: BIOCHEMICAL ENZYME TECHNIQUES USED

ENZYMES WITH ABBREVIATIONS	E.C. NUMBERS	REFERENCES
Lactate:NAD^+ oxidoreductase (LDH)	1.1.1.27	Wilson et al. (1963)
Isoenzymes of LDH		Brendel et al. (1973)
Isocitrate:$NADP^+$oxidoreductase(ICDH:$NADP^+$)	1.1.1.42	Kornberg (1955)
Phosphogluconate:$NADP^+$ oxidoreductase (PGDH)	1.1.1.44	Hohorst(1965)
Glucose-6-phophate:$NADP^+$ oxidoreductase (GPDH)	1.1.1.49	Löhr & Waller (1965); Kornberg & Horecker(1955)
Glycerol-3-phosphate:acceptor oxidoreductase(GPOX)	1.1.99.5	Dawson & Thorne (1969)
Succinate:PIDP oxidoreductase (SDH)	1.3.99.1	Neufeld et al. (1954)[1]
Transketolase (TK)	2.2.1.1	Wagner et al. (1978)
Transaldolase (TA)	2.2.1.2	Wagner et al. (1978)
Phosphofructokinase (PFK)	2.7.1.11	Opie & Newsholme (1967)
Alkaline phosphatase (ALP)	3.1.3.1	Morgenstern et al. (1965)
Isoenzymes of ALP		Sundblad et al. (1973)
Acid phosphatase (AP)	3.1.3.2	Meijer & Israël (1978)
Fructose-1,6-diphosphate 1-phosphatase	3.1.3.11	Opie & Newsholme (1967)
$NADP^+$ glycohydrolase (GH)	3.2.2.6	Bonsignore et al.(1968)
$NADP^+$ pyrophosphatase (PP)	3.6.1.9	Bonsignore et al. (1968)
D-ribulose-5-phosphate-3-epimerase (RPE)	5.1.3.1	Wagner et al. (1978)
Ribose-5-phosphate isomerase (RPI)	5.3.1.6	Wagner et al. (1978)

1 PIDP stands for 2,6-dichlorophenolindophenol

CHAPTER 3

GENERAL DISCUSSION

In this chapter the presence, distribution, activity and fluctuations in activity of the main enzymes under investigation, GPDH and PGDH, will be discussed and when necessary correlated with that of other enzymes evaluated, specifically enzymes belonging to the major energy metabolic pathways. Our observations and their significance were discussed in each separate publication . Although the capita selecta in this project form comparable items, it seems appropriate to discuss here the relevant findings under titles concerned with the type of tissue or conditions investigated.

3. 1. HUMAN AND ANIMAL SKELETAL MUSCLE FIBRES IN HEALTH AND DISEASE.

In contrast to traditional and current observations reviewed in the literature (Lehninger, 1975), the activity of the pentose phosphate pathway is in fact demonstrable in apparently normal or healthy skeletal muscle fibres. The use of reliable and more sensitive enzyme histochemical technology has demonstrated repeatedly throughout all our experiments that both PGDH and GPDH are active to a minor degree in human skeletal muscle fibres and in all other vertebrate muscles investigated. However, this activity is limited when compared with enzymes of the major metabolic pathways and is not high enough to be used for muscle typing. Experience showed that enzyme histochemical investigations are indispensable for identification and classification of the different types of skeletal muscle fibres according to their metabolic pattern. We have been able to discriminate clearly between at least

three types of fibres. Type I are equivalent to red muscle fibres, relatively rich in enzymes belonging to aerobic metabolism. These are the poorest in the activity of GPDH and PGDH. Conversely, Type II fibres are relatively rich in glycolytic or anaerobic enzymes and correspond to the designated white fibres. They are also the richest in GPDH and PGDH activity. The third type of muscle fibres was described according to the observations as "Intermediate Muscle Fibres" or IMF. Such fibres contain a variable scale of enzymes belonging to aerobic and anaerobic pathways. They demonstrate thus an intermediate type of metabolism. Under physiological or normal conditions they show an intermediate activity of GPDH and PGDH when compared with Type I or Type II fibres. Again, the activity of both enzymes is still not strong enough to be used alone for fibre typing. For this purpose we advise the investigation of different oxidoreductases beside the classically applied ATP-Ca^{++}. The activity of PGDH is apparently a little more manifest than that of GPDH in all examined muscle fibres in contrast to their activity in, for example, endothelial cells, smooth muscle fibres of the blood vessels and connective tissue. The relatively higher activity in such structures is consistent with the biochemical observations of Garcia-Buñuel & Garcia-Buñuel (1967) and Opie (1973). The biochemical findings confirmed the presence of the oxidative enzymes of the pentose phosphate pathway in muscle tissue. Though the histochemistry had the definite advantage of allowing morphologically visible distribution of these enzymes.

Considering GPDH as the rate-limiting enzyme of the pentose phosphate pathway, the findings favour the conclusion that the maximum metabolic flux of the pathway through this enzyme is very low in normal skeletal muscle fibres. Forward reactions of the pentose phosphate pathway are strongly influenced by the levels and concentration ratios of $NADP^+$/NADPH in the cells. The very low activity of GPDH and PGDH may mean a low need for NADPH or that the reduced coferment necessary for reductive processes, is primarily gained through the activity of ICDH:$NADP^+$. This can be explained by the strong activity of NADPH-dependent ICDH in apparently normal and diseased muscle fibres, especially type I.

As seen in publications: 4.2, 4.3, 4.4, 4.5, 4.6 the constancy

of activity ratios of enzymes in an equilibrium at physiological state (Pette et al., 1962; Hales, 1967; Jöbsis & Meijer, 1973; Jöbsis et al., 1976; Spamer & Pette, 1977; Pette & Hofer, 1980) is frequently lost in diseased muscle fibres. A very important feature is the rapid increase of the activity of GPDH and PGDH after nearly any insult to the muscle fibres causing either myogenic or neurogenic disease. When fibre typing is still possible, it is apparent that the increase of activity of GPDH and PGDH is demonstrable in all fibre types especially Type II where both enzymes become very active.

The distribution of the increased activity is comparable in the conditions investigated, but the extent is dependent on the nature and severity of the disease. A tremendous increase of activity is observed in skeletal muscle diseases such as Duchenne muscular dystrophy and in the atrophic fibres of infantile spinal muscle atrophy where both enzymes become very active in all pathologically altered muscle fibres irrespective of their original type. In other conditions such as myotoxic effect of drugs, (4.4, 4.7), vitamin E deficiency (4.5) and nervous injury (Elias et al, under publication 1988) the vulnerability of different muscles and muscle fibres might vary slightly and the increase of activity of GPDH and PGDH can be used as a valuable diagnostic parameter for the pathological change even before the occurrence of any observable morphological change.

Because inflammatory cells, especially leukocytes and tissue histiocytes, are rich in activity of GPDH and PGDH it was important to examine inflammatory conditions in muscle specimens histochemically for the identification of the origin of any biochemically measurable increase in activity of both enzymes. The histochemical observations showed that although the presence of inflammatory cells may contribute partially to the measured enzymatic activity in the homogenates, the muscle fibres contribute the greater part of this activity as seen in the histochemical sections. This was also manifest after cessation of the inflammatory phases of the diseased muscles. The activity of GPDH and PGDH remained high in the pathological specimens when measured biochemically or seen histochemically. Other tissue components such as blood vessel walls, endothelium, nerve fibres, connective tissue, fatty

cells or other tissue contaminants evidenced a balanced enzymatic activity of GPDH and PGDH which does not increase or decrease in health or disease.

These findings are important because at the time of our early investigations, most of the authors did not believe that the pentose phosphate pathway really contributed to the metabolism of muscle fibres or that any activity of its enzymes originated in the proper muscle fibres. The biochemically measurable activity had been attributed to the presence of other tissues such as fatty cells and connective tissue fibres (Laudahn, 1963; Laudahn & Heyck, 1963).

The activity of non-oxidative enzymes of the pentose phosphate pathway investigated, was observed to increase in some cases of Duchenne muscular dystrophy and spinal muscle atrophy. Although this increase is less marked than that of the oxidative branch of the pathway, it is possible to conclude that the activity of the pathway as a whole was enhanced (4.6).

When we take into consideration the integrated results of our investigations, it appears that the GPDH and TK are the two principal enzymes controlling the two branches of the pathway. This was confirmed by Wood (1985), who stated that there may be a control on level of PGDH and TK.

The data given in publication 4. 6. demonstrates that although the increase in activity of TK is less striking than that of GPDH or PGDH, the maximal flux of the pathway is controlled by the activity of both oxidative enzymes.

The stimulation of the pathway means an increase in the demand for the specific metabolites produced through its reactions (Wood, 1985). This is understandable in the case of muscle fibres too. In healthy subjects, the muscle fibres need to perform high energy demanding, vital activities which then become strained in pathological conditions. At the time of our early publications we tried to correlate the metabolic importance of the pentose phosphate pathway to the activity of major metabolic pathways either aerobic or anaerobic and their metabolites. Currently the occurrence and detoxication of oxygen-free radicals is receiving more attention as will be discussed further. The increasing knowledge of the free radical formation in injured tissues raises the question of any possible correlation between our observations in the

injured muscle fibres, mainly the increased activity of the NADPH regenerating enzymes of the pentose phosphate pathway, and this increase in formation of toxic free radicals as a parameter to the gravity of the lesions.

Currently it is acceptable that there are many ways to injure and to damage tissue cells. Among these the class of reactions dependent on the formation of free radicals and their intermediates which can trigger an expanding network of multifarious disturbances.

Free radicals can be defined as molecules or molecular fragments with an unpaired electron. These unpaired electrons contribute certain characteristic properties to the free radicals including their high chemical reactivity. The free radicals can be positively charged, negatively charged or electrically neutral. They are formed by processes of homolytic bond fission (reaction 1) or by electron transfer reactions (reaction 2).

$$A : B \longrightarrow A^{\circ} + B^{\circ} \qquad (1)$$

$$A^{-}: + B \longrightarrow A^{\circ} + B^{\bar{\circ}} \qquad (2)$$

In general such processes proceed either through the absorption of radiation energy or by redox reactions such as non-enzymatic electron transfer reactions, metal ion-catalyzed reactions or enzyme-catalyzed processes. Extensive studies with model systems and with biological materials in vitro have clearly shown that free radicals are able to produce chemical modifications of and damage to proteins, lipids, carbohydrates and nucleotides. Therefore, if such reactive, free radicals are produced in vivo or in cultured cells in vitro in amounts sufficient to overcome the normally efficient protective mechanisms, one can expect metabolic and cellular disturbances to occur in various ways.

Free radical-induced lipid peroxidation is known to cause changes in membrane organisation and function in skeletal muscles and in other tissues (Tappel, 1973), including increased calcium permeability (Lebedev et al., 1980; Shalev

et al., 1981). Several workers have proposed that membrane lipid peroxidation may be an important factor in the pathogenesis of Duchenne muscular dystrophy (Kar & Pearson, 1979; Hunter et al, 1981; Clark, 1984; Jackson et al., 1984). Evidence for this includes elevated concentrations of lipid peroxidation products (Kar & Pearson, 1979; Matkovics et al., 1982; Mechler et al., 1984; Omaye & Tappel, 1974) and raised activities of several antioxidant enzymes in Duchenne muscular dystrophy (Kar & Pearson, 1979). The elevated activity of these enzymes in the diseased skeletal muscles is interpreted as being an unsuccessful response to an increased peroxidative challenge. Furthermore, altered membrane properties in Duchenne muscular dystrophy are consistent with the known effects of lipid peroxidation (Baird et al., 1980).

If reactive free radicals formed inside cells can produce such a wide diversity of severely damaging reactions, life encompasses constant danger fortunately dealt with by powerful defense systems. The latter make use of different mechanisms including the use of antioxidants. An antioxidant has at least four different modes of action: quenching of electron mobility, scavenging of free radicals, breaking of free radical chain reactions and trace metal chelation. One of the most important systems is the glutathione-SH:peroxidase reaction which is illustrated in figure 3.

The role of the pentose phosphate pathway as NADPH producer is illustrated in figure 1. The inhibition of GPDH and PGDH by overflow of NADPH can be abolished by GSSG and glutathione peroxidase (Eggleston & Krebs, 1974).

The observable dramatic increase in activity of both enzymes in the muscle diseases investigated may, in a similar fashion be correlated to raised production of free radicals in the pathologically altered metabolism. This assumption has been further studied through the biochemical and enzyme histochemical evaluation of the activity of PGDH and GPDH in diseased skeletal muscles of experimental animals such as rats and rabbits.

The subcutaneous injection of N,N'-dimethyl-p-phenylenediamine (DPPD or N,N'-dimethyl-p-diamino benzidine), in rats (4.4) was sufficient to induce severe myopathy in experimental animals. In further investigations (4.7) the myotoxic effect of DPPD could be enhanced by simultaneous injection of hyalinuronidase

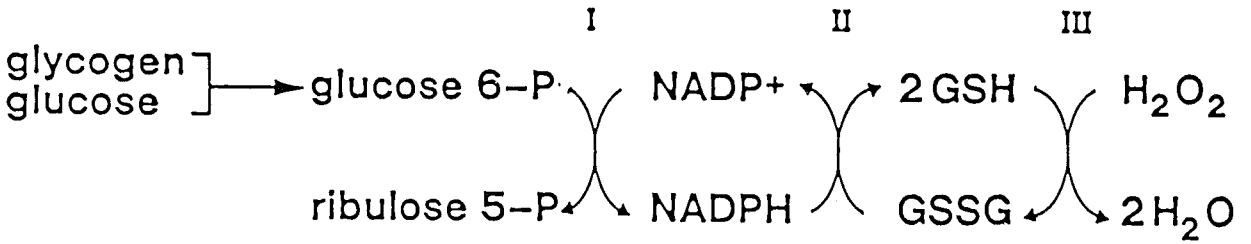

Figure 3: Coupling of hydrogen peroxide removal to the oxidative branch of the pentose phosphate pathway through the GSSG system.

which resulted in a rapid effect of the drug and faster development of the disease without any specific affect on the enzymes investigated.

The desired myopathy in rabbits (4.5) could be initiated by depletion of a-tocopherol[@] (vitamin E) through dietary deficiency.

In both types of muscle disease the pathological changes are probably the result of higher concentration of free radicals. In the case of rat muscles this might be a mechanism of higher production and in rabbit muscles a deficient scavenging system.

Electronrich substances such as N-substituted diaminobenzidines generate free radicals in metabolism. At first these substances undergo N-hydroxylation followed by an oxidation step in which a nitroxy radical can be formed (Stier et al., 1982).

The role of a-tocopherol as an important biological anti-oxidant has been actively considered (Tappel, 1973; Horwitt, 1976).

The mode of action of a-tocopherol as a free radical scavenger in the biological membrane was first proposed by Tappel as follows:

$$ROO^{\circ} + a\text{-}TH \longrightarrow ROOH + a\text{-}T^{\circ}$$

$$ROO^{\circ} + a\text{-}T^{\circ} \longrightarrow ROOH + a\text{-T chinon}$$

According to this scheme, a-tocopherol can donate hydrogen to the peroxy type free radicals resulting in formation of hydroxyperoxides. These hydroxyperoxides can be decomposed to the corresponding nontoxic hydroxy compounds by glutathione peroxidase (Chia et al, 1982).

@: "a-" stands here and throughout the following text for "alpha-".

The observations described in publication 4.4. show that the subcutaneous administration of DPPD in rats results in a myogenic myopathy. The pathological morphological and histochemical characteristics are the same in the different muscles. Only the severity of the pathological features varies. The red muscles such as m. soleus are more vulnerable to DPPD intoxication. The most important histochemical observation is the early increase in activity of GPDH and PGDH as early as 8 hours after a single injection of DPPD and long before the appearance of any noticable morphological change.

The early morphological changes in the affected muscles in experimental rats has been the appearance of scattered, rounded, often swollen or rather hypertrophic fibres which can be seen after at least one to two days. At such early phases of the experiments, fibre typing is still possible. The anaerobic or glycolytic fibres are, in this case, first affected especially in muscles which are essentially rich in red or aerobic fibres. When the disease advances with appearance of so called "ragged red" or degenerative fibres associated with inflammatory infiltrate, the typing of the fibres becomes very difficult or impossible even using a large battery of both aerobic or anaerobic enzyme reactions. The "constant proportion character" of the muscle fibres, a term used by Bücher & Pette (1965), becomes partially and then completely lost. The biochemical findings reveal a maximal increase in activity of both oxidative enzymes of the pentose phosphate pathway after 4 days of DPPD injections. GPDH shows a 13-fold increase in m. soleus. The increase of activity in white muscles: m. rectus femoris is only up to 7-fold and 8-fold in m. gastrocnemius. All compared with control specimens. The maximal activity can be attributed partially but certainly not completely to the presence of an inflammatory cellular infiltrate which is at its highest about that time.

In the last years considerable attention has been directed to the involvement of free radical intermediates in the inflammatory processes (Kuehl et al., 1979; McCord & Wong, 1979). Background information on this interesting area can be obtained from reviews by McCord et al., (1980); Autor, (1982) and Torrielli & Dianzani, (1984). A similarly active field of

study has been the role of the free radical intermediates in cell killing by leucocytes (Badwey & Karnovsky, 1980; Babior, 1982a & b; Boeksteger & Grundmann, 1985). This consists with our observation of high activity of GPDH and PGDH in the inflammatory cells especially the neutrophils. The experience with DPPD induced myopathy in rat skeletal muscles reveals a linear correlation between the severity of the morphological change, the number of the inflammatory cells present and the activity of PGDH and GPDH. Though such observations do not provide explicit evidence, they suggest a causal relationship between the production of free radicals and the increased activity of both enzymes.

In publication 4.5. the activity of GPDH and PGDH was studied in skeletal muscles with a-tocopherol deficiency induced myopathy. Again the histopathology of the diseased muscles did not show qualitative differences between muscles or fibres involved. The differences were in the vulnerability of the different fibres in various muscles. There were only quantitative differences in the severity of the disease. Early in the experiment, m. soleus and the diaphragm were the most affected muscles. The least affected was the m. plantaris. The specimens from red parts of m. gastrocnemius were more affected than the white ones. The gravity of the histological change was related to the extent of the biochemical changes and the histochemical alterations. But the histochemical changes were demonstrable much earlier before the appearance of the morphological disturbances. A marked increase in the activity of GPDH and PGDH was observable early in the experiment before the myopathy was clinically or morphologically manifest. Although this increase was present in all types of muscle fibres, it was most obvious in the anaerobic, Type II fibres. This rise could be observed microscopically as early as the 10th experimental day. The activity continued to increase steadily and was most marked in the 35 day and 45 day experimental animals. Biochemical results demonstrated in m. gastrocnemius a maximal rise of up to 8- to 9-fold for GPDH and 5- to 7-fold for PGDH. In m. plantaris this increase in activity was somewhat higher and in m. soleus it was lower. The biochemical increase in the homogenates might be partially attributed to the histologically and histochemically observed inflammatory cells

rich in both enzymes. A great resemblance between the histochemical, histological and biochemical findings in diseased rabbit muscles to those in muscles of rats treated with DPPD could be observed. Similarly a suggested positive correlation between the increased activity of the oxidative enzymes of the pentose phosphate pathway and the concentration of free radicals in vitamin E deficiency induced myopathy; beside other metabolic implications discussed in the publication.

The investigation presented in chapter 4.7 has been intended to study the nature and origin of the increased activity of the enzymes concerned.

Several possibilities can be proposed for the molecular mechanism underlying the increased activity (Watanabe & Taketa, 1973; Rifenberick et al., 1974; Wagner et al, 1978; Max & Wagner, 1979). The most likely are:

1. Enzyme induction involves formation of new messenger-RNA for the enzymes.

2. Existing messenger-RNA is activated to produce more enzyme molecules.

3. A soluble activating or inhibitory cell constituent associates reversibly with the enzyme molecules to modulate its activity.

4. Both enzymes are continuously inactivated by catabolic processes, either proteolytic (Barret & Dingle, 1971) or non-proteolytic (Francis et al, 1980), and these processes are affected by the inducing signal so that the net concentration of the enzymes is altered.

The first two possibilities lead to synthesis of new enzyme molecules. These possibilities were ascertained with the experiments described in publication 4.7.

In this study a combined administration of DPPD and protein synthesis inhibitors was applied. Due to the high toxicity of the drugs used in the experiment, especially cycloheximide and actinomycin-D, and the fact that the rats would not survive a long term experiment such as that in publication 4.4 where only DPPD was used; the experimental model was modified. The simultaneous injection of hyaluronidase was introduced for the

first time to enhance the drug effect and induce a rapid effect from the myotoxic drugs administrated. The animals were treated indiscriminatively including the control groups. The sanity of the latter was tested by using other controls which had no drug treatment at all to exclude any possible effect of the hyaluronidase itself.

The experimental rats injected with DPPD produced the same myopathy as in preceeding studies but within a shorter time and with the same biochemically and histochemically observable dramatic increase in activity of GPDH and PGDH as early as few hours after a single administration. The groups with combined administration of DPPD and cycloheximide or actinomycin-D did not show any demonstrable rise in activity of GPDH or PGDH. The actinomycin-D is known to inhibit transcription of DNA by RNA-polymerase and cycloheximide interferes with protein synthesis (Beard et al., 1969).

Our observations were in agreement with the findings obtained by the study of adaptive enzyme systems observed in mammalian tissue cells such as enhancement of GPDH activity in liver (Rudack et al., 1971; Garcia & Holten, 1975; Dao et al., 1979) and the increase of PGDH activity in liver (Procsal et al., 1976) which occur through newly synthetised enzyme proteins.

Concerning the third possibilty, the results of the activity determinations performed on homogenate mixtures of m. gastrocnemius of animals with induced myopathy, control groups and induced and repressed groups made it very unlikely that there were activators or inhibitors in the homogenates to modulate the activity of both enzymes. Modifying factors altering the kinetic properties of hepatic GPDH were described by Kahn et al. (1976) and Dao et al. (1979).

Testing for the last possibility is far from the object of our studies. From our investigations (4.7) we came to the conclusion that the rise in the activity of GPDH and PGDH is most likely the result of neosynthesis of enzyme molecules.

What is the metabolic significance of the increased activity of GPDH and PGDH in diseased muscle fibres ? At first the increase in activity of both enzymes in diseased human skeletal muscles, in particular in patients suffering of Duchenne muscular dystrophy or infantile spinal muscular

atrophy, and in pathologically altered skeletal muscles of experimental animals indicated a higher production of NADPH. As already discussed this coferment is necessary for different reductive synthetic processes and may be useful for the elimination of the free radicals.

Next, because the activity of transketolase, transaldolase, ribose-5-phosphate isomerase and ribulose-5-phosphate-3-epimerase is significantly increased in investigated diseased human skeletal muscles as well, this means that the capacity of the whole pathway is increased, (4.6). This conclusion is based on the fact that GPDH and TK are the two enzymes controlling the capacity of respectively the oxidative and nonoxidative branch of the pentose phosphate pathway. The data of Table 1 in publication 4.6 show that although the increase in activity of the studied non-oxidative enzymes is relatively less, the maximal flux of the pathway is still stipulated by GPDH. The stimulation of the whole pathway means a rise in synthetic capacity for e.g. ribose-5-phosphate necessary for the production of ATP, CoA, NAD^+, FAD, RNA and DNA. Very probably the stimulation of the pathway means an increase in the demand for specific metabolites produced through its action (Wood, 1985). This is understandable because processes such as degeneration, regeneration and inflammation are demanding energy. Through the links between the PPP and the glycolytic pathway a rise in cytoplasmic ATP regeneration is probable.

3. 2. Human heart muscle fibres and the conducting system

Two consequent studies concerned with the metabolic activities in the human heart were conducted In the first publication (4.8.) special attention was given to the histochemical evaluation of enzymes which play a role in the energy metabolism in Purkinje fibres of the conducting system correlated with the enzymatic activity of the same enzymes and histochemical components in the proper "working myocardial fibres." The fibres of the conducting system distinguished themselves from the working myocardial fibres by exhibiting a higher activity of the majority of glycolytic enzymes examined. The activity of phosphofructokinase, the glycolytic key enzyme, is noteworthy. The aerobic enzymes were slightly less active in Purkinje fibres than in working muscle fibres. The PGDH is apparently more active than GPDH, especially in Purkinje fibres, while the activity of GPDH is equally distributed in both fibres. The activity of these NADPH regenerating enzymes is not significantly high or is very low when compared to that of ICDH:$NADP^+$ in specimens from patients without manifest cardiac diseases. The finding has certain parallels to the previously discussed observations in the skeletal muscle fibres with the exception that clear-cut typing of skeletal muscles into Type I, Type II and IMF is absolutely invalid for working or conducting cardiac muscle fibres.

In the following study (4.9.) the same approach was extended to necropsy specimens obtained from patients with established clinical features of myocardial disease. The specimens were classified in three categories: 1) early ischemic change associating myocardial infarction with or without damage to the conducting system, 2) chronic ischemic changes such as cardio-vascular insufficiency, advanced coronary sclerosis, old infarctions and combinations & 3) inflammatory conditions such as myocarditis.

The findings and their metabolic implications, especially with regard to the energy metabolism are discussed in detail in the publications concerned. Some additional remarks which are considered essential are provided below.

The manifest increase in activity of PGDH and to a more extensive degree of GPDH which occurs instantly after any acute ischemic change provides in addition to the metabolic significance a reliable diagnostic measure. The chronic phases of ischemia cordis with or without infarction are also characterized by a high degree of activity of both enzymes either in the conducting system or in the working myocardiac fibres. The hypertrophic fibres in the periphery or in the vicinity of fibrotic areas exhibited more activity of such enzymes than apparently normal or less hypertrophic fibres.

The examination for activity of both enzymes is recommended as a diagnostic measure for ischemic change when inflammatory conditions are excluded. Particularly the examination for the activity of GPDH is more valuable because the rise in its activity is more striking than that of PGDH. The distribution of enzymatic change is parallel to the pathological distribution. It is, therefore, understandable that patients with a bundle branch block or ventricular fibrillation show more localized enzymatic disturbances mainly at the affected bundle branch.

GPDH in liver has a half-life of about 15 hours (Freedland, 1968; Goldberg & St.John, 1976). Although the half-life of the enzyme in heart muscle has yet to be specifically determined, the rapid response of cardiac muscles to ischemic change suggests that the half-life of GPDH in heart muscle fibres is also short. Generally speaking, regulatory enzymes with a short half-life often show the capacity to fluctuate rapidly in response to environmental changes (Goldberg et al., 1976). The strong increase in activity of GPDH & PGDH after myocardial infarction gives rise to the interesting concept that these enzymes, which play under physiological conditions with adequate oxygen supply a minor role for cardiac metabolism, creates a reserve metabolic capacity. This will be adequately and instantly stimulated to cover energy demands in life threatening conditions, particularly in working or conducting heart muscle fibres.

Damage to muscle fibres in the human heart is known to induce release and leakage of cytosolic enzymes. This phenomenon was demonstrated in our experience by a reduction of the staining intensity or early leakage from the cells in tissue sections at the time of or even before the appearance of observable microscopical changes. Also the activity of some mitochondrial enzymes as SDH decreased or disappeared completely. Such phenomena occur in a degree corresponding to the severity and extent of the lesion. The appearance of high serum levels of LDH fractions which could originate in the heart correspond to an apparent leakage from the damaged fibres in the histochemical sections. There is no a priori reason to exclude other sources of these isoenzymes in the serum of patients other than heart muscles. A noteworthy observation is that among the investigated series of enzymes with a vital role, the oxidative enzymes of the PPP were the only enzymes which showed an evident increase in activity while most of other enzymes decreased, leaked or remained unchanged in the dying cells.

The increased lipid accumulation found in the ischemic fibres is in agreement with the biochemical observations concerning lipid metabolism. Fatty acids are catabolized preferentially by the normal heart (Krebs, 1972) and oxidation of lipids supplies large quantities of ATP. Under ischemic conditions the oxidation of lipids becomes hampered and accumulation of lipids occurs. At least hypothetically, the capacity of lipid synthesis may be induced or enhanced because of the increased supply of metabolites provided through the action of GPDH and PGDH. However, the studies of Wit-Peeters et al., (1970) stated that no demonstrable "de novo" fatty acid synthesis in normal hearts of guinea pigs occurs.

Free radicals are chiefly produced in the mitochondria in the cardiac metabolism, (Loschen & Flohé, 1971; Nohl & Hegner, 1978). Lipid peroxidation, as a mechanism of free radical pathology, is increased under ischemic conditions, and has been suggested to play an important role causing irreversible membrane damage (Meerson et al., 1982). The presence of reactive leucocytic infiltrates, also after an acute ischemic change, may contribute to the delivery of free radicals. As discussed under the subject of skeletal muscle fibres (3.1),

the possibility of a correlation between the increased concentration of the free radicals in the damaged cell and the increase in activity of PGDH and GPDH might be emphasized. This will be valid, too, in case of the ischemic and inflammatory heart changes. According to Bodaness (1982), the activity of both enzymes plays a significant role in eliminating the free radicals.

3. 3. Other tissue cells in dynamic change: human endometrium and proliferative lesions in different organs.

An enzymatic histochemical approach to the cyclic changes and different patho-physiological conditions in the female reproductive system was attempted. The observations were discussed in several publications (Elias, 1981, 1984a & b, 1985b; Elias et al., 1983a, b & c). The most relevant work concerned with the endometrium and directly related to the subject of this thesis is annexed as chapter 4.10.

From this study and related presentations it was apparent that the human endometrium, and to a lesser extent the cervical mucous membrane, show a dynamic enzymatic response to physiological or pathological triggers, including the hormonal balance or the different phases of the cycle. Both stroma and glandular epithelium respond dramatically to the effect of hormones. Estrogen controls and influences the proliferative phase of a normal cycle. The progestagenic conditions initiate, influence and maintain the secretory and conceptional functions of the endometrium. This history is repeated each cycle during the reproductive life phase. Any artificial or natural change in the hormonal status influences the morphology and the function of the cells concerned. All functional, structural or morphological changes in the endometrium, including eventual changes in the subcellular construction of e.g. lysosomes, mitochondria, cell membrane or cytoplasm, have qualitative and/or quantitative enzymatic implications.

One of the interesting observations in the study is the histochemical demonstration of an obvious cyclic fluctuation of the activity of both oxidative enzymes of the pentose phosphate pathway. The enzymes reveal an abundant activity in the secretory phase in endometrial glandular epithelium and in the stroma. In pregnancy a high activity has been maintained in these structures and also in the trophoblast cells.

This high activity of GPDH and PGDH in the active cells may indicate a high capacity for synthetic processes during the secretory phase (Sawaragi & Wynn, 1969; Coulton, 1977). With regard to a possible role in the lipid biosynthesis, it is noteworthy that cells showing a high activity of both enzymes always contained a demonstrable lipid deposit. The presence of lipid material in secretory cells of the endometrium as well as in decidua cells may indicate an active lipid synthesis, an assumption which has been under discussion for many years. The concept of an active lipid synthesis is in agreement with the findings of Ascheim (1915) and Black et al. (1941) but in conflict with the opinion of Froboese (1924) and Craig & Danziger (1965) who suggested that the presence of stainable lipid material must be considered to be only a degeneration product. Since the activity of the key enzymes for the biosynthesis of lipids have not yet been studied in human endometrium, it would be premature at this moment to make definite conclusions about this subject. Nevertheless, it has been demonstrated through our observations that there is a possible association between the presence of lipids in the cells and the presence of an increased activity of PGDH and GPDH in the same cells in a parallel line. A striking fluctuation of the activity of ICDH:$NADP^+$ was observed in glandular epithelium. This too can be of a diagnostic value for the evaluation of secretory function of the glands concerned. The activity is highest in secretory cells and lowest in suppressed or inactive endometria while the proliferative phase is characterised by a rather low activity of ICDH:$NADP^+$. The surface epithelium showed a constant and low activity throughout the whole cycle.

To our knowledge, there is no literature decisive enough about the presence, activity or concentration of free radicals in human endometrium to draw a clear relation with the activity of GPDH and or PGDH.

The publications (4.11 & 4.12) are concerned with our observations during the study of benign and malignant proliferative lesions in a variety of organs, particularly the human mammary glands. Further histochemical experience with muscle pathology and material of proliferative diseases is in agreement with the published data and opens a few prospects of

further studies which are necessary to cover those constructs which are out of scope of this thesis.

In chapters 4.11 and 4.12 it was possible to conclude that malignant transformation in tissue cells is associated with a demonstrable increase in the activity of the pentose phosphate pathway enzymes examined, in particular the oxidative enzymes. This is in association with a higher activity of enzymes of the anaerobic pathways. Adenocarcinomas of almost all organs examined share, in general, many metabolic characteristics observed in mammary carcinomas. Certain differences characteristic to the differentiation expression of the tumour cells arising in a certain organ or to the malignant change in particular can be used as a diagnostic measure as discussed in the relevant publications. One of such observations is the increase in activity of non-specific esterases even in premalignant epithelium of the endometrium. Also the activity of alkaline phosphatase and its thermolabile or stable isoenzymes in adenocarcinomas of ovary and endometrium. Another example is the increased activity of aryl-sulphatase in malignant tumours and to a lesser degree in villous adenomas of the large bowel which are of potential malignant capacity. All such conditions were also characterized by a biochemically and histochemically demonstrable increase in the activity of PGDH and GPDH. Both enzymes show a tendency to increase in benign proliferative lesions too, especially in glandular epithelium. The greater the proliferation, the higher the activity. A relatively high activity of PGDH and GPDH was obvious in lactating mammary cells and adenosis. Nevertheless, the malignant cells exhibit higher activity of both enzymes than benign lesions. This was also manifested in melanomas when compared with benign naevoid tumours (Elias et al., in preparation). The same applies to the unusual metastasizing basal cell carcinoma of the skin (Elias et al., 1980); and the rather unfrequent metastasizing carcinoma of Bartholini glands (Elias et al, 1981). Cohen presented as early as 1964, a histochemical investigation of the activity of GPDH in (intra-) ductal carcinoma of the breast and their eventual metastasis in axillary lymph nodes. Despite the fact that he used conventional histochemical techniques which result in diffusion of the greatest part of enzymes

investigated into the incubation medium, as well as demonstration of the enzymes concerned through the action of auxiliary enzymes, he was able to conclude that GPDH is equally highly active in the primary as well as metastatic tumour cells. Our observations were in agreement with his findings and with the biochemical investigations on human breast tumours performed by Desphande et al. (1977 & 1981). These authors were also aware that a significantly higher concentration of PGDH is present in poorly differentiated tumour cells compared with highly differentiated ones.

Our observations often distinguished a patchy distribution of many enzymes investigated within the same tumour sample. The enzymes were more highly active in some tumour cell fields than adjacent fields. Such information can not be obtained by biochemical investigations on tissue homogenates which have too the same shortcomings experienced with muscle specimens. The biochemical assays are indiscriminative because of the presence of tissue contaminants in the homogenates of tumour samples such as stroma and blood vessels which contribute to the enzymatic activity. Conversely tissue proteins present without enzymatic activity or originating from other structures influence the statistical value and accuracy of the biochemical assays. This final handicap can be partially mitigated or avoided by measuring, not the absolute values of enzyme activities in units per weight, but the relative concentrations of enzymes present in homogenates of comparable samples of the benign and malignant tissues.

The patchy distribution of higher and lower enzymatic activity in different populations of malignant cells in the same tumour is absent in benign lesions. We have considered it as a sign of the polyclonality of the tumour cells after the malignant transformation. The differentiation or dedifferentiation of malignant cells may be associated with an uncontrolled genetic pattern as well as metabolic pattern.

The histochemically and biochemically evaluated activity of ICDH:$NADP^+$ showed a significant increase in activity in malignant lesions when compared with benign specimens of the same organs. This was not the case for the activity of

ICDH:NAD^+. We concluded that malignant lesions utilize more NADPH in their various metabolic activities produced either through the activated pentose phosphate pathway or other resources including the ICDH:$NADP^+$.

The rise in activity of TK, TA, RPI and RPE in the malignant lesions was less striking than that of the oxidative enzymes. The concentration of octulose- and heptulose diphosphates was variably increased in the malignant specimens (Meijer & Elias, 1984). These intermediate metabolites are described by Williams (1980, 1981) in the L-type of the pentose phosphate pathway.

The occurrence of free radicals in tumour tissue is still a subject of wide discussion. Many chemical carcinogens can be metabolically activated into free radical intermediates, but the true nature of these intermediates and their importance to the carcinogenesis is not fully established (Slater,1972; Ts'o et al.,1977; Demopoulos et al.,1980). Other examples of chemical carcinogens that are metabolically activated to free radicals or intermediates are documented by Mason et al. (1982) and Floyd (1982). Considerable interest arose after the suggestion of Copeland (1983) that some promotors can initiate the free radical reactions and that this type of activity may be significant to their mechanism of action in carcinogenesis.

Although several workers favour the idea of an increased formation of free radicals in tumour tissue, the work of Duchesne (1977), suggested a decrease in their concentration. He related this to a possible role of the accumulating anti-oxidants which may function in eliminating the free radicals. At present there is no final conclusion about the definite relationship between the free radical concentration and the activity of enzymes investigated, but it may be possible that the decrease in concentration of free radicals is caused by the high activity of NADPH regenerating enzymes GPDH, PGDH and ICDH:$NADP^+$.

CHAPTER 4

PAPERS DEALING WITH THE ABOVE DESCRIBED SUBJECTS.

Histochemistry 48, 257–267 (1976)

Histochemistry

CHAPTER 4.1

The Value of Enzyme Histochemical Techniques in Classifying Fibre Types of Human Skeletal Muscle

1. Adult Skeletal Muscles with no Apparent Disease of the Neuromuscular System

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Summary. Fibre-type classification of human skeletal muscle into type I and type II fibres is mostly based on their slight or strong staining with the myosin adenosine triphosphatase reaction. In order to evaluate the reliability of this screening technique a combined histochemical and biochemical study was performed on normal and diseased skeletal muscle of human subjects. In the present investigation activities of enzymes which play a role in the aerobic and anaerobic pathways and which can characterize fibre type, were examined in muscle specimens, with no apparent disease of the neuromuscular system. Special attention is given to the maximal activities of phosphofructokinase and fructose-1,6-diphosphatase, the rate limiting enzymes for the regulation of the glycolysis and glyconeogenesis, respectively. A most important feature of the biochemical findings is the constancy of the activity ratios of the examined enzymes. From these results and from the histochemical results it can be concluded that in apparently normal adult human skeletal muscle the ATP-ase technique for type I and type II typing is reliable. For fibres with an intermediate intensity of staining with the myosin ATPase technique of typing it is also necessary to apply other enzyme histochemical techniques.

Introduction

The advent of suitable enzyme histochemical techniques, and their successful application to normal and diseased skeletal muscle has not only produced a revolution in diagnosis, but has also resulted in the discovery of many new types of skeletal muscle disorders. With these techniques it is recognized that human skeletal muscle is not an uniform tissue, but is composed of different fibre types, with specific physiological and metabolic characteristics.

The metabolic characteristics of the muscle fibres have been investigated up till now by orthodox enzyme histochemical techniques. Although most conventional techniques are suitable for morphological studies, many techniques are

not reliable for metabolic studies. For instance, for the demonstration of the activity of NAD^+ and $NADP^+$ dependent oxidoreductases it is evident that the staining intensities and the sites of the staining deposits in the conventional techniques are dependent on the activity of the auxiliary enzymes NADH: tetrazolium oxidoreductase (EC 1.6.99.3) or NADPH: tetrazolium oxidoreductase (EC 1.6.99.1) respectively (Snijder and Meijer, 1970; Pearse, 1972; Meijer, 1975). Investigations of Fahimi and Amarasingham (1964); Ritter et al. (1971); Meijer (1973); Meijer and de Vries (1974, 1975) for instance, have shown that leakage of cytoplasmic non-structurally bound dehydrogenases during the incubating period may be considerable. For the purpose of classification of different types of muscle fibres the demonstration of phosphorylase activity can lead to faulty impressions. Apart from leakage of the enzyme during the incubation period another difficulty is that the demonstration needs the presence of glycogen in the tissue sections (Whelan and Cameron, 1964; Meijer, 1968). By using dextran as glucosyl acceptor (Meijer, 1968) the demonstration is rendered independent of the presence or absence of glycogen in the sections. None the less such administration of dextran does not diminish the leakage of the enzyme. Moreover, from the biochemical point of view it is very difficult to make conclusions about the real activity of the enzyme in situ (Whelan and Cameron, 1964). For these reasons only a few of the many available conventional enzyme histochemical techniques are reliable for the metabolic characterization of skeletal muscle fibres.

It is a pity that up till now the typing of human skeletal muscle fibres has been based almost exclusively on the demonstration of myosin adenosine triphosphatase (King Engel, 1974). Although in general, type I and type II fibres, as defined by myosin ATPase, correspond to those defined by activities of some oxidoreductases or α-glucan phosphorylase, we have to take into consideration that the activity of myosine ATPase will not give a *direct* impression about aerobic and anaerobic capacities of muscle fibres because myosin ATPase is not an enzyme that belongs to the glycolytic-, glyconeogenetic-, or citric acid cycle pathways. As selective influence of disease processes on one or other fibre type has been recognized and pathological changes involving the distribution of fibre types have been identified it is of the utmost importance that fibre typing based on metabolic characteristics must be performed by the use of reliable histochemical techniques.

One of the purposes of the present study is to investigate the reliability of the rather simple enzyme histochemical fibre typing for human skeletal muscle as suggested by King Engel (1974). In the study we have examined the histochemical activities of a serie of enzymes in muscle biopsies from human subjects without any apparent disease of the neuromuscular system, as well as from subjects with congenital or acquired muscular diseases. Use is made of a battery of conventional enzyme histochemical techniques and recently developed techniques in which diffusion of enzyme during incubation is hampered by using semipermeable membranes and in which the demonstration of dehydrogenases is not dependent on the activity of the diaphorases. (Meijer, 1973; Meijer and de Vries, 1974, 1975). In addition to these series, biochemical studies of assays on the important rate limiting enzymes for glycolytic and glyconeogenetic

pathways phosphofructokinase (EC 2.7.1.11) and hexosediphosphat phosphatase (EC 3.1.3.11) in muscle homogenates have been added. Such enzymes cannot be demonstrated histochemically in skeletal muscle tissue. The activities of some other enzymes important for fibre typing have been biochemically determined, and correlated as far as possible with the histochemical findings. In the present communication the results with apparently normal human skeletal muscle are presented.

Material and Methods

For the histochemical study fresh muscle biopsies of 24 adult human subjects with no apparent diseases of the neuromuscular system were rapidly frozen by immersing small tissue blocks in isopentane cooled to $-150°$ C with liquid nitrogen. The clinical investigation of the 24 human subjects was based on detailed neurological examination, including electromyography, blood and urine analysis. Moreover, for the diagnosis, microscopical examination of the muscle specimens stained with histological and histochemical techniques was performed. The biopsies were from m. quadriceps femoris (17), m. deltoideus (3), m. gastrocnemius (3), and m. latismus dorsi (1). Age of the subjects, female and male, ranged from 4 to 57 years.

Longitudinal and transverse serial sections were cut at 6 µ in a cryostat. Fixed or unfixed sections were stained using the following enzyme histochemical methods. The activities of α-glucan phosphorylase (GP) (EC 2.4.1.1.) was demonstrated according to Meijer (1968). The activities of mitochondrial adenosine triphosphatase (ATP-Mg) (Mg^{2+}-activated) (EC 3.6.1.3) and myosin adenosine triphosphatase (ATP-Ca) (Ca^{2+} activated) (EC 3.6.1.3) were demonstrated according to Wachstein and Meisel (1957a, b) and Meijer (1970) respectively. The activity of NADH: tetrazolium oxidoreductase (NADHOX) (EC 1.6.99.3) was demonstrated according to Burstone (1962) with the substrate NADH. The activity of succinate dehydrogenase (SDH) (EC 1.3.99.1) was demonstrated by the method of Nachlas et al. (1957), but with the incubation medium also containing phenazine methosulphate at a concentration of 50 $\mu g \cdot ml^{-1}$. The activity of glycerol 3-phosphate: menadion oxidoreductase (GPOX) (EC 1.1.99.5) was demonstrated according to Wattenberg and Leong (1960). The activity of cytochrome C oxidase (CO) (EC 1.9.3.1) was demonstrated according to Burstone (1962) with the substrates p-aminodiphenylamine and p-methoxy-p-aminodiphenylamine. The activity of lactate dehydrogenase (LDH) (EC 1.1.1.27) was demonstrated according to Meijer (1973). Activities of glucose-6-phosphate dehydrogenase (GPDH) (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (PGDH) (EC 1.1.1.44) were demonstrated according to Meijer and de Vries (1974). Activities of isocitrate: $NADP^+$ oxidoreductase (decarboxylating) (CDH) (EC 1.1.1.42) and malate: $NADP^+$ oxidoreductase (decarboxylating) (MDH) (EC. 1.1.1.40) were demonstrated according to Meijer and de Vries (1975).

For the biochemical study enzyme activities were determined in homogenates from a part of the frozen muscle biopsies. The tissue specimens were weighed and immediately after thawing cut finely in a tenfold volume 50 mM Tris-buffer (pH 8.2, 4° C) according to Opie and Newsholme (1967). After homogenizing for 30 s at 0° C in an all glass tight-fitting homogenizer, the homogenates were centrifuged for 10 min ($175 \times g$, 4° C) and the determinations were performed in a fixed sequence directly from the supernatant. Only the LDH reaction was determined from the supernatant after repeated centrifugation (45 min $20{,}000 \times g$, 4° C) of the first supernatant. The reaction rate ($20 \pm 1°$ C) was deduced from the linear part of extinction change after a time elapse of 10 min (Zeiss, Spectral photometer PMQ II). The reactions were followed for at least 15 min and carried out in quadruplicate; the supernatant in the four cuvettes being added in a volume ratio of 0:1:2:3. When the spread of the values, with regard to the average value, amounted to more than 10%, the reaction was repeated. The average value of the three observations formed the starting point for the calculation of the enzyme activity expressed in µmol converted substrated. $min^{-1} \cdot g\ tissue^{-1}$ ($20 \pm 1°$ C).

The activities of SDH (Neufeld et al., 1954) and of GPOX (Dawson and Thorne, 1969) were determined by means of the reduction of phenol indo-2,6 dichlorophenol (PIDP).

The extinction coefficient of the PIDP used amounted to 16.3 $cm^2 \cdot \mu mol^{-1}$ at 600 nm. The

LDH-activity was measured at 2 pyruvate concentrations (0.28 mM and 0.8 mM) as the quotient of these two LDH-activity values reflects the degree of predominance of the glycolysis (Wilson et al., 1963). The activities of phosphofructokinase (PFK) and fructose 1,6-diphosphatase (FDP) were determined according to Opie and Newsholme (1967).

Considering that histochemical techniques can only be performed on frozen tissue specimens, it was checked, in a parallel study, if freezing of the tissues caused any inactivation of the enzymes examined. In order to do this, enzyme activities were estimated in homogenates of fresh muscle specimens and in homogenates of the same specimens after freezing. All chemicals and added enzyme preparations were of the highest commercially available purity. The test reagents were dissolved in double glass distilled water.

Results

Histochemistry. (Fig. 1–3). In Table 1 the histochemically determined enzyme activities in type I, type II and intermediate muscle fibres (IMF) are presented. The data are from 24 apparently normal muscle specimens of adult human subjects. The enzyme activities in endothelial cells of the blood vessels (EC), smooth muscle fibres (SM), connective tissue (CT) and in the nervous fibres (NF) are also presented.

Biochemistry. The biochemical incubation conditions for the SDH and the GPOX reactions were such that the reaction ratios were optimal and directly proportional to the amount of enzyme added. The SDH-reaction was inhibited by 60 mM malonate 90% and by 10 mM NaF 75%. The inhibition with the

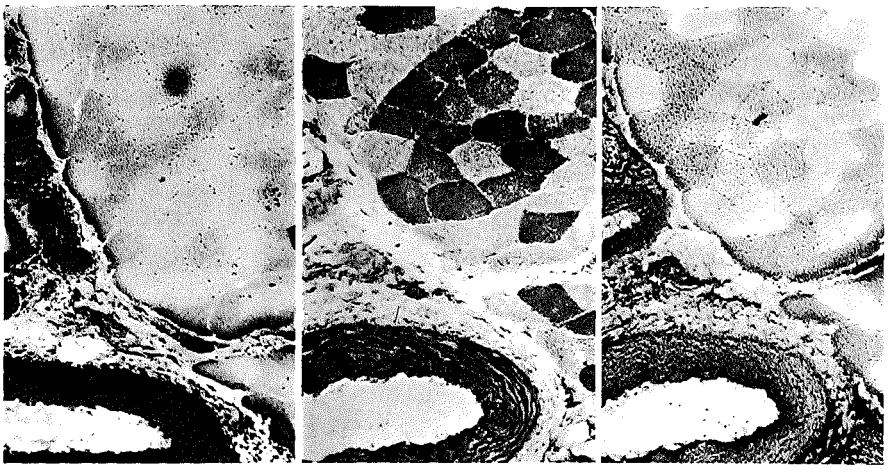

Fig. 1. Demonstration of the activity of glucose 6-phosphate dehydrogenase (left) and 6-phosphogluconate dehydrogenase (right) in human quadriceps femoris muscle with no apparent disease of the neuromuscular system. In the middle the activity of NADH: tetrazolium oxidoreductase is reproduced. Both pentose phosphate pathway enzymes show a higher activity in the anaerobic type II fibres. The NADH: tetrazolium oxidoreductase shows a higher activity in the aerobic type I fibres. In the muscle fibres the activity of glucose 6-phosphate dehydrogenase is less than the activity of 6-phosphogluconate dehydrogenase, contrary to the activity in endothelial cells, smooth muscle fibres and connective tissue. Magnification $\times 140$

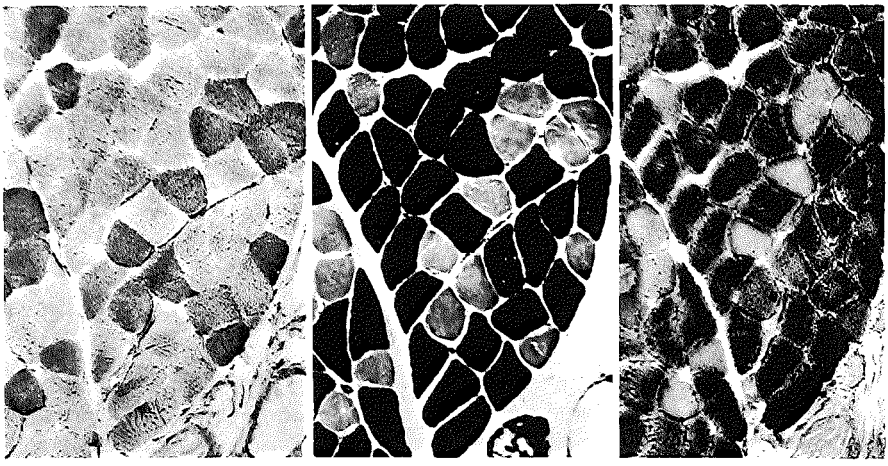

Fig. 2. Demonstration of the activity of NADH: tetrazolium oxidoreductase (left) and lactate dehydrogenase (right) in the absence of amytal in m. quadriceps femoris. Owing to the absence of amytal a part of the aerobic type I fibres shows very slight staining (Meijer, 1973). The other part of the type I fibres still reveals a rather high activity of lactate dehydrogenase. In the middle the activity of myosin adenosine triphosphatase is reproduced. Magnification $\times 140$

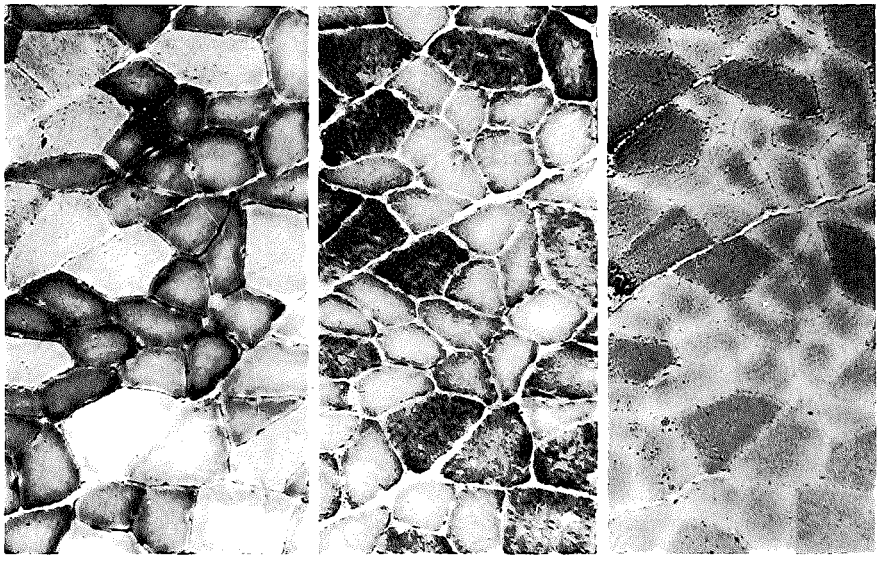

Fig. 3. Demonstration of the activity of cytochrome c oxidase (left), mitochondrial glycerol 3-phosphate: menadion oxidoreductase (middle) and 6-phosphogluconate dehydrogenase (right) in m. quadriceps femoris. In general there is a reciprocal enzyme activity between the cytochrome c oxidase with the mitochondrial glycerol 3-phosphate: menadion oxidoreductase and 6-phosphogluconate dehydrogenase. There are many fibres however with intermediate enzyme activities. Magnification $\times 200$

Table 1. Histochemically determined enzyme activities in muscle fibres and other morphological structures

Enzyme	Type I	Type II	IMF[a]	EC	SM	CT	NF
SDH	3+	1+	2+	−	1+	−	−
NADHOX	3+	1+	2+	1+	2+	1+	−
CO	3+	1+	2+	1+	2+	1+	3+
GPOX	1+	3+	2+	±	±	−	−
MDH	4+	2+	2+	±	±	±	1+
CDH	3+	1+	2+	−	−	−	1+
LDH	2+	3+	2+/3+	1+	1+/2+	1+	1+
GPDH	±	1+	1+	4+	4+	3+	3+
PGDH	±	2+	1+	3+	3+	2+	3+
GP	2+	4+	3+	3+	3+	1+/2+	1+/2+
ATP-Ca	1+	3+	1+/2+	3+	4+	−/1+	−

4+ = very strong activity; 3+ = strong activity; 2+ = moderate activity; 1+ = low activity; ± = slight activity; − = no activity; / = changing activity

[a] Figures concerning IMF are approximate, bearing in mind that these fibres are variable in staining capacities

Table 2. Mean quotient values of enzyme activities in m. quadriceps femoris

$\frac{\text{SDH}}{\text{GPOX}}$	$\frac{\text{PFK}}{\text{SDH}}$	$\frac{\text{LDH 0.28 mM}}{\text{LDH 0.8 mM}}$
5.3 ± 2.7	32 ± 12	2.1 ± 0.8

The enzyme activities were calculated in μmol substrate. min^{-1}. g $tissue^{-1}$ (20 ± 1° C)

GPOX-reaction by means of 5 mM 3-phosphoglycerate amounted to 70% (Tung et al., 1952). The PFK-, FDP,- and the LDH-activities were also determined under circumstances in which the reaction rates were directly proportional to the enzyme concentration. With the applied LDH-techniques the oxidation rate of NADH could not be observed without the addition of pyruvate. The control findings for PFK, FDP and LDH were in agreement with those of Opie and Newsholme (1967) and Wilson et al. (1963).

Kendall's rank correlation test (1955) was performed to find if there was a correlation between the activities of GPOX and PFK, GPOX and FDP, and finally GPOX and the quotient of the LDH activities. With a reliability limit of $\alpha = 0.05$ it was proved that there was a positive correlation between GPOX activity and PFK and FDP activity. There was a negative correlation between GPOX activity and the quotient of LDH activity. It was proved moreover, that there was a negative correlation between SDH and GPOX activity, and SDH and PFK activity.

In Table 2 the mean ratio of the activity between SDH and GPOX, the mean ratio between SDH and PFK and the mean ratio of the activity of

LDH determined by the two pyruvate concentrations found in the m. quadriceps femoris are presented.

From the results of the parallel study it could be concluded that freezing of the muscle specimens did not have any important influence on the activities of the enzymes examined, as far as fibre typing was concerned.

Discussion

This study concerned the histochemical demonstration chiefly of those electron transferring enzymes which give reliable information about the metabolism of muscle fibres and whose activity can be reliably visualised in the sections. The problem of leakage was removed either by selecting enzymes which are tightly bound to the mitochondria or by using semipermeable membranes to prevent leakage of soluble enzymes. Furthermore, to increase the reliability of the techniques, the histochemical systems used were modified such that the demonstration of the activity of the enzymes concerned was independent of auxiliary enzymes or other rate limiting factors.

From the results of the parallel study could be concluded that the histochemical results are representative for the actual enzymatic pattern of the different types of muscle fibres in adult (mature) human skeletal muscles without apparent disease of the neuromuscular system.

The histochemical results obtained by the techniques used could readily be tabulated in Table 1 since it was found that the activity of each enzyme investigated was practically the same in each of the three types of muscle fibres in all the investigated muscle specimens. Their activity was therefore independent of the type of muscle and of the age and sex of the patient. The interrelation between the whole series of the enzymes investigated could therefore be correlated at individual fibre level.

It can be seen from the results presented in Table 1 that the histochemical techniques for the demonstration of the activity of SDH, NADHOX, CO and CDH all give the same staining intensity ratios in the three types of muscle fibres. For fibre typing normal human skeletal muscle it is therefore sufficient to demonstrate activity of only one of these four enzymes. Since these enzymes play an important role in the aerobic part of metabolism, the aerobic type I fibres, stain intensely (Fig.s 1, 2 and 3).

Although the activity of MDH is also strongest in the aerobic fibres, the staining intensity ratios in the three types of muscle fibres are different. The intermediate fibres frequently show a MDH activity similar to that seen in the anaerobic type II fibres (Table 1). The enzymes GPOX, LDH and GP play an important role in the anaerobic part of metabolism. The anaerobic type II fibres stain intensely for these enzymes (Figs. 2 and 3). As can be seen from the staining intensity ratios presented in Table 1, the method used for demonstrating GPOX activity gives the most obvious differences in the staining intensities with regard to fibre typing.

Considering the fact that the demonstration of GP activity produces less obvious contrasts in staining intensity and the fact that this enzyme partly

diffuses out of the sections during incubation, the technique for the demonstration of GP activity has little value in fibre typing. Of these three enzymes, the demonstration of GPOX activity is by far the most suitable.

These histochemical findings regarding the metabolism of the muscle types investigated were confirmed by concurrently performed biochemical investigations. These investigations demonstrated both positive and negative correlations between the activities of the enzymes GPOX, PFK, FDP, and SDH, and the correlation between the ratios of the LDH activity estimated with the two pyruvate concentrations. These results seem to indicate the presence of "constant proportion groups" (Bücher and Pette, 1965) in human skeletal muscle. By comparing the data presented in Table 2 with the data presented by Jöbsis and Meijer (1973 b) it can be concluded that a muscle such as the human quadriceps femoris muscle cannot be classified as essentially aerobic or essentially anaerobic. The biochemical data obtained from the homogenates of biopsies from deltoid muscle specimens and gastrocnemius muscle specimens were not mentioned in Table II since the number of biopsies available was too small to permit satisfactory statistical calculations. In general, however, it can be said that these findings tended to indicate an aerobic metabolic pattern in the case of the m. deltoideus and an anaerobic metabolic pattern in the case of the m. gastrocnemius. These observations indicate that the biochemical and histochemical findings are certainly compatible if not entirely consistent.

In particular the histochemical technique for the demonstration of the GPOX activity gave a reliable impression of the activity of the rate limiting enzymes PFK and FDP. The results as regards the reliability of the enzymhistochemical techniques applied are consistent with the findings found in the study by Jöbsis and Meijer (1973a, b) of rabbit skeletal muscles. The muscle specimens with a high GPOX activity for example almost always showed a relatively high activity of PFK and FDP and a relatively low SDH activity. These findings also indicate the presence of the constant proportions phenomenon in normal human skeletal muscle specimens.

The biochemical assays done on homogenates of m. quadriceps femoris specimens showed definite differences in the activity levels ($\pm 20\%$ above or below the mean values) of certain enzymes. These differences in metabolic patterns could not be demonstrated histochemically. This was only possible when the differences amounted to more than 30%. From these results can be concluded that the enzyme histochemical findings can only give coarse impressions about the metabolism.

The anaerobic type II fibres of human m. quadriceps femoris, m. deltoid, m. gastrocnemius and m. latismus dorsi exhibited more activity of GPDH and PGDH than the aerobic type I fibres (Fig. 1). Both types of fibres showed a higher degree of activity of PGDH than GPDH. Conversely this last enzyme shows a higher degree of activity in the endothelium of blood vessels, smooth muscles and, to a certain degree, in connective tissue fibres. The high activity of these enzymes in these structures is consistent with biochemical findings of Garcia-Buñuel and Garcia-Buñuel (1967) and of Opie (1973). The enzymes PGDH and GPDH, together with MDH, play an important role in the production of NADPH which es required for the production of many synthetic

processes such as lipogenesis and steroid synthesis (Gumaa et al., 1971). It is worth mentioning that activity of MDH in distinction from the activity of the two pentosephosphate pathway enzymes is higher in type I fibres than in type II fibres. Although these two pentose phosphate pathway enzymes are evidently present in mainly anaerobic fibres, as proved by the semipermeable membrane techniques, the staining intensities are so low in human muscle fibres relative to that of other dehydrogenases that they are not suitable for use alone for the purpose of characterization of different types of muscle fibres.

The oxidative phosphorylation is a mechanism by which the decrease in free energy, accompanying the transfer of electrons along the respiratory chain is coupled with the formation of ATP-high energy phosphate bonds. This mechanism is present mainly in type I fibres where ATP-Mg is abundant and plays an active role in oxidative phosphorylation. It was found however that the staining intensities of different muscle fibres are not only dependent on the oxidative phosphorylation capacities but also on the coupling state of phosphorylation. Considering that the coupling state is greatly dependent on freezing effects, the results are therefore not very suitable for characterizing the type of muscle fibres. Hence the histochemical demonstration of mitochondrial ATP activity for fibre typing has no purpose.

Although the histochemical method for the demonstration of ATP-Ca provide a reliable technique for the identification of type II fibres, the use of this enzyme technique alone may give faulty impressions, at least in the identification of the intermediate muscle fibres, which often show a rather low activity of this enzyme. This may lead to misidentification of these fibres as type I fibres (Table 1).

The percentage of the different types of muscle fibres was found to be dependent on the type of muscle. The specimens from quadriceps femoris muscle for example, generally contained more or less equal numbers of type I and type II fibres. Specimens from m. deltoideus however, showed a preponderance of type I fibres, suggesting that the m. deltoideus is more adapted to aerobic metabolism. On the other hand biopsies from gastrocnemius muscle tended to show a predominance of type II fibres, but this was found to depend to a certain extent on the site of the biopsy. This would appear to concur with the theory that one head of the m. gastrocnemius is more adapted to anaerobic metabolism than the other.

There was a large variability in the percentage of intermediate muscle fibres. The percentage was depending on the type of muscle, on the site of biopsy and to a certain degree depending on the age of patient. The younger the patient, the more intermediate fibres. The number of investigated m. quadriceps femoris was such that could be stated that there was a large variability in the percentage in the different specimens. The intermediate fibres in some locations accounted for from 10 to 50% of the fibres present. The highest percentage was in a specimen from rectus abdominis from a child, 4 years old. The lowest percentage was in a specimen from the deltoid muscle of a middle aged male. The percentage of intermediate muscle fibres also differed with regard to the enzyme investigated. The application of the lactic acid dehydrogenase technique, for example, revealed very few or even no intermediate fibres. They usually

showed the same staining intensity as type II fibres (Table 1). Comparing the staining intensities with those obtained with other enzyme histochemical techniques, the metabolic pattern of the intermediate fibres is evidently different from that of either type I or type II fibres (Table 1).

It is important to point out that the intermediate fibres are not an uniform or identical group but more or less they represent a spectrum of fibres with different degrees of specialization towards aerobic (type I) or anaerobic (type II) metabolic pattern.

The observation that the ATP-Ca intermediate fibres show in general a relatively high activity of LDH and GPOX and a low activity of MDH (Table 1) is an indication that these fibres have a relatively intensive anaerobic metabolism. On the other hand the rather high activity of some other oxidoreductases implies a relatively intensive aerobic metabolism. These observations confirm the findings of Ringqvist (1973) who found that human m. masseter fibres with a moderate ATP-Ca activity exhibited a strong or moderate NADHOX and also a strong GPOX activity.

From the results of a preliminary investigation with human skeletal muscle specimens from subjects younger than 4 years of age it was found that histochemical attempts to characterize the different types of muscle fibres in these muscles, especially with the use of ATP-Ca are not always successful. There was some evidence that skeletal muscles at such a young age may have a "different" metabolic pattern. These results will appear in full detail in another communication.

From the above mentioned findings, it can be concluded that for adult human skeletal muscles with no apparent disease of the neuromuscular system in order to classify muscle fibres into type I. Type II and intermediate fibres, it is recommended that in addition to the technique for demonstration of ATP-Ca activity, reliable histochemical techniques for the demonstration of activity of oxidoreductases should be applied.

For the characterisation of the more aerobic facets of metabolism one can demonstrate the activity of one of the aerobic enzymes SDH, NADHOX, CO or CDH, and for the characterisation of the more anaerobic facets of metabolism one can demonstrate the activity of GPOX and LDH.

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Histochemistry

CHAPTER 4.2

The Value of Enzyme Histochemical Techniques in the Classification of Fibre Types of Human Skeletal Muscle

3. Human Skeletal Muscles with Inherited or Acquired Disease of the Neuromuscular System

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Summary. Classification of human skeletal muscle into type I and type II fibres is frequently based on their weak or strong staining with the myosin adenosine triphosphatase reaction. In order to evaluate the reliability of this screening technique a combined histochemical and biochemical study was performed on normal and diseased skeletal muscle of human subjects. In the present investigation activities of enzymes which play a role in the aerobic and anaerobic pathways and which can characterize fibre type, were examined in human muscle specimens with disease of the neuromuscular system.

Special attention is given to the maximal activities of phosphofructokinase and fructose-1,6-diphosphatase, the rate limiting enzymes for the regulation of the glycolysis and gluconeogenesis respectively. Moreover the activities of enzymes of the pentose phosphate pathway are determined.

A most important feature of the biochemical findings is that the constancy of activity ratios of the examined enzymes, as is found apparently normal human skeletal muscle, was frequently not present in diseased human skeletal muscle. From these results and from the histochemical results it can be concluded that for fibre classification in diseased human skeletal muscle the histochemical demonstration of myosin ATPase activity exclusively is not sufficient, but that it is necessary to apply other enzyme histochemical techniques too.

Moreover it was found that in diseased human skeletal muscles the activity of the NADPH regenerating enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was strongly increased. A third observation was the relative decrease of the activity of the examined aerobic enzymes in affected muscle fibres of neurogenic muscle diseases.

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Introduction

The successful application of suitable enzyme histochemical techniques to normal and diseased human skeletal muscles has opened a new era of diagnostic facilities and a greater understanding of the physiological and metabolic characteristics of different types of muscle fibres. The classification of skeletal muscle fibres into type I and type II is based almost exclusively on the demonstration of activity of myosin adenosine triphosphatase. In order to evaluate the reliability of this screening technique a combined histochemical and biochemical study was performed on normal and diseased human skeletal muscle. The study concerned the histochemical demonstration of those electron transferring enzymes which give reliable information about the metabolism of muscle fibres and whose activity can be reliably demonstrated in the sections. The activity of the myosin adenosine triphosphatase is also demonstrated.

For the biochemical study activity of rate limiting enzymes for the regulation of the glycolysis and gluconeogenesis, and moreover electron transferring enzymes are examined.

Results obtained with apparently normal human skeletal muscles were presented in a previous study (Meijer and Elias, 1976). The biochemical findings indicate the presence of "constant proportion groups" (Bücher and Pette, 1965) of the activities of the examined enzymes. Based on the histochemical and biochemical findings it could be concluded that in apparently normal adult human skeletal muscle the histochemical myosin adenosine triphosphatase technique for type I and type II classification is reliable. However for fibres with an intermediate intensity of staining with the myosin ATPase technique it is necessary to apply other enzyme histochemical techniques.

In the present investigation the reliability of the myosin ATPase screening technique is evaluated for human skeletal muscles with disease of the neuromuscular system.

Material and Methods

For the histochemical study fresh muscle biopsies of 80 patients with inherited or acquired diseases of the neuromuscular system were rapidly frozen by immersing small tissue blocks in isopentane cooled to −150° C with liquid nitrogen. The clinical investigations of the patients were based on detailed neurological examination, including electromyography, blood and urine analysis.

For blood analysis activities of creatine kinase (EC 2.7.3.2), glutamic-oxaloacetic transaminase (EC 2.6.1.1), glutamic-pyruvic transaminase (EC 2.6.1.2), aldolase (EC 4.1.2.13) and the levels of K^+-, Na^+-, Ca^{2+}-, and Cl^--ions in sera were determined. Blood lactate levels before and after ischemic exercise were also determined. In urine the determination of creatine and creatinine excretions were carried out. Furthermore for the diagnosis, microscopical examination of the muscle specimens stained with histological and histochemical techniques was performed. The muscle biopsies were chiefly from patients with muscular dystrophy, dystrophia myotonica, polymyositis, myotubular and centronuclear myopathy, amyotrophic lateral sclerosis, Werdnig-Hoffmann disease and Wohlfahrt-Kugelberg-Welander disease. The age of the patients, male and female, ranged from 1 to 65 years. The biopsies were from m. quadriceps femoris (69), m.deltoideus (5), m.gastrocnemius (2), and m.tibialis anterior (4).

Longitudinal and transverse serial sections were cut at 6 μ in a cryostat. In fixed or unfixed sections activities of α-glucan phosphorylase (GP) (EC 2.4.1.1), Mg^{2+}-activated mitochondrial ade-

nosine triphosphatase (ATP-Mg) (EC 3.6.1.3), Ca^{2+}-activated myosin adenosine triphosphatase (ATP-Ca) (EC 3.6.1.3), NADH: tetrazolium oxidoreductase (NADH) (EC 1.6.99.3), succinate dehydrogenase (SDH) (EC 1.3.99.1), glycerol-3-phosphate: acceptor oxidoreductase (GPOX) (EC 1.1.99.5), cytochrome c oxidase (CO) (EC 1.9.3.1), lactate dehydrogenase (LDH) (EC 1.1.1.27), glucose 6-phosphate dehydrogenase (GPDH) (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (PGDH) (EC 1.1.1.44), isocitrate: $NADP^+$ oxidoreductase (decarboxylating) (IDH) (EC 1.1.1.42) and malate: $NADP^+$ oxidoreductase (decarboxylating) (MDH) (EC 1.1.1.40) were demonstrated according to the procedures described in the previous communication (Meijer and Elias, 1976). For the biochemical study, enzyme activities were determined in homogenates from a part of the frozen muscle biopsies. The homogenizing procedures and the determination of activities of succinate dehydrogenase, glycerol-3-phosphate: acceptor oxidoreductase, lactate dehydrogenase, phosphofructokinase (PFK) (EC 2.7.1.11) and hexosediphosphatase (FDP) (EC 3.1.3.11) were performed as previously described (Meijer and Elias, 1976).

In some of the muscle specimens activities of glucose 6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase were also determined according to Löhr and Waller (1965) and Hohorst (1965) respectively.

The activities were determined from the supernatant after repeated centrifugation (45 min $20{,}000 \times g$, 4° C) of the first supernatant. All chemicals and added enzyme preparations were of the highest commercially available purity. The test reagents were dissolved in double glass distilled water.

Results

All investigated muscle specimens showed invariably the well documented and the well known morphological changes characteristic of the concerned diseases in the involved muscles. In about 70 percent of the muscle specimens the biochemical findings demonstrated both positive and negative correlations between the activities of the enzymes PFK, FDP, GPOX, SDH, and between the ratios of the lactate dehydrogenase activity estimated with the two pyruvate concentrations (Wilson et al., 1963). These results seem to indicate the presence of "constant proportion groups" (Bücher and Pette, 1965). In these muscle specimens histochemical classification into type I and type II fibres did not give any difficulty, because in the muscle fibres there was a positive correlation between the activities of GP, ATP-Ca, LDH and GPOX, a positive correlation between the activities of NADH, SDH, MDH, IDH, and CO, and a negative correlation between the enzyme activities of both groups (Figs. 1 and 2).

In some of these muscle specimens occasional fibres react strongly or weakly with both aerobic and anaerobic enzymes, giving rise to some confusion with regard to defining the fibre type (Figs. 1 and 2). In about 30 percent of the muscle specimens the biochemical findings could not clearly demonstrate the presence of "constant proportion groups". For instance some specimens showed a low activity of SDH, PFK, FDP, GPOX and a LDH activity ratio in agreement with an anaerobic metabolism. In general the specimens in which the "constant proportion groups" could not be revealed, were more severely affected.

The histochemical findings clearly demonstrated that the sections contained a large number of fibres for which it was not possible to classify the fibre type (Figs. 3, 4 and 5). For instance in infantile spinal muscular atrophy hypertrophic fibres frequently revealed a low activity of ATP-Ca, however with the GPOX, SDH and NADH reactions these fibres could clearly be differentiated

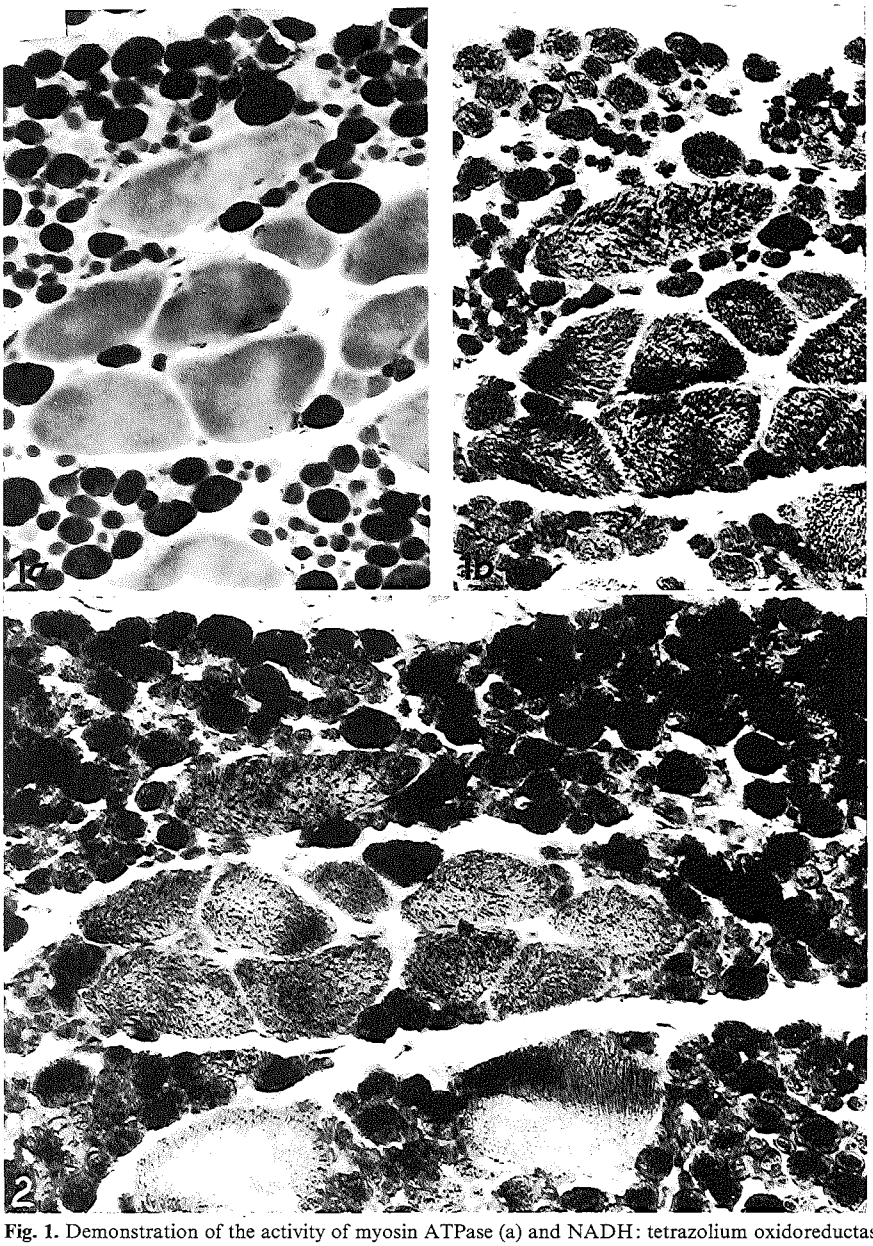

Fig. 1. Demonstration of the activity of myosin ATPase (a) and NADH: tetrazolium oxidoreductase (b) in m.quadriceps femoris of a patient with infantile spinal muscular atrophy. The great difference in staining intensity between atrophic fibres and hypertrophic ones demonstrated by the myosin ATPase reaction could not be shown with the diaphorase reaction. With the demonstration of activities of other enzymes however, the atrophic fibres revealed a more anaerobic metabolism. (See for comparison Fig. 2), so the myosin ATPase reaction in this case gave rather good information about the aerobic and anaerobic aspects of the muscle fibres. The hypertrophic fibres showed an irregular density of staining ($\times 65$)

Fig. 2. Serial section to sections demonstrated in Figure 1. A high activity of glycerol 3-phosphate: menadion oxidoreductase was present in the small atrophic fibres. With the exception of a few fibres, the staining pattern was the same as that shown by the myosin ATPase ($\times 65$)

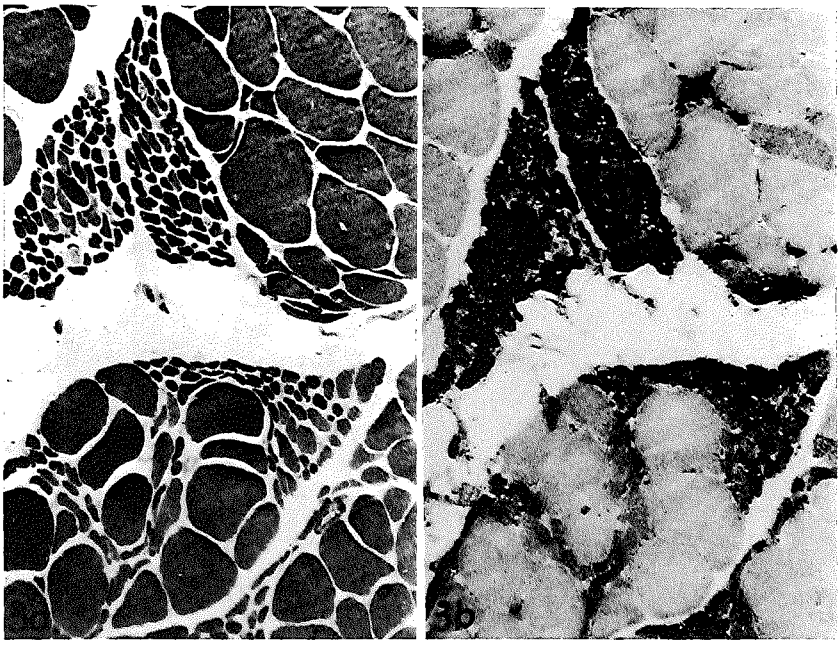

Fig. 3. Demonstration of the activity of myosin ATPase (a) and glycerol 3-phosphate: menadion oxidoreductase (b) in m.tibialis anterior of a patient with amyotrophic lateral sclerosis. The myosin ATPase reaction did not give good information about the aerobic and anaerobic capacities of the atrophic fibres because with the exception of NADH: tetrazolium oxidoreductase, these fibres revealed a pronounced anaerobic metabolism with all investigated enzymes ($\times 140$)

Fig. 4. Serial section to sections demonstrated in Figure 3. The atrophic fibres showed the same activity with the NADH: tetrazolium oxidoreductase reaction as the hypertrophic fibres. Because these fibres in comparison to the hypertrophic ones, revealed an anaerobic metabolism with the other investigated enzymes, it follows that in these atrophic muscle fibres the constant proportion phenomenon is not present ($\times 140$)

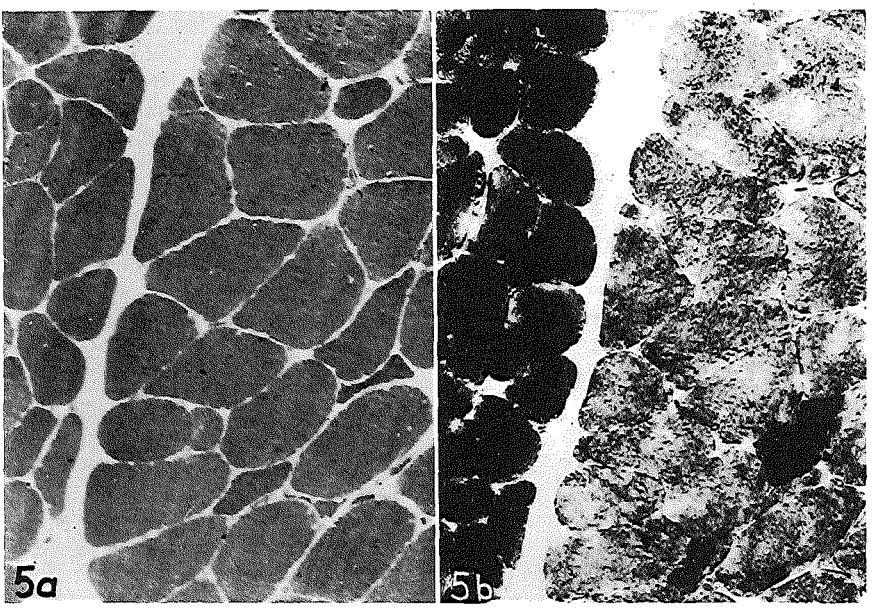

Fig. 5. Demonstration of the activity of myosin ATPase (a) and α-glucan phosphorylase (b) in m.tibialis anterior of a patient with Charcot-Marie-Tooth disease. There was a low activity of myosin ATPase of muscle fibres in both fascicles. With the phosphorylase reaction the activity was high in one fascicle and low in most fibres of the other fascicle. In this muscle specimen the constant proportion phenomenon was completely absent, making it difficult to characterize the aerobic and anaerobic metabolism (×90) of the muscle fibres

into two types. In all types of muscle diseases it could be seen with histochemical techniques that atrophic fibres frequently showed a shift to a more anaerobic metabolism. Also in hypertrophic fibres there is a certain degree of decrease in activity of aerobic enzymes, especially of BDH, ADH, IDH, and CO. The activity of NADH in contrast to the other aerobic enzymes remained markedly unaffected except in advanced forms of the disease. In hypertrophic fibres activities of anaerobic enzymes are frequently decreased too. Typing of hypertrophic fibres was frequently difficult because certain parts of the fibres stained more intensely than others (Figs. 1 and 2).

It is very striking that muscle specimens (7 cases) showing, with all used enzyme histochemical techniques used, an uniform staining of the fibres, revealed without exception a marked aerobic (4 cases) or anaerobic (3 cases) metabolic pattern with the used biochemical techniques. It is noteworthy that muscle specimens (8 cases) with an inclination towards uniform staining of the fibres, frequently still have a distinct difference in staining intensity with the GPOX-technique for the muscle fibres. With the biochemical techniques these muscle specimens never revealed a distinct aerobic or anaerobic metabolism.

In pathologically altered fibres in all types of muscle disease, there is frequently a marked increase of both pentose phosphate enzymes (Figs. 6 and 7). The increase in activity of these enzymes, especially of GPDH, which is

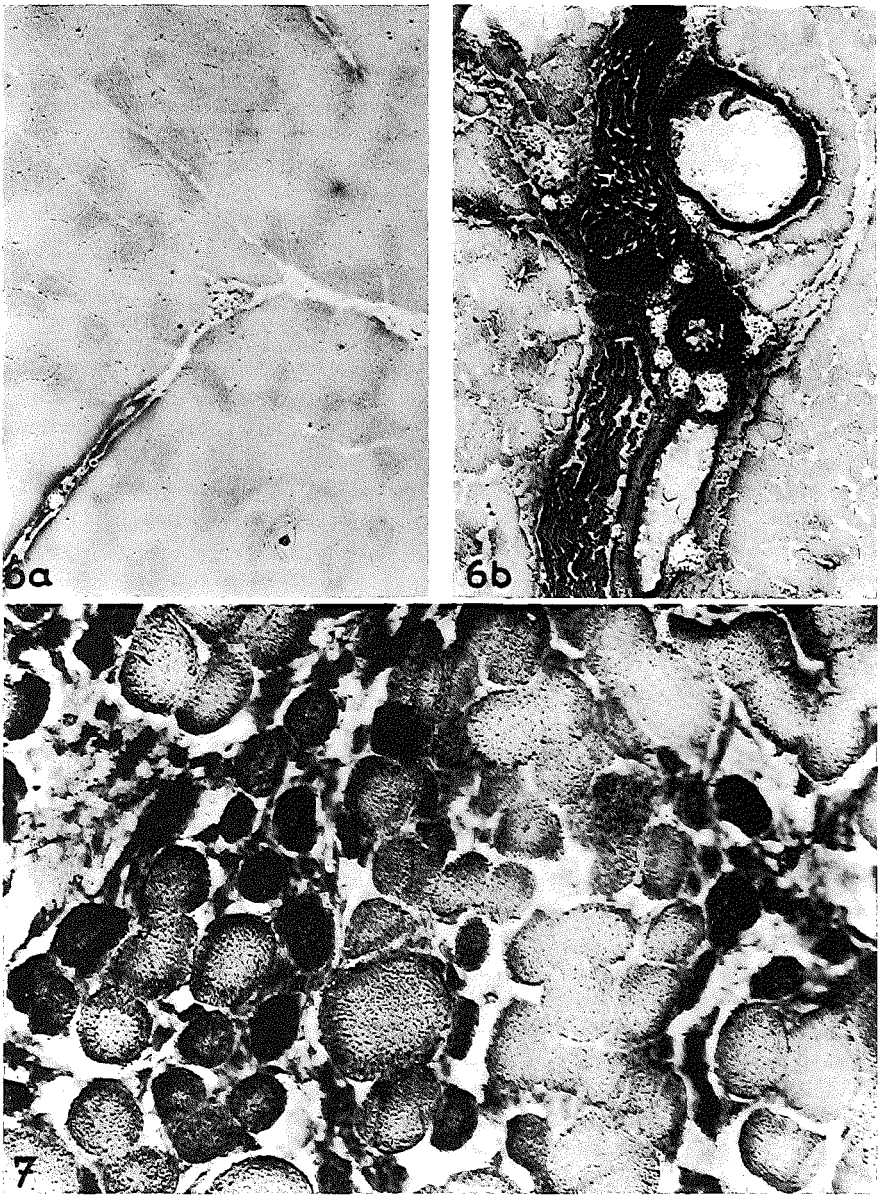

Fig. 6. Demonstration of the activity of glucose-6-phosphate dehydrogenase in m.quadriceps femoris. To the left (a) activity in apparently normal skeletal muscle is demonstrated, to the right (b) activity in skeletal muscle affected by infantile spinal muscular atrophy is demonstrated. Atrophic fibres especially revealed an enormous increase in activity of both pentose phosphate shunt enzymes ($\times 90$)

Fig. 7. Demonstration of the activity of glucose-6-phosphate dehydrogenase in m.quadriceps femoris of a patient with infantile spinal muscular atrophy. Muscle fibres with weak and high enzyme activity in both types of fibres ($\times 230$)

more activated than PGDH, is observable in all stages and forms of disease. The hypertrophic fibres, are more active then normal sized ones. The atrophic fibres showed a very high degree of activity in comparison to the hypertrophic and apparently normal fibres. Atrophic fibres in specimens of infantile spinal muscular atrophy especially revealed an enormous increase in activity of both enzymes. Frequently, the normal sized fibres in the affected muscles showed a slight but evident increase too.

Discussion

From the results of the combined histochemical and biochemical study it can be concluded that the histochemical findings give reliable impressions of the metabolic characteristics of the muscle fibres. The histochemical findings clearly indicate that in affected muscle tissues a loss in reciprocal staining properties of the fibres may be present. As moreover in many of these pathologically altered muscle specimens the constancy of the activity ratios of the biochemical examined enzymes disappears, it is evident that in affected human skeletal muscle the performing of the myosin ATP-ase technique exclusively for type I and type II classification is not sufficient. It can be recommended that for characterization of the more aerobic facets of metabolism the activity of SDH, NADH and CO and for the anaerobic facets the activity of GPOX and LDH should be examined.

The activity of GPOX especial will give valuable applied metabolic information, because in this study it is proved that in comparison to other enzymes in diseased human skeletal muscle there is a good correlation between the activity of the GPOX and the activity of the two key enzymes PFK and FDP. In other studies we have found this good correlation too (Jöbsis and Meijer, 1973a, b; Jöbsis et al., 1976).

The marked increase in activity of the two pentose phosphate shunt enzymes in diseased human skeletal muscles has also been observed by Laudahn and Heyck (1963) and Heyck et al., (1963) whereas McCaman (1963), Manchester et al. (1970) and Dhalla et al. (1972) have found the same phenomenon in skeletal muscles from mice with hereditary muscular dystrophy, rat diaphragma, and hind leg muscles of genetically dystrophic hamsters, respectively. In diseased muscles the percentage of connective tissue and fat tissue, which are characterized by a high activity of both pentose phosphate shunt enzymes, may be prominently increased. Homogenized muscle fractions may be contaminated by connective tissue and fat tissue, therefore Laudahn and Heyck (1963), Heyck et al. (1963) and Stengel-Rutkowski and Barthelmai (1973) have the opinion that the increased activity of GPDH and PGDH is not from activity originated from muscle fibres. However with the applied semipermeable membrane techniques we have clearly demonstrated that the increase in activity of GPDH and PGDH in muscle fibres is a reality. Because both enzymes are involved in biosynthetic processes required for cell proliferation (Coulton, 1977), the application of the semipermeable membrane techniques for the demonstration of activity of GPDH and PGDH can be valuable to characterize this phenomenon in muscle fibres.

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CHAPTER 4.3

Die Aktivität der Glucose-6-phosphat-Dehydrogenase und 6-Phosphogluconat-Dehydrogenase in Skelettmuskelgewebe von Patienten mit Muskelkrankheiten[1])

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Mit 5 Abbildungen im Text

In pathologisch veränderten Skelettmuskeln von Patienten mit Muskelkrankheiten sind die Aktivitäten von weitaus den meisten untersuchten Enzymen im Vergleich zu den Aktivitäten in ähnlichen, aber gesunden Skelettmuskeln signifikant verringert. Die wichtigsten Ausnahmen sind die Aktivitäten der Glucose-6-phosphat-Dehydrogenase (EC 1.1.1.49)
und der 6-Phosphogluconat-Dehydrogenase (EC 1.1.1.44). Die Aktivitäten dieser zwei Pentosephosphatzyklus-Enzyme sind in erkranktem Muskelgewebe sehr stark vermehrt (Laudahn und Heyck 1963, Heyck et al. 1963, Manchester et al. 1970, Dhalla et al. 1972).

Aus diesen biochemischen Befunden könnte zunächst geschlossen werden, daß in erkranktem Muskelgewebe der Kohlenhydratabbau über den Pentosephosphatweg kompensatorisch gesteigert ist, weil die Funktion des Embden-Meyerhof-Weges infolge der starken Abnahme der meisten hierzu gehörenden Enzyme stark eingeschränkt ist. Dagegen sprechen die Beobachtungen von Heyck et al. (1963) und von Laudahn (1963), die eine echte Korrelaton zwischen dem Fett- und dem Bindegewebegehalt der untersuchten Muskulatur und der Aktivität dieser zwei Enzyme gefunden haben.

Mit steigendem Fett- und Bindegewebegehalt nehmen die Aktivitäten der Pentosephosphatzyklus-Enzyme zu, da die spezifische Aktivität beider Enzyme in Fett- und Bindegewebe ca. 60–100fach höher ist als in reinem Muskelgewebe. In pathologischer Muskulatur hat man meistens eine deutliche Vermehrung des Binde- und Fettgewebes gefunden. Da die Trennungsmethoden für Homogenatuntersuchungen nicht einwandfrei sind, hat man jetzt die Einsicht, daß die Aktivitätsvermehrung dieser beiden Enzyme auf Kontaminierung des Homogenates mit Fett- und Bindegewebe zurückzuführen sein dürfte. Darüber hinaus hat man mit konventionellen enzymhistochemischen Methoden in Muskelfasern von normalem und pathologisch verändertem Gewebe fast gar keine Aktivität dieser zwei Enzyme gefunden (Suchenwirth et al. 1970). Es muß jedoch betont werden, daß die konventionellen Methoden keine zuverlässigen Befunde ergeben, da während der Inkubation die Enzyme teilweise in das Inkubationsmedium diffundieren und überdies die Farbstoffmengen abhängig sind von der Aktivität des Hilfsenzyms der Diaphorase (Meijer und de Vries 1974).

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[1]) Die Muskelproben wurden in dankenswerter Weise von dem Skelettmuskelforschungszentrum (Prof. Dr. J. Bethlem, Amsterdam) zur Verfügung gestellt.

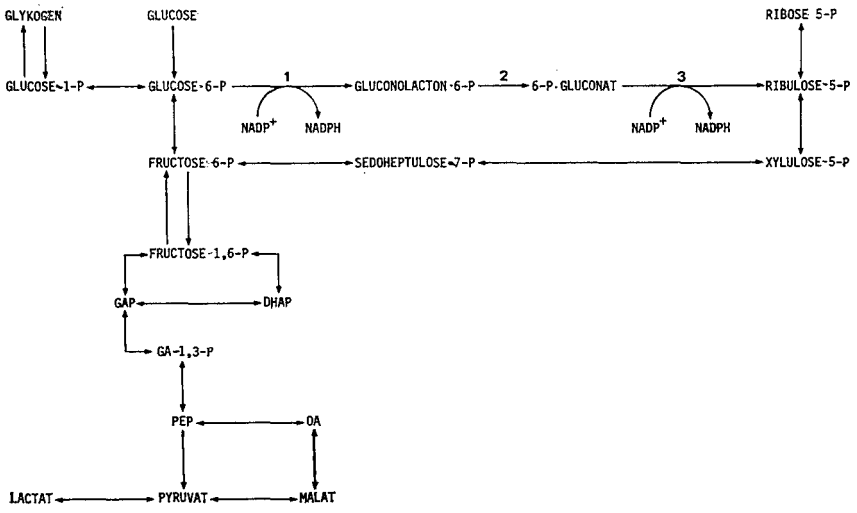

Abb. 1. Vereinfachtes Schema des Embden-Meyerhof- und Pentosephosphatzyklus. 1 = Glucose-6-phosphat-Dehydrogenase; 2 = Lactonase; 3 = 6-Phosphogluconat-Dehydrogenase; GAP = Glycerinaldehydphosphat; DHAP = Dihydroxyacetonphosphat; GA-1,3-P = Glycerinsäure-1,3-diphosphat; PEP = Phosphoenolpyruvat; OA = Oxalacetat.

Die Pentosephosphatzyklus-Enzyme sind im Stoffwechsel als Lieferant von NADPH von besonderer Bedeutung (Abb. 1). Durch die Oxydation der Substrate wird aus $NADP^+$ die Substanz NADPH gebildet. Die reduzierte Verbindung NADPH wird für viele synthetische Prozesse benötigt, unter anderem für den Aufbau von Fettsäuren, von Steroiden und für Hydroxylierungen. Aus Abb. 1 ist gleichfalls ersichtlich, daß zur Pentosesynthese das Gewebe nicht ausschließlich abhängig ist von den Pentosephosphatzyklus-Enzymen, wie man früher gemeint hat.

Für unsere Muskelstoffwechseluntersuchungen ist es von großer Bedeutung, die NADPH-Synthesekapazität in pathologisch veränderten Skelettmuskeln von Patienten mit Muskelkrankheiten nachzuprüfen. Deswegen werden in erkranktem Muskelgewebe die Aktivität und die Lokalisierung der Glucose-6-phosphat-Dehydrogenase, der 6-Phosphogluconat-Dehydrogenase und anderer Enzyme, die als NADPH-Lieferant eine Rolle spielen können, biochemisch und histochemisch untersucht. Die Resultate werden verglichen mit Befunden an normalem menschlichem Muskelgewebe.

Es werden semipermeabele Membrantechniken für enzymhistochemische Darstellungsmethoden verwendet (Meijer und de Vries 1974, 1975). Durch die Verwendung von Membranen ist die Enzymdiffusion beseitigt. Da darüber hinaus mit dem Zusatz von Phenazinmethosulfat und Menadion die Farbreaktionen unabhängig von der Diaphorase-Aktivität vor sich gehen, sind die histochemischen Befunde zuverlässiger als mit den konventionellen Darstellungsmethoden.

Dieser Vortrag steht in Beziehung zu dem workshop „Spezifität histochemischer Enzymnachweise und Kontrollreaktionen", in dem die Membranprinzipien und die Anwendungsmöglichkeiten eingehend erwähnt worden sind. Deshalb wird in diesem Vortrag ausschließlich auf die enzymhistochemischen Befunde und der bemessenen Zeit wegen nur auf die Befunde der zwei Pentosephosphatzyklus-Enzyme eingegangen.

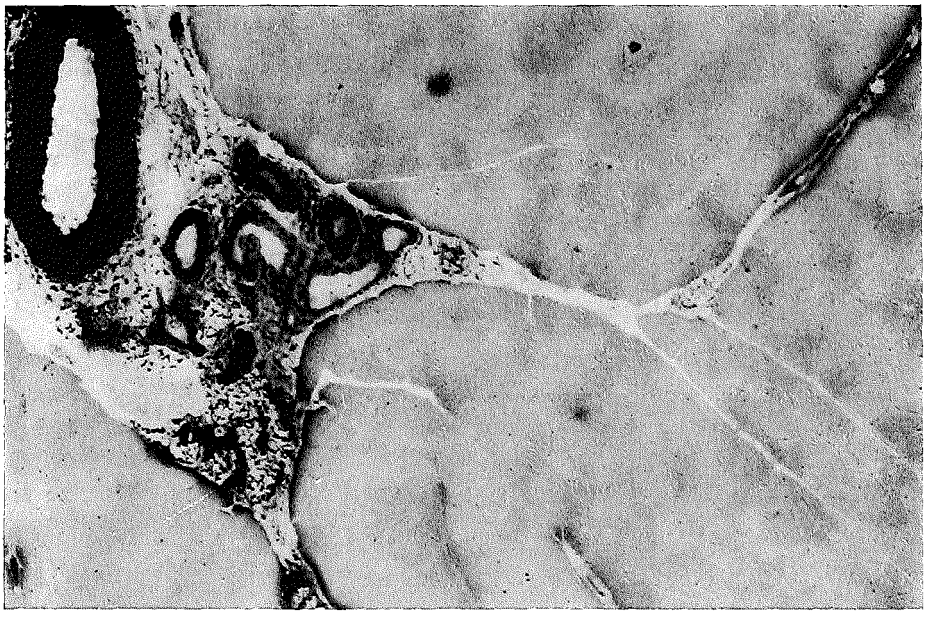

Abb. 2. Glucose-6-phosphat-Dehydrogenase-Aktivität im normalen M. quadriceps femoris des Menschen. Besonders Blutgefäße zeigen eine stark vermehrte Enzymaktivität. Im Lipidgewebe hat sich das Formazan grobkristallin rekristallisiert. Die anaeroben Fasern zeigen schwache Enzymaktivität. 90×.

Das Biopsiematerial entstammte dem M. quadriceps femoris von 60 Patienten mit erblichen oder erworbenen Krankheiten der Skelettmuskulatur. Entsprechende Gewebsproben von 24 offensichtlich muskelgesunden Personen lieferten die normalen Vergleichswerte. Die klinischen Diagnosen basierten auf detaillierter neurologischer Untersuchung der Patienten, einschließlich Elektromyographie, Blut- und Harn-Analysen. Überdies wurde in Schnittpräparaten mittels histologischer und histochemischer Farbreaktionen die Morphologie des Muskelgewebes untersucht. Die verwendeten histochemischen Techniken zur Darstellung der Aktivität der Glucose-6-phosphat-Dehydrogenase und der 6-Phosphogluconat-Dehydrogenase sind an anderer Stelle ausführlich beschrieben worden (Meijer und de Vries 1974).

In normalen menschlichen Skelettmuskeln zeigen die anaeroben Typ-II-Fasern im Gegensatz zu den aeroben Typ-I-Fasern für beide Enzyme mehr Aktivität (Abb. 2 und 3). Diese Ergebnisse stimmen mit denen überein, die wir im M. gastrocnemius, M. soleus und Diaphragma von verschiedenen Laboratoriumstieren gefunden haben. Es ist merkwürdig, daß, obgleich die höchste Aktivität in den Typ-II-Fasern gefunden wird, im allgemeinen Muskelgewebe mit einem relativ aeroben Stoffwechsel wie Herz und M. soleus eine höhere Aktivität für die zwei Enzyme zeigt als die mehr anaeroben Skelettmuskeln. Da wir mit einigen anderen Dehydrogenasen ähnliche Befunde erhalten haben, ergibt sich, daß der Stoffwechsel der Typ-I- respektive der Typ-II-Fasern z. B. des M. soleus nicht dem der Typ-I- respektive der Typ-II-Fasern z. B. des M. gastrocnemius ähnlich sein kann. Die Ergebnisse, daß die Pentosephosphatzyklus-Enzyme mittels der Membrantech-

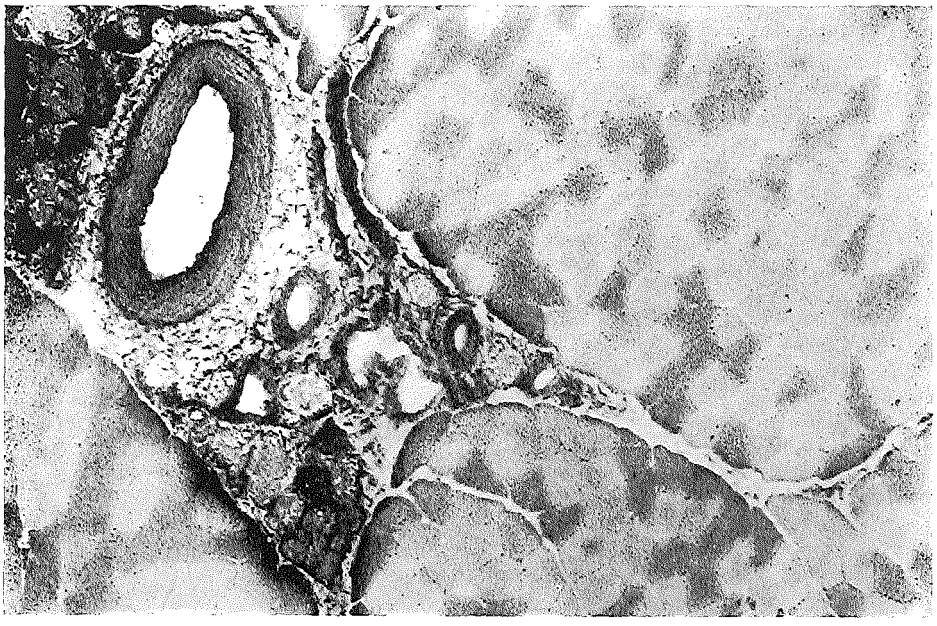

Abb. 3. 6-Phosphogluconat-Dehydrogenase-Aktivität im gleichen M. quadriceps femoris wie in Abb. 2. Die Blutgefäße zeigen etwas weniger Aktivität der 6-Phosphogluconat-Dehydrogenase im Vergleich zur Glucose-6-phosphat-Dehydrogenase. Hingegen zeigen die Muskelfasern und besonders die anaeroben Fasern deutlich mehr Enzymaktivität. 90×.

niken eine höhere Aktivität in den mehr aeroben Muskeltypen zeigen, stimmen völlig überein mit unseren biochemischen Ergebnissen und mit denen von Pette und Bücher (1963) und Dawson und Romanul (1964).

Histochemisch und auch biochemisch haben wir gefunden, daß normale menschliche Skelettmuskeln deutlich mehr Aktivität der 6-Phosphogluconat-Dehydrogenase- als der Glucose-6-phosphat-Dehydrogenase zeigen (Abb. 2 und 3).

In Übereinstimmung mit biochemischen Ergebnissen von Garcia-Buñuel und Garcia-Buñuel (1967) sowie Opie (1973) findet man mit den Membrantechniken eine starke Aktivität in Bindegewebe, Venen und Arterien. Aus den Abb. 2 und 3 ist ersichtlich, daß die Aktivität der 6-Phosphogluconat-Dehydrogenase in diesen Gewebsstrukturen etwas geringer ist.

Die Befunde mit Membrantechniken stehen in großem Gegensatz zu denen, die mit den nicht zuverlässigen konventionellen Techniken erhalten werden. So haben Rudolph und Klein (1964) nur einen schwachen Reaktionsausfall in Gefäßen und Bindegewebe beobachtet.

In Muskelfasern von pathologisch veränderten menschlichen Skelettmuskeln wird oft eine starke Aktivitätszunahme der zwei Pentosephosphatzyklus-Enzyme gefunden (Abb. 4 und 5).

Die Aktivitätszunahme in einem erkrankten Skelettmuskel kann nur in vereinzelten Fasern oder in allen Fasern vorkommen und in beiden Fasertypen beobachtet werden. Ohne Ausnahme zeigen die kleinen, sogenannten atrophischen

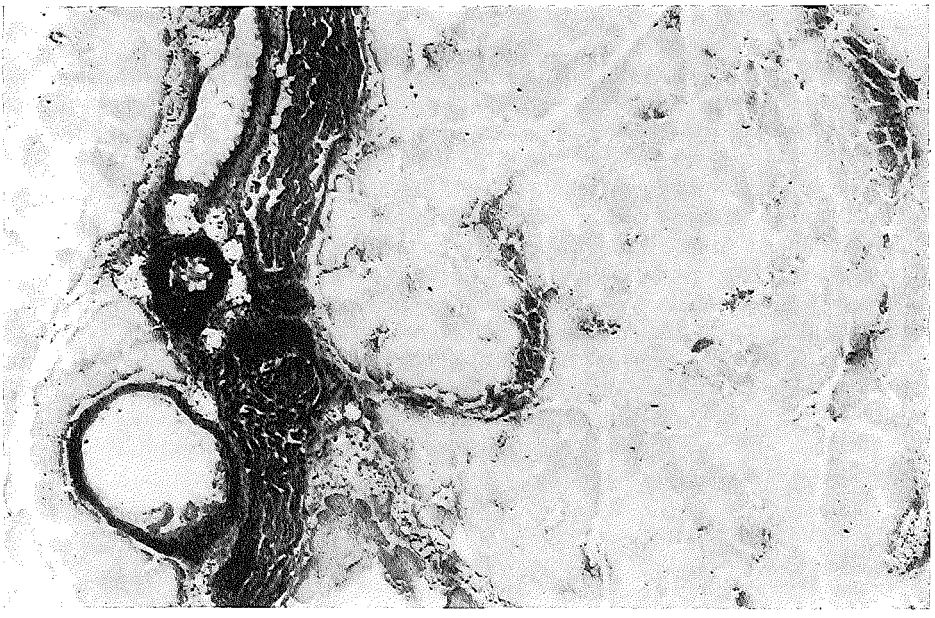

Abb. 4. Besonders die pathologisch betroffenen Muskelfasern zeigen stark vermehrte Aktivität der Glucose-6-phosphat-Dehydrogenase. Infantile spinale Atrophie. 90×.

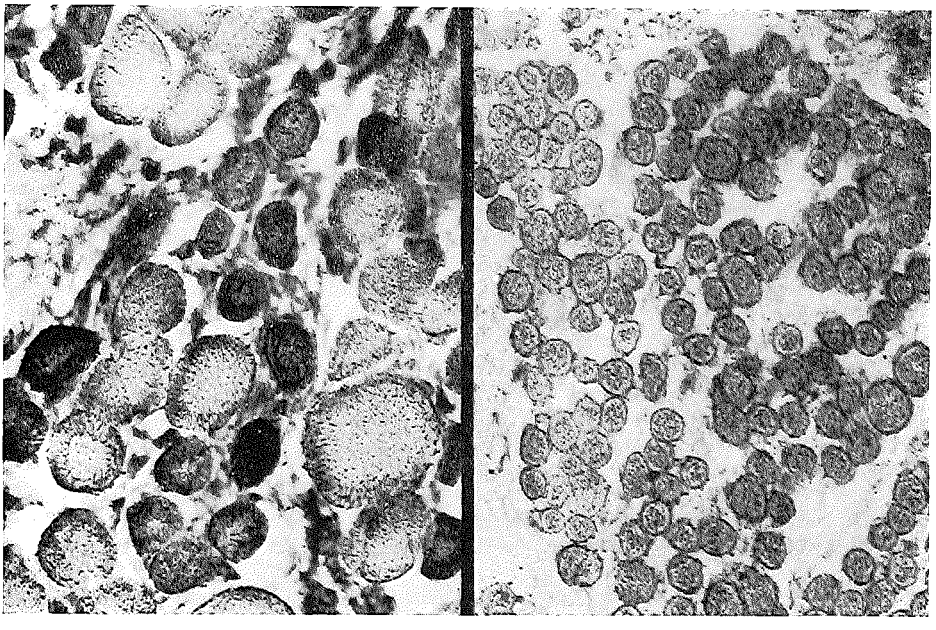

Abb. 5. Links: pathologische Muskelfasern mit wechselnder Aktivität der Glucose-6-phosphat-Dehydrogenase. Progressive Muskeldystrophie. 230×.

Rechts: kleine atrophische Muskelfasern mit vermehrter Aktivität der Glucose-6-phosphat-Dehydrogenase. Infantile spinale Atrophie. 230×.

Muskelfasern eine starke Enzymaktivität (Abb. 5). Besonders in erkrankten Muskeln des Duchenneschen Typs und bei benignen oder malignen Typen der infantilen spinalen Atrophie wird ein starker Anstieg der Enzymaktivität gefunden. Pathologische Fasern mit einer stark erhöhten Aktivität der Glucose-6-phosphat-Dehydrogenase zeigen auch immer eine erhöhte Aktivität von 6-Phosphogluconat-Dehydrogenase. Ebenso zeigen Fasern mit erhöhter 6-Phosphogluconat-Dehydrogenase-Aktivität immer eine angestiegene Glucose-6-phosphat-Dehydrogenase-Aktivität. Die Enzymaktivitäten in Bindegewebe und Gefäßen pathologisch veränderter Skelettmuskeln haben sich im Vergleich zu den Aktivitäten in ähnlichen, aber gesunden Skelettmuskeln nicht geändert.

Die enzymhistochemischen Befunde, erhalten mit Membrantechniken, haben also deutlich gezeigt, daß die mit Homogenatuntersuchungen gefundenen Aktivitätszunahmen nicht auf Kontaminierungsartefakte zurückzuführen sein können und deshalb die Aktivitätssteigerung der Pentosephosphatzyklus-Enzyme ein bemerkenswertes Phänomen ist.

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Histochemistry

CHAPTER 4.4

The Increase in Activity of Glucose-6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase in Skeletal Muscles of Rats after Subcutaneous Administration of N,N′-Dimethyl-Para-Phenylenediamine

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Summary. After subcutaneous administration of N,N′-dimethyl-para-phenylenediamine (DPPD) in rats, a myogenic myopathy was produced in the skeletal muscles. In this communication, the results of the application of various histochemical techniques for the localization of oxidoreductases, transferases, hydrolases and isomerases and biochemical techniques for the estimation of activities of oxidoreductases in the experimental skeletal muscles are presented. The most striking result was the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase which increased dramatically during the early phase of the muscle disease. The increase in activity of the pentose phosphate shunt enzymes was the first pathological alteration and was present as early as 8 h after a single injection of DPPD. Histochemical techniques for demonstration of activity of both enzymes are therefore highly suited for the detection of minor diseases and the early onset of major diseases of the neuromuscular system. Some glycolytic enzymes as well as some enzymes of the aerobic part of the metabolism showed an early decrease or increase in activity indicating a metabolic imbalance in the muscle fibres. There were more fibres with an intermediate pattern of the energy yielding enzymes in the experimental muscle specimens then in specimens from the control groups. The activity of the catabolic hydrolytic enzymes was strongly increased in pathological muscles. The aerobic muscles were more vulnerable to DPPD than the anaerobic muscles.

Introduction

The successful application of suitable enzyme histochemical techniques to normal and diseased human muscles has opened a new era of diagnostic facilities and a greater understanding of the physiological and metabolic characteristics of

the different types of diseases of the neuromuscular system. In previous histochemical and biochemical studies, Meijer and Elias (1976), Meijer and Vossenberg (1977) and Meijer et al. (1977) the reliability of the rather simple and tissue-saving enzyme histochemical techniques for characterising pathological disorders in human skeletal muscles has been investigated. Use was made of a battery of conventional enzyme histochemical techniques and recently developed techniques in which diffusion of enzymes during the incubation is hampered by semipermeable membranes and in which the demonstration of oxidoreductases is not dependent on the activity of auxiliary enzymes.

A striking feature of the findings was the strong increase in activity of the NADPH regenerating enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which was frequently present in biopsies of diseased muscle. Moreover this increase in activity was sometimes present in skeletal muscles of patients with muscle complaints where the morphology, the activity and localization of other enzymes and the quantity and localization of glycogen and lipids were within normal limits (Meijer and Elias 1977; Meijer 1979)

The aim of the present investigation was to further study the activity of both pentose phosphate shunt enzymes in order to establish if the histochemical techniques for the demonstration of the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase can in principle be of value in the detection of minor diseases and early onset of major diseases of the neuromuscular system. Moreover the activity and localization of other enzymes was investigated.

In the combined histochemical and biochemical study, use was made of an experimentally induced myopathy in rats. The myopathy was induced by subcutaneous administration of the toxic substance N,N′-dimethyl-para-phenylenediamine (DPPD) (Meijer and Israël 1979a, b). In different stages of the myopathy the location and the activity of both pentose phosphate shunt enzymes and other enzymes were determined.

Material and Methods

Rats Treated with DPPD

Six-weeks old male Wistar rats with an average start weight of about 110 g were injected twice daily with a freshly prepared solution of DPPD. 2 HCl (20 $mg \cdot kg^{-1}$ body weight, with a solution of 10 mg $DPPD \cdot ml^{-1}$ distilled water). The injections were given subcutaneously in the left body side after routine weight registration at 8 a.m. and 5 p.m. precisely. For the study 10 controls rats, 2 rats receiving 1 injection (8 a.m.), 2 rats receiving 2 injections (1 day), 2 rats receiving 4 injections (2 days), 10 rats receiving 8 injections (4 days), 10 rats receiving 14 injections (7 days), 10 rats receiving 18 injections (9 days), and 10 rats receiving 22 injections (11 days) were used. The two rats receiving 1 injection were sacrificed 8 h after the single injection of DPPD, the other animals were sacrificed at 8 a.m. on the day following the last injection, which was administrated at 5 p.m. The animals were sacrificed under ether narcosis without use of barbiturate containing anaesthetics.

Preparation of the Muscle Specimens. After sacrificing the animals, specimens from m. biceps femoris, m. gastrocnemius, m. rectus femoris and m. soleus were removed and freed from fat, tendinous and fibrous tissue. The specimens were from the right body side (free from injection lesion). The muscle specimens of m. gastrocnemius, m. rectus femoris and m. soleus were divided into 4 parts

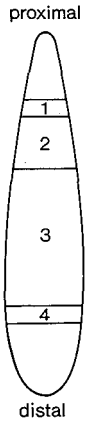

Fig. 1. Scheme of dissection

(Fig. 1). Blocks 1 and 4 were used for histological examination; block 2 for histochemical investigation and block 3 for the biochemical precedures. The muscle specimens of m. biceps femoris were divided into 3 parts. Blocks 1 and 3 were used for histological examination and block 2 for the histochemical preocedures.

Histology. The muscle blocks for the histological study were fixed in "Suza" solution (Romeis 1968). After paraffin embedding, 7 µm sections were cut and stained with haematoxylin-eosin, with the trichrome technique of Gomori and with the PAS technique.

Histochemistry. The muscle specimens were rapidly frozen by immersing the tissue blocks in isopentane cooled to −150° C with liquid nitrogen and stored at −96° C. Transverse serial sections at a thickness of 7 µm were cut in a cryostat. Unfixed sections were stained by the following techniques.

The activity of *succinate dehydrogenase* (EC 1.3.99.1) was demonstrated according to Nachlas et al. (1957), but with the addition of 50 µg phenazine methosulphate · ml^{-1}. The activity of *NADH: tetrazolium oxidoreductase* (EC 1.6.99.3) was demonstrated according to Barka and Anderson (1963) with the substrate NADH. The activity of *glycerol-3-phosphate: menadione oxidoreductase* (EC 1.1.99.5) was demonstrated according to a substrate modification of the method of Wattenberg and Leong (1960). In place of succinate an equimolar concentration of glycerol-3-phosphate was used. The activity of *cytochrome C oxidase* (EC 1.9.3.1) was demonstrated according to Burstone (1962) with the substrates p-aminodiphenylamine and p-methoxy-p-aminodiphenylamine. The activity of *lactate dehydrogenase* (EC 1.1.1.27) was demonstrated according to Meijer (1973). The methods described by Barka and Anderson (1963) were used to demonstrate the activity of *3-hydroxybutyrate dehydrogenase* (EC 1.1.1.30), *malate: NAD^+ oxidoreductase* (EC 1.1.1.37) and *isocitrate: NAD^+ oxidoreductase* (EC 1.1.1.41). The activity of *glucose-6-phosphate dehydrogenase* (EC 1.1.1.49) and *6-phosphogluconate dehydrogenase* (EC 1.1.1.44) was demonstrated according to Meijer and de Vries (1974). The activity of *isocitrate: $NADP^+$ oxidoreductase* (decarboxylating) (EC 1.1.1.42) and *malate: $NADP^+$ oxidoreductase* (decarboxylating) (EC 1.1.1.40) was demonstrated according to Meijer and de Vries (1975) The activity of *monoamine oxidase* was demonstrated according to Glenner et al. (1957), but using epinephrine as substrate instead of tryptamine. The activity of *glyceraldehyde phosphate dehydrogenase* (EC 1.2.1.12) was demonstrated according to de Vries et al. (1980). The activity of *α-glucan phosphorylase* (EC 2.4.1.1) was demonstrated according to Meijer (1968). The activity of *phosphofructokinase* (EC 2.7.1.11) was demonstrated according to Meijer and Stegehuis (1980). The activity of *phosphoglucomutase* (EC 2.7.5.1) and *glucose-6-phosphate isomerase* (EC 5.3.1.9) was demonstrated according to de Vries and Meijer (1976). The activity of *mitochondrial adenosine triphosphatase* (Mg^{2+}- and 2,4-dinitrophenol-activated) (EC 3.6.1.3) and *myosin adenosine triphosphatase* (Ca^{2+}-activated) (EC 3.6.1.3) was demonstrated according to Meijer and Vloedman (1980) and Meijer (1970) respectively. The activity of *acid phosphatase* (EC 3.1.3.2) and *non specific esterase* (EC 3.1.1.1 and 3.1.1.2) was demonstrated according to Meijer (1972) and Meijer and

Vloedman (1973) respectively. The activity of *myeloperoxidase* (EC 1.11.1.7) was demonstrated at pH 6.0. The incubating medium contained the same substrates as used for cytochrome c oxidase and hydrogen peroxide 10^{-2} M. Oil red O was used to demonstrate *lipids*. The method of von Kossa and the alizarin red s stain were used to demonstrate *calcium* deposits.

Biochemistry. The tissue specimens from the m. soleus, m. rectus femoris and m. gastrocnemius of control rats and from the rats receiving 8 injections or more were weighed and immediately after thawing were cut finely in a tenfold volume 50 mM Trisbuffer (pH 8.2, 4° C) according to Opie and Newsholme (1967). After homogenising for 30 s at 0° C in an all glass tight-fitting homogeniser, the homogenates were centrifuged for 10 min ($175 \times g$, 4° C) and the determinations were performed in a fixed sequence directly from the supernatant. Only the *lactate dehydrogenase* reaction was determined from the supernatant after repeated centrifugation (45 min, $20{,}000 \times g$, 4° C) of the first supernatant. The reaction rate (20° ± 1° C) was deduced from the linear part of extinction change after a time elapse of 10 min. The reactions were followed for at least 15 min and carried out in quadruplicate; the supernatant in the four cuvettes being added in a volume ratio of 0:1:2:3.

When the spread of the values, amounted to more than 10%, of the average value, the reaction was repeated. The average value of the three observations formed the starting point for the calculation of the enzyme activity expressed in μmol converted substrate·min^{-1}·g $tissue^{-1}$ (20° C ± 1° C). The muscle blocks of m. soleus were too small to estimate enzyme activity in each tissue block. Enzyme activity in m. soleus was therefore performed in homogenates prepared from all muscle blocks of each group of rats.

The activities of *succinate dehydrogenase* (Neufeld et al. 1954) and *glycerol-3-phosphate:acceptor oxidoreductase* (Dawson and Thorne 1969) were determined by means of the reduction of phenol indo-2,6-dichlorophenol. The extinction coefficient of the electron acceptor amounted to 16.3 $cm^2 \cdot \mu mol^{-1}$ at 600 nm. The activity of *lactate dehydrogenase* was measured at 2 pyruvate concentrations (0.28 mM and 0.80 mM) since the quotient of these two LDH activity values reflects the degree of predominance of the glycolysis (Wilson et al. 1963). The activity of *glucose-6-phosphate dehydrogenase* and *6-phosphogluconate dehydrogenase* was determined according to Löhr and Waller (1965) and Hohorst (1965), respectively. All chemicals and enzyme preparations were of the highest commercial purity available. The test reagents were dissolved in double glass distilled water.

Results

Histology

Specimens of the different muscle types from the control groups showed no histopathological alterations.

The histopathological changes in the various muscle types in the experimental animals were the same in each type, but varied in severity. Generally speaking the most severely affected muscle specimens were those from m. soleus, followed by m. rectus femoris, then m. biceps femoris. The intensity of changes in the red parts of the m. gastrocnemius was similar to that in the m. soleus. These parts were more affected than the white parts of the same muscle and the white muscles in general. Morphological alterations in the experimental muscle specimens were observed as early as one day after commencement of the DPPD injections, increasing steadily to reach a maximum at 7 days. After 9 days the lesions became steadily less extensive, suggesting a progressively developing refractory state.

From 2 days an inflammatory reaction could be observed with, in the early stages, a relative high component of neutrophil leucocytes. At 4 days the infiltrate was marked (Fig. 2) and at 7 days extensive. This pathological component de-

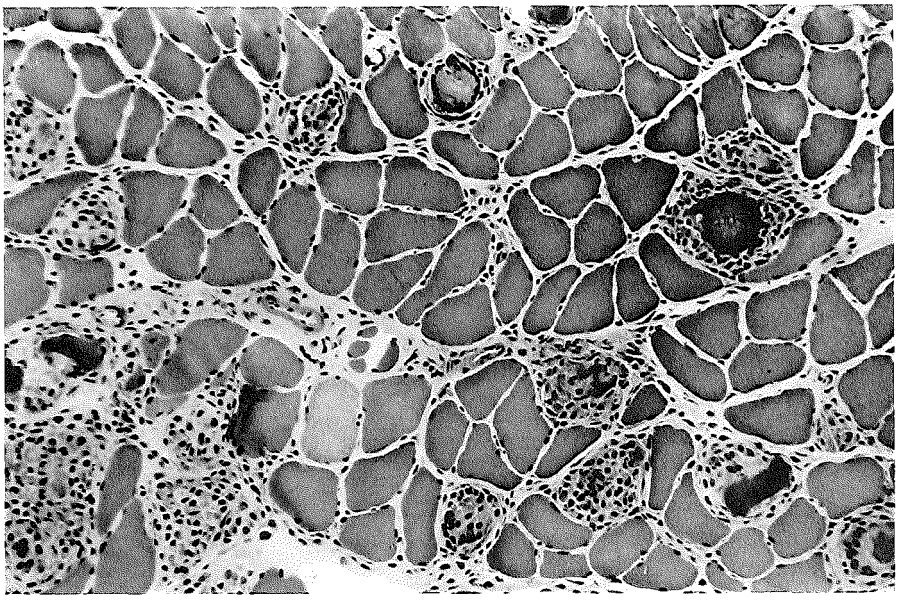

Fig. 2. Transverse section of m.soleus of a rat treated with 8 DPPD injections. Rounded fibres with eosinophilic cytoplasm, surrounded by an inflammatory infiltrate. The pathological process results in a floccular appearance of some fibres. Haematoxylin-Eosin

clined at 9 days and became quite insignificant at 11 days. The macrophages and monocytic cells of the inflammatory infiltrate appeared at about the fourth day, increasing gradually and replacing the declining neutrophil population. Maximum concentration of these components was reached at 9 days and was already markedly decreased at 11 days. Signs of regeneration and muscle spindles appeared at 7 days, but were marked at 9–11 days.

The morphological changes in the muscle fibres of the experimental rats observed as early as one day consisted of an increased variation in the diameter, the fibres having rounded contours, and in the size of individual scattered muscle fibres. These rounded fibres with eosinophilic cytoplasm contained vesicular nuclei with prominent nucleoli. The pathological changes in the muscle fibres gradually increased with a maximum at 7 days. Moth eaten and ragged red fibres appeared in the diseased muscles. Some fibres showed loss of cross-striation with cloudy and granular or floccular aspects.

Histochemistry

In the muscle specimens of the control groups no pathological changes could be observed and fibre typing was always possible with good identification of type I (aerobic), type II (anaerobic) and intermediate muscle fibres. In agreement with the histologic findings in all muscle specimens of the experimental groups the same histochemical changes were present. The m. soleus was the most affected; m. rectus femoris and m. biceps femoris were less affected. Sometimes

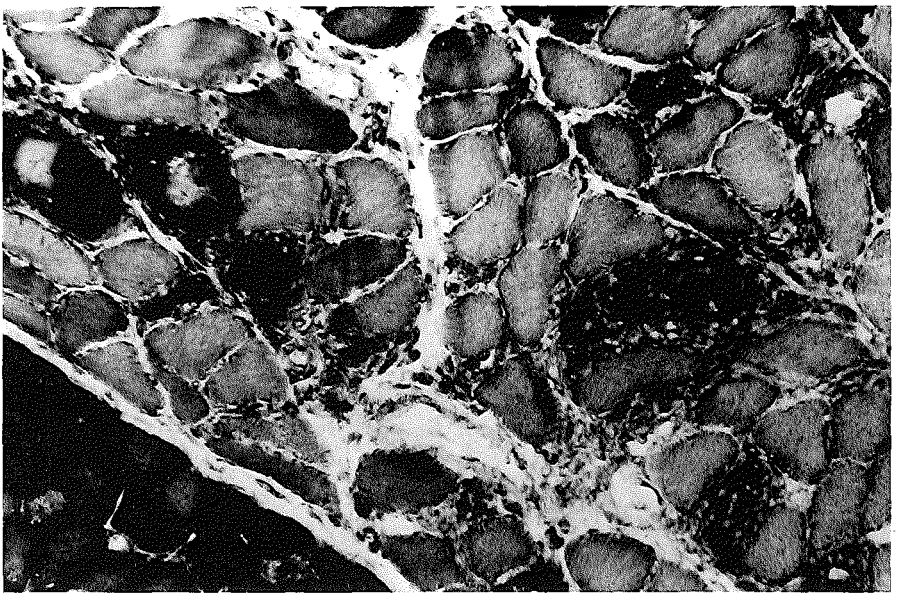

Fig. 3. Transverse section of m.soleus of a rat treated with 8 DPPD injections. There is a high activity of mitochondrial glycerol-3-phosphate dehydrogenase in the inflammatory infiltrate. The muscle fibres in the affected regions of the diseased muscle show a decrease in enzyme activity. In the bottom left of the illustration an apparently less affected part of the muscle with normal enzyme activity

the anaerobic fibres tended to be affected earlier, in other specimens the aerobic fibres, although both were ultimately involved in the process. An important feature of the affected fibres was a loss of the constancy of activity ratios of the enzymes belonging to the energy metabolism, such as is found in non-affected skeletal muscles (Bücher and Pette 1965). Therefore, it was frequently impossible to define the fibre type, especially after 2 days of the beginning of the injections. There were more fibres with an intermediate enzyme pattern in the experimental muscle specimens than in specimens from the control groups. Moreover, in the diseased muscles the percentage of intermediate fibres among affected fibres was higher then among the apparently non-affected fibres.

Microscopic examination of the staining intensities in the muscle sections of the DPPD-rats showed a moderate decrease in the activity of succinate dehydrogenase, NAD^+ and $NADP^+$ dependent malate dehydrogenase and isocitrate dehydrogenase and mitochondrial glycerol-3-phosphate dehydrogenase (Fig. 3). On the other hand, the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, monoamine oxidase, 3-hydroxybutyrate dehydrogenase, myeloperoxidase, acid phosphatase and non-specific esterase was increased. There was an intense increase in activity of both pentose phosphate shunt enzymes, whilst the activity of the other enzymes showed only a moderate increase. These activity

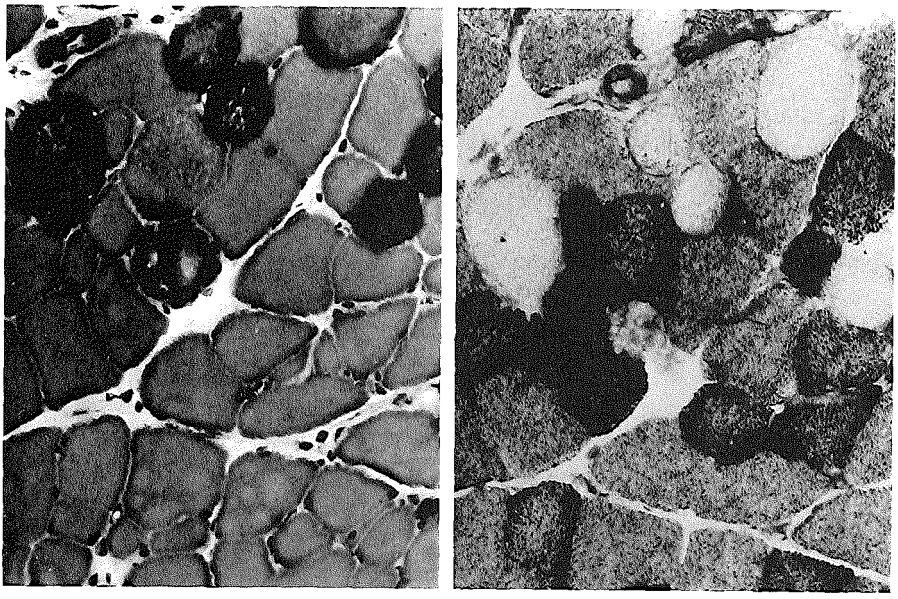

Fig. 4. Transverse serial sections of m.rectus femoris of a rat treated with 8 DPPD injections. Haematoxylin-Eosin (left). The rounded fibres with eosinophilia have no activity of succinate dehydrogenase (right)

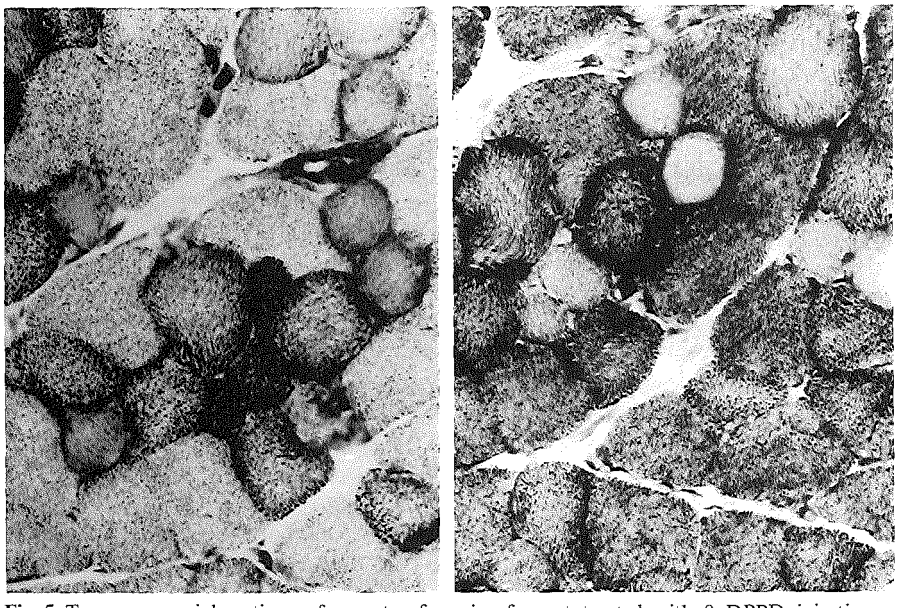

Fig. 5. Transverse serial sections of m.rectus femoris of a rat treated with 8 DPPD injections. The rounded fibres reveal activity of NADH: tetrazolium oxido-reductase (left) and most rounded fibres reveal only a slight activity of mitochondrial glycerol-3-phosphate dehydrogenase (right). Figures 4 and 5 show a metabolic imbalance in the muscle fibres

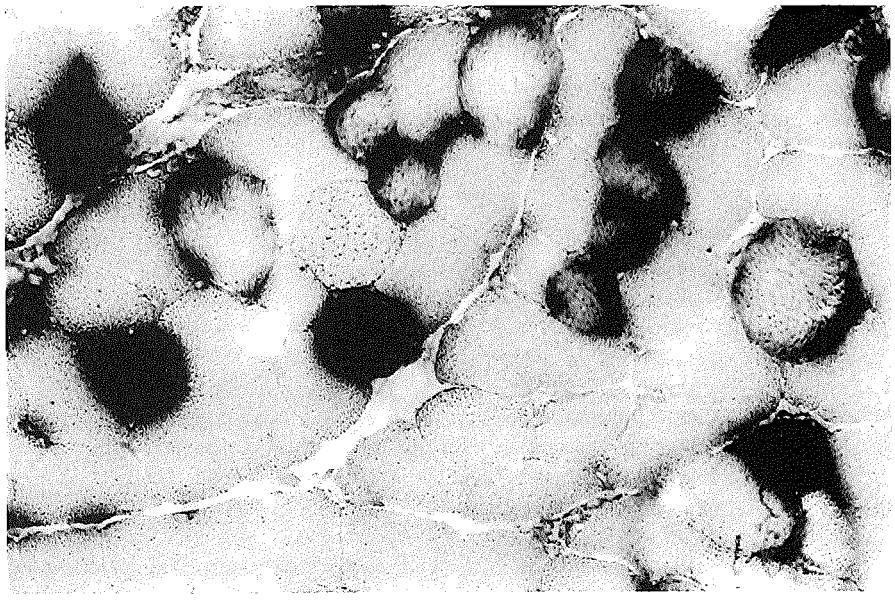

Fig. 6. Transverse serial section of m. rectus femoris of a rat with 8 DPPD injections. Activity of glucose-6-phosphate dehydrogenase. Owing to the very high activity the incubation time was 20 min instead of 90 min that is necessary for normal skeletal muscles

changes were maximal at 4 and 7 days and they gradually disappeared at 9 and 11 days.

Microscopic examination of the staining intensity produced by the activity of other enzymes which play a role in oxidation-reduction reactions, as well as phosphofructokinase, phosphoglucomutase and glucosephosphate isomerase showed no distinct change in the mean activity.

In the rounded fibres with eosinophilia the activity of succinate dehydrogenase, NAD^+ dependent malate dehydrogenase and isocitrate dehydrogenase was absent (Fig. 4). The activity of mitochondrial glycerol-3-phosphate dehydrogenase, $NADP^+$ dependent malate dehydrogenase and isocitrate dehydrogenase was decreased in these fibres (Fig. 5) whereas the activity of both pentose phosphate shunt enzymes was enormously increased (Fig. 6). In many fibres this activity was present in the entire fibre, whilst in other fibres a patchy distribution of the activity was present with the greatest activity in the peripheral zone of the fibres. The increase in activity of the pentose phosphate shunt enzymes was present as early as 8 h after a single injection of DPPD and was the first pathological change to be observed. This increase in activity was present in the peripheral zone of some fibres which still maintained normal morphological features. The activities of the other enzymes belonging to the energetic part of the metabolism were not obviously altered in the rounded fibres.

The accumulation of granular material, containing calcium phosphates as demonstrated by both the von Kossa and alizarine red s stain accompanied the initial stages of degeneration of the rounded fibres with eosinophilic cytoplasm. These deposits gave rise to false positive staining results with the metal-salt techniques for the demonstration of mitochondrial and myosin adenosine triphosphatase activity. The results obtained with these techniques proved therefore unsuitable for fibre typing of the affected rounded fibres.

In muscle specimens from control rats, only a slight activity of acid phosphatase, non-specific esterase and myeloperoxidase was present in the subsarcolemmal regions of the muscle fibres. In the muscle fibres from the DPPD-rats, activity of the hydrolytic enzymes was present in the inflammatory infiltrate and in fibres showing severe pathological changes. In these fibres, this activity was mainly located in the subsarcolemmal regions with both a diffuse and a granular pattern. The rounded fibres with eosinophilic cytoplasm contained an increase in lipids and a decrease in glycogen. In the diseased muscles, no type grouping or other neurogenic aspects could be observed.

In the experimental animals some changes were present in other tissues. Both cardiac muscle fibres and the conducting system showed no prominent changes, either histologically or histochemically. Some minimal perivascular infiltrations could occasionally be seen. The metabolic pattern of cardiac muscle fibres was the same as that of the control groups. The activity of acid phosphatase was not increased. Liver cells showed some cloudy swelling. A few cells were degenerated. Sinusoidal leukocytes and minimal infiltrates were encountered. No fatty degeneration was evident. The spleen showed a transient congestion in comparison with the control group. This was most obvious in the 7 days DPPD rats. The pancreas did not show any signs of pancreatitis or degeneration whilst in the suprarenal no evident changes or lipoid depletion was evident. The kidneys showed slight signs of transient tubular degeneration early in the experiment. This was less pronounced or absent in the later stages.

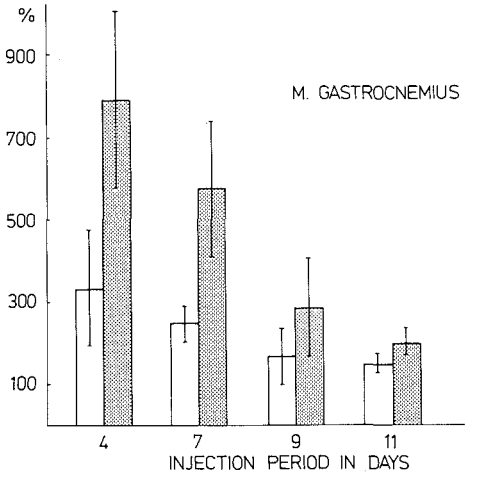

Fig. 7. The increase in percentage activity of glucose-6-phosphate dehydrogenase ▨ and 6-phosphogluconate dehydrogenase ▭ with SD in m.gastrocnemius after DPPD injections. Activity of control specimens is 100%

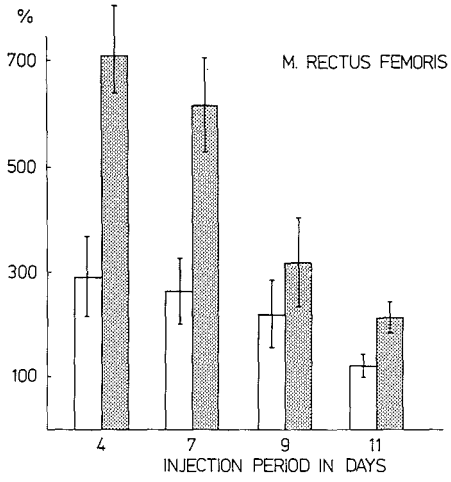

Fig. 8. The increase in percentage activity of glucose-6-phosphate dehydrogenase ▨ and 6-phosphogluconate dehydrogenase □ with SD in m.rectus femoris after DPPD injections

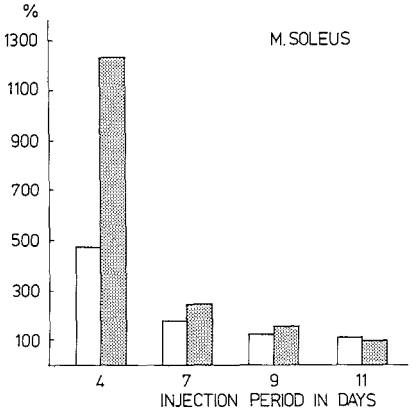

Fig. 9. The increase in percentage activity of glucose-6-phosphate dehydrogenase ▨ and 6-phosphogluconate dehydrogenase □ in m.soleus after DPPD injections

Biochemistry

The biochemical findings concerning the activity of glucose-6-phoshate dehydrogenase and 6-phosphogluconate dehydrogenase are presented in Figs. 7–9. The marked increase in activity was maximal after 8 injections of DPPD. Glucose-6-phosphate dehydrogenase showed a 13-fold increase in m. soleus, whilst in m. rectus femoris the increase was 7-fold and in m. gastrocnemius 8-fold when compared with control activities. The maximal activity is partly related to the presence of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase positive leucocytes – histochemically observed – in the inflammatory infiltrate of the experimental muscle specimens. The density of this infiltrate is at its highest at this stage of the experiments. After four days the elevation in activity of these enzymes gradually diminished and for some muscle specimens had already disappeared after 11 days. Contrary to the effects on the activity of both pentose phosphate shunt enzymes, the activity of succinate dehydroge-

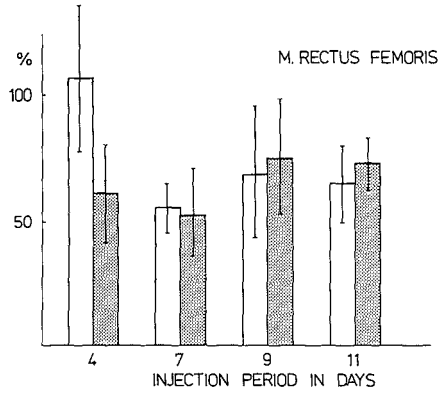

Fig. 10. The decrease in percentage activity of mitochondrial glycerol-3-phosphate dehydrogenase ▨ and succinate dehydrogenase ▭ with SD in m.rectus after DPPD injections

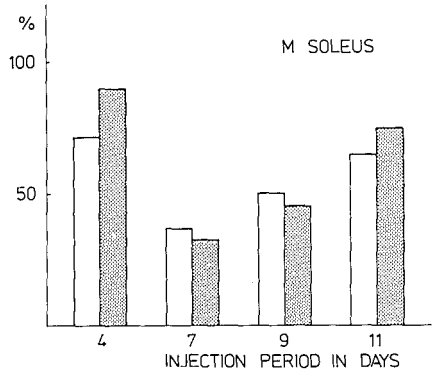

Fig. 11. The decrease in percentage activity of mitochondrial glycerol-3-phosphate dehydrogenase ▨ and succinate dehydrogenase ▭ in m.soleus after DPPD injections

nase and mitochondrial glycerol-3-phosphate dehydrogenase decreased as a result of the DPPD injections. This decrease was maximal at 7 days. The results for m. soleus and m. rectus femoris are presented in Figs. 10 and 11. For m. gastrocnemius this decrease was identical and by 7 days had reached approximately 50% of the control activity. The activity of lactate dehydrogenase measured at 0.28 mM and 0.80 mM pyruvate was not significantly altered in the skeletal muscles of the experimental rats.

Discussion

The mode of pathological reaction of human and animal skeletal muscle tissue to injury is well understood by virtue of the large number of experimental studies and the wide variety of techniques applied to this phenomenon. The most notable aspect of the reaction of the injured skeletal muscle is the remarkably stereotyped character of the resulting changes as demonstrated by the fact that many diverse agents produce similar results. The only exception is experimental denervation, which gives rise to typical neurogenic lesions. Therefore the pathological changes and especially the marked increase in activity of both pentose phosphate shunt enzymes found in skeletal muscles of rats in response

to the DPPD injections may be considered representative of myogenic changes in human skeletal muscles. The histological, histochemical and biochemical findings concerning the pathological changes in the experimental muscles induced by the administration of DPPD corresponded completely with each other, as far as these changes can be compared. The pathological characteristics were the same in the different muscle types. Only the severity of the pathological features varied.

Since no type grouping or other neurogenic aspects could be observed in the diseased muscles, the histopathological findings clearly demonstrated a myogenic pattern of the lesion. The generalized myopathic changes with sarcoplasmic degeneration, necrosis, phagocytosis, perivascular and interstitial inflammation, internalization of vesicular nuclei with prominent nucleoli and fibre diameter changes with eosinophilia are suggestive of a form of myositis.

The m. soleus is characterized by a more sustained activity with a relatively intensive aerobic energy-metabolism; the m. gastrocnemius is adapted to sudden activity and function for short periods with a relatively anaerobic energy-metabolism. M. rectus femoris and m. biceps femoris have a mixed type of energy-metabolism. Since the m. soleus was the most severely affected and the white (anaerobic) parts of m. gastrocnemius the least, the histochemical findings revealed a correlation between the degree of severity of the pathological processes and the metabolic type of the muscles investigated. The aerobic muscles are clearly more vulnerable to DPPD. This correlation was further supported by the fact that the most severe pathological alterations in the m. gastrocnemius were mainly present in the aerobic parts of the muscle.

The appearance of the rounded, mostly hypertrophic fibres was the first morphological change that could be observed in the experimental muscles. Despite the fact that the activity of a great number of enzymes was demonstrated, it was difficult, or even impossible, to determine the histochemical type of these fibres, owing to a complete loss of the so-called constant proportion groups of enzymes (Bücher and Pette 1965) and irregular staining of the fibres. The decrease in activity of succinate dehydrogenase and NADH: tetrazolium oxidoreductase tended to be observed first in the aerobic fibres, whilst the marked increase in activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase tended to be found first in the anaerobic fibres. These results clearly demonstrate a metabolic imbalance in the muscle fibres. In comparison with the control-muscles, the experimental muscles did not appear to have become more aerobic or more anaerobic. Also important is the fact that the activity of the glycolytic and gluconeogenetic rate limiting enzyme phosphofructokinase is not changed. Moreover the activity and the isoenzyme pattern of lactate dehydrogenase is unaltered in the experimental muscles, indicating that the metabolic pattern is not changed as a result of the DPPD injections. The estimation of lactate dehydrogenase activity in the presence of 0.28 mM pyruvate whereby the activity of the H-type of LDH is measured, provides information about the aerobic capacities of the muscles, whereas the estimation in the presence of 0.80 mM pyruvate, whereby the activity of the M-type of LDH is estimated, provides information about the anaerobic capacities of the muscles (Kaplan and Goodfriend 1964).

The results demonstrate a dramatic early enhancement (within 8 h) of both rate-limiting enzymes of the pentose phosphate pathway. A marked increase in activity of these enzymes in diseased skeletal muscles has also been observed by Laudahn and Heyck 1963; Heyck et al. 1963; McCaman 1963; Manchester et al. 1970; Dhalla et al. 1972. Glucose-6-phosphate dehydrogenase in liver has a half-life of about 15 h (Freedland 1968; Goldberg and St. John 1976). Although the half-life of glucose-6-phosphate dehydrogenase in skeletal muscle has yet to be determined, the rapid increase in activity in response to DPPD suggests that the half-life will be relatively short in skeletal muscles. The same holds for 6-phosphogluconate dehydrogenase whereby an increase in activity could also be observed as early as 15 h after one single injection of DPPD. Short half-life enzymes are considered to be regulatory enzymes whose activity can fluctuate rapidly in response to environmental changes (Goldberg and St. John 1976). Since the pentose phosphate shunt is only a negligible pathway in normal skeletal muscle, the marked increase in activity of both enzymes can be detected very easily. The results of the present study indicate that the histochemical techniques are highly efficient in detecting minor diseases and early onset of major diseases of the human neuromuscular system. It should be noted that regulation of glucose-6-phosphate dehydrogenase and 6-phospho gluconate dehydrogenase is complex and the process differs among experimental systems. For example, the enhancement of glucose-6-phosphate dehydrogenase activity in liver (Rudack et al. 1971; Garcia and Holten 1975), uterus (Smith and Barker 1974) and hepatoma (Selmeci and Weber 1976) and 6-phosphogluconate dehydrogenase activity in liver (Procsal et al. 1976) and mammary gland (Betts and Mayer 1977) occurs via newly synthesized enzyme protein. On the other hand, increased glucose-6-phosphate dehydrogenase activity may be accomplished without synthesis of enzyme protein, although the increase is blocked by actomyosin D or cycloheximide (Yagil et al. 1974; Hizi and Yagil 1974). In these situations, enhanced activity apparently results from modification of pre-existing enzyme molecules (Hizi and Yagil 1974). It has been postulated that post-transcriptional regulation of glucose-6-phosphate dehydrogenase synthesis occurs in liver after carbon tetrachloride injury (Watanabe and Taketa 1973) and in erythrocytes and leukaemic granulocytic cells (Kahn et al. 1976).

Since short half-life enzymes regulate biochemical pathways we attempt in a subsequent study to discover how these pentose phosphate shunt enzymes are regulated. Understanding of this process may give insight into the pathological processes in skeletal muscles.

The biosynthesis of fatty acids is related to the oxidative part of the pentose phosphate shunt where both enzymes produce NADPH. In connection with this, it was striking that muscle fibres having a high activity of both enzymes always contained increased lipid accumulations. However the biosynthesis of fatty acids is complex and since the activity of many important enzymes such as citrate lyase, fatty acid synthetase and acetyl CoA carboxylase have not yet been studied in skeletal muscles, it is too early to suggest a connection between lipid storage and increase in activity of both pentose phosphate shunt enzymes.

The calcium deposits in the rounded fibres were similar to those described in Duchenne dystrophy and in other muscle diseases (Bödensteiner and Engel

1977), and are therefore not specific. It is presumed that at least some, if not all of these granular deposits were associated with mitochondria and may correspond to the dense particles seen in the mitochondrial matrix (Peachy 1964). Muscle fibres showing calcium accumulations were not seen in the controls. In the diseased skeletal muscles the presence of mitochondria with loosely-coupled mitochondria could not be observed (Meijer and Vloedman 1980).

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CHAPTER 4.5

THE INCREASE IN ACTIVITY OF THE NADPH-REGENERATING ENZYMES OF THE PENTOSE PHOSPHATE PATHWAY IN VITAMIN E DEFICIENCY INDUCED MYOPATHY IN RABBITS. A HISTOCHEMICAL AND BIOCHEMICAL STUDY

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Abstract—Vitamin E deficiency induced myopathy in rabbits has been studied histochemically and biochemically. In this communication alterations in the activity of enzymes which play a role in the aerobic and anaerobic energy metabolism as well as the key enzymes of the pentose phosphate pathway are presented and discussed. The most important finding of the experiment was a marked increase in the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase early in the experiment, even before the myopathy was clinically manifest. This result emphasizes the importance of studying the activity of these enzymes as an indicator of metabolic imbalance or to detect the early stages of diseases affecting the neuromuscular system, or primary or secondary myopathies. A second important finding is the loss of constant proportion characteristics of the metabolism in the diseased muscle fibres, which is expressed in an increase in the number of fibres which show an intermediate type of metabolism.

Key words: $NADP^+$, pentose phosphate pathway, vitamin E deficiency, myopathy, muscular diseases, neuromuscular diseases, skeletal muscles, rabbit, enzyme histochemistry

L'AUGMENTATION DE L'ACTIVITE DES ENZYMES REGENERANT LE NADPH DE LA VOIE DES PENTOSES PHOSPHATES AU COURS DE LA MYOPATHIE INDUITE PAR LA DEFICIENCE EN VITAMINE E CHEZ LES LAPINS

Résumé—La myopathie induite par la déficience en vitamine E chez le lapin été étudiée histochimiquement et biochimiquement. Dans cette communication, des altérations dans les activités des enzymes, qui jouent un rôle dans le métabolisme de l'énergie aérobique et anaérobique aussi bien que les enzymes-clefs de la voie des pentoses phosphates, sont présentées et commentées. Le fait saillant le plus important de cette recherche a constitué dans une élévation marquée dans l'activité de la glucose-6-phosphate deshydrogénase et de la 6-phosphogluconate deshydrogénase, dès les premiers stades de l'expérimentation, même avant une manifestation clinique de la myopathie. Ces résultats mettent l'accent sur l'importance de l'étude de l'activité de ces enzymes comme indicatrices du désordre métabolique ou pour détecter les étapes initiales de maladies affectant le système neuromusculaire, ou des myopathies primaires ou secondaires. Une deuxième constatation importante réside dans la perte de la proportion constante des caractéristiques du métabolisme dans les fibres musculaires atteintes, ce qui s'exprime par une augmentation du nombre des fibres révélant un type métabolique intermédiaire.

Mots-clefs: $NADP^+$, shunt de pentoses phosphates, carence en vitamine E, myopathie, maladies neuromusculaires, muscles squelettiques, lapin, histochimie des enzymes.

INTRODUCTION

At the present time, data are accumulating in the literature about the metabolic role of the pentose phosphate pathway enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in cancer metabolism and in many diseases where the living cell must resort to subsidiary means of energy supply to maintain its existence under adverse conditions.

In previous communications we discussed the increase in the activity of these enzymes in

conditions affecting the neuromuscular system in human subjects (Meijer and Elias, 1977; Meijer *et al.*, 1977). A similar increase was noted during a study of the early and late ischaemic changes in the conducting system and fibres of the myocardium proper in the human heart, as compared to the non-ischaemic metabolic pattern of these fibres (Elias *et al.*, 1980 b, 1982). In an experimental study of the toxic effect of *N, N'*-dimethyl-*p*-phenylenediamine in rats we concluded that there is an early increase in the activity of these enzymes, even before the onset of clinical myopathy (Elias and Meijer, 1981). In other communications we discussed the presence and role of these enzymes in many cancer cells (Elias *et al.*, 1980a, 1981). The present investigation was intended as a further study of the activity of both enzymes of the pentose phosphate pathway in the type of myopathy induced by vitamin E deficiency. Use was made of a battery of conventional enzyme histochemical techniques and of the recently developed semipermeable membrane techniques which are designated to hamper the diffusion of soluble enzymes during the incubation.

MATERIALS AND METHODS

Rabbits

Thirty-five male white New Zealand rabbits were subjected to *ad libitum* administration of a specially prepared synthetic diet (Hope Farms, Woerden, The Netherlands) containing all necessary ingredients except vitamin E. Another 7 rabbits served as a control group. The control group received the same diet after addition of an appropriate and balanced amount of vitamin E, (α-tocopherol) and a supplementary dose of vitamins A and D. All animals received sulphamerazine at a concentration of 2 g/litre added to the drinking water as a protective measure against infective diarrhoea. When necessary, the sulphonamide agent was injected intravenously. The experimental animals were between 28 and 39 days old. The starting weight ranged from 440 to 840 g, with a mean of 570 g. The experimental animals were observed and weighed daily. Of the 35 experimental animals 24 were killed when the clinical condition was such that the experiment could not be continued (Telford, 1971; stage 3). The remaining 11 experimental animals were killed at different stages without showing any clinical disablement. These included two animals which were killed at 48 and 130 days respectively. Blood specimens from the ear veins were collected for biochemical evaluation of serum levels of vitamin E immediately before killing the animals. The rabbits were killed by a blunt blow on the back of the neck after ether anaesthesia. No barbiturate-containing drugs were used.

Tissue specimens

Representative skeletal muscle specimens were taken from both the white and red heads of m. gastrocnemius, m. soleus and m. plantaris, and were dissected free of fatty tissue, tendons or fibrous tissue. The muscle specimens were cut into four blocks according to the scheme shown in Fig. 1. Blocks 1 and 4 were used for histological examination. Block 2 was used for histochemistry and block 3 was reserved for biochemical assays. From each rabbit, three specimens from diaphragm muscles were prepared. These were ribbon-shaped and were rolled. One was prepared for histochemistry, one for histology and the third was reserved for biochemical assays. Heart muscle specimens were transversely dissected from the posterior cardiac wall and included left ventricular wall, septum and a part of the right ventricular wall. From each heart one specimen was used for histochemistry, a second for histology and a third for biochemistry. Representative specimens from other organs including kidney, suprarenal, spleen, liver, stomach and intestine were prepared for histological examination alone.

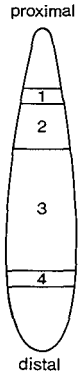

Fig. 1. Scheme of dissection.

Histology

All specimens intended for histological examination were fixed in buffered formalin 8% solution adjusted at pH 7.2:7.4 for 24 hr, processed and embedded in parawax. Tissue sections were cut at 6 μm and stained with modified Harris's haematoxylin and eosin (Harris, 1900), Gomori trichrome (modified after: Gomori, 1950) and modified periodic acid Schiff reagent as carried on in our laboratories (after Bancroft and Stevens, 1975). Oil red-O was used to demonstrate lipids (modified after Lillie and Ashburn, 1943). The methods of Von Kossa (1901) and Alizarin Red-S stain (modified after McGee-Russel, 1958) were used to demonstrate calcium deposits.

Histochemistry

The muscle specimens were rapidly frozen by immersing the tissue blocks in isopentane precooled to -150°C with liquid nitrogen and were stored at -96°C. Transverse serial sections at a thickness of 7 μm were cut in a cryotome. Unfixed sections were stained by the following techniques. The activity of succinate dehydrogenase (SD, EC 1.3.99.1) was demonstrated according to Nachlas *et al.* (1957), but with the addition of 50 mg phenazine

methosulphate ml^{-1}. The activity of NADH: tetrazolium oxidoreductase (NADH, EC 1.6.99.3) was demonstrated according to Barka and Anderson (1963) with the substrate NADH. The activity of glycerol-3-phosphate: menadione oxidoreductase G3P-OR, EC 1.1.99.5) was demonstrated according to a substrate modification of the method of Wattenberg and Leong (1960). In place of succinate an equimolar concentration of glycerol-3-phosphate was used. The activity of lactate dehydrogenase (LD, EC 1.1.1.27) was demonstrated according to Meijer (1973). The methods described by Barka and Anderson (1963) were used to demonstrate the activity of NAD^+ dependent malate dehydrogenase (MDH:NAD^+, EC 1.1.1.37) and isocitrate dehydrogenase (ICD:NAD^+, EC 1.1.1.41). The activity of glucose-6-phosphate dehydrogenase G6P-D, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-PGD, EC 1.1.1.44) was demonstrated according to Meijer and de Vries (1974). The activity of isocitrate: $NADP^+$ oxidoreductase (decarboxylating) (ICD:$NADP^+$, EC 1.1.1.42) and malate: $NADP^+$ oxidoreductase (decarboxylating) (MD:$NADP^+$, EC1.1.1.40) was demonstrated according to Meijer and de Vries (1975). The activity of calcium activated myosin adenosine triphosphatase (ATP–Ca^{2+}, EC 3.6.1.3) was demonstrated according to Meijer (1970). The activity of acid phosphatase (AP, EC 3.1.3.2) was demonstrated according to Meijer (1972).

Biochemistry

The tissue specimens from m. soleus, m. plantaris, m. gastrocnemius, as well as cardiac and diaphragm muscles, from the control group and the experimental animals were weighed and, immediately after thawing, were cut finely in a tenfold volume of 50 mM Tris buffer (pH 8.2, 4°C) according to the method of Opie and Newsholme (1967). After homogenising for 30 sec at 0°C in an all-glass tight-fitting homogeniser, the homogenates were centrifuged for 10 min (175 × *g*, 4°C) and the determinations were performed in a fixed sequence directly from the supernatant. Only the lactate dehydrogenase reaction was determined from the supernatant after repeated centrifugation (45 min 20.000 × *g*, 4°C) of the first supernatant. The reaction rate (20°C ± 1°C) was deduced from the linear part of extinction change after a time elapse of 10 min. The reactions were followed for at least 15 min and carried out in quadruplicate, the supernatant in the four cuvettes being added in a vol. ratio of 0:1:2:3. When the spread of the values amounted to more than 10% of the average value, the reaction was repeated. The average value of the three observations formed the starting point for the calculation of the enzyme activity expressed in μmole converted substrate/min/g tissue (20°C ± 1°C). The activities of succinate dehydrogenase (Neufeld *et al.*, 1954) and glycerol-3-phosphate: acceptor oxidoreductase (Dawson and Thorne, 1969) were determined by means of the reduction of phenol indo-2.6-dichlorophenol. The extinction coefficient of the electron acceptor amounted to 16.3 cm^2/μmole at 600 nm. The activity of lactate dehydrogenase was measured at two pyruvate concentrations (0.28 mM and 8.0 mM) since the quotient of these two LD activity values reflects the degree of predominance of the glycolysis (Wilson *et al.*, 1963). The activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was determined according to Löhr and Waller (1965) and Hohorst (1965). The activity of acid phosphatase was determined according to Meijer and Israël (1978, 1979). The technique is a modification of the method of Robinson and Willcock (1969). The degree of vitamin E deficiency was estimated from the serum levels of α-tocopherol, according to the method described by Duggan (1959).

RESULTS

Course of clinicopathological changes

The delay between the commencement of the vitamin E deficient diet and the onset of clinical symptoms in the test-animals varied. The shortest period was 10 days and the longest 35 days. Four rabbits showed early symptoms at about the 10th or 12th day and were killed. A further four symptom-free rabbits receiving the same deficient diet were killed at the same time. Of the 35 test-animals 14 were clinically disabled between the 16th and the 23rd experimental day. These were killed together with three animals which had remained symptom-free. Another 8 animals, (6 disabled and 2 symptom-free) were killed between 25 and 35 days. Two animals failed to show any clinical disablement and continued to grow normally. These rabbits were killed, one at the 48th and one at the 130th experimental day, in order to terminate the experiment. All experimental animals, with or without clinical symptoms showed the same type of histochemical and biochemical changes. All showed a low concentration of vitamin E levels in the serum. The levels varied from 0.0 μg/ml to 10.5 μg/ml irrespective of the length of the experiment.

The mean concentration was 3.95 μg/ml. The vitamin E level in sera of the control group varied from 20.0 μg/ml to 89 μg/ml, with a mean value of 38.8 μg/ml. Although no linear relation could be established between serum levels, duration of the experiment, the extent of the histological changes and the development of clinical symptoms, nonetheless all experimental animals were histologically affected and all showed a deficient level of vitamin E in their sera. It could also be seen that the longer the period of deficiency, the greater was the extent of the histochemical and histological changes. Another important observation is that the experimental animals always tended to lose weight about two to three days before the sudden onset of the clincial 'disablement'. The latter was defined in our experiment as failure to stand up, or as stage 3 vitamin E deficiency (Telford, 1971).

Histology

The skeletal muscle specimens from the control group showed no histological alterations. The

histopathological changes in the various muscles from the experimental group showed no qualitative differences beween the different muscles investigated. They differed only in the severity of the lesions as measured by the number of degenerated muscle fibres. Early in the experiment, m. soleus and the diaphragm were the most severely affected muscle specimens. The least affected was m. plantaris. The changes in m. gastrocnemius specimens differed according to the site of the specimen. The specimens from the red parts showed earlier and more extensive changes than the white parts. The red head of gastrocnemius was almost as severely affected as m. soleus, while the white parts were as little, or less affected than m. plantaris. The muscles showing the most extensive histological changes also showed the most severe histochemical and biochemical alterations. Later in the experiment, the white muscle specimens became as severely affected as the red muscles and sometimes they even seemed more intensely affected. A fibrotic component was also present in the advanced stages of the disease, sometimes being observed as early as the 20th experimental day. The presence or absence of clinical disablement did not show a direct relation to the extent of the histological and histochemical alteration, at any stage of the experiment. The most extensive changes were seen at 48 experimental days followed by the animal that survived to the 130th day. The morphological changes at 35 days were less severe than at 48 days and the 10-day animals were the least affected. The morphological changes in the affected muscle fibres began with a change in the caliber of individual muscle fibres and the appearance of rounded fibres, progressing to ragged or 'moth-eaten' fibres (Fig. 2). The changes were almost always associated with an infiltration of mixed leucocytic inflammatory cells especially just after the onset of the degenerative changes. In the later stages, the infiltration contained a monocytic, histiocytic or macrophage component which replaced the decreasing neutrophil component. Some focal perivascular infiltrate was often observed. This consisted of neutrophils, plasma cells and macrophages. Areas of small-group atrophy were not uncommon. Large-group atrophy,

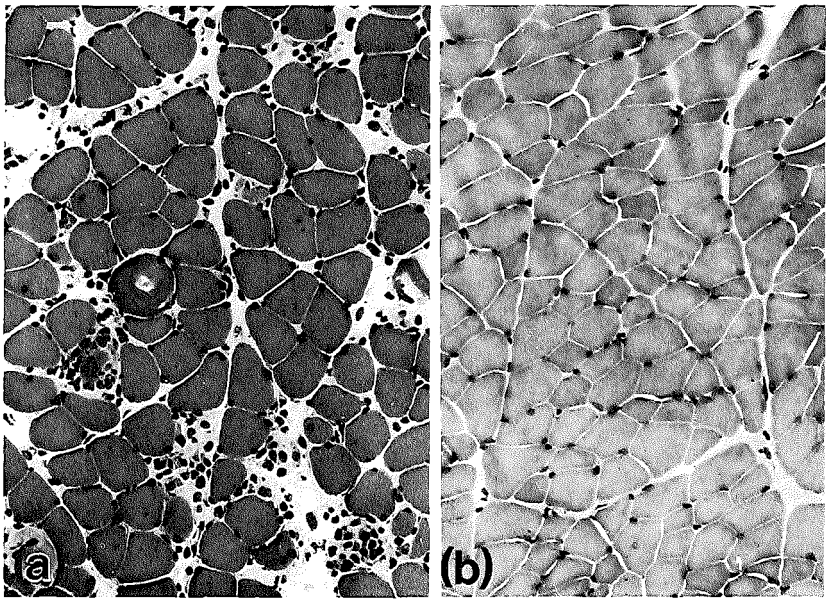

Fig. 2. Transverse section of m. soleus of a rabbit (a) fed with vitamin E deficient diet during 30 days. The pathological changes include: rounding of some fibres, variation of size, moth-eaten fibres, or completely degenerative fibres. Often a mixed inflammatory infiltrate is present. An increase of connective tissue component is also seen. (b) shows normal muscle fibres of a similar specimen from a control rabbit. × 140.

as is usually seen in neurogenic muscular dystrophies was observed in only a few specimens and sometimes involved a whole fascicle. The later stages of the disease showed scattered small and large rounded fibres among a proliferative connective tissue stroma. Evidence of regeneration was sometimes observed with the appearance of muscle spindles, especially where the vascularity was still adequate. The cardiac involvement was relatively limited, less than half of the experimental animals being involved. Small foci of lymphocytic and histiocytic cells were found in the vicinity of some slightly degenerated cardiac muscle fibres, or fibres with an increased number of nuclei. Major changes, or extensive cardiac myopathy, were not observed in our material. The morphological myocardial involvement was random and not related to the intensity of the histochemical changes or to the duration of the experiment. Pancreas, stomach, liver, kidney, suprarenals and intestines were macroscopically and microscopically without significant alterations and could not be distinguished from the control group. The spleen, however, was somewhat affected. Small focal aggregates of inflammatory cells, mainly eosinophils and plasma cells were found.

Histochemistry

The muscle specimens of the control group showed no apparent histopathological or histochemical changes. The process of fibre typing was always possible with good identification of type I (aerobic), type II (anaerobic) and intermediate muscle fibres. The histopathological changes in all muscle specimens from the experimental animals were always associated with histochemical changes in the affected muscle fibres and also in the apparently non-affected fibres in the same specimens. The sequence of the histochemical changes followed that of the histological changes. In the animals killed early in the experiment, m. soleus and the diaphragm muscles were most affected. M. plantaris and the white head of m. gastrocnemius were the least affected. The red parts of m.

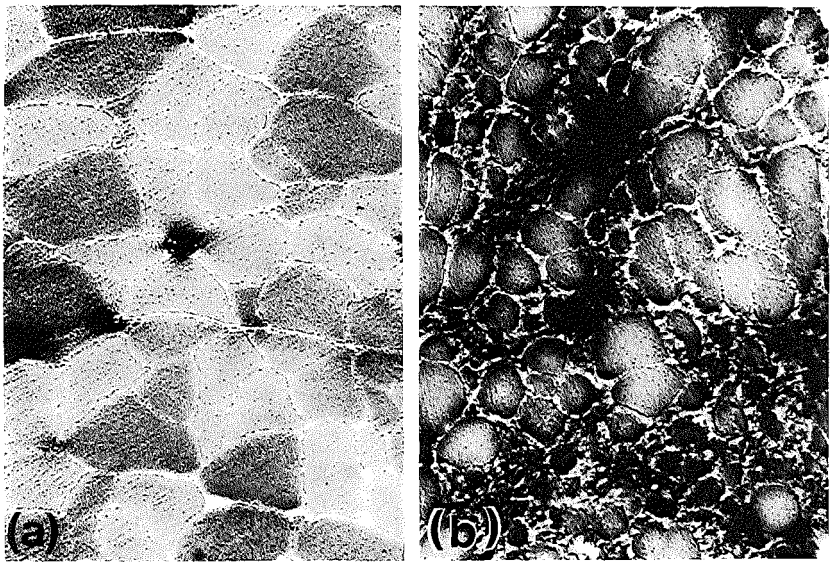

Fig. 3. Transverse sections of m. gastrocnemius of a rabbit fed with vitamin E deficient diet during 14 days showing activity of glucose-6-phosphate dehydrogenase. However the determination of the original type of the fibres is difficult, many anaerobic type II fibres show a higher activity than the aerobic type I fibres. The anaerobic white part of the muscle reveals only a slight increase in activity in both types of fibres (a). In the early stages of the disease some muscle fibres surrounding small capillaries showed an increased activity probably coming from damaged capillaries. The enzymes diffuse into the surrounding muscle fibres. Incubation time is 90 min. The aerobic part of the muscle (b) reveals a strong increase in the activity of GPDH in especially the small fibres. Owing to the very high activity the incubation time was reduced to 20 min instead of 90 min that is necessary for normal skeletal muscle specimens. × 140.

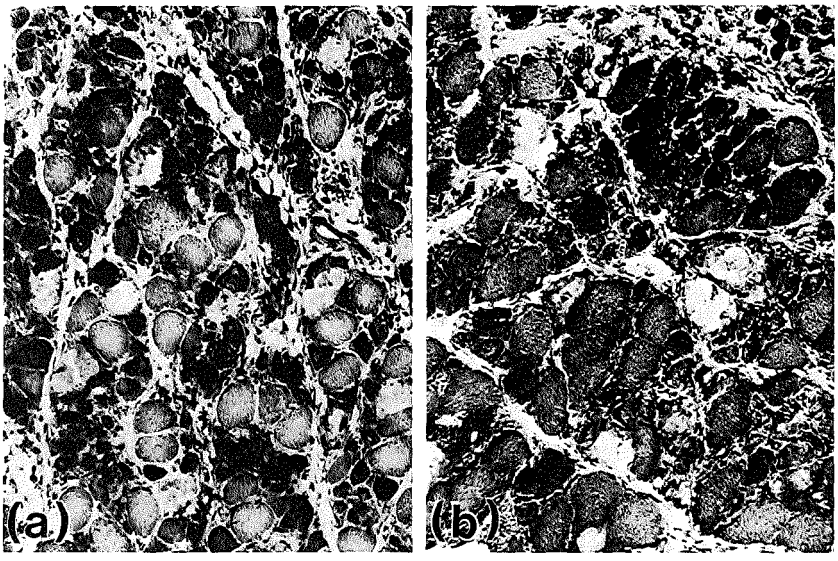

Fig. 4. Transverse sections of m. soleus of a rabbit fed with vitamin E deficient diet during 30 days. Most small fibres show a high activity of mitochondrial glycerol-3-phosphate dehydrogenase (a) and a moderate activity of succinate dehydrogenase (b). Some rounded fibres reveal no succinate dehydrogenase activity. The pictures reveal a metabolic imbalance in the muscle fibres. The inflammatory infiltrate show a high degree of activity of the mitochondrial glycerol-3-phosphate dehydrogenase. × 140.

gastrocnemius were affected to almost the same extent as m. soleus and were affected earlier and more extensively than the white parts (Fig. 3). In the late stages of the experiment the white and red muscles appeared equally affected and sometimes the white muscles even seemed to be more severely affected. The longer the experimental period the higher was the numerical percentage of fibres showing an intermediate type of metabolism (Fig. 4). This important feature indicates the loss of constancy of activity ratios of enzymes belonging to any given aerobic or anaerobic metabolic pathway. This constancy is a characteristic of non-affected skeletal muscle fibres, (Bücher and Pette 1965; Meijer and Elias 1976, 1977). It was frequently impossible to define the original type of the muscle fibres in the pathological specimens, especially when morpho-pathological changes had also taken place. No apparent differences were observed between clinically disabled and non-disabled experimental animals provided they had a deficient level of tocopherol compounds in their sera. The morphologically apparently non-affected fibres in the pathological specimens showed not only the above mentioned increase in intermediate type of metabolism, but also a general increase in the activity of both of the pentose phosphate pathway enzymes. Although this increase was present in all types of apparently intact muscle fibres, it was most obvious in the anaerobic type II fibres. Both the morphologically affected fibres and the regenerative muscle spindles always showed a high degree of activity of these enzymes. Generally speaking, the muscle spindles attained an intermediate type of metabolism containing a high degree of activity of all enzymes examined, including the pentose phosphate pathway enzymes. The increase in the activity of the pentose phosphate pathways enzymes could be observed microscopically as early as the 10th experimental day. The activity continued to increase steadily and was most marked in the 35- and 48 -day experimental animals (Fig. 5). The 130-day animal showed an almost equally high degree of activity of these enzymes. The activity of G3P-OR, NADH and LD was not constant. There was an irregular increase or decrease in activity in some fibres, the extent of these changes differing from fibre to fibre even in the same specimen. More fibres showed an increase rather than a decrease in the activity of G3P-OR. On the other hand more fibres showed a

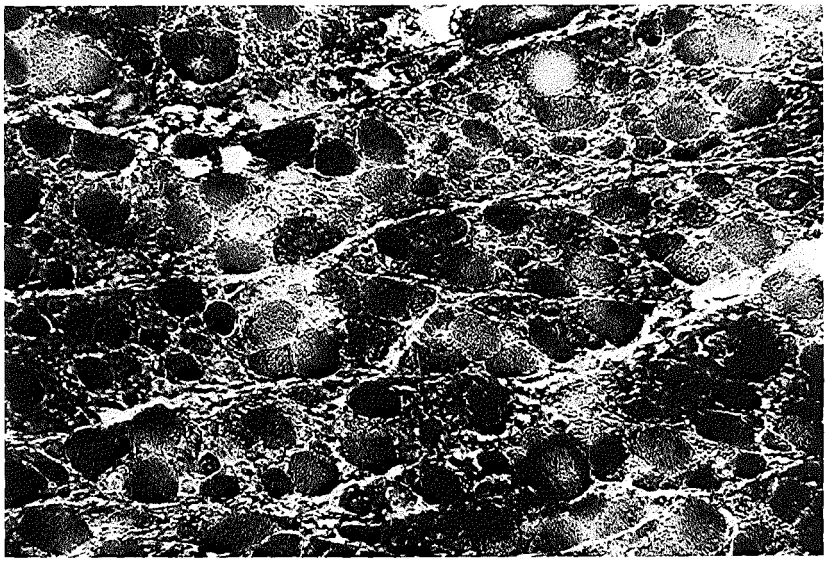

Fig. 5. Transverse section of m. soleus of a rabbit fed with vitamin E deficient diet during 30 days. Small and large muscle fibres show a strong increase in activity of glucose-6-phosphate dehydrogenase. Incubation time 20 min. × 140.

decrease rather than an increase in the activity of NADH. The number of fibres which showed increased LDH activity was almost equal to the number of fibres with decreased activity. The activity of SD was decreased or completely absent in most of the affected fibres. In the later stages, some restoration to normal was frequently observed. The activity of $NADP^+$ dependent ICD and of the NAD^+ dependent MD, was often decreased in pathological fibres both early and late in the experiment. The activity of the ATP–Ca was also altered, but more fibres showed an increase than a decrease in activity. An increased activity of acid phosphatase was an early marker of the occurrence of any pathological change in the affected muscle fibres. The cardiac muscle fibres were much less affected than the skeletal muscle fibres. The metabolic pattern of the myocardial fibres, with the exception of the following changes, was similar to that in the control group and to the normal pattern of metabolism in cardiac muscles which we described in an earlier communication (Elias *et al.*, 1980b). The most important change was a marked increase in the activity of AP, limited to the sites of focal degeneration and inflammation. This was associated with a general diffuse, but slight increase in the activity of the pentose shunt enzymes. The

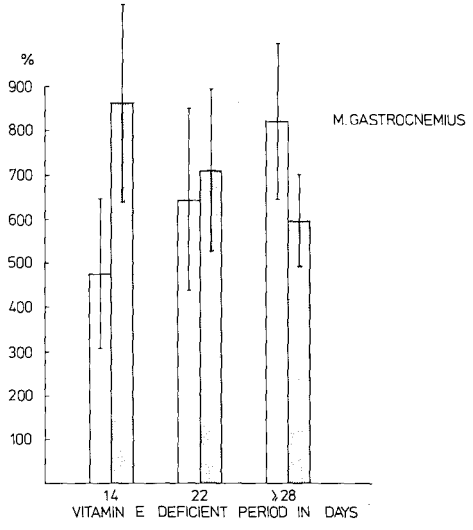

Fig. 6. The increase in percentage activity of glucose-6-phosphate dehydrogenase ▭ and 6-phosphogluconate dehydrogenase ▭ with SD in m. gastrocnemius of rabbits with vitamin E deficiency. Activity of control specimens is 100%.

latter group of enzymes was markedly increased in the vicinity of degeneration and inflammation. A certain diffusion or decrease in activity of LD, SD

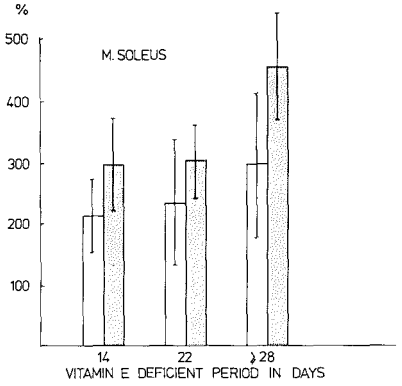

Fig. 7. The increase in percentage activity of glucose-6-phosphate dehydrogenase [ ] and 6-phosphogluconate dehydrogenase [ ] with SD in m. soleus of rabbits with vitamin E deficiency.

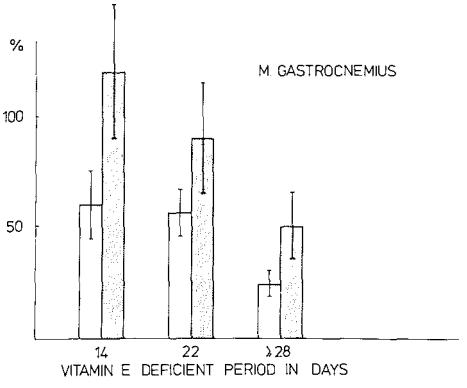

Fig. 8. The alteration in percentage activity of succinate dehydrogenase [ ] and mitochondrial glycerol-3-phosphate dehydrogenase [ ] with SD in m. gastrocnemius of rabbits with vitamin E deficiency.

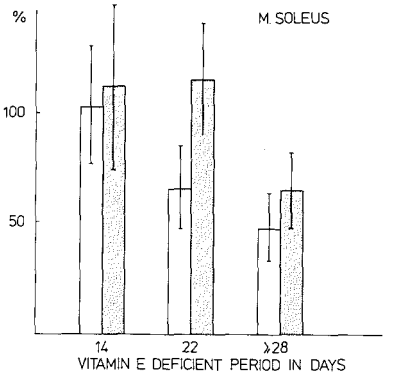

Fig. 9. The alteration in percentage activity of succinate dehydrogenase [ ] and mitochondrial glycerol-3-phosphate dehydrogenase [ ] with SD in m. soleus of rabbits with vitamin E deficiency.

and NADH-TR was observable in the small focal areas of degeneration. The Von Kossa and Alizarin stains showed fine deposits of calcium salts in the degenerated fibres. The same fibres also showed a slight increase in positivity for lipid content as seen with the O.R.O stain.

Biochemistry

The biochemical increase in the activity of G6PD and 6-PGD is presented in Fig. 6 for m. gastrocnemius and in Fig. 7 for m. soleus. G6PD showed an increase of up to 8 to 9-fold in m. gastrocnemius, and of up to 4 to 5-fold in m. soleus. 6-PGD showed an increase of up to 5 to 7-fold in m. gastrocnemius and of up to 3-fold in m. soleus. A similar increase in activity for both enzymes could be found in other muscles but mainly in m. plantaris. The increase in activity of G6PD and 6-PGD is partly related to their presence in the positive leucocytes which were histochemically and histologically observed in the inflammatory infiltrate of the experimental muscle specimens. These cells were most numerous in the early phase of the experiment. In contrast to the elevation of the pentose phosphate pathway enzymes, vitamin E deficiency resulted in a slight decreased activity of SD and G3PDR, as seen in Figs 8 and 9. The activity of LD measured at 0.28 mM and 8.0 mM pyruvate and of ICD:$NADP^+$ was not significantly altered in the skeletal muscles after 14 and 22 days. In the final stages, the activity of ICD:$NADP^+$ and of LD for both substrate concentrations was diminished.

DISCUSSION

Much knowledge of the reaction of muscle fibres to disease may be gained by studying animal models with myopathic conditions (Blaxter, 1968), such as α-tocopherol responsive myopathy (Telford, 1971). One of the functions of α-tocopherol is very probably to protect highly unsaturated fatty acids in the lipids of biological membranes against the deleterious effects of molecular oxygen. Under normal conditions autoxidation products of unsaturated fats do not occur in the tissues, but in tocopherol deficiency they can be detected in the fat

depots, liver, and other organs (Wasserman and Taylor, 1972). In many species, a vitamin E-deficient diet leads to the development of acute muscular dystrophy. The anatomical lesions consist of hyaline degeneration, loss of muscle striations, irregular distribution of sarcolemmal nuclei, swelling and vacuolization of the sarcoplasm and inflammatory reactions in the interstitial connective tissue. Metabolic abnormalities are also found Hulstaert *et al.*, 1975; Meijer and Israël, 1978). The lesions produced in skeletal muscle by a deficiency of vitamin E extend to cardiac muscle (Mason and Telford, 1947) and other organs (Vos, 1972). The younger the animal, the more rapid is the growth, and the greater is the need for vitamin E. Furthermore the stores of vitamin E are smaller in younger animals than in adult (Vos, 1972). For this reason young rabbits were used in this investigation.

Our histopathological findings clearly demonstrate a myogenic pattern of the lesion. However, in some muscle specimens a pattern of group atrophy of a whole or a large part of a fascicle was observed. Such changes are usually found in denervation models or neurogenic muscular disease. This observation was limited to a small number of animals, but its presence indicates a possible neurogenic injury in addition to the developing myopathy. On the other hand, the histochemical findings in such areas were the same as in areas where the lesion showed a more myogenic distribution.

All specimens examined revealed the same pattern of changes in all affected animals, with no qualitative differences. Only quantitative differences were found. Some muscles proved to be more extensively affected than others. The m. soleus is normally characterized by a more sustained activity with a relatively intense aerobic energy metabolism; the white part of the m. gastrocnemius is adapted to sudden activity and function for short periods with a relatively anaerobic energy metabolism. In the animals killed early in the experiment the m. soleus was the most severely affected and the anaerobic white part of the m. gastrocnemius the least, indicating a correlation between the degree of severity of the pathological process and the metabolic type of the muscles investigated. In the beginning of the experiment the aerobic fibres proved more vulnerable to vitamin E deficiency.

Despite the fact that the activity of a great number of enzymes was histochemically examined, in the later stages it was difficult, or even impossible to determine the histochemical type of these fibres, owing to a complete loss of the so-called constant proportion groups of enzymes (Bücher and Pette, 1965) and irregular staining of the fibres. Some fibres showed a decrease in activity of SD and of GPOX, whilst the activity of PFK and NADH was not altered and the activity of G6PD and 6-PGD was markedly increased. Moreover in the affected muscles there was a great increase in intermediate type fibres. These results clearly demonstrate a metabolic imbalance in the muscle fibres. The biochemical experiment showed that the experimental muscles did not appear to have become more aerobic or more anaerobic when compared with the control group. For instance, the activity and the isoenzyme pattern of lactate dehydrogenase is unaltered in the experimental muscles, indicating that the metabolic pattern is not changed. The estimation of lactate dehydrogenase activity in the presence of 0.28 mM pyruvate whereby the activity of the H-type of LD is measured, provides information about the aerobic capacities of the muscles. The estimation in the presence of 8.0 mM pyruvate, whereby the activity of the M-type of LD is estimated, provides information about the anaerobic capacities of the muscles (Kaplan and Goodfriend, 1964).

An interesting finding is the marked increase in the activity of G6PD and 6-PGD in the muscle specimens of the vitamin E deficient rabbits. These enzymes belong to the oxidative part of the pentose phosphate pathway, which is barely active in normal skeletal muscles. The dramatic increase in activity of G6PD and 6-PGD can therefore be very easily detected. The increase in the activity of both these enzymes could be demonstrated early in the experiment, even before the myopathy was clinically manifest. The findings of the present study therefore indicate that the histochemical techniques for both enzymes are highly efficient in detecting minor diseases and the early onset of major diseases of the neuromuscular system. Since the activity of G6PD in normal skeletal muscles is lower than that of 6-PGD and the increase of activity in the affected muscle specimens is higher than that of G6PD, the histochemical technique would seem to be particularly suitable for demonstrating changes in the activity of G6PD.

We also found a marked increase in the activity of both enzymes in skeletal muscles of human subjects with diseases of the neuromuscular system (Meijer and Elias, 1976, 1977; Meijer *et al.*, 1977). A similar increase in activity of both enzymes was demonstrated by Elias and Meijer (1981) in muscle fibres of

rats suffering from a *N,N'*dimethyl-*p*-phenylenediamine induced myopathy. Moreover a marked increase in activity of both enzymes, demonstrated biochemically in diseased skeletal muscles has also been observed by Laudahn and Heyck, 1963; Heyck *et al.*, 1963; McCaman, 1963; Manchester *et al.*, 1970 and Dhalla *et al.*, 1972. The histochemical technique recommended is therefore based on the results of different studies. The histochemical findings in the heart specimens showed that the application of the G6PD technique for the study of metabolic alterations in heart tissues may also be useful. In a previous communication we described an evident increase in the activity of both pentose phosphate pathway enzymes in human cardiac muscle fibre under conditions of deficient or unsuitable oxygen supply, either chronic or acute (Elias *et al.*, 1982). The same observations were found in minor infarcted areas of cardiac fibres of rats (Ciplea *et al.*, 1982). It is interesting to note that the same histochemical observations were found in proliferative lesions, mainly various types of cancer cells (Elias *et al.*, 1980 a, 1981). The practice of applying Alizarin stain, Von Kossa stain and ORO stain to the muscle specimens resulted in the demonstrations of positive fine granular calcium containing deposits and tiny droplets of lipid material in the degenerated fibres. Similar results were observed by Bodensteiner and Engel (1977) in Duchenne dystrophy and other muscle diseases. Our previous experience with the type of myopathy induced by administration of *N,N'*-dimethyl-*p*-phenylenediamine, demonstrated similar results (Elias and Meijer, 1981). The presence of calcium phosphate deposits in the degenerated cells may give rise to false-positive staining results with the metal salt technique for the demonstration of mitochondrial and myosin adenosine triphosphatase. The results obtained with these enzyme reactions proved therefore unsuitable for fibre typing in the affected and rounded fibres.

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CHAPTER 4.6

A. E. F. H. MEIJER und E. A. ELIAS

Kapazitätszunahme des Pentosephosphatzyklus in pathologisch veränderten Skelettmuskelfasern

Einführung

In pathologisch veränderten Skelettmuskeln von Patienten mit Muskelkrankheiten hat man mittels biochemischer Homogenat-Techniken gefunden, daß die Aktivität von weitaus den meisten untersuchten Enzymen im Vergleich zu der Aktivität in ähnlichen, aber gesunden Skelettmuskeln signifikant verringert ist [4, 19]. Ausnahme ist die Aktivität der Glukose-6-Phosphat-Dehydrogenase (GPDH) (EC 1.1.1.49) und der 6-Phosphogluconat-Dehydrogenase (PGDH) (EC 1.1.1.44). Die Aktivität dieser zwei Pentosephosphatzyklus-Enzyme ist in erkranktem Muskelgewebe sehr stark vermehrt [4, 10, 15, 16, 19]. Aus diesen biochemischen Befunden könnte geschlossen werden, daß in erkranktem Muskelgewebe der Kohlenhydratabbau über den Pentosephosphatweg kompensatorisch gesteigert ist, weil die Funktion des Embden-Meyerhof-Weges infolge der starken Abnahme der meisten hierzu gehörenden Enzyme stark eingeschränkt ist.

Dagegen sprechen Beobachtungen die eine Korrelation zwischen dem Fett- und dem Bindegewebegehalt der untersuchten Muskulatur und der Aktivität dieser zwei Enzyme ergeben hatten [10, 15]. Mit steigendem Fett- und Bindegewebegehalt der Skelettmuskeln nimmt die Aktivität der Pentosephosphatzyklus-Enzyme in Homogenaten von Muskelgeweben zu, da die spezifische Aktivität beider Enzyme in Fett- und Bindegewebe mehrfach höher ist als in reinem nicht erkranktem Muskelgewebe. Trennungsmethoden für Homogenat-Untersuchungen sind nämlich nicht einwandfrei. Deswegen, weil die pathologische Muskulatur oft eine deutliche Vermehrung des Binde- und Fettgewebes enthält, war man zur Einsicht gekommen, daß die Aktivitätsvermehrung dieser beiden Enzyme auf Kontaminierung des Muskelhomogenates mit Fett- und Bindegewebe zurückzuführen sein dürfte. Darüber hinaus hat man mit konventionellen enzymhistochemischen Methoden in Muskelfasern von normalem und auch von pathologisch verändertem Gewebe fast gar keine Aktivität dieser zwei Enzyme gefunden [26, 27].

Jedoch die angewendeten histochemischen Methoden konnten keine zuverlässigen Befunde ergeben haben, da während der Inkubation die Enzyme größtenteils in das Inkubationsmedium hineindiffundieren und die Farbstoffmengen abhängig sind von der Aktivität des Hilfsenzyms der Diaphorase [22].

Es werden für beide Enzyme neue histochemische Techniken entwickelt [20]. Durch die Verwendung von semipermeablen Membranen ist die Enzymdiffusion beseitigt. Da überdies mit dem Zusatz von Phenazinmethosulfat und Menadion die Farbreaktionen unabhängig von der Diaphorase-Aktivität vor sich gehen, sind die histochemischen Befunde zuverlässiger als mit den konventionellen Darstellungsmethoden. Ergebnisse, erhalten mit diesen neuen histochemischen Techniken, haben eindeutig herausgestellt, daß der Aktivitätsanstieg dieser zwei Enzyme im erkrankten Muskelgewebe nicht nur beruht auf einer Vermehrung von Binde- und Fettgewebe, sondern auch auf einer bemerkenswerten Aktivitätsvermehrung in den Muskelfasern [6, 7, 8,

21]. Mittels Modelltierversuche, in denen Cycloheximide als Hemmstoff der Proteinsynthese und Actinomycin D als Hemmstoff der Matrizen-RNS-Biosynthese angewendet wurden, haben wir feststellen können, daß die erhebliche Aktivitätsvermehrung, gefunden in den Muskelfasern von verschiedenen Typen von Muskelkrankheiten, sich auf eine Neusynthese der zwei Enzyme in den Muskelfasern selber gründet und nicht auf eine Aktivierung bereits anwesender Enzymmoleküle. Überdies war eine postduplizierende Regulierung der Proteinsynthese, oder eine Hineindiffusion von den diesbezüglichen Enzymen aus dem Binde- oder Fettgewebe in die Muskelfasern nicht vorhanden [23].
Die vorzustellende Untersuchung erfolgte mit dem Ziel, nachzuspüren ob die Aktivitätsvermehrung der zwei oxydativen Pentosephosphatzyklus-Enzyme verknüpft ist mit einer Aktivitätsvermehrung der nicht oxydativen Enzyme dieses Zyklus. Zuletzt wird näher eingegangen auf die mögliche Bedeutung des Pentosephosphatzyklus für den Stoffwechsel im Muskelgewebe.

Methodik

Das Biopsiematerial entstammte dem M. quadriceps femoris von Patienten mit erblichen oder erworbenen Krankheiten der Skelettmuskulatur. Entsprechende Gewebsproben von offensichtlich muskelgesunden Personen lieferten die normalen Vergleichswerte. Die klinischen Diagnosen basierten auf detaillierter neurologischer Untersuchung des Patienten, einschließlich Elektromyographie, Blut- und Harn-Analysen. Überdies wurde in Schnittpräparaten mittels histologischer und histochemischer Farbreaktionen die Diagnose bestimmt. Muskelmaterial von Tieren wurde verwendet, in denen eine Muskelkrankheit mittels einspritzenden von N,N'-Dimethyl-p-Phenylendiamin [6] oder mittels eines Vitamin E-Mangels [8] erzeugt worden war.

Histochemie. Die verwendeten histochemischen Techniken zur Darstellung der GPDH- und PGDH-Aktivität sind an anderer Stelle ausführlich beschrieben worden [20].

Biochemie. In Homogenaten von eingefrorenen Muskelgewebe-Blöckchen, wurde die Aktivität der GPDH nach [17], der PGDH nach [11] und die Aktivität der Transaldolase (EC 2.2.1.2.), der Transketolase (EC 2.2.1.1.), der Ribose-5-Phosphat-Isomerase (EC 5.3.1.6.) und der D-Ribulose-5-Phosphat-3-Epimerase (EC 5.1.3.1.) nach [28] bestimmt. Zur Charakterisierung des Typus der Pentophosphatzyklus wurde die Konzentration von Sedoheptulose-1,7-Diphosphat und Octulose-1,7-Diphosphat in den Geweben nach [12, 24] bestimmt. Mittels histochemischer Kontrolle wurde dafür gesorgt, daß die für die Bochemie verwendeten Gewebe-Blöckchen so viel wie möglich frei waren von Binde- und Fettgeweben.

Ergebnisse

Histochemie. Die Aktivität der GPDH und der PGDH ist in Fasern von normalen Skelettmuskeln von Mensch und Tier gering. Für beide Enzyme ist die Aktivität in den anaeroben Typ-II-Fasern etwas stärker als in den aeroben Typ-I-Fasern. Den Abbildungen 1 und 2 sind gleichfalls zu entnehmen, daß die Aktivität der Enzyme in Bindegewebe, Venen und Arterien, im Gegensatz zu den Muskelfasern, erheblich ist. In Muskelfasern von pathologisch veränderten Skelettmuskeln wird oftmals eine starke Aktivitätszunahme der zwei Pentosephosphatzyklus-Enzyme gefunden (Abb. 3, 4 und 5). Die Aktivitätszunahme kann in vereinzelten Fasern oder in allen Fasern vorkommen und in beiden Fasertypen beobachtet werden. Vor allem erkankte Muskelfasern des Duchenneschen Typus und der infantilen spinalen Atrophie zeigen einen starken Anstieg der Enzymaktivität beider Enzyme. Überdies zeigen Muskelfasern, eingebettet in Entzündungsinfiltrat, immer eine sehr stark vermehrte Aktivität der diesbezüglichen Enzyme. Fasern mit einer erhöhten Aktivität der GPDH zeigen

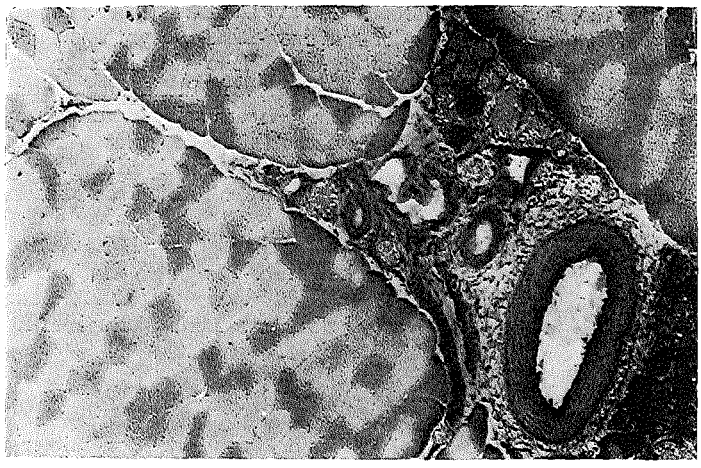

Abb. 1 *6-Phosphogluconat-Dehydrogenase-Aktivität im normalen M. quadriceps femoris des Menschen. Die anaeroben Fasern zeigen mehr Aktivität als die aeroben Fasern. Blutgefäße zeigen deutlich mehr Aktivität als die Muskelfasern. 72×*

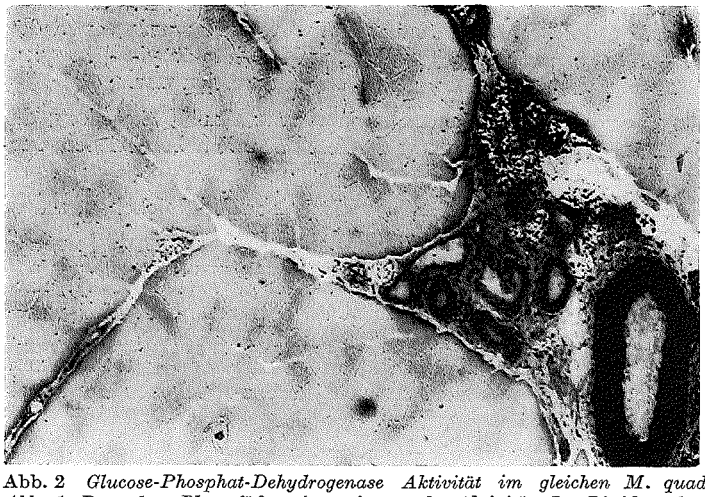

Abb. 2 *Glucose-Phosphat-Dehydrogenase Aktivität im gleichen M. quadriceps femoris wie in Abb. 1. Besonders Blutgefäße zeigen eine starke Aktivität. Im Lipidgewebe hat sich das Formazan grobkristallin rekristallisiert. Die anaeroben Fasern zeigen sehr schwache Aktivität. 72×*

immer eine erhöhte Aktivität der PGDH und umgekehrt. Die Enzymaktivitäten in Bindegewebe und Gefäßen pathologisch veränderter Skelettmuskeln haben sich im Vergleich zu den Aktivitäten in ähnlichen aber gesunden Skelettmuskeln nicht geändert.

Biochemie. In menschlichen erkrankten Muskeln kam es zu einem dreifachen bis zu einem zehnfachen Anstieg der GPDH und zu einem zweifachen bis zu einem sechsfachen Anstieg der PGDH (Tabelle 1). Ebenfalls war die Aktivität der nicht oxydativen Enzyme des Zyklus gestiegen. Die Aktivitätszunahme war dennoch wesentlich geringer und betrug höchstens einen dreifachen Anstieg im Vergleich mit der Aktivität in den diesbezüglichen normalen Skelettmuskeln. Es werden in einigen erkrankten

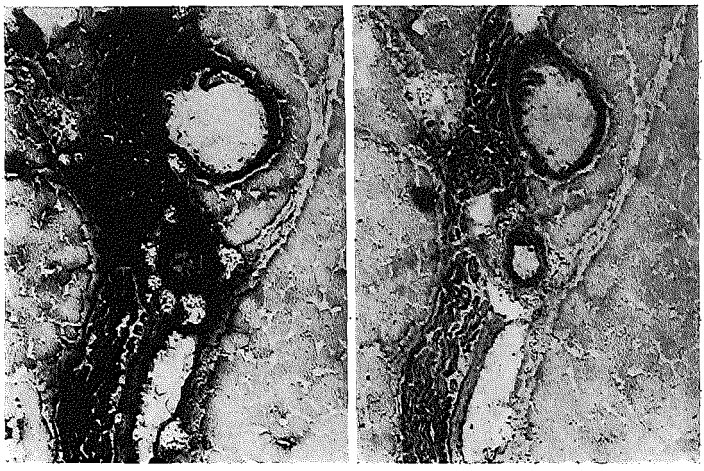

Abb. 3 *Besonders die pathologisch betroffenen Fasern zeigen vermehrte Aktivität der Glucose-6-Phosphat-Dehydrogenase (links) und weniger vermehrte Aktivität der 6-Phosphogluconat-Dehydrogenase (rechts). Infantile spinale Atrophie. M. quadriceps femoris des Menschen. 72×*

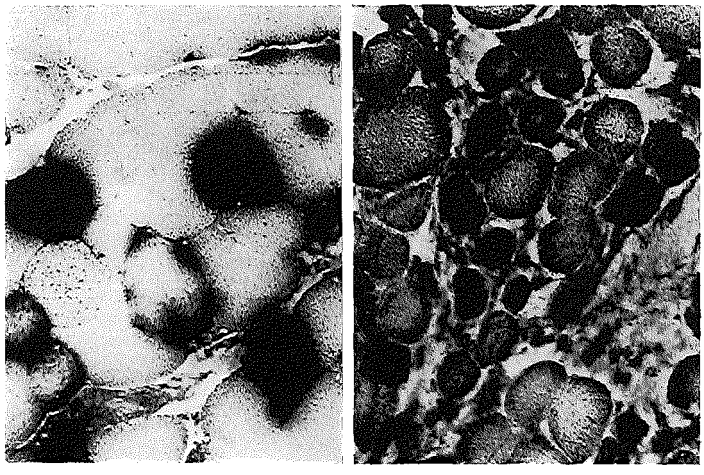

Abb. 4 *Links: pathologische Muskelfasern mit einer sehr stark vermehrten Aktivität der Glucose-6-Phosphat-Dehydrogenase. N_1N^1-Dimethyl-p-Phenylendiamin erzeugte Myopathie. M. rectus femoris einer Ratte. 184×*

Rechts: pathologische Muskelfasern mit wechselnder Aktivität der Glucose-6-Phosphat-Dehydrogenase. Progressive Muskeldystrophie. M. quadriceps femoris des Menschen. 184×

menschlichen und tierischen Skelettmuskeln geringe Quantitäten von Octulose-1,7-Diphosphat und Sedoheptulose-1,6-Diphosphat (respektive 5 und 17 nMol · g^{-1} Gewebe) gefunden. In normalen Muskeln konnten diese Substanzen nicht entdeckt werden.

Diskussion

Aus den Daten, dargestellt in Tabelle 1, ist ersichtlich, daß die Aktivität der GPDH in normalen Muskeln im Vergleich mit der Aktivität anderer Enzyme des Pentose-

Abb. 5 *Wechselnde, oft stark vermehrte Aktivität der Glucose-6-Phosphat-Dehydrogenase. Rectus femoris einer Ratte mit einer Myopathie. Auch die entzündlichen Infiltrate zeigen eine starke Enzymaktivität. 72×*

Tabelle 1 *Enzymaktivität* des Pentosephosphatzyklus — Enzyme im löslichen Anteil des M. quadriceps femoris — homogenates von Menschen*

Normale Skelettmuskeln		Progressive Muskeldystrophie Duchenne
Glucose-6-Phosphat Dehydrogenase	1,2 ± 0,2 (10)	6,4 ± 3,8 (8)
6-Phosphogluconat-Dehydrogenase	1,8 ± 0,2 (10)	7,2 ± 4,1 (8)
Transketolase	15,5 ± 1,4 (10)	28,9 ± 7,6 (7)
Transaldolase	16,1 ± 0,9 (10)	30,4 ± 6,2 (7)
Ribose-5-Phosphat Isomerase	22,7 ± 3,2 (10)	41,3 ± 10,5 (7)
Ribulose-5-Phosphat 3-Epimerase	47,8 ± 6,1 (10)	71,2 ± 16,1 (6)

*) Aktivitäten mit Standardabweichung und Anzahl Biopsien zwischen Klammern.

phosphatzyklus am geringsten ist. Obwohl die Aktivitätsvermehrung der GPDH, gefunden in Muskeln mit neuromuskulären Erkrankungen, relativ am stärksten ist, hat das Enzym ebenso in erkrankten Muskeln eine kapazitätsbestimmende Tätigkeit im Pentosephosphatzyklus. Überdies kann darauf hingewiesen werden, daß im Gegensatz zu den nicht oxydativen Enzymen, die GPDH-Reaktion in Geweben nicht reversibel ist, weil das Enzym vom Produkt NADPH gehemmt wird, hat die GPDH im Stoffwechsel gleichfalls eine regulierende Funktion. Diese Ergebnisse zeigen, daß die Anwendung der gewebeschonenden histochemischen Technik zur Darstellung der Aktivität der GPDH in Skelettmuskeln zu zuverlässigen Einsichten der Kapazität des Pentosephosphatzyklus führt.

In verschiedenen Geweben von Kaninchen und Ratten kommt die Aktivität der Enzyme des Glykogenstoffwechsels und des Krebszyklus in bestimmten Aktivitätsproportionen vor [3, 25].

Besonders im Muskelgewebe sind die proportionskonstanten Enzymgruppen eingehend untersucht worden. Obwohl wir diese bestimmten Proportionen ebenfalls in normalem menschlichem Muskelgewebe gefunden haben, haben unsere biochemischen und histochemischen Befunde deutlich gemacht, daß in pathologisch verändertem Muskelgewebe die Aktivitätsproportionen der Enzyme des Glykogenstoffwechsels

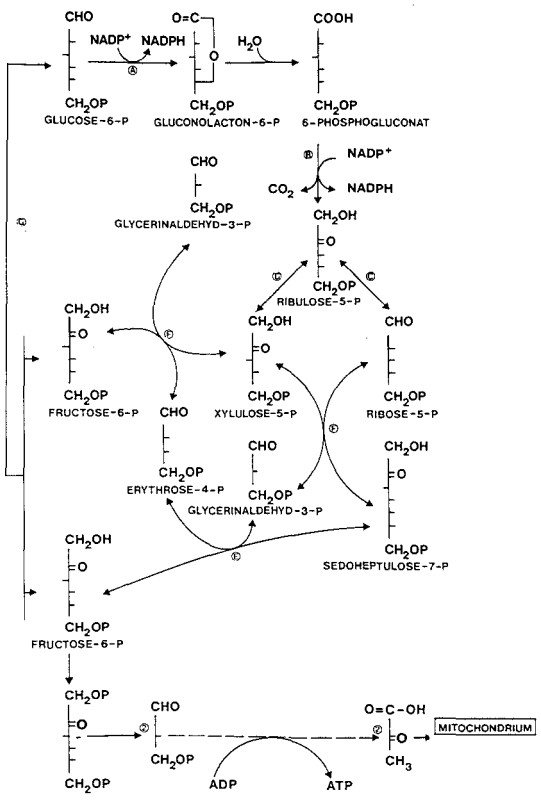

Abb. 6 *Schema des Pentosephosphatzyklus nach Horecker*
A. Glucose-6-Phosphat-Dehydrogenase; B. 6-Phosphogluconat-Dehydrogenase; C. Pentose-5-Phosphat-Isomerase; D. Pentose-5-Phosphat-3-Epimerase; E. Transaldolase; F. Transketolase; G. Phosphohexose-Isomerase. Ab Fructose-6-Phosphat ist der Embden-Meyerhof-Stoffwechsel vereinfacht dargestellt und sind die Namen der glykolytischen Metaboliten nicht aufgeführt worden.

und des Zitronensäurezyklus oft nicht mehr anwesend sind [21]. Deswegen ist es bemerkenswert, daß im Gegensatz dazu die Aktivitätsproportionen der Pentosephosphatzyklus-Enzyme im Muskelgewebe mit neuromuskulären Krankheiten noch anwesend sind.

Der Pentosephosphatzyklus hat im Stoffwechsel als Lieferant von NADPH eine besondere Bedeutung. Durch die Aktivität von GPDH und PGDH wird aus $NADP^+$ das Koferment NADPH gebildet (Abb. 6, 7). Die reduzierte Verbindung NADPH wird für viele synthetische Prozesse unter anderem für den Aufbau von Fettsäuren, von Steroiden und für Hydroxylierungen benötigt [5, 13]. Eine andere wichtige Rolle des Kofermentes NADPH ist die Reduzierung der Disulfidform des Tripeptides Glutathion. Diese Reaktion dient zur Entfernung schädlicher Peroxide und freier Radikale wie zum Beispiel nach folgenden Vorgängen [1, 9]:

$$O_2 + e \rightarrow O_2^-$$
$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$
$$H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O$$
$$H^+ + NADPH + GSSG \rightarrow 2\ GSH + NADP^+.$$

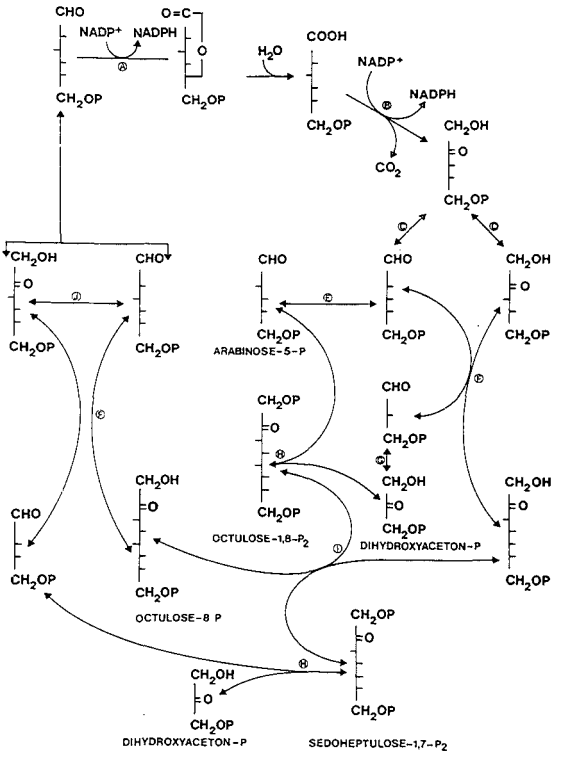

Abb. 7 *Schema des Pentosephosphatzyklus nach Williams*
A. Glucose-6-Phosphat-Dehydrogenase; B. 6-Phosphogluconat-Dehydrogenase; C. Pentose-5-Phosphat-Isomerase; D. Pentose-5-Phosphat-3-Epimerase; E. Pentose-5-Phosphat-2-Epimerase; F. Transketolase; G. Triosephosphat-Isomerase; H. Aldolase; I. Phosphotransferase; J. Phosphohexose-Isomerase

Schließlich hat das NADPH eine Funktion bei der Beseitigung des „singlet" Sauerstoffs [2]:

$$O_2 + h_\nu \rightarrow {}^1O_2$$
$${}^1O_2 + H^+ + NADPH \rightarrow H_2O_2 + NADP^+.$$

All diese Substanzen werden im Stoffwechsel erzeugt, besonders durch Entzündungsreaktionen. Im Zusammenhang mit der obengenannten Funktion des NADPH ist es interessant, daß wir in erkrankten Skelettmuskeln mit Entzündungsinfiltraten immer eine erhebliche Aktivität der GPDH und der PGDH im Infiltrat und in den Muskelfasern darin eingebettet gefunden haben (Abb. 5). Aus diesen Befunden geht hervor, daß die zwei oxydativen Enzyme des Pentosephosphatzyklus möglicherweise eine Schutzwirkung ausüben.
Weiterhin führt die Oxydation der Substrate in Pentosephosphatzyklus zu Pentosephosphaten. Aus den Abbildungen 6 und 7 ist ersichtlich, daß zur Pentosephosphatsynthese das Gewebe nicht ausschließlich abhängig ist von dem Stoffwechselweg über die zwei oxydativen Enzyme. Abbildung 6 gibt eine Darstellung des Zyklus im Fettgewebe. Das Schema ist größtenteils aus Resultaten von Untersuchungen von *Horecker* entwickelt worden [13]. Biochemische Untersuchungen von *Williams* haben es wahrscheinlich gemacht, daß im Lebergewebe und Herzgewebe ein anderer Typus des

Pentosephosphatzyklus vorhanden ist [29]. Die Reaktionsvorgänge dieses Typus sind in Abbildung 7 veranschaulicht. Aus Fließgleichgewichten und Schrittmacherreaktionen der verschiedenen Enzyme des Pentosephosphatzyklus geht hervor, daß besonders der Leber-Typus imstande ist, Glukose-Moleküle abzubauen. Im Lebergewebe [18] wird ungefähr 25 % und im Herzgewebe [14] ungefähr 80 % der verabreichten Glukose-Moleküle über den Leber-Typus zum CO_2 abgebaut. Diese interessanten Befunde zeigen, daß der Zyklus im Energie-Stoffwechsel von großer Bedeutung sein könnte. Der Zyklus ermöglicht nämlich eine zytoplasmatische ATP-Synthese über Glyzerinsäure-1,3-Diphosphat und Enolbrenztraubensäure-Phosphat. Von großer Bedeutung ist, daß die ATP-Synthese für beide Typen des Zyklus ungehemmt erfolgen kann, weil die Phosphofruktokinase, das Schlüsselenzym der Glykolyse umgangen wird.

Im Unterschied mit dem Fett-Typus erzeugt nach Abbildung 7 des Leber-Typus Sedoheptulose-1,7-Diphosphat und Octulose-1,7-Diphosphat. Es ist interessant, daß in Muskelgeweben mit einer Aktivitätsvermehrung der Pentosephosphatzyklus-Enzyme wir geringe Quantitäten von dieser Substanz gefunden haben.

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Zusammenfassung

Mittels histochemischer Techniken wurde gefunden, daß die Aktivität der oxydativen Pentosephosphatzyklus-Enzyme in Fasern von gesunden Skelettmuskeln sehr gering ist und daß die Aktivität in Muskelfasern von Patienten mit neuromuskulären Erkrankungen stark vermehrt sein kann. Besonders in erkrankten Muskelfasern des Duchenneschen Typus und der verschiedenen Typen der infantilen spinalen Atrophie wird ein starker Anstieg der Enzymaktivität gefunden. Auch Muskelfasern, eingebettet in Entzündungsinfiltrat, zeigen eine starke Vermehrung der Enzymaktivität. Biochemische Befunde zeigen, daß diese Aktivitätszunahme mit einer Aktivitätszunahme der nichtoxydativen Enzyme des Pentosephosphatzyklus verknüpft ist. Es wird über die mögliche Bedeutung der Kapazitätszunahme des Pentosephosphatzyklus für den Stoffwechsel in neuromuskulären Erkrankungen berichtet.

Резюме

С помощью гистохимических техник установилось, что активность окислительных энзимов пентозофосфатного цикла в волокнах здоровых скелетных мышц очень низка и что активность в мышечных волокнах у больных нервно-мышечными заболеваниями может быть резко повышенной. Особенно в заболевших волокнах типа Дюшенна и разных типов инфантильной атрофии наблюдается резкое нарастание активности энзимов. И мышечные волокна, находящиеся в воспалительном инфильтрате, показывают резкое увеличение энзимной активности. Биохимические данные показывают, что это нарастание активности не сопряжено с нарастанием активности неокислительных энзимов пентозофосфатного цикла. Излагается возможное значение повышения мощности пентозофосфатного цикла для обмена веществ при нервно-мышечных заболеваниях.

Summary

With histochemical techniques the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was demonstrated in skeletal muscles with and without apparent disease

of the neuromuscular system. Moreover with biochemical techniques the activity of both oxidative and of the nonoxidative enzymes transketolase, transaldolase, ribose-5-phosphate isomerase and ribulose-5-phosphate-3-epimerase of the pentose phosphate pathway was measured.
The activity of the oxidative enzymes was strongly increased in the muscle fibres of Duchenne muscular dystrophy and of infantile muscular atrophy. Moreover the activity of all nonoxidative enzymes was increased in these diseased skeletal muscles, but to a much smaller extent than of the oxidative enzymes. The significance of the increase in capacity of the pentose phosphate pathway for the metabolism of the diseased muscles is shortly discussed.

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Histochemical Journal 16, 971–982 (1984)
CHAPTER 4.7

The inhibitory effect of actinomycin D and cycloheximide on the increase in activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in experimentally induced diseased skeletal muscles

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Summary

The myotoxic effect of the subcutaneous administration of N,N^1-dimethyl-*p*-phenylenediamine (DPPD) in rats was enhanced by the simultaneous administration of hyaluronidase. The resulting myopathy was associated with an early and dramatic increase in activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Administration of actinomycin D or cycloheximide prior to the combined DPPD and hyaluronidase treatment prevented the increase in activity of both pentose phosphate pathway enzymes, indicating that the increase in activity requires RNA synthesis and protein synthesis. The possibility that the increase in activity of both NADPH-regenerating enzymes results from the modification by effectors of existing less active forms of these enzymes leading to more highly active forms was refuted.

Introduction

In previous investigations, we demonstrated the induction of a myogenic myopathy in the skeletal muscles of rats after subcutaneous administration of DPPD. By applying various histochemical and biochemical techniques for the demonstration and estimation of the activities of several oxidoreductases, transferases, hydrolases and isomerases, it was found that the most striking result was the dramatic increase in activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase during the early phase of the muscle disease (Meijer & Israel, 1979a,b; Elias & Meijer, 1981).

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The increase of both pentose phosphate pathway enzymes was the first pathological alteration and was present as early as 8 h after a single injection of DPPD.

A marked increase in the activity of these enzymes in diseased skeletal muscles has also been observed by McCamen (1963), Manchester *et al.* (1970) and Dhalla *et al.* (1972). Moreover, Wagner *et al.* (1978) and Max & Wagner (1979) found an early induction of both enzymes in regenerating skeletal muscles. More recently, Elias & Meijer (1983) found in a vitamin E deficiency-induced myopathy in rabbits, a marked early increase in the activity of these enzymes, even before the myopathy was clinically manifest.

Glucose-6-phosphate dehydrogenase in the liver of the mouse has a half-life of only 15 h (Freedland, 1968; Goldberg & St. John, 1976). Although the half-life of glucose-6-phosphate dehydrogenase in skeletal muscles has not yet been determined, the rapid increase in activity in response to DPPD suggests that the half-life in this tissue will also be relatively short. The same is true for 6-phosphogluconate dehydrogenase; an early increase in its activity was also observed after a single injection of DPPD.

Short half-life enzymes are considered to be regulatory enzymes whose activity can fluctuate rapidly in response to environmental changes (Goldberg & St. John, 1976). Since short half-life enzymes regulate biochemical pathways we attempted, in a subsequent study, to discover the mechanisms of regulation of these pentose phosphate enzymes. In this histochemical study, a myopathy in rats was induced by subcutaneous administration of the myotoxic substance DPPD. The DPPD injections were augmented by hyaluronidase (EC 3.2.1.35) in most experimental animals. In order to establish if the increase in activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase is mediated by a process which can be blocked by inhibitors of protein synthesis and ribonucleic acid synthesis, the activity of both enzymes was examined in the presence and absence of cycloheximide and actinomycin D. In the present communication, we report the results of this investigation.

Materials and Methods

Rats treated with DPPD, hyaluronidase, actinomycin D and cycloheximide

Six-week-old male Wistar rats with an average starting weight of about 110 g were injected twice daily (8 am and 5 pm) with a freshly prepared solution of DPPD.2HCl (20 mg/kg body weight; 10 mg DPPD/ml in distilled water). Some animals were also injected with a freshly prepared solution of hyaluronidase (1% aqueous solution of hyaluronidase at a dose of 10 mg/kg body weight). A freshly prepared solution of actinomycin D (0.015% of a sterilized aqueous solution of actinomycin D at a dose of 1.5 mg/kg body weight) was also injected in some cases and a freshly prepared solution of cycloheximide (0.5% cycloheximide at a dose of 50 mg/kg body weight) in others. The injections of DPPD and hyaluronidase were given subcutaneously in the left body side. The injections of actinomycin D and cycloheximide were administered intraperitoneally. Due to the high toxicity of the substances used, the experimental animals were sacrified on the second morning, 90 min after the last (third) injection.

For this study, the experimental animals were divided into the following groups: two control, untreated rats (group 1); three rats receiving three injections of hyaluronidase (group 2); two rats receiving three injections of DPPD (group 3); three rats receiving three injections of DPPD, each

injection directly followed by a hyaluronidase injection (group 4); three rats receiving the same treatment as group 4, but with intraperitoneal injections of actinomycin D 90 min prior to the DPPD and hyaluronidase injections (group 5); and three rats receiving the same treatment as group 4, but with intraperitoneal injections of cycloheximide 90 min prior to the DPPD and hyaluronidase injections (group 6).

Preparation of the muscle specimen

After sacrificing the animals, specimens from m.biceps femoris, m.gastrocnemius, m.rectus femoris, m.soleus, the diaphragm and heart muscle were removed and freed from fat, tendinous and fibrous tissue. The skeletal muscle specimens were taken from the right body side (free from injection lesions). The heart specimens were dissected transversely in such a manner that the specimens contained right and left ventricular walls as well as the interventricular septum. They were taken from just below the annulus fibrosus, thereby allowing the study of possible changes in the conducting system.

Histology

The muscle blocks for the histological study were fixed in 'Suza' solution (Romeis, 1968). After paraffin embedding, 7 μm sections were cut and stained with Harris' Haematoxylin–Eosin, with the Gomori trichrome technique, and with the PAS technique.

Histochemistry

The tissue specimens were rapidly frozen by immersing the tissue blocks in isopentane cooled to $-150°$ C with liquid nitrogen, and stored at $-96°$ C. Transverse serial sections at a thickness of 7 μm were cut in a cryostat. Unfixed sections were used for the demonstration of the activity and localization of 24 enzymes belonging to the glycolytic, gluconeogenetic, and citric acid cycle pathways according to the techniques described by Elias *et al.* (1982). Oil red O was used to demonstrate lipids.

Biochemistry

The activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was determined according to Löhr and Waller (1965) and Hohorst (1965) respectively.

To investigate the possibility that the activity of both enzymes might be regulated by activators and inhibitors, the enzyme activity was determined in mixtures of homogenates of m.gastrocnemius in various proportions of induced and repressed animals. To estimate the activity of catabolic processes that may inactivate glucose-6-phosphate dehydrogenase and possibly 6-phosphogluconate dehydrogenase, the activity of $NADP^+$-glycohydrolase (EC 3.2.2.6) and $NADP^+$-pyrophosphatase (EC 3.6.1.9) was determined in homogenates of the skeletal muscle specimens according to Bonsignore *et al.* (1968). The total activity of the $NADP^+$-cleaving enzymes was assayed by following the rate of disappearance of free $NADP^+$ in a system incubated at 37° C and containing 30 mM potassium phosphate buffer, pH 7.0, and 2 mM $NADP^+$, in a final volume of 2 ml; at different time intervals, $NADP^+$ was assayed by means of yeast glucose-6-phosphate dehydrogenase.

DPPD was obtained from BDH, UK. Hypnorm was obtained from Philips Duphar, The Netherlands. The enzyme substrates, actinomycin D, cycloheximide, hyaluronidase and yeast glucose-6-phosphate dehydrogenase were obtained from Sigma, USA. All other chemicals were of the highest degree of purity commercially available. The test reagents were dissolved in double-distilled water.

Results

Histology

Specimens of the different skeletal muscle types and heart muscle from the control rats (group 1) and the hyaluronidase rats (group 2) showed no histological or histochemical alterations. The subcutaneous injections of hyaluronidase did not, therefore, produce any morphological or histochemical change.

Skeletal muscle specimens from groups 3 and 4 showed severe pathological alterations indicating a myogenic myopathy with inflammatory infiltrate and catabolic processes. The pathological changes were the same in each type of skeletal muscle, but varied in severity and were as described in a previous communication (Elias & Meijer, 1981). The myopathy showed centrally located nuclei, and an increased number of subsarcolemmal nuclei. There was variation in size of muscle fibres, and rounded and degenerative fibres with a 'moth-eaten' appearance were seen (Fig. 1). Most rounded fibres with eosinophilic cytoplasm were surrounded by inflammatory infiltrate. Type 1 fibres were morphologically more vulnerable than type II fibres to the toxic and degenerative effects of DPPD and DPPD combined with hyaluronidase. The aerobic

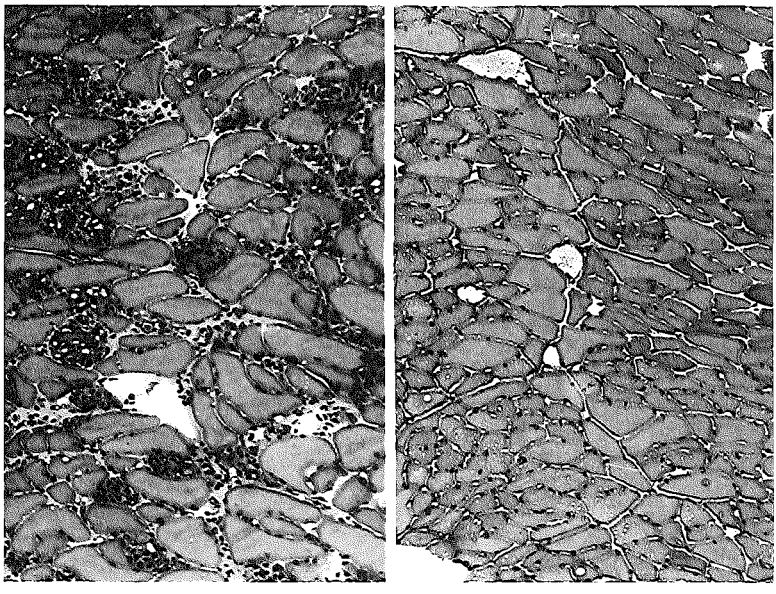

Fig. 1. Transverse section of diaphragm. Left, rat injected with DPPD and hyaluronidase (group 4). Rounded fibres with eosinophilic cytoplasm, surrounded by an inflammatory infiltrate. The pathological process results in a floccular appearance of some fibres. Right; rat injected with DPPD, hyaluronidase and cycloheximide (group 6). Only some fibres showed an eosinophilic appearance. These fibres are not surrounded by an inflammatory infiltrate. Haematoxylin–Eosin. × 90.

soleus muscle and the aerobic regions of muscles such as m.gastrocnemius, were more affected by DPPD than anaerobic muscles. The diaphragm specimens exhibited an intermediate vulnerability. On the other hand, aerobic heart muscle was only minimally affected.

The most affected group of skeletal muscles were from animals treated with combined DPPD and hyaluronidase injections (group 4). The hyaluronidase injections which, when administered alone in the doses used in this study were not myotoxic (group 2), clearly enhanced the myotoxic effect of DPPD on the muscle fibres. On the other hand, the number of inflammatory cells in rats of group 4 was somewhat less than in rats of group 3. It is remarkable that the intraperitoneal injections of the very toxic antibiotics, cycloheximide and actinomycin D, reduced the myotoxic effect of DPPD.

Histochemistry

In the muscle specimens of the control groups 1 and 2, no pathological changes were observed and fibre type differentiation was always possible. In agreement with the histological findings, the same histochemical changes were present in all skeletal muscle specimens of the experimental groups 3 and 4. The soleus muscle was the most affected and the anaerobic region of m.gastrocnemius was the least affected.

Most glycolytic and aerobic enzymes showed some increase in activity in the rounded and highly variable muscle fibres. The number of muscle fibres with an intermediate pattern of energy-yielding enzymes was strongly increased, indicating a metabolic imbalance in the muscle fibres. The metabolic effect of DPPD was more obvious in the anaerobic fibres, which showed an increase in activity of glycolytic enzymes. In contrast to the limited changes in activity of these enzymes, the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase increased dramatically in about 30% of the muscle fibres (Fig. 2). Most of these fibres were surrounded by an inflammatory infiltrate showing a high activity of both oxidative pentose phosphate pathway enzymes. Some fibres revealing a high enzyme activity of both enzymes were free from inflammatory infiltrate (Fig. 3). The activity of $NADP^+$-dependent isocitrate dehydrogenase was also increased, but to a lesser degree. The rounded fibres with eosinophilic cytoplasm contained an increase in lipids and a decrease in glycogen. The pathological fibres and the inflammatory infiltrate showed acid phosphatase activity, indicating the presence of catabolic processes. (Meijer & Israel, 1978a,b). In contrast to the skeletal muscles, heart muscle showed only minor alterations in its enzyme activities.

The protective effect of actinomycin D and cycloheximide on the induction of the myogenic myopathy was evident and was shown by the strong inhibition of increase in activity of both pentose phosphate pathway enzymes in the muscle specimens. Less than 5% of the muscle fibres showed an increase in enzyme activity. The morphological alterations were similarly much less severe (Fig. 1). Isocitrate dehydrogenase showed only a small decrease in activity after the administration of actinomycin D and cycloheximide. The activity of the other oxidoreductases, transferases and isomerases

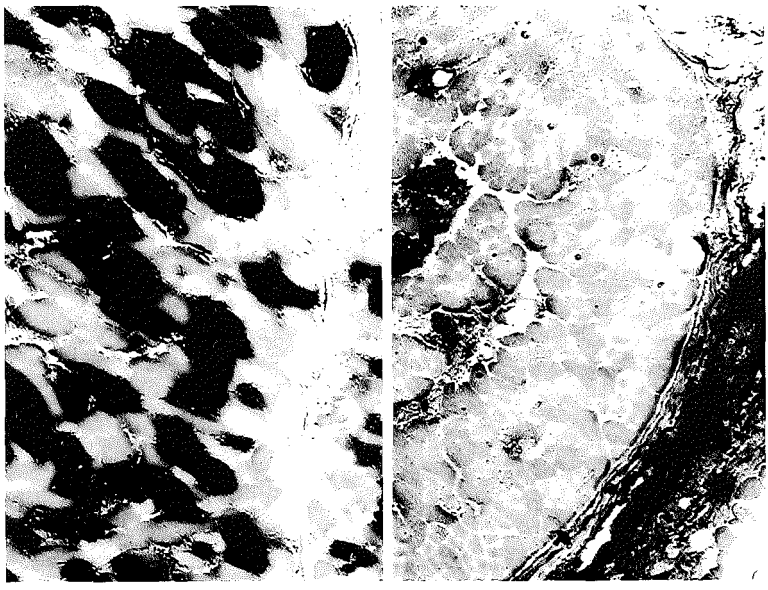

Fig. 2. Demonstration of activity of 6-phosphogluconate dehydrogenase in transverse sections of diaphragm. Left; rat injected with DPPD and hyaluronidase (group 4). A very high activity in the inflammatory infiltrate, in muscle fibres surrounded by the inflammatory infiltrate and in some other muscle fibres. Owing to the very high activity, the incubation time was 20 min instead of the 90 min that is necessary for normal skeletal muscles. Muscle fibres with less pathological alterations revealed a low activity. There is more activity in the anaerobic type II fibres than in the aerobic type I fibres. Right; rat injected with DPPD, hyaluronidase and cycloheximide (group 6). The muscle fibres showed the normal low activity of 6-phosphogluconate dehydrogenase. Rather strong activity in connective tissue. × 55.

studied was not changed and the activity of acid phosphatase was strongly reduced. The degree of protection offered by actinomycin D and cycloheximide was inversely related to the vulnerability of the muscle type concerned to the myotoxic substance DPPD, the anaerobic muscles being the most protected. Actinomycin D exhibited a greater protective effect than cycloheximide.

Biochemistry

The activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in homogenates of m.gastrocnemius increased 6-fold and 3-fold respectively after DPPD and hyaluronidase injections (group 4). The activity of both enzymes in homogenates of m.gastrocnemius was not significantly increased or decreased in animals of groups 5 and 6 (Table 1).

The results of the activity determinations in homogenate mixtures of m.gastrocnemius

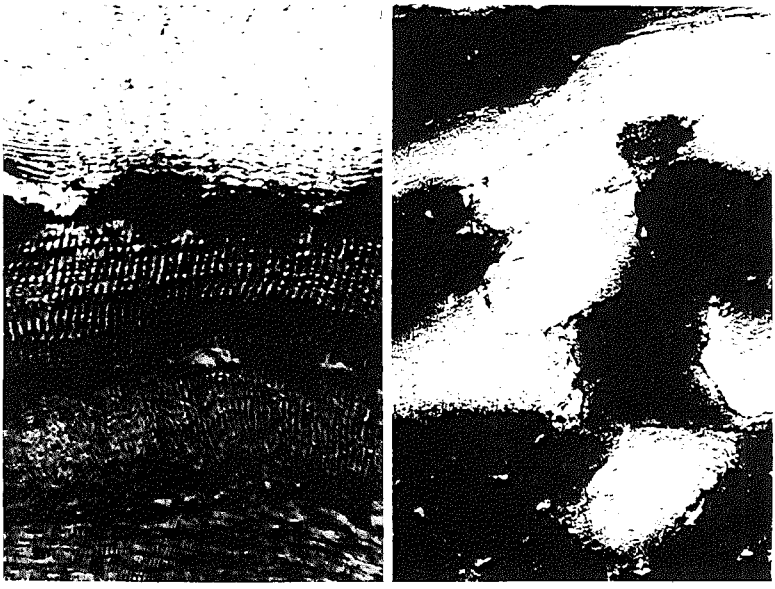

Fig. 3. Muscle fibres with a high activity of 6-phosphogluconate dehydrogenase in a small area of m.gastrocnemius without inflammatory infiltrate. Incubation time, 20 min. Left, × 600, right, × 350.

of induced (group 4) and control (group 1) animals are presented in Fig. 4. The activity of the homogenate mixtures was found to be the arithmetic mean of the activities of the two homogenates, indicating that there are no effectors in the skeletal muscles of the induced animals. Moreover, the same arithmetic results were obtained with homogenate mixtures of m.gastrocnemius of induced (group 4) and repressed (groups 5 and 6) animals. These results made it very unlikely that there are repressor substances

Table 1. Mean activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in (I) normal, (II) DPPD–hyaluronidase and (III) DPPD–hyaluronidase–cycloheximide treated m.gastrocnemius of rats.

	*Units of activity per g tissue**		
	I	*II*	*III*
Glucose-6-phosphate dehydrogenase	0.32 ± 0.03(5)	19.7 ± 2.4(5)	0.39 ± 0.07(4)
6-Phosphogluconate dehydrogenase	0.41 ± 0.01(5)	13.4 ± 3.7(5)	0.47 ± 0.04(4)

*Units of activity are μ-mol product formed per g tissue per min at 25° C and each figure is the mean ± S.D. The number of muscle specimens is shown in parenthesis. In groups I and III, activity is derived from muscle fibres and some connective tissue and in group II from cellular infiltrate as well.

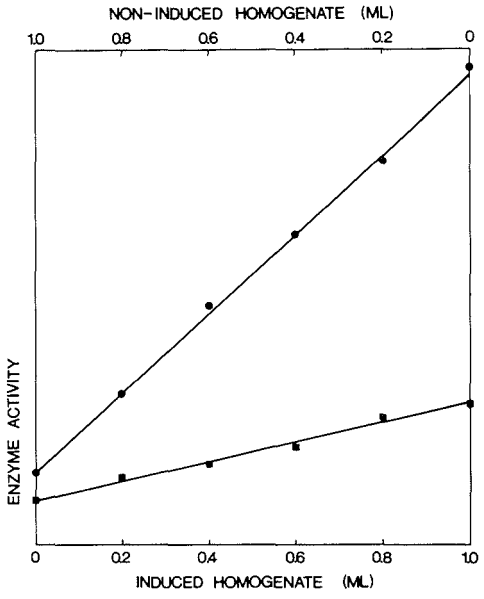

Fig. 4. Activity of glucose-6-phosphate dehydrogenase (●) and 6-phosphogluconate dehydrogenase (■) in mixtures of homogenates of skeletal muscle of control animals (non-induced) and of animals injected with DPPD and hyaluronidase (induced). Mixtures of the homogenates were prepared in the ratios 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 v/v. The activity of the homogenate mixtures was the arithmetic mean of the activity of the two homogenates.

which combine reversibly with both pentose phosphate pathway enzymes to modulate the enzyme activity in the skeletal muscles of groups 5 and 6.

Further, it was found that the activity of enzymes inactivating glucose-6-phosphate dehydrogenase was practically the same in the skeletal muscles of control and experimental animals. The activity was rather low and varied between 0.09 to 0.11 unit/mg muscle tissue.

Discussion

As far as these changes can be compared, the histological and histochemical findings in the pathological changes in the skeletal muscles of the experimental animals corresponded completely with each other. Since no neurogenic changes were observed in the diseased muscles, the histopathological findings clearly demonstrated that the lesion was myogenic. The soleus muscle was the most severely affected and the anaerobic regions of the gastrocnemius muscle the least. The histochemical findings, therefore, demonstrated a correlation between the degree of severity of the pathological processes and the metabolic type of the muscles investigated. Aerobic skeletal muscles

are clearly more vulnerable to DPPD. It is thus remarkable that the aerobic heart muscle of the experimental animals of groups 3 and 4 showed only minor morphological and enzyme histochemical changes.

The enhancement of the DPPD myopathy by hyaluronidase is in accordance with the findings of Rifenberick *et al.* (1974) and Hall-Craggs (1974). These authors found that metabolic changes induced by pathological processes were enhanced by hyaluronidase. The aggravation of the pathological processes may be caused by a facilitation of the entry of the myotoxic drugs into the muscle cells.

Several possibilities can be proposed for the molecular mechanism underlying the increased activity of both oxidative pentose phosphate pathway enzymes in the skeletal muscles of groups 3 and 4.

1. Enzyme induction involves formation of new messenger-RNA for the enzymes.
2. Existing messenger-RNA is activated to produce more enzyme molecules (post-transcriptional control).
3. A soluble activating or inhibitory cell constituent associates reversibly with the enzyme molecules to modulate its activity.
4. The enzymes are continuously degraded or inactivated by catabolic processes, and these processes are affected by the inducing signal so that the net steady-state of the enzyme activity is altered.

The first two possibilities lead to synthesis of new enzyme molecules. This has been found to take place in most mammalian adaptive enzyme systems, for example, for a number of amino acid catabolizing enzymes, (Schimke *et al.*, 1965; Granner *et al.*, 1968; Levitan & Webb, 1970; Lee & Kenney, 1971; Jost *et al.*, 1968) and the lipogenic liver enzymes acetyl CoA carboxylase (Majerus & Kilburn, 1969; Nakanishi & Numa, 1970), citrate lyase and fatty acid synthetase (Gibson *et al.*, 1972). The enhancement of glucose-6-phosphate dehydrogenase activity in the liver (Rudack *et al.*, 1971; Garcia & Holten, 1975; Dao *et al.*, 1979), the uterus (Smith & Barker, 1974) and a hepatoma (Selmeci & Weber, 1976), and the increase in 6-phosphogluconate dehydrogenase activity in the liver (Procsal *et al.*, 1976) and the mammary gland (Betts & Mayer, 1977), occurs through newly synthesized enzyme protein. It has been postulated that post-transcriptional regulation of glucose-6-phosphate dehydrogenase synthesis occurs in the liver after carbon tetrachloride injury (Watanabe & Taketa, 1973).

With regard to the third possibility, the modification of pre-existing molecules has been found to be of importance in a number of other systems (Cox *et al.*, 1971; Holzer & Duntze, 1971; de Wulf & Hers, 1968). For example, the presence of glucose-6-phosphate dehydrogenase modifying factors altering the kinetic properties of the enzyme has been demonstrated in leukaemic cells (Kahn *et al.*, 1976) and in rat liver (Dao *et al.*, 1979). As for the fourth possibility, Bonsignore *et al.* (1968) have demonstrated that $NADP^+$-ases are involved in the increased breakdown of glucose-6-phosphate dehydrogenase during repression. These authors showed that two $NADP^+$-cleaving enzymes, namely $NADP^+$

glycohydrolase and $NADP^+$ pyrophosphatase, may inactivate glucose-6-phosphate dehydrogenase. The possibility that these enzymes can also inactivate phosphogluconate dehydrogenase cannot be excluded.

Cycloheximide interferes with protein synthesis and actinomycin D inhibits transcription of DNA by RNA-polymerase (Beard *et al.*, 1969). The results of our investigation show that both antibiotics completely prevent the increase in activity of both pentose phosphate pathway enzymes. Our results therefore indicate that protein and RNA synthesis seems to be required for the elevation of the enzyme activities. It can be further concluded from the results described in Fig. 4 that it is highly unlikely that a molecular entity exists in the skeletal muscles of the DPPD animals which combines reversibly with the enzymes to modulate their activity. If reversibly combining inhibitors were present in the noninduced homogenate, or an activator existed in the induced homogenate, deviations from the arithmetic mean would have been expected (Max & Wagner, 1979).

Since the activities of the various $NADP^+$-ases in skeletal muscles of all the experimental groups did not differ significantly, our results clearly indicate that the alterations in activity of both pentose phosphate pathway enzymes could not be caused by varying activity of degradation processes effected by these enzymes (Bonsignore *et al.*, 1968).

The final conclusion that can be drawn is that the findings of this study suggest that the striking increase in activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase results mainly from the synthesis of new enzyme molecules. Further studies are under way to assess the biological significance of the increase in activity of both pentose phosphate pathway enzymes.

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CHAPTER 4.8

Enzyme histochemical studies on the conducting system of the human heart

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Summary

In this communication, the results of applying various histochemical techniques for the localization of oxidoreductases, transferases, hydrolases and isomerases in the human heart are presented. The Purkinje fibres of the atrioventricular conducting system of the human heart differ from the myocardium proper in containing a slightly higher activity of most of the glycolytic and gluconeogenetic enzymes investigated. The relatively higher activity of 6-phosphofructokinase, the key enzyme in anaerobic carbohydrate metabolism, is especially noteworthy. On the other hand, the activities of some of the enzymes that play a part in the aerobic energy metabolism is slightly less than those in the myocardium fibres.

As for the activity of the NADPH regenerating enzymes, the activity of 6-phosphogluconate dehydrogenase and malate dehydrogenase (oxaloacetate-decarboxylating) is somewhat higher, and the activity of glucose-6-phosphate dehydrogenase similar, in the Purkinje fibres compared to that in the myocardial fibres. The activity of myosin ATPase is similar for both types of fibre. Likewise, the fibres of the conducting system and of the myocardium show a similar activity of acid phosphatase, β-glucuronidase, non-specific naphthylesterase and peroxidase. The neurogenic function of the conducting system of the human heart was demonstrated by the high activity of acetylcholinesterase in the Purkinje fibres and in the atrioventricular node. All these histochemical findings in Purkinje fibres are similar at widely differing levels of the conducting system.

Introduction

Histochemical techniques have been used in studying heart tissues of various species. These techniques have revealed differences in activity of enzymes that play a role in the energetic aerobic and anaerobic pathways and in the amount of glycogen in the Purkinje fibres of the atrioventricular conducting system when compared

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with common myocardial fibres. Histochemical studies in ungulates and rodents (Schiebler, 1953, 1955, 1961, 1963; Schiebler *et al.*, 1956), cattle, sheep, dogs and rats (Carbonell, 1955a, b), rats (Alcine *et al.*, 1965) and dogs (Otsuka *et al.*, 1967), have demonstrated a greater amount of glycogen and a higher activity of α-glucan phosphorylase in the Purkinje fibres when compared with that in the normal myocardial cells. On the other hand, some enzymes that play a role in aerobic energy metabolism showed less activity in the Purkinje fibres than in the myocardial cells (Schiebler, 1963; Otsuka *et al.*, 1967). Moreover, Gossrau (1968, 1971) found that Purkinje fibres in the hearts of birds, hamsters, mice and rats are characterized by a relatively low activity of some glycolytic enzymes. Although a species-dependent difference in the metabolism of the atrioventricular conducting system cannot be excluded, studies by Snijder & Meijer (1970) and Meijer & de Vries (1978), working with bovine and porcine hearts, have shown that the cause of these conflicting metabolic findings may be due to the use of enzyme histochemical techniques that are not suitable for metabolic studies. Using improved enzyme histochemical techniques, these authors found a higher rate of anaerobic carbohydrate metabolism and a lower rate of aerobic energy metabolism in the cells of the conducting system when compared with the common myocardial fibres.

Although a few enzyme histochemical studies have been performed on the human heart in which the activity of the Purkinje fibres of the atrioventricular conducting system was compared with that in the common myocardial fibres (Carbonell, 1955a, b; Morales & Fine, 1965), the findings give no clear indication of the capacity of the anaerobic and aerobic energy metabolism. This stems from the fact that the histochemical techniques that were used at that time cannot produce reliable results (Snijder & Meijer, 1970; Meijer & de Vries, 1978).

The present investigation is aimed at deciding whether similar metabolic differences exist in the human heart between the Purkinje fibres and the common myocardial fibres. In this study, the activity and localization of 16 enzymes that play a role in the aerobic and anaerobic part of energy metabolism were examined. In addition, the activity and localization of some other enzymes and lipid were studied.

Materials and methods

Representative specimens from the atrioventricular node, left bundle branch and right bundle branch of the conductive system of the human heart were dissected in the course of routine autopsies. To avoid *post mortem* changes, only material taken within 60 min after death was used. The specimens were selected from patients showing no *ante* or *post mortem* signs of cardiac involvement. Heart specimens were studied from 12 cases, mean age of 60 years, varying from 40–80 years, of both sexes.

Tissue blocks of about $1 \times 1 \times 0.5$ cm^3 containing the endocardium, the conducting system and the adjacent myocardial tissue were rapidly frozen by immersion in isopentane, pre-cooled to $-150°$ C with liquid nitrogen. Frozen tissue blocks were stored at $-95°$ C. Sections, 6 μm thick, were cut in a cryostat.

For morphological studies, sections were stained with Haematoxylin–Eosin. For metabolic studies, fixed or unfixed sections were stained by the following histochemical techniques. The activity of succinate dehydrogenase (EC 1.3.99.1) was demonstrated by the method of Nachlas *et al.* (1957), with the addition of phenazine methosulphate at a concentration of 50 μg/ml to the incubation medium. The activity of NADH dehydrogenase (or NADH: tetrozolium oxidoreductase, EC 1.6.99.3) was demonstrated according to Burstone (1962) using NADH as substrate. Mitochondrial glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate: menadione oxidoreductase, EC 1.1.99.5) was demonstrated according to a substrate modification of the method of Wattenberg & Leong (1960); instead of succinate, an equimolar concentration of glycerolphosphate was used. The methods described by Barka & Anderson (1963) were used to demonstrate activity of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) and isocitrate dehydrogenase *threo*-D_s-isocitrate: NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41. To distinguish the mitochondrial NAD^+ malate dehydrogenase from the extramitochondrial NAD^+ malate dehydrogenase the activity of the enzyme was also demonstrated according to a substrate modification of the lactate dehydrogenase technique (Meijer, 1973); instead of lactate, an equimolar concentration of malic acid was used. In addition, the activity was demonstrated with and without 5 mM oxaloacetate in the incubating medium. The activity of cytochrome *c* oxidase (EC 1.9.3.1) was demonstrated according to Burstone (1962) with the substrates *p*-aminodiphenylamine and *p*-methoxy-*p*-aminodiphenylamine. Activity was demonstrated with and without cytochrome *c* added to the incubation medium at a concentration of 0.5 mg/ml. The activity of isocitrate dehydrogenase ($NADP^+$) (EC 1.1.1.42) and malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$) (EC 1.1.1.40) was demonstrated according to Meijer & de Vries (1975). The activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) was demonstrated according to Meijer & de Vries (1974), and that of lactate dehydrogenase (EC 1.1.1.27) as described by Meijer (1973). The method of de Vries *et al.* (1980) was used for demonstrating glyceraldehyde phosphate dehydrogenase activity (EC 1.2.1.12) and that of Meijer & Stegehuis (1980) for revealing the activity of 6-phosphofructokinase (EC 2.7.1.11). The activities of glucosephosphate isomerase (EC 5.3.1.9) and phosphoglucomutase (EC 2.7.5.1) were localized according to de Vries & Meijer (1976). Meijer's (1968) method was employed for the demonstration of α-glucan phosphorylase (EC 2.4.1.1). The activity of amine oxidase (EC 1.4.3.4) (more usually known as monoamine oxidase) was demonstrated as described by Glenner *et al.* (1957), but using adrenaline as substrate instead of tryptamine. Peroxidase activity (EC 1.11.1.7) was demonstrated at pH 6, the incubation medium containing 10 mM H_2O_2 and the same substrate as that used for revealing cytochrome *c* oxidase. Mitochondrial Mg^{2+}-activated ATPase (EC 3.6.1.3) and myosin Ca^{2+}-activated ATPase (EC 3.6.1.3) were demonstrated using the methods of Wachstein & Meisel (1957a,b) and Meijer (1970) respectively. Acid phosphatase activity (EC 3.1.3.2) was localized according to Meijer (1972), and non-specific esterase (EC 3.1.1.1 and 3.1.1.2) and β-D-glucuronidase (EC 3.2.1.31) according to Meijer & Vloedman (1973). Karnovsky & Root's (1964) method was followed for demonstrating acetylcholinesterase (EC 3.1.1.7). Oil red 0 was used for staining lipids.

Results

The histochemical activities of the various enzymes in common myocardial fibres and in Purkinje fibres of the atrioventricular conducting system of the human heart are presented in Table 1 and in Figs. 1–4. The activities of the various enzymes in

Table 1. Enzyme activities in common myocardial fibres, Purkinje fibres*, blood vessels and connective tissue of the human heart.

Enzyme (EC recommended name)	*MF*	*PF*	*BV*	*CT*
Succinate dehydrogenase	4+	3+	1+	±
NADH dehydrogenase	3+	3+	±	±
Isocitrate dehydrogenase (NAD^+)	3+	3+	±	±
Isocitrate dehydrogenase ($NADP^+$)	4+	2+	1+	±
3-Hydroxybutyrate dehydrogenase	3+	2+	±	±
Cytochrome *c* oxidase	2+	1+	±	±
Mitochondrial ATPase	2+	1+	±	±
6-Phosphofructokinase	1+	2+	±	±
α-Glucan phosphorylase	2+	3+	±	±
Glucosephosphate isomerase	2+	3+	3+	2+
Phosphoglucomutase	2+	3+	3+	2+
Glyceraldehyde phosphate dehydrogenase	1+	2+	1+	±
Glycerol-3-phosphate dehydrogenase	1+	1+	1+	±
Lactate dehydrogenase	2+	2+	3+	1+
Malate dehydrogenase†	3+	3+	±	±
Malate dehydrogenase oxaloacetate-decarboxylating ($NADP^+$)	1+	2+	±	±
Myosin ATPase	1+	1+	±	–
Amine oxidase (flavin-containing)	1+	2+	±	±
Glucose-6-phosphate dehydrogenase	±	±	2+	2+
6-Phosphogluconate dehydrogenase	±	1+	3+	2+
Acid phosphatase	1+	1+	–	±
β-D-Glucuronidase	±	±	–	–
Non-specific esterase	1+	1+	±	1+
Peroxidase	±	±	±	±
Acetylcholinesterase	–	3+	–	–

Abbreviations: MF, common myocardial fibres; PF, Purkinje fibres; BV, blood vessels; CT, connective tissue. 4+, very strong activity; 3+, strong activity; 2+, moderate activity; 1+, low activity; and ±, slight activity.

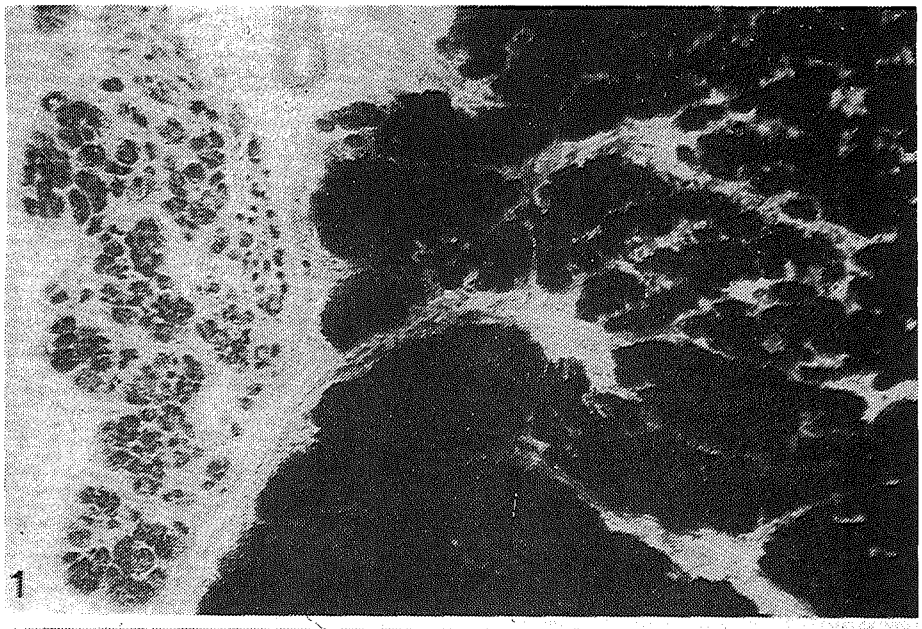
*The atrioventricular node gave almost the same enzyme activities; only the activity of acetylcholinesterase in the node was stronger.

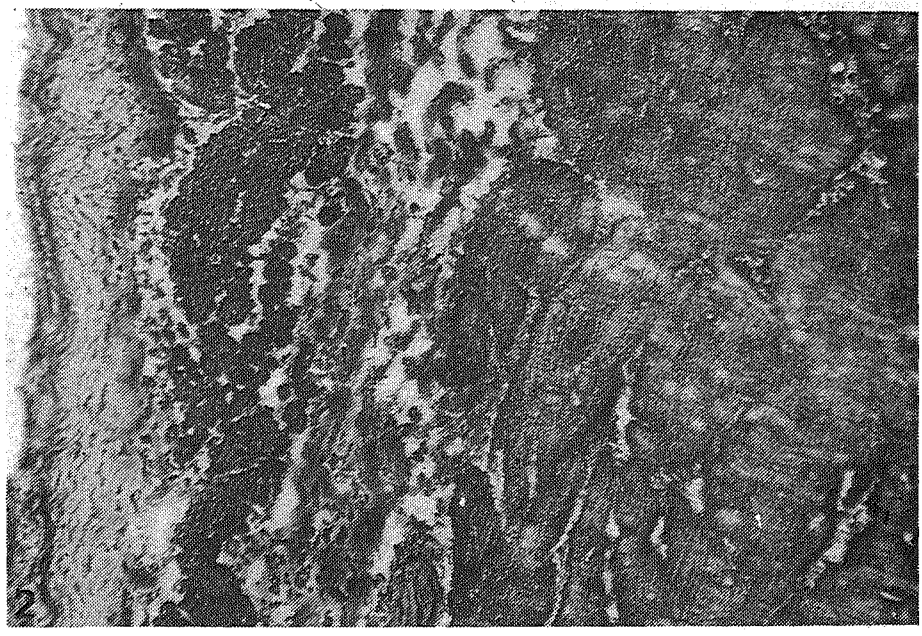
†Activity of the mitochondrial and extramitochondrial enzyme.

connective tissue and blood vessels are also given in Table 1. The findings are the same for all the specimens examined and are independent of age and sex. With the exception of lactate dehydrogenase, all the glycolytic and glycogenolytic enzymes investigated show a higher maximal activity in the Purkinje fibres of the atrioventricular conducting system than in the common myocardial fibres (Figs. 2, 4).

Fig. 1. Intramyocardial human Purkinje fibres showing a lower activity of isocitrate dehydrogenase ($NADP^+$) compared to that in common myocardial fibres. The staining of the connective tissue is very slight. × 99.

Fig. 2. Intramyocardial human Purkinje fibres showing a higher activity of phosphoglucomutase than in common myocardial fibres. The activity in connective tissue is moderate. × 99.


1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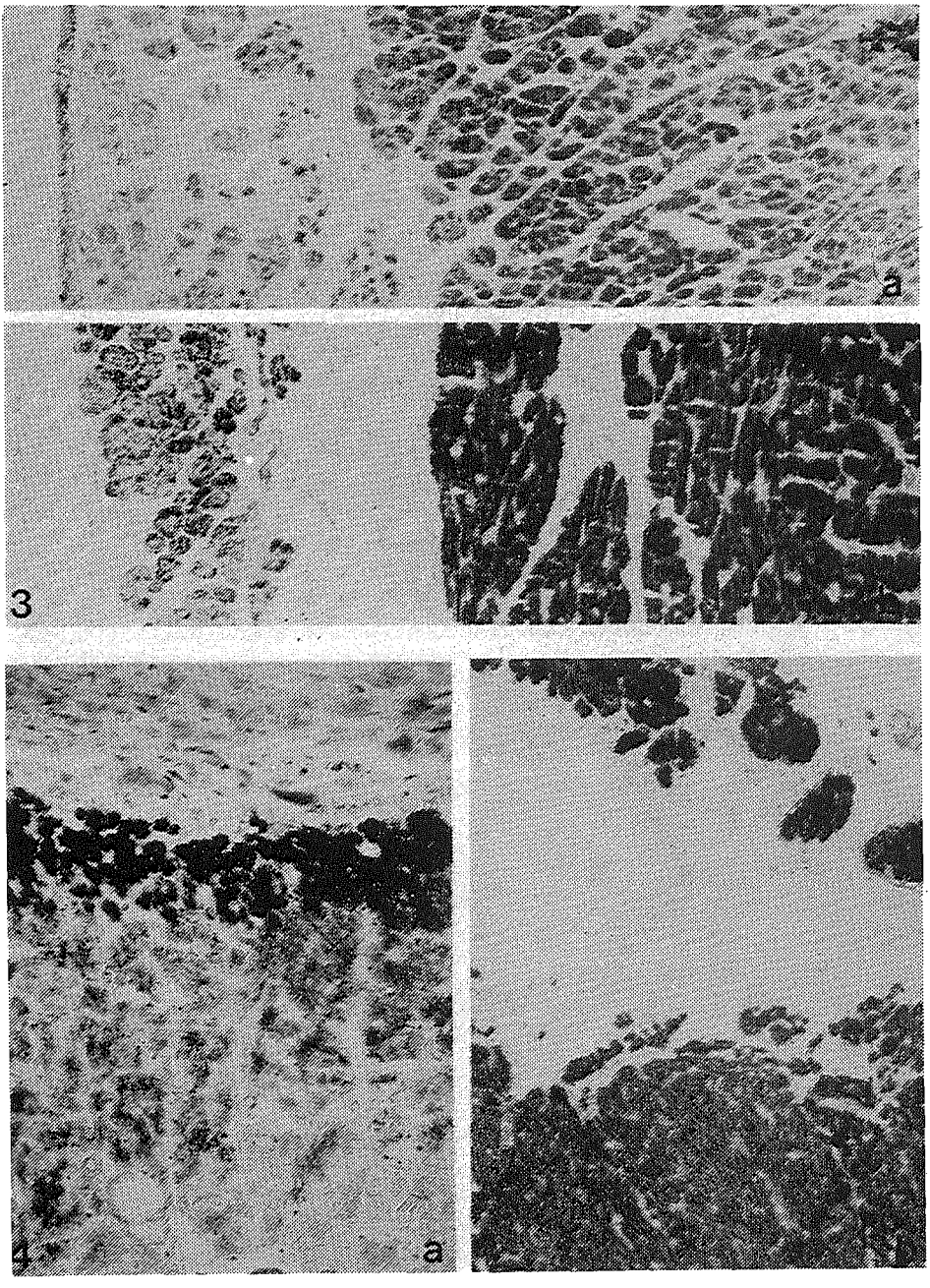
a
3
4
a

The histochemical techniques applied are reliable since the results of control experiments with hearts of experimental animals have shown that a period of 60 min between death and freezing of the tissue specimens did not have any influence on the activity and localization of the enzymes examined. Moreover, with the sole exception of 6-phosphofructokinase, no significant inactivation occurred during the histochemical freezing procedures (Meijer *et al.*, 1977; Meijer & Stegehuis, 1980). A diminishment of 6-phosphofructokinase activity of 10–20% cannot be excluded by the freezing procedure.

In general, there were no qualitative nor quantitative differences in enzyme activity in the left and right bundle branches. Apart from some minor differences, the atrioventricular node gave almost the same reactions as the Purkinje fibres. The main difference between the bundle branches and the atrioventricular node was that the node contained more acetylcholinesterase activity than the Purkinje fibres. the acetylcholine esterase was always located in relation to cell membranes. The fat content of the common myocardial fibres and of the Purkinje fibres is low, yet it is still demonstrable in the form of tiny cytoplasmic droplets which are more intense in the vicinity of the nuclei. The blood vessels and connective tissue are characterized by a low aerobic metabolism profile and, with the exception of some enzymes, by a low anaerobic energy metabolism as well. In contrast with these findings, the activities of both the pentose shunt enzymes are rather high.

Discussion

The glycolytic and gluconeogenetic pathways are catalysed by the consecutive action of a group of enzymes of which, in this study, α-glucan phosphorylase, phosphoglucomutase, glucosephosphate isomerase, 6-phosphofructokinase, glyceraldehyde-phosphate dehydrogenase and lactate dehydrogenase were investigated. The point of regulation of the rates of these pathways is governed by α-glucan phosphorylase, 6-phosphofructokinase and pyruvate kinase. Since 6-phosphofructokinase is the most important control point of the glycolytic and glycogenolytic sequence (Passonneau & Lowry, 1964), it can be concluded that these pathways have a higher capacity in the Purkinje fibres than in the common heart fibres.

Most of the reaction steps from pyruvate to glucose-6-phosphate, the

Fig. 3. Demonstration of cytochrome *c* oxidase (a) and β-hydroxybutyrate dehydrogenase (b). Human Purkinje fibres show less activity for both enzymes than do common myocardial fibres. The activity of the connective tissue is low for both enzymes. × 99.

Fig. 4. Demonstration of α-glucan phosphorylase activity (a) and 6-phosphofructokinase activity (b). Human Purkinje fibres show higher activity for both enzymes than do common myocardial fibres. The activity in the connective tissue and in the endothelial cells of the blood vessels is weak. × 99.

gluconeogenetic pathway, are catalysed by enzymes of the glycolytic sequence and thus produced by reversal of steps employed in glycolysis. However, there are two irreversible steps which cannot be utilized in the conversion of pyruvate to glucose-6-phosphate (Krebs *et al.*, 1964). In the biosynthetic direction, these steps are by-passed by alternative reactions which are thermodynamically biased in the direction of the synthesis. The first of these by-pass steps is the phosphorylation of pyruvate to phosphoenolpyruvate through some cytosol and mitochondrial enzymes of which pyruvate carboxylase is the rate-limiting enzyme. The second step is the conversion of fructose-1,6-diphosphate to fructose-6-phosphate by fructose-bisphosphatase. Unfortunately it is not possible at present to demonstrate the activity of both these key enzymes by histochemical methods. However, it has been established that groups of enzymes exist whose activities are found in constant proportion groups in a variety of tissues such as skeletal and heart muscle. These constant proportion groups have been described for the glycolytic, gluconeogenetic, tricarboxylic acid cycle, fatty acid oxidation and respiratory chain enzymes (Pette & Dölken, 1975). It is, therefore, very probable that the human Purkinje fibres of the atrioventricular conducting system will display a higher gluconeogenetic capacity. The co-enzyme NAD^+ in the extramitochondrial cytoplasm is continuously reduced by glyceraldehyde-3-phosphate dehydrogenase of the glycolytic pathway. Since NADH cannot penetrate through the mitochondrial membrane, as first reported by Lehninger (1951), the co-enzyme is reoxidized in the cytoplasm by at least two routes: either by pyruvate to form lactate, by the action of extramitochondrial lactate dehydrogenase, or alternatively by the mitochondrial respiratory chain, via the glycerolphosphate shuttle (Boxer & Devlin, 1961). Since the glycerolphosphate shuttle requires the mitochondrial glycerol-3-phosphate dehydrogenase as an essential link in its operation, it is evident that this mitochondrial enzyme is more active in tissues with high anaerobic capacities. From this, one would have expected that, in the light of the constant proportion groups hypothesis (Pette & Dölken, 1975), the activities of both enzymes in the human Purkinje fibres would be greater than in the common myocardial fibres as was the case in the Purkinje fibres of the bovine and porcine heart (Snijder & Meijer, 1970). This, however, was not found in the present study in the Purkinje fibres of the human heart.

Another possibility for the transfer of electrons or reducing equivalents from cytosol NADH to molecules that could penetrate the mitochondrial membrane is the malate–aspartate shuttle (Digerness & Reddy, 1976). This shuttle involves the participation of cytosol and mitochondrial forms of NAD^+-specific malate dehydrogenase and aspartate aminotransaminase. In heart tissue, about 60% of the NAD^+-specific enzyme has an extramitochondrial location (Lowenstein, 1967) and this could indicate that this shuttle is important in heart tissue. The extra- and intramitochondrial enzymes have different kinetic properties. For instance, the mitochondrial enzyme is inhibited by oxaloacetate, whereas the extramitochondrial enzyme is not (Englard & Breiger, 1962). In Table 1, the total activity of both enzymes

is included. Although the findings obtained with the various enzyme histochemical techniques obviously show that a cytoplasmic NAD^+-specific malate dehydrogenase is present both in the common myocardial fibres and in the Purkinje fibres, the results were not precise enough to be able to investigate possible differences in the percentage of extramitochondrial enzyme in the two types of fibres.

Some of the tricarboxylic acid cycle enzymes investigated show a higher maximal activity in the common myocardial fibres. The condensation of acetyl CoA with oxaloacetate to yield citrate is the primary control point of the citric acid cycle in most tissues (Mehlman & Hanson, 1972; Lowenstein, 1976). However, the activity of citrate synthase cannot be demonstrated histochemically. Other reactions in the cycle which are under allosteric regulation, thus indicating a regulation function, are isocitrate dehydrogenase (NAD^+) (Goebell & Klingenberg, 1964), succinate dehydrogenase (Mehlman & Hanson, 1972; Lowenstein, 1967) and maybe also the mitochondrial ATPase (Chappell, 1964). Isocitrate is oxidized to 2-oxoglutarate and CO_2 by two enzymes, one specific for NAD^+, the other for $NADP^+$. The overall reaction is identical for both enzymes, though the reaction catalysed by the $NADP^+$-linked enzyme is reversible and the reaction catalysed by the NAD^+-linked enzyme is not reversible. Both enzymes occur in the mitochondria of mammalian tissues, but the NAD^+-linked isocitrate dehydrogenase is found only in mitochondria whereas the other enzyme is present both in mitochondria and in the cytosol. The NAD^+-linked enzyme is the major catalyst for isocitrate oxidation in the tricarboxylic acid cycle and is subjected to allosteric activitation by ADP. The $NADP^+$-specific enzyme is not considered to be subject to regulation. However, any controls exerted on the oxidative decarboxylation of isocitrate must necessarily involve both enzymes (Colman, 1975).

Moreover, since the respiratory chain enzyme cytochrome *c* oxidase also shows a higher activity in the common myocardial fibres it is very probable that the maximal flux capacity of the tricarboxylic cycle and the respiratory chain is less in the human Purkinje fibres. In CO_2 fixation processes, malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$) in combination with the mitochondrial malate dehydrogenase and phosphoenolpyruvate carboxykinase (the NADPH transferring malate shuttle) plays an important part in the synthesis of glycogen from pyruvate via malate and oxaloacetate (Wood & Utter, 1965). In this way, the energetically unfavourable mechanism of pyruvate kinase in phosphoenolpyruvate synthesis (Krebs, 1954; Utter, 1959) can be by-passed. This shuttle connects the extramitochondrial carbohydrate metabolism with the mitochondrial part of carbohydrate metabolism. Also glucose-6-phosphate dehydrogenase and phospho-gluconate dehydrogenase, both enzymes of the pentose phosphate pathway, play a role in the production of NADPH. The activity of both these enzymes is relatively high in the human Purkinje fibres in comparison with the slight activity in the myocardium proper.

3-Hydroxybutyrate dehydrogenase is one of the key enzymes of ketone body

utilization. There is an important connection with the aerobic tricarboxylic acid cycle via 3-oxoacid-CoA transferase and acetyl-CoA acetyltransferase. The lower activity of 3-hydroxybutyrate dehydrogenase without doubt indicates a lesser flux of acetyl-CoA into the tricarboxylic acid cycle of the Purkinje fibres, indicating a lower aerobic capacity than the common myocardial fibres. Boxer & Devlin (1961) proposed that the enzyme also regulates the acetoacetate-3-hydroxybutyrate shuttle but some doubt about this mechanism was raised by Lehninger (1965).

From the above findings, it follows that in general the staining results obtained with the human heart correspond with those observed for the porcine and bovine heart (Meijer & de Vries, 1978). However, with the histochemical techniques applied, the human Purkinje fibres of the atrioventricular conducting system showed less differences in enzyme activity when compared with the myocardium proper.

The relatively high activity of acetylcholinesterase, mainly at the cell membranes of the Purkinje fibres as well as in the atrioventricular node, demonstrates that the conducting system is influenced by the nervous system (Mommaerts *et al.*, 1960; Kent *et al.*, 1974; Mochet *et al.*, 1975). The relatively high activity of amine oxidase in human Purkinje fibres of the atrioventricular conducting system offers confirmation of the observations of Müller & Pearse (1965) and of Snijder & Meijer (1970), who also showed that in the Purkinje fibres of various species the activity of the enzyme is relatively high.

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CHAPTER 4.9

Early and late changes in the metabolic pattern of the working myocardial fibres and Purkinje fibres of the human heart under ischaemic and inflammatory conditions: an enzyme histochemical study

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Summary

In this communication, the results of an enzyme histochemical study on the working myocardial fibres and Purkinje fibres of the atrioventricular conducting system of the human heart under ischaemic and inflammatory conditions are presented. The material was selected from patients showing changes which could be classified in three major groups: (1) early changes due to acute ischaemia either in the myocardial fibres or in the conducting system or in both; (2) chronic ischaemic changes due to cardiovascular insufficiency, such as old infarction, or coronary arteriosclerosis or both; and (3) inflammatory conditions such as myocarditis.

The activity and location of about 20 enzymes that play a role in the aerobic and anaerobic pathways of energy metabolism were examined. The activity and location of some hydrolytic enzymes and the glycogen and lipid content were also studied.

The most important findings were an obvious depletion of the glycogen reserves under acute ischaemic changes in both types of fibre. This was associated with a transient or permanent reduction in activity of many enzymes that play a role in aerobic and anaerobic metabolism. Further, there was an instantaneous and persistent increase in the activity of the NADPH-regenerating enzymes of the pentose phosphate pathway and of glyceraldehyde-3-phosphate dehydrogenase, the rate-limiting enzyme of glycolysis under ischaemic conditions. Chronic ischaemic changes were characterized by a gradual long-term increase in the activity of many anaerobic glycolytic enzymes. Moreover, there was an absence of activity of acetylcholine esterase immediately after the onset of infarction in the fibres of the conducting system. Lastly, a slight increase in lipid content was found in the hypertrophic chronic ischaemic fibres and in old infarcted areas. Cardiac fibres in inflamed areas showed a marked

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increased activity of the pentose phosphate shunt enzymes and a less pronounced increased activity of most anaerobic and hydrolytic enzymes. In contrast to the cardiac fibres in infarcted areas, the fibres in inflamed areas did not reveal a decrease or absence of activity of aerobic enzymes such as succinate dehydrogenase.

Introduction

The application of histochemical techniques to heart tissues of various animal species has revealed differences in enzyme activities and in the content of glycogen and lipids between the Purkinje fibres of the atrioventricular conducting system and the common myocardial fibres. The Purkinje fibres differ from the myocardium proper in containing a larger amount of glycogen and greater activity of enzymes, which play a role in anaerobic energy pathways. Enzymes that play a role in aerobic energy metabolism, however, are less active in the Purkinje fibres than in the myocardial cells (Alcine *et al.*, 1965; Meijer & de Vries, 1978; Otsuka *et al.*, 1967; Schiebler, 1961, 1963; Schiebler *et al.*, 1956; Snijder & Meijer, 1970). In a previous communication (Elias *et al.*, 1980a), similar results were obtained with histochemical techniques performed on human heart tissues.

The aim of the present investigation was to gain an insight into possible changes in the aerobic and anaerobic metabolism of Purkinje fibres of the conducting system and the common myocardial fibres under ischaemic and inflammatory conditions of the human heart. Histochemical studies which have been performed in the past on the human heart under ischaemic conditions have concentrated exclusively on the myocardial fibres. These studies have revealed that the activity of some enzymes is diminished in working myocardial fibres under ischaemic conditions (Nachlas & Shnitka, 1963; Bajusz & Jasmin, 1964a,b; Fine *et al.*, 1966; Morales & Fine, 1966). However, some of the enzyme histochemical techniques used in these investigations were not suitable for metabolic studies. Recently, better enzyme histochemical techniques have become available. In the present investigation, possible changes in the activities of enzymes in the heart under these pathological conditions were studied using these improved techniques.

Materials and methods

Representative material from the atrioventricular node, left bundle branch and right bundle branch of the conducting system of the human heart, as well as areas suspected of early or chronic ischaemic changes, was dissected during the course of routine post mortem examination. The time lag between the clinical manifestation of the cardiac shock and the death of the patient was noted. The specimens of the patients were grouped according to the duration of the ischaemia in hours, days, weeks or years. Material from 52 patients of both sexes was examined. Tissue from four patients who died from acute inflammatory conditions (myocarditis) was also studied. The age of the patients ranged from 15 to 78 years. In order to exclude autolytic changes, only material taken within 60 min of death was considered. Tissue blocks, measuring at least $1 \times 1 \times 0.5$ cm^3, and containing endocardium, conducting system fibres and the adjacent myocardial tissue were

Table 1. Enzyme histochemical techiques used.

Enzyme	*EC number*	*References*
Lactate: NAD^+ oxidoreductase	1.1.1.27	Meijer (1973)
3-Hydroxybutyrate: NAD^+ oxidoreductase	1.1.1.30	Barka & Anderson (1963)
Malate: NAD^+ oxidoreductase	1.1.1.37	Barka & Anderson (1963)
Malate: $NADP^+$ oxidoreductase	1.1.1.40	Meijer & De Vries (1975)
Isocitrate: NAD^+ oxidoreductase	1.1.1.41	Barka & Anderson (1963)
Isocitrate: $NADP^+$ oxidoreductase	1.1.1.42	Meijer & De Vries (1975)
Phosphogluconate: $NADP^+$ oxidoreductase	1.1.1.44	Meijer & De Vries (1974)
Glucose-6-phosphate: $NADP^+$ oxidoreductase	1.1.1.49	Meijer & De Vries (1974)
Glycerol-3-phosphate: menadione oxidoreductase	1.1.99.5	Lojda *et al.* (1979)
Glyceraldehyde-3-phosphate: NAD^+ oxidoreductase	1.2.1.12	De Vries *et al.* (1980)
Succinate: PMS oxidoreductase	1.3.99.1	Nachlas *et al.* (1957)*
Amine: O_2 oxidoreductase	1.4.3.4	Glenner *et al.* (1957)†
NADH: Nitro BT oxidoreductase	1.6.99.3	Burstone (1962)
Cytochrome *c* oxidase	1.9.3.1	Burstone (1962)‡
Peroxidase	1.11.1.7	Christie & Stoward (1978)
α-Glucan phosphorylase	2.4.1.1	Meijer (1968)
Phosphofructokinase	2.7.1.11	Meijer & Stegehuis (1980)
Phosphoglucomutase	2.7.5.1	De Vries & Meijer (1976)
Aryl esterase	3.1.1.2	Meijer & Vloedman (1973)
Acetylcholine esterase	3.1.1.7	Karnovsky & Roots (1964)
Acid phosphatase	3.1.3.2	Meijer (1972)
Mitochondrial ATPase (Mg^{2+}-activated)	3.6.1.3	Meijer & Vloedman (1980)
Myosin ATPase	3.6.1.3	Meijer (1970)
Glucosephosphate isomerase	5.3.1.9	De Vries & Meijer (1976)

Notes:
*Phenazine methosulphate was added to the incubation medium at a final concentration of 50 μg/ml.
†Epinephrine was used as substrate.
‡*p*-Aminodiphenylamine and *p*-methoxy-*p*-aminodiphenylamine were used as substrates. The reactions were performed with and without cytochrome *c* added to the incubation medium at a final concentration of 0.5 mg/ml.

frozen rapidly by immersion in isopentane, precooled to −150° C with liquid nitrogen. The frozen tissue blocks were stored at −95° C. Sections were cut at a thickness of 6 μm in a cryostat at −30° C.

Sections obtained with Haematoxylin and Eosin were used for morphological studies, with the periodic acid–Schiff technique to demonstrate glycogen and with Oil Red O for demonstrating lipids. For the metabolic studies, sections were subjected to the histochemical techniques listed in Table 1.

Results

The morphological alterations which occur in the Purkinje fibres of the atrioventricular conducting system and in the common myocardial fibres under pathological conditions,

and in the changes in the activity of enzymes and in the glycogen and lipid content, represent a dynamic process with a changing character. The different intensities of the staining reactions are not, therefore, presented here. The histochemical findings were independent of age and sex. In the early stages of acute myocardial infarction, the damaged muscle fibres in the ischaemic areas revealed a release and leakage of mitochondrial enzymes and enzymes located in the non-particulate fraction of the cytoplasm. This phenomenon was demonstrated by a reduction in the intensity of the staining reactions and could be seen before any gross morphological change was evident. Some non-structurally bound enzymes, such as lactate dehydrogenase, showed an early diffusion out of the infarcted fibres into the surrounding tissue structures. During this period of 1–2 days, the serum of these patients showed a rise in activity of the heart enzymes, which were released into the circulation. Generally speaking, there was a correlation between the degree of the enzyme activity elevation in the serum and the size of the infarcted area.

The observed reduction in the intensity of the staining reactions of many enzymes at a given time may show in other cases of longer duration an intensity comparable to that observable in normal tissue. This may suggest a restoration of activity within a variable period of time. For many enzymes, the increase in activity began within a few days after the onset of the infarction and the activity returned to normal in the infarcted areas after about two weeks. In some cases, a residual variation from the normal pattern remained for an extended period of time or, in a few cases, permanently. Some enzymes, such as the pentose phosphate shunt enzymes, showed an increase in activity in the muscle fibres after the onset of infarction. The same increase was also observed in chronic ischaemic areas, even without infarction. The glycogen content in the infarcted areas showed an instant depletion after the onset of the infarction. In the early stages of infarction, the lipid content of the ischaemic fibres remained unchanged. However, a higher lipid content was observed in areas of chronic ischaemia, or in areas with old and organized infarction.

The above histochemical findings were present to the same extent in all types of affected fibres, irrespective of whether they were in the working myocardium or in the conducting system. The only exception was that the glycogen reserves in the affected conducting system were depleted at a higher rate than in the adjacent myocardial fibres. Specimens from patients dying with acute myocardial shock as a result of complete heart block or ventricular fibrillation almost always showed histochemical alterations restricted to the fibres of the conducting system. The pattern of activity changes in the infarcted fibres was the same in all the enzymes studied. Only the degree of the activity changes was different.

Mitochondrial enzymes

Succinate dehydrogenase showed an instant decrease in activity in the affected fibres, visible in the sections as early as 3–6 h after the onset of the infarction (Figs. 1–3, 5). Within one day after the onset, the activity in the affected fibres had completely

disappeared, and remained permanently negative for this enzyme. Chronic ischaemia without infarction did not have an apparent influence on the degree of activity. The pattern of change in the activity of NADH: tetrazolium oxidoreductase was more or less similar to that of succinate dehydrogenase, yet a complete absence of activity, such as observed with succinate dehydrogenase, was never observed. The same applies to the other mitochondrial oxidoreductases studied and to cytochrome *c* oxidase; an early decrease due to leakage out of the cells was noted but the activity never disappeared completely. Furthermore, the activity in the affected areas was restored to normal within 1–2 weeks. Hypertrophic muscle fibres at the periphery of the fascicles, or in the vicinity of fibrotic areas showed an increase in activity of glycerol-3-phosphate menadione oxidoreductase. The activity of the mitochondrial Mg^{2+}-activated ATPase showed an early decrease which lasted throughout the breakdown processes in the affected fibres. The staining patterns showed no defect in the coupling state of oxidative phosphorylation.

Non-mitochondrial enzymes

Enzymes that play a role in glycolysis and gluconeogenesis showed a decrease in activity in the fibres of the infarcted areas less than one day after the onset of infarction. This decrease in activity was particularly striking for lactate dehydrogenase. The decrease is rapidly followed by a restoration within a few days. At about two weeks, no difference in activity between infarcted and non-infarcted areas could be demonstrated. In cases of chronic ischaemic changes, whether or not associated with infarction, an increase was evident in the activity of these enzymes in the hypertrophic muscle fibres. The activity of glyceraldehyde-3-phosphate dehydrogenase was especially strongly increased. The two pentose phosphate shunt enzymes examined showed a gradual increase in activity after the onset of infarction. There is an early diffusion of the enzymes out of the infarcted fibres into the adjacent tissue structures, followed within one day by a gradual increase in activity in the entire heart tissue. The increase in activity was enormous in the affected areas (Figs. 4, 6). The rise in activity of phosphogluconate dehydrogenase is less striking than that of glucose-6-phosphate dehydrogenase. Chronic myocardial ischaemia, with or without infarction, showed a higher activity of these enzymes in all muscle fibres of the working heart and of the atrioventricular conducting system. The hypertrophic fibres at the periphery of the fascicles or in the vicinity of fibrotic areas contained a remarkably high degree of activity. The activity of myosin ATPase showed an early decrease which lasted throughout the breakdown processes in the affected fibres of the infarcted areas. The activity of acetylcholine esterase is exclusively present in the Purkinje fibres and located at the periphery of the fibres under the sarcolemma. This activity is completely absent directly after infarction of these fibres. The shortest post-infarction period in material examined was 1 h. The enzyme was not demonstrable in the Purkinje fibres of this case.

The activity of acid phosphatase, β-glucuronidase, non-specific esterase and peroxidase was increased to some degree in the fibres of the infarcted areas. Only acid phosphatase

showed an increase in activity in adjacent heart tissue after an initial leakage from infarcted fibres. The associated leucocytic and histiocytic cellular infiltrates, which appeared in the later stages of infarction, exhibited a certain degree of activity of these hydrolytic enzymes. The fibres in chronic ischaemic and non-infarcted areas did not reveal a significant decrease or increase of glycogen content.

In contrast to the cardiac fibres in infarcted areas, the fibres in inflamed areas showed no apparent change in activity in the vast majority of the enzymes examined. However, the activity of glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase and lactate dehydrogenase showed a marked increase in the inflamed fibres. The activity of glyceraldehyde-3-phosphate dehydrogenase, phosphoglucomutase, glucose phosphate isomerase and phosphofructokinase showed similar, but less pronounced, changes. Moreover, the activity of acid phosphatase showed an increase in many of the inflamed fibres. This increase in activity was mainly perinuclear. β-Glucuronidase, non-specific esterase and peroxidase showed only a minor increase in activity.

Discussion

The enzyme histochemical findings are reliable because previous control experiments with hearts of experimental animals (Elias *et al.*, 1980a) have shown that a period of 60 min between death and freezing of the tissue specimens, the maximum interval in this study, does not have any influence on the activity or location of the enzymes examined. Furthermore, no destruction of enzymes by autolytic processes after this relatively short post mortem interval could be demonstrated. With the exception of

Fig. 1. Cryostat section of a one-day-old infarction incubated according to the succinate dehydrogenase technique. Normal myocardial fibres show a high activity whereas the affected fibres in the infarcted area (*) exhibit a complete absence of activity. The staining of the connective tissue is slight. × 99.

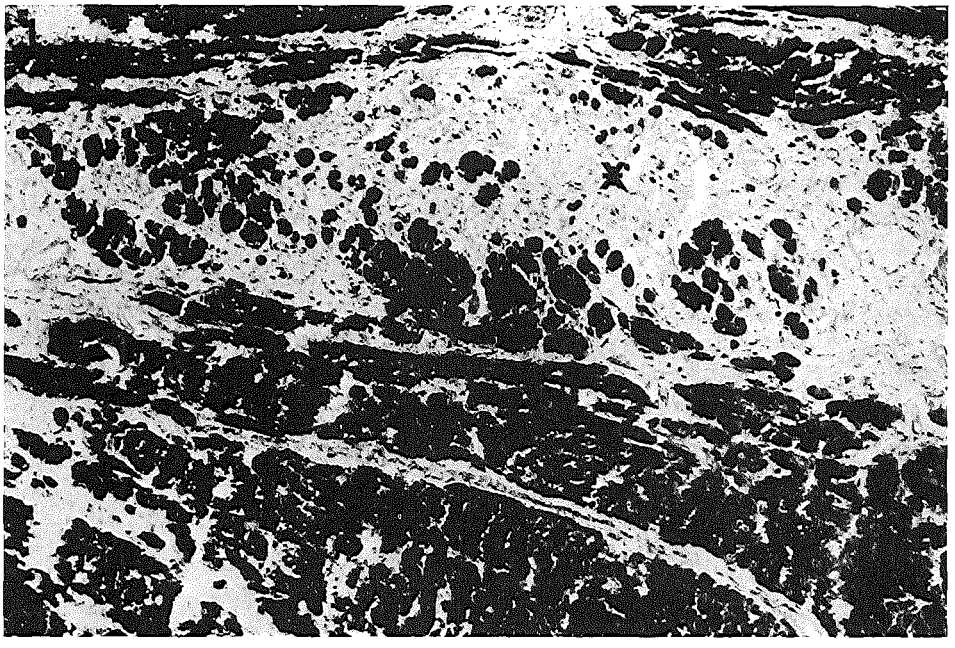
Fig. 2. Cryostat section of a 6 h-old infarction incubated for succinate dehydrogenase. In this case, the affected fibres belong to the atrioventricular conducting system (*) and show an irregular reduction of activity. × 99.

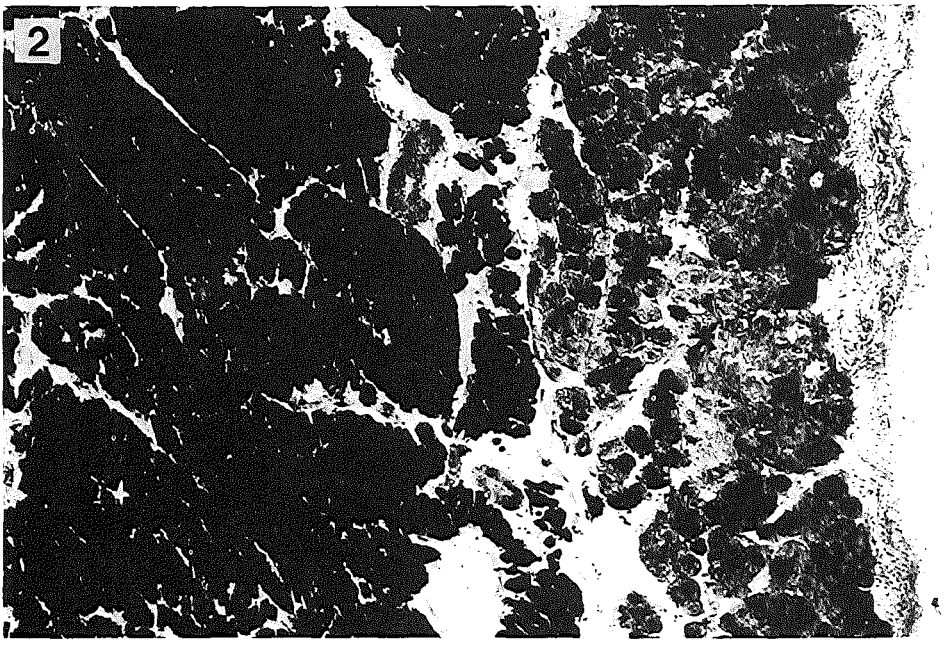
Fig. 3. Demonstration of a 1–2-day-old infarction. Here the ischaemic changes are more pronounced in the conducting system. The structurally altered Purkinje fibres show a complete absence of succinate dehydrogenase activity. A few adjacent myocardial fibres reveal a decreased activity of succinate dehydrogenase. a, Purkinje fibres; b, connective tissue. × 99.

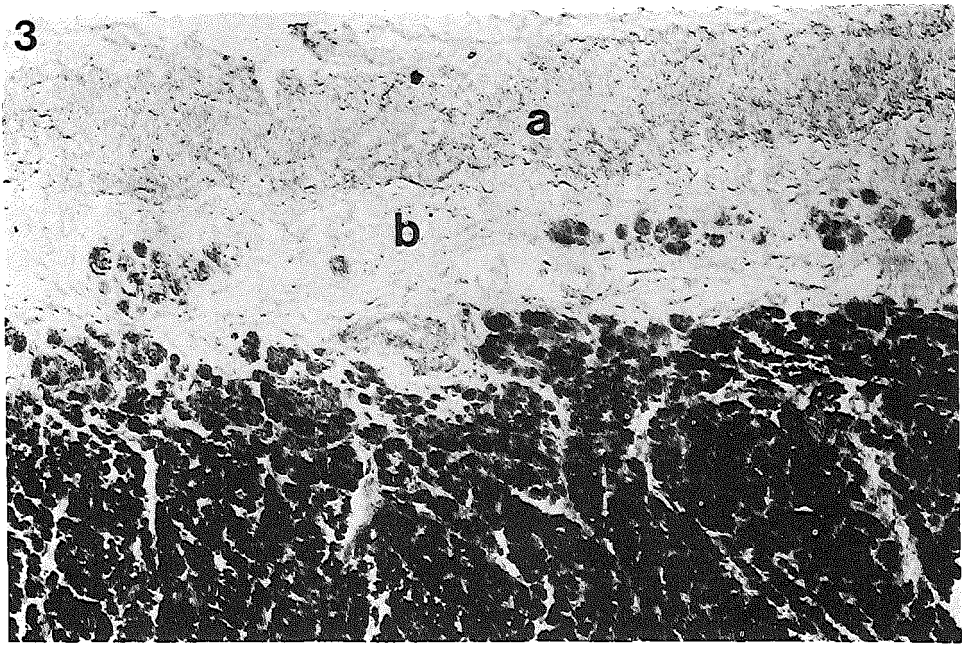
Fig. 4. Demonstration of a non-infarcted area of a chronic ischaemic heart. The working myocardial fibres show only a slight activity of glucose-6-phosphate dehydrogenase, whereas the activity in the structurally altered Purkinje fibres is strikingly increased. a, Affected Purkinje fibres; b, connective tissue; c, common myocardial fibres. × 99.

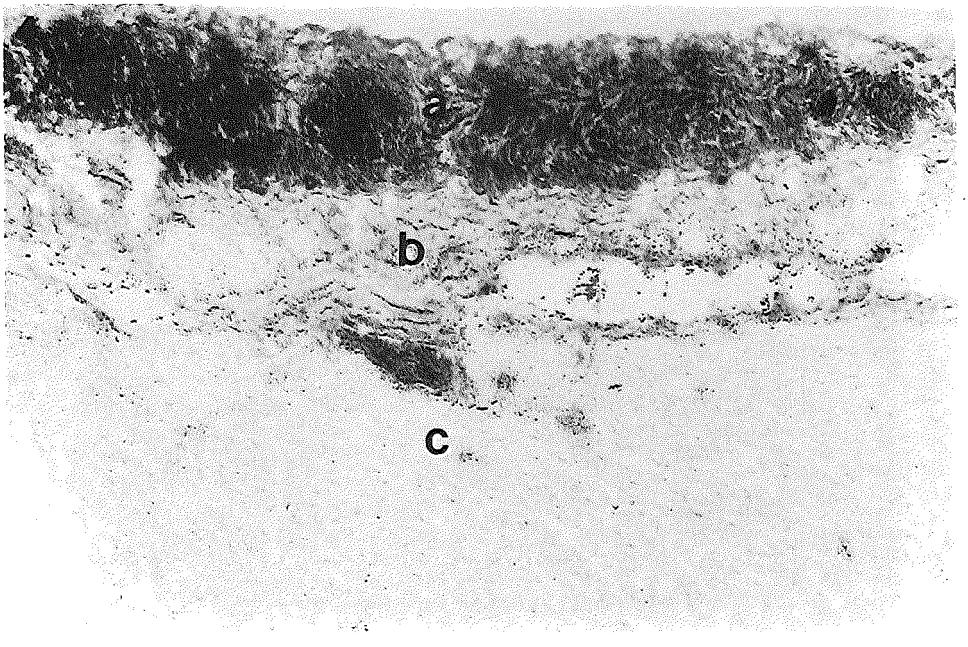
Fig. 5. Affected myocardial fibres show a complete absence of succinate dehydrogenase activity. × 16.5.

Fig. 6. The activity of glucose-6-phosphate dehydrogenase is strongly increased in these altered fibres. The blood vessels also show a high activity. × 16.5.




2


3
a
b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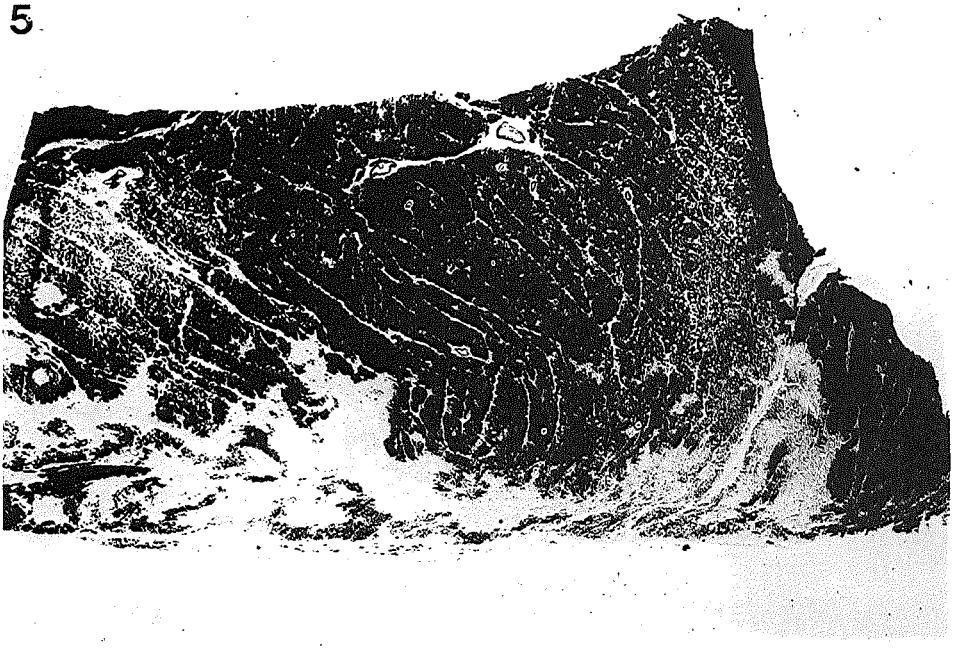
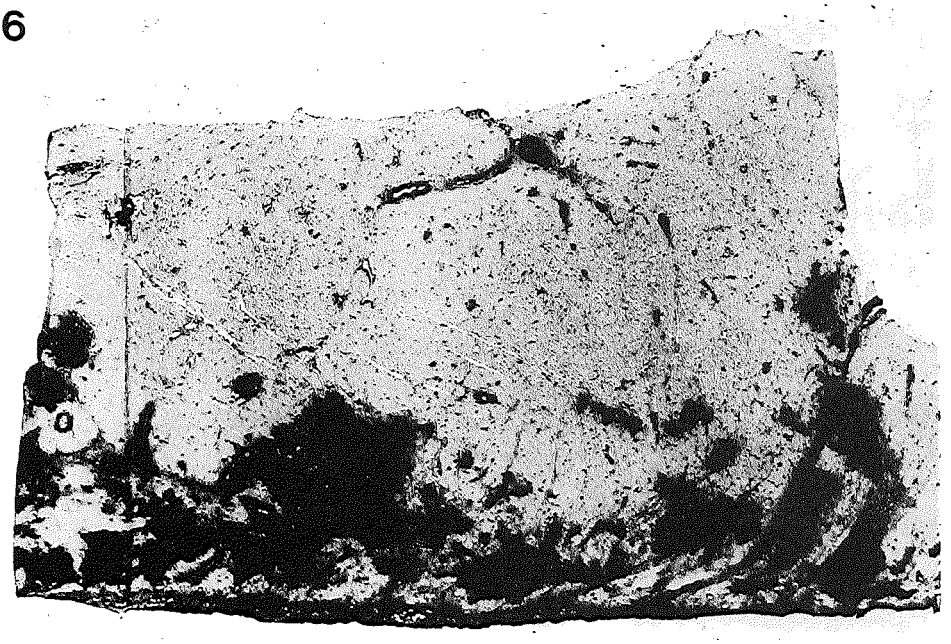
5



6



phosphofructokinase, no significant inactivation occurred during the histochemical freezing procedures (Meijer *et al.*, 1977a; Meijer & Stegehuis, 1980). The activity of phosphofructokinase is possibly reduced by about 15% as a result of the freezing procedure. However, the percentage reduction in activity is fairly constant for heart specimens. The technique employed in this study for qualitative studies, therefore, appears to be suitable.

Other control experiments with hearts of experimental animals have, however, demonstrated that findings obtained with the PAS reaction are less reliable. This results from the fact that glycogen is metabolized after death by glycolytic enzymes which are still active during the post mortem interval. The extent of the breakdown of glycogen is, therefore, strongly dependent on the temperature of the cadaver.

For the morphopathologist, one of the major problems is the difficulty in determining the early phases of myocardial infarction with a reliable degree of accuracy. Unequivocal gross morphological changes are not evident for 24–48 h following the occlusion of a major coronary artery in man (Mallory *et al.*, 1939; Gould, 1960). The same difficulty faces the clinician. It is also very difficult to determine with accuracy the time lag between the actual onset of a proper infarction of the myocardial fibres and the death of the patient, for the simple reason that one cannot exactly measure the time lag between the onset of pain or cardiac shock and the actual onset of the infarction. To characterize the myocardial pathological conditions, the findings of many techniques are available. For instance, ECG findings, especially the rise and depression of the ST segment or development of a Q wave, findings of echo visualization of the left ventricular wall and of end-diastolic pressure, findings with the isotope Thallium 201, and rises in serum lactate dehydrogenase and creatine kinase levels may be helpful. However, inaccuracy may still be possible. To avoid this, we resorted to a baseline measuring the time of onset of the clinical cardiac shock as an indication of the ischaemic change whether associated at the same moment or later with infarction.

The selection of the site of the specimens is also a product of a complex of factors involving the entire clinical and pathological data available. In the late stages, the selection is simple, being based on the macroscopic or morphological change. In the early phases, a wide selection of many representative specimens is obligatory. In this study, these included three specimens in a fixed pattern from the A–V node, left bundle branch and right bundle branch about 1 cm distal to the node. Other representative specimens from the anterior and posterior walls of the heart were taken, including left and right ventricular wall as well as the septum. When necessary, additional specimens from the lateral wall of both sides were taken. In practice, three or four specimens in general were sufficient to determine the site of the lesion. The details of the clinical data, autopsy findings and individual variations between one patient and another are deliberately avoided in this communication since it does not deal with a homogeneous group of experimental material. Every patient has his own clinical and pathological picture. The stress is made only on the pattern of changes as a whole. A tabulation and grouping of data and patients was avoided as we are dealing with a dynamic process of post-

ischaemic change affecting the myocardial fibres, and every patient must be regarded as an individual case occupying a certain point in this continuous process.

The application of enzyme histochemical techniques might be able to demonstrate infarction of shorter duration before any morphological change manifests itself (Wachstein & Meisel, 1955; Jestädt & Sandritter, 1959; Shnitka & Nachlas, 1963; Anderson *et al.*, 1979). When myocardial fibres in infarcted areas are damaged, enzymes are released into the circulation, giving rise to an elevation in activity of the enzymes in the serum. The histochemical techniques are based on this reduction or loss of enzyme activity in the infarcted areas. However, even if the problem of obtaining fresh tissue for reliable diagnostic examination can be overcome, there still remains the difficulty in finding the changes, especially in the very early phase of myocardial infarction. Unfortunately, this is precisely the phase which is of the greatest interest to the clinician, the medico-legal examiner and the pathologist. In the enzyme histochemical studies just cited, it is principally the activity of the succinate dehydrogenase and, in exceptional cases, the activity of a few other mitochondrial enzymes which were investigated.

In the present study, about 25 enzymes were examined. The results clearly show that the technique for demonstration of succinate dehydrogenase activity is one of the most suitable for demonstrating ischaemic damage in the myocardial fibres. The activity of this enzyme is strongly reduced and often completely absent in affected myocardial fibres. As distinct from the other enzymes studied, no apparent restoration of the activity could be demonstrated. The infarcted areas remain permanently negative for this enzyme.

The elevation in activity of the myocardial enzymes in the serum after the onset of an infarct is transient. For most enzymes, it reaches a peak at about 24 h after the onset of a myocardial infarction and generally returns to normal on about the third day (Dreyfus *et al.*, 1960). The transient elevation suggests that after initial damage of the cell membranes, and which gives rise to a release of enzymes, there is a gradual repair of membranes in the later stages. It is interesting that the transient elevation in the activity of the enzymes in the serum corresponds with the transient drop in activity of most enzymes studied in the damaged fibres of the infarcted areas. This transient drop in activity was not found in inflammatory processes.

The dramatic increase in activity of phosphogluconate dehydrogenase, and more especially of glucose-6-phosphate dehydrogenase, even during the early stages of acute myocardial infarction is very striking. Since the activity of this enzyme in normal myocardial fibres is slight, the marked increase in activity can be detected very easily. The histochemical technique for the demonstration of the activity of glucose-6-phosphate dehydrogenase is, therefore, useful in the detection and delineation of myocardial infarction. On the other hand, the increase in the activity of both pentose phosphate shunt enzymes seems to be unspecific and occurs in ischaemic changes, either acute (and associated with infarction) or chronic, and also in inflammatory conditions. Glucose-6-phosphate dehydrogenase in liver has a half-life of about 15 h (Freedland, 1968; Goldberg & St John, 1976). Although the half-life time of this enzyme in

heart muscle has yet to be determined, the rapid increase in activity in response to myocardial infarction suggests that the half-life time will also be relatively short in heart fibres. Short half-life enzymes are considered to be regulatory enzymes whose activity can fluctuate rapidly in response to environmental changes (Goldberg & St John, 1976). The strong increase in activity in myocardial infarction, therefore, gives rise to the interesting concept that these activity changes may represent the stimulation of a pathway which, under normal conditions of adequate oxygen supply, plays a subsidiary or insignificant metabolic role. Stimulation of this pathway has also been demonstrated in diseased skeletal muscles (Meijer & Elias, 1977; Meijer *et al.*, 1977b; Elias & Meijer, 1981) and in malignancies and other proliferative diseases (Elias *et al.*, 1980b, 1981).

Until now there have been no descriptions in the literature of histochemical investigations on the conducting system under ischaemic and inflammatory conditions. The results of this study clearly show that, in principle, the same activity changes can occur in the fibres of the conducting system. Moreover, the fibres of the conducting system invariably showed histochemical changes when the patients died from complete heart block or ventricular fibrillation. The histochemical findings reveal, in infarcted regions, that the ratio of activity of the enzymes which play a role in the aerobic and anaerobic pathways is altered in favour of a more anaerobic metabolism. However, from the biochemical findings described in the literature, the metabolical changes found with the histochemical techniques under ischaemic conditions are very complex. It is, therefore, not possible at present to draw clear conclusions from these changes in energy metabolism. Immediately after the onset of myocardial infarction, utilization of tissue glycogen is accelerated (Morgan *et al.*, 1959; Cornblatt *et al.*, 1963) and oxidation of fatty acids is inhibited (Evans, 1964). Acceleration of glycolysis has been related to decreased tissue levels of creatine phosphate and ATP and to increased tissue levels of inorganic phosphate, ADP and AMP (Cornblatt *et al.*, 1963; Williamson, 1966), activating the activity of the key enzymes α-glucan phosphorylase and phosphofructokinase. However, after some duration of the ischaemic conditions there is a decrease in glycolytic flux when the coronary flow rate falls sufficiently (Rovetto *et al.*, 1975). The inhibition of glycolysis develops at these levels of phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase and is related to the accumulation of H^+, NADH and lactate (Kubler & Spieckermann, 1970; Rovetto *et al.*, 1975). In ischaemic regions, glyceraldehyde-3-phosphate dehydrogenase is the glycolytic rate-limiting enzyme instead of phosphofructokinase (Opie, 1976).

The increase in activity of acid phosphatase in damaged myocardial fibres of infarcted and inflamed regions are in agreement with the 'lysosomal hypothesis' of Wildenthal (1975). According to this hypothesis, lysosomal enzymes are significantly involved in the degradation of myocardial proteins. The increased lipid accumulation found in the infarcted fibres is in agreement with biochemical findings concerning the lipid metabolism in normal and ischaemic heart tissue. Fatty acids are catabolized preferentially by the normal heart (Krebs, 1972) and oxidation of lipids supplies large quantities of ATP. Under ischaemic conditions, oxidation of lipids is hampered and accumulation of lipids occurs. Moreover, the capacity of lipid synthesis may be increased under ischaemic

conditions because of an increased activity of the pentose phosphate shunt enzymes. These enzymes produce the NADPH necessary for the lipid synthesis.

Finally, we would like to suggest the following features as a diagnostic aid for the pathologist seeking reliable findings for the histochemical demonstration of myocardial disease, especially acute ischaemic changes or early infarction. (1) Absence of activity of acetylcholine esterase as a marker of cellular damage in the infarcted fibres of the conducting system. The disappearance in activity occurs the instant the infarction takes place. (2) Absence or decrease in activity of succinate dehydrogenase in the infarcted fibres. (3) Increase in activity of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase, indicating an adaption mechanism in regions with a poor oxygen supply, whether acute or chronic. In addition, an increase in activity occurs in the fibres of inflamed areas.

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CHAPTER 4.10

Histochemistry

Fluctuations in the Enzymatic Activity of the Human Endometrium

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Summary. Cyclic fluctuations were studied in the activity of oxidoreductases playing a role in the major energy metabolic pathways, lysosomal and non-lysosomal hydrolases and some non-enzymatic cytochemical components demonstrable in different developmental physiological or pathophysiological phases of human endometrium. The total scope of the study involved 170 tissues and cytological specimens. The cytological material included microcurettings, aspirates, brush preparations and tissue prints. An evaluation of the usefulness of the application of enzyme cytochemistry to cytological material is included. The most important results were a cyclic fluctuation and a progestagenic controlled increase in the activity of many oxidoreductases, especially the NADPH regenerating enzymes of the pentose phosphate pathway, and of the $NADP^+$ dependent isocitrate dehydrogenase. The histochemical evaluation of the activity of these $NADP^+$ linked enzymes can therefore be recommended for the evaluation of the physiological status of the endometrial cells, especially in patients with infertility problems.

Introduction

Since the early days of microscopy, the cyclic changes in the human endometrium have been a subject of interest to histopathologists. The data accumulated provide clear evidence of correlation or interrelation between the hormonal status, morpho-developmental changes, cytochemical contents, subcellular construction and enzymatic activity in the endometrial cells. For example Fuhrmann (1961) found that the biological effect of many steroid hormones is reflected in the endometrium as a target organ. This interaction may prove to be a valuable diagnostic aid in evaluating the functional state of the endometrium as was pointed out by Dallenbach-Hellweg (1981)

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in her book of the known data on this subject. However, according to Dallenbach-Hellweg, a great deal of the enzyme histochemical techniques used by many researchers did not yield results that are reliable for metabolic interpretations. Results obtained with the used techniques were often equivocal owing to diffusion artefacts, because the non-structural bound and the partially or loosely structural bound enzymes tend to diffuse out of the cells during the incubation period.

During the past years the development of numerous new histochemical techniques have been described in literature. For instance semipermeable membrane techniques have been developed for the demonstration of activities of hydrolases, oxidoreductases, transferases and isomerases (Meijer 1978; Lojda et al. 1979; Meijer 1980). By application of semipermeable membranes, which are interposed between the incubation solution and the tissue sections, diffusion of enzymes is prevented.

The great progress of enzyme histochemistry prompted us to re-study the enzyme histochemical characteristics of the human endometrium in different phases of the cycle, and in some different pathophysiological conditions. The option was made to present an useful information to the clinicopathologist concerned mainly in evaluating the developmental or the hormonal status of a given endometrium especially when dealing with infertility problems. To avoid complicating the subject the evaluating of the main components was stressed ignoring the irrelevant findings or evaluating the results in some types of cells present in endometrium which do not have a significant diagnostic aid.

In preparing the present communication, use was made of the most reliable conventional techniques and of membrane techniques. In these techniques the demonstration of the oxidoreductases is rendered independent on the activity of auxilliary enzymes (Meijer 1980). Special attention was paid to the demonstration of the activity of enzymes which formerly could not be reliable demonstrated.

Materials and Methods

Endometrial curettings and operative material representative of the different stages of the normal cycle, physiological or pathophysiological conditions of the human endometrium, excluding malignancy and inflammatory conditions, were examined for the activity of a battery of enzymes and for some metabolic products. The study involved endometrial material from 130 patients and 10 non-pathological pregnancy endometria with trophoblast. Cervical mucosa was not included in the study. In a parallel study, cytological material, mainly obtained as brush preparations, microcuretting, aspirates and sometimes as tissue prints, in total from 30 patients, were spreaded directly onto glass slides or semipermeable membranes and incubated for the demonstration of the same series of enzymes.

Histochemistry: To avoid any lytical changes or unreliability of the quality of material used, the tissue specimens were immediately after sampling frozen in a fresh unfixed state in isopentane precooled to $-150°$ C in liquid nitrogen and stored at $-95°$ C. Serial sections were cut at 6 µm in a cryostat adjusted to $-25°$ C. The tissue sections were utilized fixed or unfixed according to the prescriptions of the techniques used. The sections were then incubated at 37° C to demonstrate the activity of the following enzymes. The activity of NADH: tetrazolium oxidoreductase (NADH, EC 1.6.99.3) was demonstrated according to Barka and Anderson

(1963) using NADH as substrate. The activity of glycerol-3-phosphate: menadione oxidoreductase (GPOX, EC 1.1.99.5) was demonstrated according to a substrate modification of the method of Wattenberg and Leong (1960). Instead of succinate, an equimolar concentration of glycerol-3-phosphate was used. The activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was demonstrated according to Meijer (1973). The method described by Barka and Anderson (1963) was used to demonstrate the activity of malate: NAD^+ oxidoreductase (MDH: NAD^+, EC 1.1.1.37) and isocitrate: NAD^+ oxidoreductase (ICDH: NAD^+, EC 1.1.1.41). The activity of glucose-6-phosphate dehydrogenase (GPDH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (PGDH, EC 1.1.1.44) was demonstrated according to Meijer and de Vries (1974). The activity of isocitrate: $NADP^+$ oxidoreductase (decarboxylating) (ICDH: $NADP^+$, EC 1.1.1.42) was demonstrated according to Meijer and de Vries (1975). The activity of β-glucuronidase (GLUC, EC 3.2.1.31) and of the non-specific esterases (NE, EC 3.1.1.1. and EC, 3.1.1.2) was demonstrated according to Meijer and Vloedman (1973). The activity of aryl sulphatase (AS, 3.1.6.1.) was demonstrated according to Koudstaal (1975). The activity of acid phosphatase (ACP, EC 3.1.3.1) was demonstrated according to Meijer (1972). The activity of alkaline phosphatase (ALP, EC, 3.1.3.1.) was demonstrated by using naphthyl AS-MX phosphate and fast red violet LB (Pearse 1972). The specificity of the enzyme reactions was tested with control reactions as described in the mentioned literature of the applied techniques.

Oil red-O was used to demonstrate lipoid deposits. The PAS stain was applied to sections cut from the fresh frozen material and from fixed tissue specimens as mentioned further on.

Histology: Material destined for routine histological examination was fixed in a buffered 8% solution of formaldehyde adjusted to a pH of 7.2–7.5, processed and embedded in parawax. Sections were cut at 6 µm and stained with Harris' haematoxyline and eosin, alcian blue at pH 2.4, mucicarmin, PAS with and without pretreatment with diastase and when necessary other stains.

The results were listed for each specimen separately. This was followed by a correlation with the clinical data, oestrogen and progesteron levels, and the histological and cytological appearance. After the individual evaluation of each specimen all data of the total scope of experience were correlated together. The final results were listed together not as individual cases or types of lesions, but according to the findings in the separate components of the endometrium and their stage of development.

Results

The reliability of the applied histochemical techniques was tested with control experiments. This was necessary because most applied techniques were developed for muscle tissue with completely different metabolic characteristics as endometrium. These control experiments have shown that the applied histochemical techniques are reliable for human endometrium. Furthermore it was proved that the period between sampling and freezing of the tissue specimens did not have any influence on the activity and localization of the examined enzymes.

The histochemical results are presented in Table 1, considering the endometrium as separate components and presenting the observations as histochemical characteristics of glandular epithelium or stromal cells in different stages of development. The description involved some pathophysiological conditions thought to be influenced either by a balanced or imbalanced hormonal status in the female subjects. These conditions include cystic glandular hyperplasia, as well as the small non-functional glandular structures which are seen with increasing frequency these days, especially in women using oral contraceptives or other drugs which have a suppressive effect on the normal biphasic cycle.

Table 1. Distribution of enzyme activities and some metabolic products of human endometrium

Enzyme	Endometrial epithelium					Stroma		Tro-pho-blast
	Prolif-erative	Secre-tory	Sur-face	Non-active	Hyper-plastic	Prolif-erative	Proges-tagenic	
NADH	2+	2+/3+	2+	2+	2+	2+	2+	2+
MDH:NAD$^+$	2+	2+	2+	1+	2+	2+	3+	3+
ICDH:NAD$^+$	2+	2+	2+	1+	2+	2+	3+	3+
ICDH:NADP$^+$	1+	4+	1+	1+	2+	−	1+stroma 3+decidua	3+
GPOX	3+	3+	3+	2+	3+	±	+	4+
LDH	1+/2+	2+/3+	2+	1+	2+	1+/2+	3+	1+
GPDH	3+	4+	2+	2+	3+/4+	2+/3+	4+	4+
PGDH	2+	4+	2+	2+	3+/4+	2+	4+	4+
ACP	1+	2+	phasic	1+	1+	1+	1+	1+
GLUC	2+	4+	3+	2+	1+/2+	±	1+/2+	1+
NE	2+	2+ apical	−	1+/2+	1+	±	1+	1+
AS	−	±	−	−	−	−	−	2+
ALP	3+	2+ apical	phasic	1+/2+	1+/2+	−	3+ perivascular	1+
Glycogen	±	2+	±	±	±	±	±	±
Acid MP	±	2+	1+	±	1+	−	1+	−
Neutral MP	−	2+	1+	−	1+	−	1+	+

NADH=NADH: tetrazolium oxidoreductase; MDH:NAD$^+$=malate: NAD$^+$ oxidoreductase; ICDH:NAD$^+$=isocitrate: NAD$^+$ oxidoreductase; ICDH:NADP$^+$=isocitrate: NADP$^+$ oxidoreductase; GPOX=glycerol-3-phosphate: menadione oxidoreductase; LDH=lactate dehydrogenase; GPDH=glucose-6-phosphate dehydrogenase; PGDH=6-phosphogluconate dehydrogenase; ACP=acid phosphatase; GLUC=β-glucuronidase; NE=non-specific esterase; AS=aryl sulphatase; ALP=alkaline phosphatase; MP=mucopolysaccharides.
−Absent; ±slight; 1+ low; 2+ moderate; 3+ strong; 4+ abundant; / variable activity.
Phasic: The fluctuation in activity is equal to the phasic fluctuation in glandular epithelium.

With some exceptions, the post-menopausal atrophic endometrium was found to have the same histochemical characteristics as the non-functional glandular epithelium described above and which is found under certain conditions during reproductive life. The exceptions consisted of the absence of alkaline phosphatase and a lower activity of non-specific esterases in the atrophic post-menopausal epithelium when compared with the non-functional epithelium. The surface epithelium was found to have its own enzymatic pattern which differed from other glandular epithelium. For this reason, the surface epithelium has been tabulated separately. With regard to the stromal component, only two phases are described: proliferative or oestrogenic and progestagenic stroma which was found to be similar to that of normal pregnancy or to that in the premenstrual phase of an ovulatory cycle. To complete the study, the histochemical appearances of the trophoblast cells of normal pregnancy were also tabulated, although they do not belong to the endometrium itself but to the developing foetus. No substantial differences were observed between both types of trophoblast cells.

As can be seen from the data presented in Table 1, the activity of the mitochondrial enzymes NADH, MDH: NAD^+, ICDH: NAD^+, and GPOX reveals moderate or little cyclic fluctuation (Fig. 1). On the other hand the activity of ICDH: $NADP^+$, which is located in the mitochondrial and non-particulate fractions of the cytoplasm reveals a strong cyclic fluctuation: increasing greatly in secretory cells (Fig. 2) and decreasing to a minimum in atrophic cells and remaining at low activity in the relatively non-functional or proliferative epithelium. The surface epithelium revealed a low activity of this enzyme throughout the cycle. It is striking that the activity pattern of LDH is completely different from the activity pattern of GPOX, though both enzymes play a role in the anaerobic energy metabolism. The activity of the pentose phosphate cycle enzymes GPDH and PGDH reveals a very strong cyclic fluctuation, with an abundant activity in the secretory and hyperplastic phase of the endometrial epithelium, progestagenic phase of the stroma and in the trophoblast. There are only slight fluctuations in the activity of ACP. The most striking observation concerning this enzyme was not the variation in activity, but the location of the enzyme in the cells concerned. In the proliferative phase, the activity was equally distributed throughout the cytoplasm. In the early secretory phase, there was a basal subnuclear accumulation of activity, followed by a gradual migration to the apex (Fig. 3). The activity of GLUC showed no cyclic migration at cellular level, but a clear increase in activity in the secretory phase (Fig. 3). The surface epithelium showed a constant activity of this enzyme throughout the cycle. The stromal cells showed a slight variation in the activity of both ACP and GLUC, but the decidual cells, despite their tabulation in Table 1 under the same catagory as progestagenic stroma (of the secretory phase) showed a relatively higher activity of both enzymes, especially of GLUC. The stromal macrophages which appear late in the secretory phase were very rich in activity of both enzymes, and to a lesser extent in activity of NE. A moderate activity of AS was exclusively shown in the trophoblast. The distribution of ALP activity in the endometrium throughout the cycle was strikingly variable. The most important finding in connection with ALP was the relatively high activity in proliferative epithelium: a characteristic feature which will remain specific for endometrial epithelium even after malignant transformation. The secretory epithelium showed a much lower activity, or showed in a few specimens no activity at all (Fig. 4). When some activity was encountered, it was only found in the apical position. The non-active small glandular structures seen in the endometrium of women using contraceptive drugs showed a relatively high activity of ALP. The small atrophic glands most often seen in post menopausal but which are morphological similar to the glands suppressed by oral contraceptives, proved to be much less active, or contained no activity of ALP at all. In contrast to the slightly increased activity of ACP, activity of ALP showed a clear decrease in activity, but with a similar migration to the apical position in the secretory cells. More glycogen and acid and neutral mucopolysaccharides were found in the surface epithelium and glandular epithelium during the secretory phase than in the non-active or proliferative cells. The presence of lipid deposits could be demonstrated in the secretory and decidual cells.

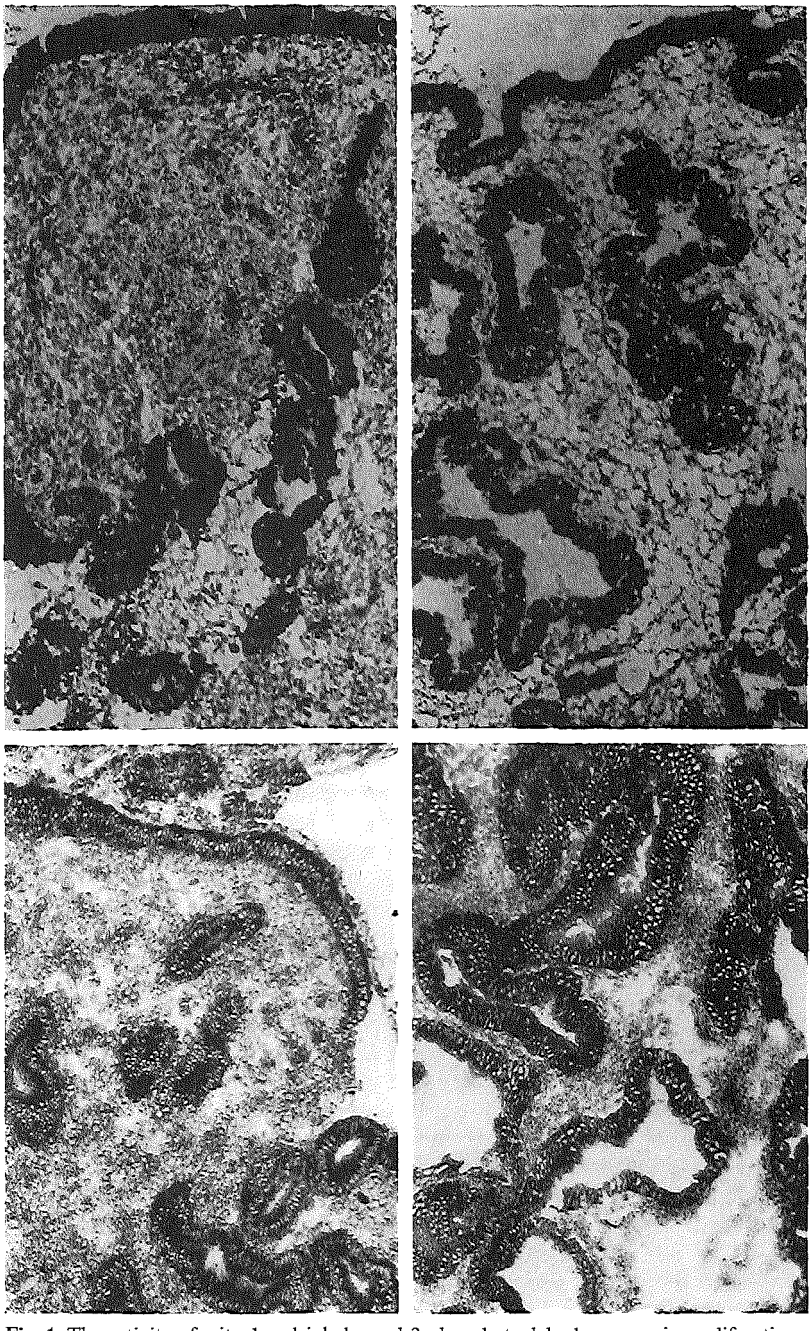
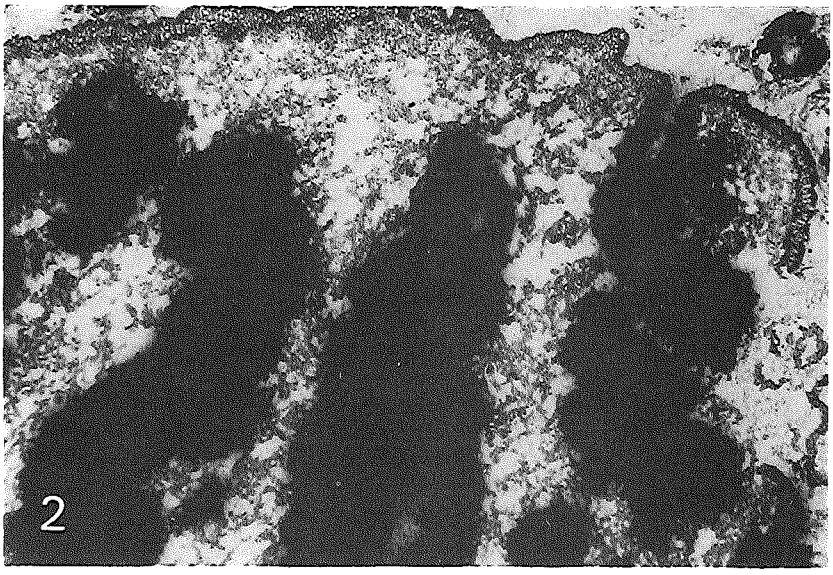


Fig. 1. The activity of mitochondrial glycerol-3-phosphate dehydrogenase in proliferative endometrium (upper left) and in secretory endometrium (upper right). The activity of NAD^+ dependent malate dehydrogenase in proliferative endometrium (below left) and in secretory endometrium (below right). Both enzymes show slight or no cyclic variations and are equally active in surface and glandular epithelium. $\times 90$



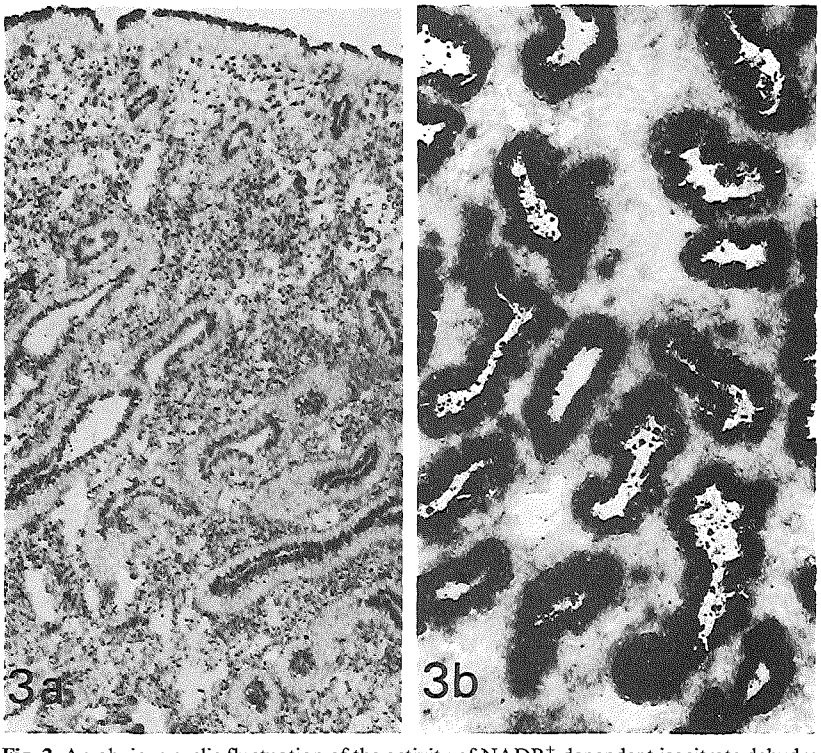
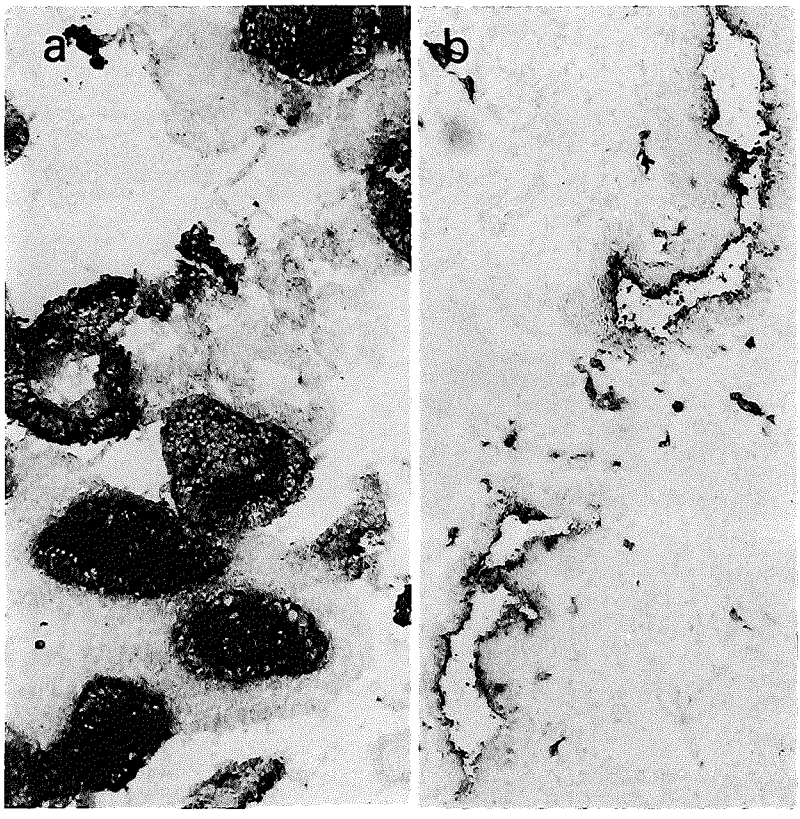


Fig. 2. An obvious cyclic fluctuation of the activity of $NADP^+$ dependent isocitrate dehydrogenase is observable. In contrast to the slight activity in the non active as well as in the proliferation phase the secretory phase exhibits a very high activity. ×90

Fig. 3a, b. The activity of β-glucuronidase (**a**) in contrast to that of acid phosphatase (**b**) remain evenly distributed in the glandular epithelium. The acid phosphatase shows a tendency of migration to apical position in the secretory phase. ×90



Figs. 4a, b. The activity of alkaline phosphatase is relatively high in the proliferative phase (**a**). An obvious decrease in activity associate the secretory phase. Late in the cycle the activity becomes only demonstrable at apical position (**b**). Pregnancy endometrium shows the same characteristic features as seen in the secretory phase. × 90

Discussion

One of the most significant findings in the present study is the demonstration of a very strong activity of the oxidative part of the pentose phosphate pathway in the secretory cells and in the decidua when compared with the proliferation endometrium. The latter, on the other hand, showed a greater activity of GPDH and PGDH than the non functional glandular epithelium. A very important role of both enzymes in the energy metabolism is the reduction of $NADP^+$ into NADPH which is required for many reductive synthetic processes in cell proliferation and for fatty acid and steroid synthesis. The activity of both enzymes therefore indicates a high capacity for synthetic processes during the secretory phase (Sawaragi and Wynn 1969; Coulton 1977). With regard to the role in the biosynthesis of fatty acids, it is noteworthy that cells having a high activity of both enzymes

always contained lipid deposits. The presence of lipid material in the secretory cells as well as in decidual cells may indicate an active lipid synthesis. This concept of active lipid synthesis is in agreement with the findings of Aschheim (1915) and Black et al. (1941), but in conflict with the opinion of Froboese (1924) and Craig and Danziger (1965) who suggested that the presence of lipids must be considered as a mere degeneration product. However the consideration of lipids as a degeneration product is from the biochemical point of view not understandable. Since the biosynthesis of fatty acids is complex and the activity of many important enzymes such as citrate lyase, fatty acid synthetase and acetyl CoA carboxylase have not yet been studied in the human endometrium, it would be premature to suggest an established connection between lipid synthesis and increase in activity of both pentose phosphate pathway enzymes.

Another function of the pentose phosphate pathway is the production of ribose-5-phosphate necessary for the synthesis of 5-phosphoribosyl-1-pyrophosphate. This substance is an essential substrate for the conversion of the purine basis adenine and hypoxanthine into the corresponding mononucleotides, as well as for the biosynthesis of purine and pyrimidine nucleotides (Henderson 1972). The production of the pentoses may proceed via the oxidative steps and the non-oxidative steps of the pentose phosphate pathway. The activity of GPDH and PGDH gives an impression of exclusively the capacity of the oxidative steps. The direction of flow and the path taken by glucose-6-phosphate after entry into the pentose phosphate pathway reactions are determined largely by the relative requirements of the cells for NAPH and ribose-5-phosphate. GPDH is the key enzyme in the control of these reactions. Enzymes playing a role in the non-oxidative steps of the pentose phosphate pathway cannot be demonstrated with enzyme histochemical techniques. We have established with biochemical studies that the activity of the non-oxidative enzymes ribose-5-phosphate ketol-isomerase (EC 5.3.1.6.), ribose-5-phosphate-3-epimerase (EC 5.3.3.1.), sedoheptulose-7-phosphate: glyceraldehyde-3-phosphate dihydroxyacetone transferase (EC 2.2.1.2.) and sedoheptulose-7-phosphate: glyceraldehyde-3-phosphate glycolaldehyde (EC 2.2.1.1.) run parallel with the increase in activity of GPDH (Meijer and Elias 1983).

Lastly the cycle plays a role in the production of triose phosphate for energy metabolism and its activity serves to bypass the rate limiting steps of glycolysis (Kapuscinski and Williams 1981).

In previous communications we described a similar strong increase in the activity of GPDH and PGDH in many pathological conditions, such as diseases of the neuromuscular system (Meijer and Elias 1977; Meijer et al. 1977; Elias and Meijer 1981), myocardial ischaemic conditions (Elias et al. 1982) and in malignancies (Elias et al. 1980, 1981).

The ICDH: $NADP^+$ is another enzyme with the ability to convert $NADP^+$ into NADPH. The activity of this enzyme may therefore provide information on the capacity of NADPH production as well (Goebell and Kadenbach 1964; Lardy et al. 1965).

Liebig and Stenger (1977) described an increase in the number of mito-

chondria in the secretory phase. From this, one might expect a clear increase of the mitochondrial enzymes investigated NADH, MDH: NAD^+ and ICDH: NAD^+. However, this increase proved to be very limited in this study. Similarly, no fluctuation could be found in the activity of the mitochondrial GPOX. The minimal or total absence of activity of this enzyme in stromal cells when compared with the high activity in glandular epithelium, may prove important to researchers looking for a means of differentiating between carcinomas, carcino-sarcomas and pure sarcomas.

The premenstrual decrease in the activity of ACP in the secretory cells is a result of either decapitation secretion which is usually observable in the premenstrual phase or diffusion out of cells (Dallenbach-Hellweg 1981). The same will apply to many other lysosomal enzymes. A study by Bitenski and Cohen (1965) showed that the premenstrual fall in progesteron is coupled with a leakage or release of relaxin as a result of increased permeability of the lysosomes.

It is likely that the variation of location of ACP and ALP in the cells might correspond to a certain pattern of distribution in the subcellular structures which might vary in location in the different phases of the cycle.

Our experience with the application of the enzyme histochemical techniques to cytological material often proved these to be reliable techniques giving satisfactory results similar to those obtained with histological techniques. The same results were reported working with tumour cells (Elias et al. 1980, 1981). However, these techniques have serious limitation in the evaluation of the hormonal status or the stage of development of the endometrium since one cannot determine with any certainty the anatomical site of origin of the epithelial cells examined in a cytological preparation. The brushing technique, microcurettage, aspiration biopsies, or even tissue prints of histological specimens, will yield a proportion of surface epithelium, morphologically indistinguishable from glandular epithelium. The different enzymatic histochemical properties of surface epithelium and glandular epithelium, also reported by Schmidt-Matthiesen (1968) and Ferenczy et al. (1972), will lead to erroneous evaluation of results obtained from cytological material. This is certainly true for enzymes which show a variable distribution. Other enzymes, which are equally distributed in both types of endometrial epithelium, will be yield more reliable, but this will depend on the magnitude of their cyclic fluctuation.

The absence of aryl sulphatase in both healthy and diseased endometrial cells is by no means a negative finding. The investigation of the activity of this enzyme in, for example, lesions or cells containing malignant effusions will assist in differentiating between primary tumours of the endometrium and ovary and primary lesions originating in the large bowel. A differentiation which can prove exceedingly difficult on the histology alone. Endometrial and ovarian tumours do not exhibit any activity of this enzyme, while a considerable percentage of, for example, colonic carcinomas, in our experience do exhibit such activity.

Finally it can be concluded from this study that the enzymes GPDH, PGDH and ICDH: $NADP^+$, reveal a marked cyclic fluctuation in activity.

The application of the semipermeable membrane techniques for the demonstration of their activity can therefore be highly recommended as a reliable diagnostic measure for evaluating the hormonal, pathophysiological or developmental status of the human endometrium and for studying the metabolic condition of the endometrium.

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CHAPTER 4.11

Die Bedeutung der Kapazitätszunahme des Pentosephosphatzyklus in malignen Tumoren für den Energiestoffwechsel

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Mit 6 Abbildungen im Text

Einleitung

Die Ergebnisse mehrerer biochemischer Homogenat-Untersuchungen haben gezeigt, daß die Aktivität der zwei Pentosephosphatzyklus-Enzyme Glucose-6-phosphat-Dehydrogenase und 6-Phosphogluconat-Dehydrogenase in pathologisch veränderten Skelettmuskeln von Mensch und Tier oft stark vermehrt ist (Laudahn, 1963; Laudahn und Heyck, 1963; Heyck et al., 1963; Manchester et al., 1970; Dhalla et al., 1972; Rifenberick et al., 1974; Wagner et al., 1978). Histochemische Befunde haben eindeutig ergeben, daß der Aktivitätsanstieg dieser zwei Enzyme im Muskelgewebe nicht nur auf einer Vermehrung von Bindegewebe beruht, das eine relativ hohe Aktivität der diesbezüglichen Enzyme enthält, sondern auch auf einer Aktivitätsvermehrung in den Muskelfasern (Meijer und Elias 1976; Meijer und Elias, 1977; Meijer et al., 1977; Elias und Meijer, 1981; Abb. 1).

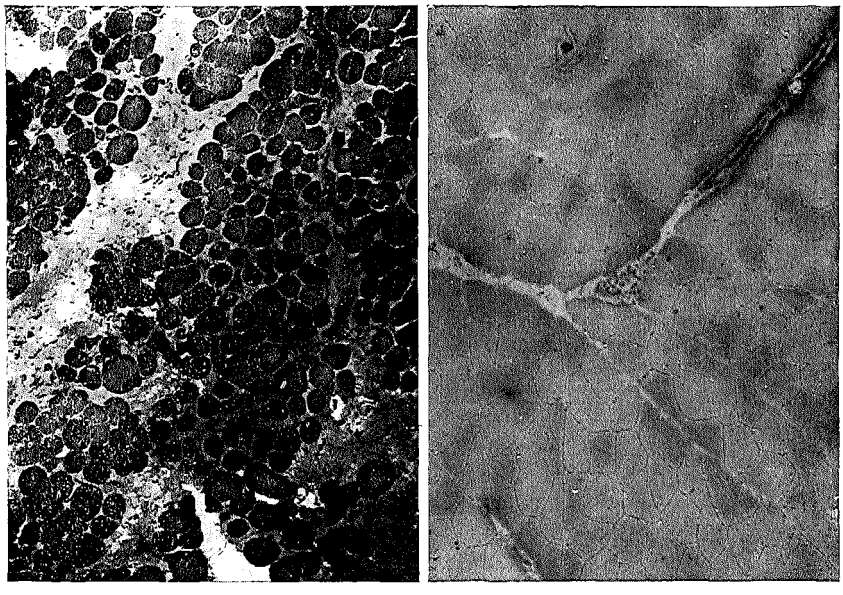

Abb. 1. Rechts: Im normalen M. quadriceps femoris des Menschen zeigen die anaeroben Muskelfasern eine schwache Aktivität der Glucose-6-phosphat-Dehydrogenase. Links: abgerundete Muskelfasern im M. quadriceps femoris des Menschen mit wechselnder Aktivität der Glucose-6-phosphat-Dehydrogenase. Mehrere Fasern zeigen eine sehr stark vermehrte Enzymaktivität; nur mäßige Aktivität im Bindegewebe. Dystrophia musculorum progressiva (Duchenne). 90×.

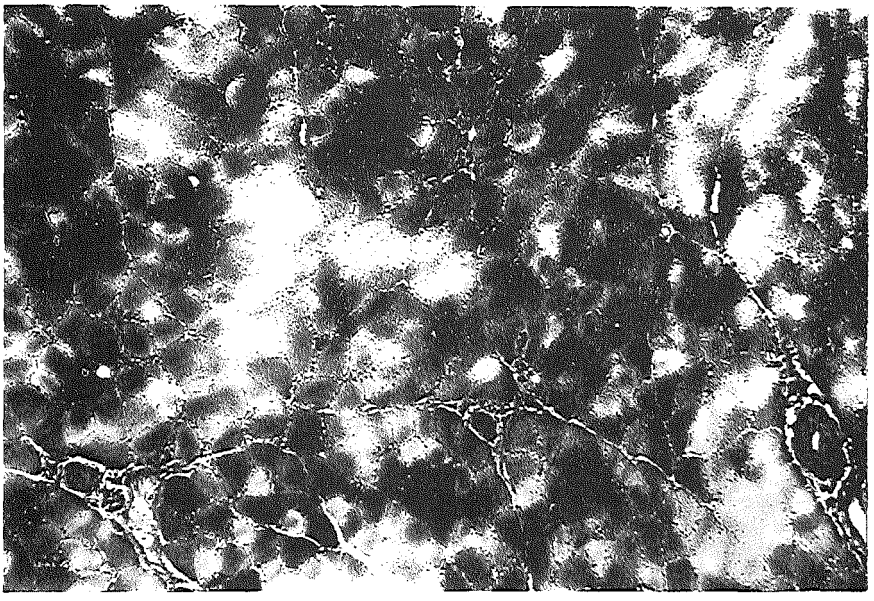

Abb. 2. Wechselnde, oft stark vermehrte Aktivität der Glucose-6-phosphat-Dehydrogenase im Rectus femoris einer Ratte, der N,N^1-Dimethyl-p-Phenylendiamin injiziert wurde. Auch die entzündlichen Infiltrate zeigen eine starke Enzymaktivität. Mittels Hemmstoffen der Proteinsynthese oder der Matrizen-DNS-Synthese konnte die durch Dimethylphenylendiamin hervorgerufene Aktivitätsvermehrung völlig verhindert werden. 90×.

Mittels Modelltierversuchen, in denen Cycloheximid als Hemmstoff der Proteinsynthese und Actinomycin D als Hemmstoff der Matrizen-RNS-Biosynthese angewendet wurden, haben wir feststellen können, daß die in den Muskelfasern verschiedener Typen von Muskelkrankheiten gefundene erhebliche Aktivitätsvermehrung durch eine Neusynthese der zwei Enzyme in den Muskelfasern und nicht durch eine Aktivierung bereits anwesender Enzymmoleküle zustande kommt (Abb. 2). Überdies war eine postduplizierende Regulation der Proteinsynthese oder eine Diffusion der diesbezüglichen Enzyme aus dem Bindegewebe in die Muskelfasern nicht vorhanden. Außerdem stellte sich heraus, daß diese Aktivitätszunahme mit einer Aktivitätszunahme der nichtoxidativen Enzyme des Pentosephosphatzyklus verknüpft war (Meijer und Elias, 1982).

Vor kurzem haben wir feststellen können, daß auch der Stoffwechsel in malignen Tumoren durch eine bedeutsame Aktivität der zwei oxidativen Pentosephosphatzyklus-Enzyme und der nichtoxidativen Enzyme dieses Zyklus gekennzeichnet wird. Die Befunde jener Untersuchungen werden in dieser Veröffentlichung erörtert, wobei insbesondere der Bedeutung der Kapazitätszunahme des Pentosephosphatzyklus für den Energiestoffwechsel dieser Tumoren Aufmerksamkeit gewidmet wird.

Material und Methoden

Histochemie und Histologie: Frisches Gewebe von menschlichen Mamma- und Kolonkarzinomen wurde mit stickstoffgekühltem Isopentan eingefroren. Zum Vergleich diente normales eingefrorenes Gewebe. In unfixierten Schnitten wurde die Aktivität der Glucose-6-phosphat-Dehydrogenase

(EC 1.1.1.49) und der 6-Phosphogluconat-Dehydrogenase (EC 1.1.1.44) nach Meijer und De Vries (1974) dargestellt. Die Hämatoxylin-Eosin-Färbung diente zur morphologischen Kennzeichnung der Tumoren.

Biochemie: In Homogenaten von eingefrorenen Gewebeblöckchen, hergestellt nach Meijer und Elias (1976), wurde die Aktivität der Glucose-6-phosphat-Dehydrogenase nach Löhr und Waller (1965), der 6-Phosphogluconat-Dehydrogenase nach Hohorst (1965) und die Aktivität der Transaldolase (EC 2.2.1.2), der Transketolase (EC 2.2.1.1), der Ribose-5-phosphat-Isomerase (EC 5.3.1.6) und der D-Ribulose-5-phosphat-3-Epimerase (EC 5.1.3.1) nach Wagner et al. (1978) bestimmt. Die Konzentration von D-Sedoheptulose-1,7-Diphosphat in den Geweben wurde nach Horecker (1965) bestimmt. Als Hilfsenzym diente Aldolase (EC 4.1.2.13). Es spaltet den nachzuweisenden Metabolit nach folgender Reaktion:

$$\text{D-Sedoheptulose-1,7-Diphosphat}^{-4} \rightarrow \text{Dihydroxyacetonphosphat}^{-2} + \text{D-Erythrose-4-Phosphat}^{-2}$$

Mittels hinzugefügter NADH und Glycerol-3-phosphat-Dehydrogenase (EC 1.1.1.8) erfolgte die Reaktion:

$$\text{Dihydroxyacetonphosphat}^{-2} + \text{NADH} + \text{H}^{+} \rightarrow \text{1-Glycerolphosphat}^{-2} + \text{NAD}^{+}$$

Die Gewebeblöckchen enthalten ebenso Fructose-1,6-Diphosphat. Diese Substanz reagiert genauso mit Aldolase, wobei sich Dihydroxyacetonphosphat bildet. Deswegen wurde die Konzentration dieser Substanz gleichfalls ermittelt. Als Hilfsenzym diente Glycerinaldehyd-3-phosphat-Dehydrogenase (EC 1.2.1.12):

a) $\text{D-Fructose-1,6-Diphosphat}^{-4} \rightarrow \text{Dihydroxyacetonphosphat}^{-2} + \text{D-Glycerinaldehyd-3-Phosphat}^{-2}$

b) $\text{D-Glycerinaldehyd-3-Phosphat}^{-2} + \text{NAD}^{+} \rightarrow \text{3-Phosphoglycerat}^{-2} + \text{NADH} + \text{H}^{+}$

Spektrophotometrisch wurde die Quantität des NADH oder des NAD^+ ermittelt. Der Nachweis von Octulose-1,7-Diphosphat erfolgte auf eine ähnliche Weise. Es bildet sich bei diesem Nachweisverfahren mit Aldolase D-Arabinose-5-Phosphat statt D-Erythrose-5-Phosphat. Die Zusammensetzung des Gemisches wurde mittels Fructose-1,6-Diphosphatase nach Paoletti et al. (1979) untersucht.

Ergebnisse und Diskussion

Die histochemischen Befunde zeigen eindeutig eine wesentliche Aktivitätsvermehrung der Glucose-6-phosphat-Dehydrogenase und der 6-Phosphogluconat-Dehydrogenase in den malignen Tumorzellen im Vergleich zu den normalen Zellen (Abb. 3 und 4). Diese Ergebnisse stehen in Übereinstimmung mit den mehr quantitativen Werten, die mit den biochemischen Techniken erhalten wurden. In den Tumoren kam es zu einem 5—10fachen Anstieg der Glucose-6-phosphat-Dehydrogenase und zu einem 3—6fachen Anstieg der Aktivität der 6-Phosphogluconat-Dehydrogenase. Ebenfalls war die Aktivität der nichtoxidativen Enzyme des Pentosephosphatzyklus gestiegen. Die Aktivitätszunahme war dennoch weniger und betrug höchstens einen 3fachen Anstieg im Vergleich mit der Aktivität in den normalen Gewebeblöckchen. Diese histochemischen und biochemischen Befunde deuten auf eine eindeutige Kapazitätszunahme des Pentosephosphatzyklus hin, vor allem weil das Schlüsselsystem des Zyklus, die Glucose-6-phosphat-Dehydrogenase, eine ausgeprägte Vermehrung der Aktivität zeigte.

Der Pentosephosphatzyklus hat im Stoffwechsel als Lieferant von NADPH eine besondere Bedeutung. Durch Oxidation der Substrate wird aus $NADP^+$ das Koferment NADPH gebildet (Abb. 5 und 6). Die reduzierte Verbindung NADPH wird für viele synthetische Prozesse benötigt, u. a. für den Aufbau von Fettsäuren, von Steroiden und für Hydroxylierungen. Besonders schnell wachsende Tumoren erfordern relativ viel NADPH. Weiterhin führt die Oxidation zu Pentosephosphaten. Aus Abb. 5 und 6 ist gleichfalls ersichtlich, daß zur Pentosesynthese das Gewebe nicht ausschließlich vom Stoffwechselweg über die zwei oxidativen Enzyme ab-

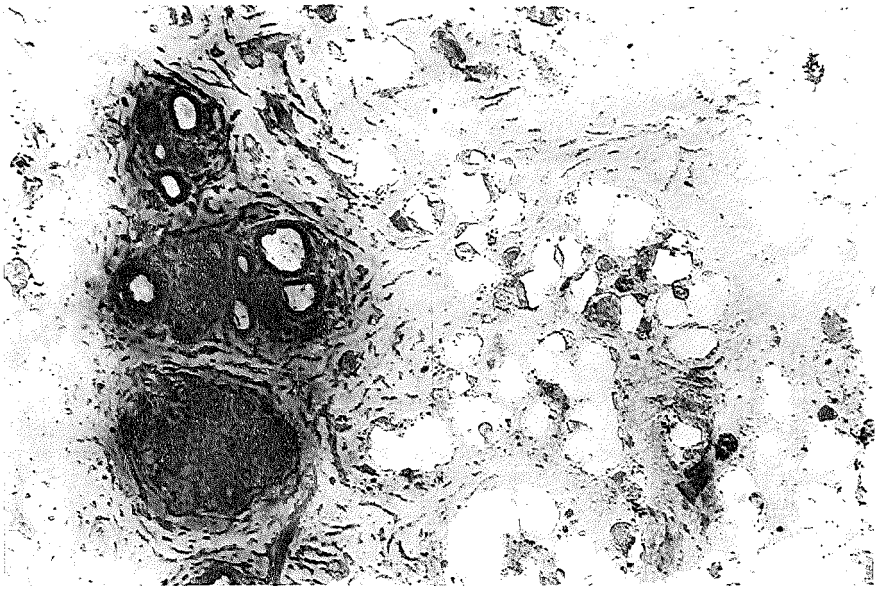

Abb. 3. Mäßige Aktivität der 6-Phosphogluconat-Dehydrogenase in der Mamma bei Mastopathie. 55×.

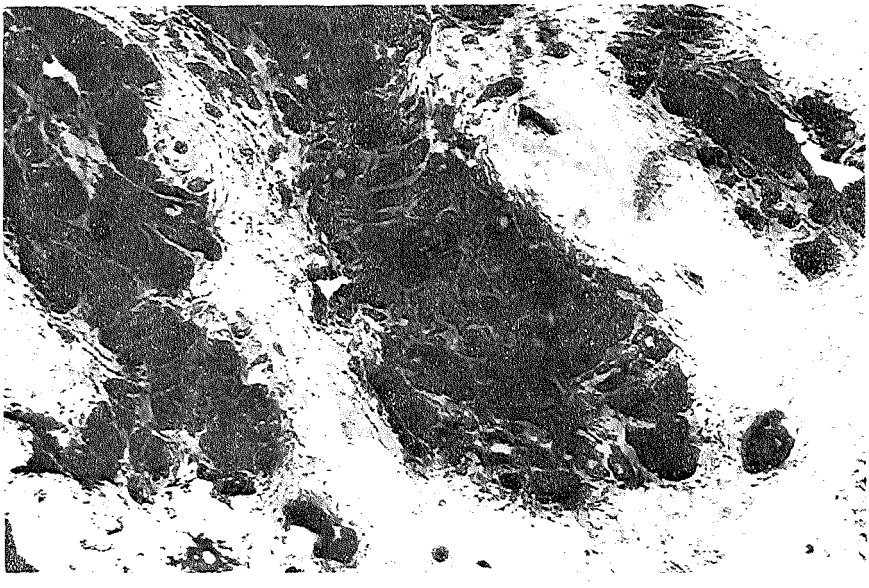

Abb. 4. Erhebliche Aktivität der 6-Phosphogluconat-Dehydrogenase in den soliden Epithelwucherungen im Adenokarzinom einer Mamma. Schwache Aktivität in den Stromazellen. Wegen der beträchtlichen Enzymaktivität war die Inkubationszeit etwas verkürzt. 55×.

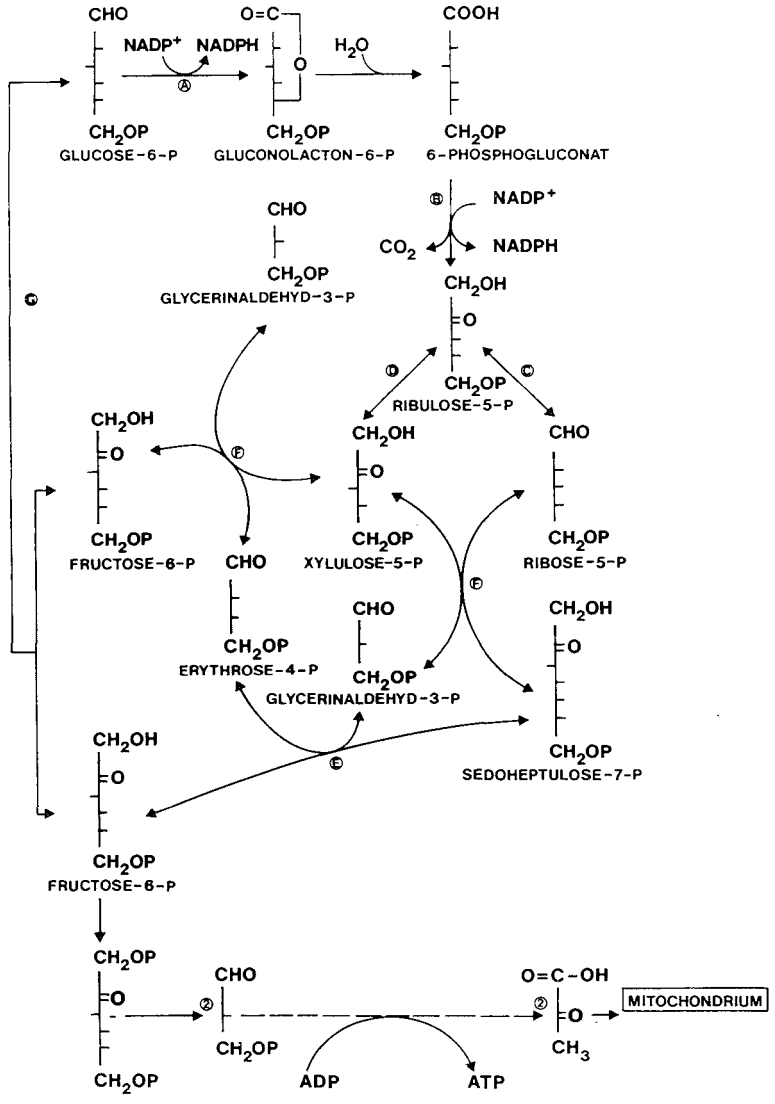

Abb. 5. Schema des Pentosephosphatzyklus im Fettgewebe nach Horecker. A. Glucose-6-phosphat-Dehydrogenase; B. 6-Phosphogluconat-Dehydrogenase; C. Pentose-5-phosphat-Isomerase; D. Pentose-5-phosphat-3′-Epimerase; E. Transaldolase; F. Transketolase; G. Phosphohexose-Isomerase. Ab Fructose-6-Phosphat ist der Embden-Meyerhof-Stoffwechsel vereinfacht dargestellt und sind die Namen der glykolytischen Metabolite nicht aufgeführt worden.

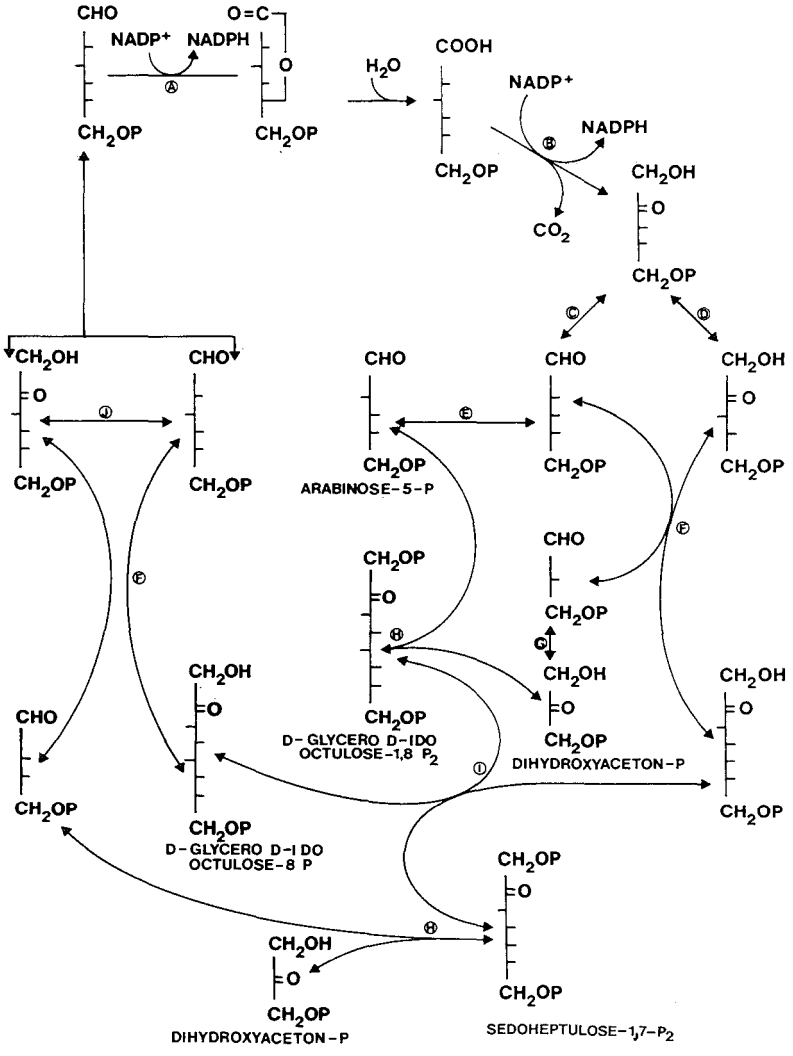

Abb. 6. Schema des Pentosephosphatzyklus im Lebergewebe nach Williams. A. Glucose-6-phosphat-Dehydrogenase; B. 6-Phosphogluconat-Dehydrogenase; C. Pentose-5-phosphat-Isomerase; D. Pentose-5-phosphat-3′-Epimerase; E. Pentose-5-phosphat-2′-Epimerase; F. Transketolase; G. Triosephosphat-Isomerase; H. Aldolase; I. Phosphotransferase; J. Phosphohexose-Isomerase.

hängig ist. Mittels einer Kombination von Transaldolase- und Transketolase-Reaktionen kann Fructose-6-Phosphat ebenso in Pentosephosphate umgewandelt werden (Kit et al., 1957). Abb. 5 gibt eine anschauliche Darstellung des Zyklus im Fettgewebe. Das Schema ist größtenteils aus Resultaten von Untersuchungen von Horekker entwickelt worden (Horecker et al., 1954). Vor kurzem haben biochemische Untersuchungen von Williams (1980) gezeigt, daß im Lebergewebe ein anderer Typus des Pentosephosphatzyklus vorliegt. Die Reaktionsvorgänge dieses Typus sind in Abb. 6

veranschaulicht. Zum Unterschied von dem im Fettgewebe vorkommenden Zyklus enthält der Lebertypus Octulosephosphat, Arabinose-5-Phosphat, Pentose-5-phosphat-2-Epimerase und D-Glycero-D-ido-Octulose-1,8-Biphosphat:D-altro-Heptulose-7-Phosphotransferase. Neuerdings ist dieser Typus auch im pathologisch veränderten Herzgewebe entdeckt worden (Kapuscinski und Williams, 1981).

Resultate von Perfusionsversuchen, in denen ^{14}C-Glucose-Präparate angewendet wurden, haben ergeben, daß im Lebergewebe (Longenecker und Williams, 1980) ungefähr 25% und im Herzgewebe (Kapuscinski und Williams, 1980) ungefähr 80% der verabreichten Glucosemoleküle über den Lebertypus des Pentosephosphatzyklus zum CO_2 abgebaut werden. Diese interessanten Befunde zeigen, daß der Zyklus im Energiestoffwechsel von großer Bedeutung sein könnte. Der Zyklus ermöglicht nämlich eine zytoplasmatische ATP-Production über Glycerinsäure-1,3-Diphosphat und Enolbrenztraubensäure-Phosphat.

Die Verfasser haben ausgerechnet, daß diese ATP-Bildung in Geweben mit einer hohen Zykluskapazität sehr bedeutsam ist. Überdies hat sich herausgestellt, daß der Fett-Typus des Pentosephosphatzyklus eine ebenso wichtige Rolle bei der zytoplasmatischen ATP-Synthese spielen kann. Von großer Bedeutung ist, daß die ATP-Synthese für beide Typen des Pentosephosphatzyklus unbeschränkt erfolgt, weil die Phosphofructokinase, das Schlüsselenzym der Glykolyse, deren Intensität es hemmen kann, umgangen wird. Infolgedessen weist die hemmungsfreie Reaktion auf eine ungehemmte Möglichkeit zur zytoplasmatischen ATP-Synthese hin. In den schnell wachsenden Tumoren erfordert der Stoffwechsel viel ATP, deshalb kommt die hohe zytoplasmatische ATP-Synthese-Kapazität den Tumoren zunutze. Vollständigkeitshalber soll erwähnt werden, daß einige andere Aussagen von Williams und Mitarbeitern über den neuen Typus des Pentosephosphatzyklus, die in dieser Veröffentlichung nicht erörtert werden, einigermaßen umstritten sind (Katz et al., 1981).

Auf jeden Fall konnten wir nachweisen, daß die Tumoren geringe Quantitäten Octulose-1,7-Diphosphat und D-Sedoheptulose-1,6-Diphosphat enthalten (zwischen 0 und 21 nmol $\cdot$ g^{-1} Gewebe). Diese Befunde geben einen Hinweis auf die Anwesenheit eines Lebertypus des Pentosephosphatzyklus in den untersuchten Tumoren. In den Blöckchen der entsprechenden normalen Gewebe konnten wir diese Metabolite nicht nachweisen. Die Zeitspanne zwischen Entnahme und Einfrieren des Patientengewebes war verhältnismäßig lang. Für quantitative Aussagen zum Metabolismus war die Qualität der untersuchten Gewebeblöckchen sehr wahrscheinlich nicht geeignet.

Zusammenfassend kann darauf hingewiesen werden, daß die Ergebnisse dieser Untersuchung eindeutig eine lineare Beziehung zwischen der Aktivität der Glucose-6-phosphat-Dehydrogenase, der 6-Phosphogluconat-Dehydrogenase und der Kapazität des Pentosephosphatzyklus erwiesen haben. Daher kann die Schlußfolgerung gezogen werden, daß die Anwendung der gewebeschonenden histochemischen Techniken zur Darstellung der Aktivität der Glucose-6-phosphat-Dehydrogenase und der 6-Phosphogluconat-Dehydrogenase nicht nur in Skelettmuskeln, sondern auch in Tumoren zu zuverlässigen Einsichten in die zytoplasmatische ATP-Synthese-Kapazität führt.

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CHAPTER 4.12

METABOLIC STUDIES AS A DIAGNOSTIC MEASURE FOR CANCER—I. ADENOCARCINOMAS OF DIFFERENT ORGANS, ESPECIALLY THE HUMAN MAMMA

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Abstract—In order to evaluate the usefulness of modern enzyme histochemical techniques as a diagnostic measure for malignancy, at least 22 enzymes active in major metabolic pathways were studied in a large series of histological and cytological specimens, representing proliferative lesions, mainly malignant originating in many organs. The study and results were confirmed by a parallel biochemical study performed on tissue homogenates of the same material. In this communication, a special emphasis is paid to the glandular malignancies. The study demonstrated that adenocarcinomas share some general characteristics and that some metabolic activities characteristic of a target organ, or cell, remain identifiable in lesions arising in that organ, even after malignant transformation and in metastatic tumours at other sites. This will prove helpful in the identification and classification of the malignant cells and the identification of their origin. The patchy distribution of many enzymes within the same tumour tissue raises some doubt about the monoclonality of the cancer cells. Some general metabolic changes were almost always found to associate with the malignant transformation. A higher capacity for NADPH regeneration with an increase in the activity of some anaerobic enzymes was observed. The anaerobic oxidoreductases, phospho-glucomutase and glucose-phosphate isomerase and glyceraldehyde phosphate dehydrogenase were very active in cancer cells. In the meantime the increase in activity of many aerobic enzymes of the oxidative pathways, lactate dehydrogenase and the rate limiting enzyme of glycolysis, phosphofructokinase was less pronounced. The findings, especially the last observation, could be seen as an adaptation mechanism of energy supply and glycolysis to an increased demand for energy at a time when the normal capacity of oxygen consuming pathways becomes inadequate to satisfy the needs of the proliferating malignant cells. This will be compensated for by an increase in some subsidiary anaerobic mechanisms and adaptation of glycolysis. Interrelations between fat metabolism, glycolysis, protein metabolism and synthesis of components such as ATP essential for the proliferating cells, have yet to be reviewed (Meijer and Elias, *Acta histochem. Suppl.* **29**, 141–148 (1984) and in: *IV Jenaer Myologie—Colloquium., Mathematisch Natur Wissenschaftiche Reihe der Friedrich—Schiller—Universitat* (in print, 1985).

Key words: Cancer enzymology, cancer metabolism, adenocarcinomas, cytochemistry, glycolysis, pentose phosphate shunt, diagnosis of cancer, enzyme, NADP metabolism

ETUDES METABOLIQUES COMME MESURE DIAGNOSTIQUE DU CANCER—I. ADENOCARCINOMES DES DIFFERENTS ORGANES EN PARTICULIER LE SEIN HUMAIN

Résumé—Afin d'évaluer l'utilité des techniques modernes de l'histochimie enzymatique comme mesure diagnostique de la malignité, plus de 22 enzymes actives de diverses voies métaboliques ont été étudiées dans une grande série (environ 3000) de spécimens histologiques et cytologiques montrant des lésions prolifératives, principalement malignes d'origine humaine dans e.a.: le sein, le colon, rectum, poumon, estomac, endomètre, ovaire, prostate et reins. L'étude et les résultats ont été confirmés par une étude biochimique parallèle exécutée sur des homogénats du même matériel. Cette communication met l'accent sur les malignités glandulaires. Les résultats de cette étude démontrent que les adénocarcino mes présentent des caractères généraux d'activités métaboliques spécifiques pour l'organe ou la cellule d'origine. Ces caractères restent identifiables dans les lésions apparaissant dans cet organe, même apres la transformation maligne et même dans les métastases. Ceci s'avère d'une aide fructueuse dans l'identification et la classification des cellules malignes et l'identification de leur site d'origine. La distribution inégale de l'activité enzymatique dans la même tumeur distribution ou plaques, soulève certains doutes sur la monoclonalité des cellules cancéreuses. La transformation maligne a toujours été trouvée associée à d'austres modifications métaboliques spécifiques telles une augmentation de la régénération du NADPH ou un accroissement de l'activité des enzymes anaérobiques, alors que l'activité de la

phosphofructokinase et de plusieurs enzymes des voies aérobiques ou oxydatives augmente peu. Ces changements peuvent signifier un mécanisme d'adaptation pour satisfaire l'augmentation de la demande d'énergie par les cellules malignes prolifératives quand la capacité des voies aérobiques ordinaires est insuffisante. Cette insuffisance sera compensée par l'augmentation d'activité de mécanismes subsidiaires anaérobiques et par l'adaptation de la glycolyse. L'inter-relation entre le métabolisme des lipides, la glycolyse, le métabolisme des protéines et la synthèse des composants essentiels tels que l'ATP doit attirer davantage l'attention (Meijer et Elias, *Acta histochem. Suppl.* **29,** 141–148 (1984) and in: *IV Jenaer Myologie—Colloquium., Mathematisch Natur Wissenschaftische Reihe der Friedrich—Schiller—Universitat* (in print, 1985).

Mots-clefs: Enzymologie du cancer, métabolisme du cancer, adénocarcinomes, cytochimie, histochimie, glycolyse, NADP, metabolisme, enzyme, diagnostic du cancer, shunt du pentose phosphate

INTRODUCTION

Metabolic studies, including the study of energy metabolism in cancer cells, have been subjected to many 'multidisciplinary' approaches. Our knowledge is still far from complete. The use of unreliable enzyme histochemical techniques, especially for the demonstration of non-structurally bound or partially-structurally bound enzymes including many oxidoreductases, has limited the value and usefulness of enzyme histochemistry as a diagnostic measure and in the field of metabolic studies (Dallenbach-Hellweg, 1981; Meijer and Elias, 1984; Elias, 1984). The introduction of modern and more reliable enzyme histochemical techniques such as the semipermeable membrane (Meijer, 1980) and the polyacrylic membrane techniques (Elias *et al.*, 1984a, 1984b) permitted further steps towards a better metabolic orientation in the living cells, especially for workers concerned with malignant transformation (Elias *et al.*, 1980a, b. 1981, 1983).

The present and subsequent communication give the results of a project set up to study some enzyme groups active in energy metabolism and other major metabolic pathways thought to be of a diagnostic value or necessary for the growth and maintenance of the proliferating cells, mainly malignant. Special attention has been paid to the metabolic role and significance of the NADPH regenerating enzymes of the pentose phosphate pathway and other related enzymes. The total scope of the histochemical experience involves a continuously increasing number of cytological and tissue specimens which at present exceeds 3000 cases. These represent either primary lesions in the organs described or metastases. The results with adenocarcinomas arising in a variety of organs especially the breast will be emphasized in this communication. The metabolic activity characteristic of squamous cell carcinomas, especially of the lung and oesophagus and other types of malignancies will be presented separately.

MATERIALS AND METHODS

Specimens

Tissue specimens usually obtained during operative procedures and as diagnostic punch or needle biopsies, and also cytological material, including brush preparations, tissue prints, fine needle aspiration punctures and malignant body effusions, were collected in a fresh state. To avoid autolytic changes or inaccuracies due to any possible metabolic change after collection, only specimens subjected to immediate freezing after collection were considered. The series of benign and malignant lesions from the human breast consisted of representative specimens from: 350 carcinomas, 50 metastatic tumours which proved to be from primary mammary cancers, 450 cases of variants of fibrocystic disease and 50 fibroadenomas. The mammary series further included 250 cytological specimens consisting of tissue prints and fine needle punctures in an almost equal ratio of malignant and benign lesions. A further 20 fine needle aspiration biopsies of metastatic tumours and 200 specimens of malignant body effusions, mainly ascites and pleural fluids as well as five cerebrospinal fluid specimens which proved to contain malignant cells complicating mammary lesions, were also available. About 50 cases of gynaecomasty of the male breast were added for comparison. Representative cytological and histological material from adenocarcinomas in other organs were collected in the same way, either at the primary site or from metastases. The histological material of non-mammary cancers comprised 50 (primary) adenocarcinomas of the lung, 75 gastric cancers, 75 endometrial and ovarian carcinomas, 150 colon and rectum carcinomas, 50 (benign-) villous adenopapillomas of the large intestine, 50 prostate carcinomas and 25 renal carcinomas.

As far as possible, the malignant samples were examinec together with samples of benign tissue from the organs involved serving as control material. Occasional cases of adenocarcinomas of other organs were also examined including, for example, five pancreatic cancers. The cytological material involved more than 500 specimens obtained as mentioned above and in a more or less similar distribution. About 200 of these were malignant body effusions.

One or more representative blocks of each specimer were cut free of fat, frozen in a fresh unfixed state ir isopentane precooled to about −150°C in liquid nitrogen and stored at −95°C. Tissue sections were cut at 6 μm in a cryostat adjusted to −26°C. The details of techniques

Table 1. Enzyme histochemical and cytochemical reactions used

Enzyme	Designation	EC number	References
3-D-isocitrate: $NADP^+$ oxidoreductase (decarboxylating)	ICDH:$NADP^+$	1.1.1.42	Meijer and de Vries (1975)
L-malate:NAD^+ oxidoreductase	MDH:NAD^+	1.1.1.37	Barka and Anderson (1963)
L-isocitrate:NAD^+ oxidoreductase	ICDH:NAD^+	1.1.1.41	Barka and Anderson (1963)
D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase	GPDH	1.1.1.49	Meijer and de Vries (1974)
6-phosphogluconate:$NADP^+$ 2-oxidoreductase (decarboxylating)	PGDH	1.1.1.44	Meijer and de Vries (1974)
Malate:$NADP^+$ oxidoreductase	MDH:$NADP^+$	1.1.1.40	Meijer and de Vries (1975)
Glycerol-3-phosphate:menadione oxidoreductase	GPOX	1.1.99.5	Lojda *et al.* (1979) Elias *et al.* (1984b)*
Orthophosphoric monoester phosphohydrolase, alkaline optimum	ALP	3.1.3.1	Pearse (1972)† Elias *et al.* (1984a)*
Orthophosphoric monoester phosphohydrolase, acid optimum	ACP	3.1.3.2	Meijer (1972) Elias *et al.* (1984b)*
Mg^{2+} activated, mitochondrial ATP phosphohydrolase	ATP-Mg	3.6.1.3	Meijer and Vloedman (1980)
Calcium activated, myosin ATP-phosphohydrolase	ATP-Ca	3.6.1.3	Meijer (1970)
L-lactate:NAD^+ oxidoreductase	LDH	1.1.1.27	Meijer (1973)
NADH:nitro BT oxidoreductase	NADH	1.6.99.3	Pearse (1972) Elias *et al.* (1984b)*
NADPH:tetrazolium oxidoreductase	NADPH	1.6.99.1	Meijer (1980) Elias *et al.* (1984b)*
Aryl-ester hydrolase and Carboxylic-ester hydrolase (known as non-specific esterases)	NE	3.1.1.2 & 3.1.1.1	Meijer and Vloedman (1973) Elias *et al.* (1984b)*
Aryl-sulphate sulphohydrolase (arylsulphatase)	AS	3.1.6.1	Koudstaal (1975)
β-D-glucuronide glucuronosohydrolase	Gluc.	3.2.2.31	Meijer and Vloedman (1973) Elias *et al.* (1984b)*
Alkaline phosphomonoesterase, sp. to hydrolyse: Nucleotide-5-phosphate group (5-nucleotidase)	AMP	3.1.3.5	Pearse (1972)
Phosphofructokinase	PFK	2.7.1.11	Meijer and Stegehuis (1980)
α.D.glucose-1, 6-biphosphate:x.D.glucose-1-phosphate phosphotransferase (phospho-glucomutase).	PGM	2.7.5.1	de Vries and Meijer (1976)
D-Glucose-6-phosphate ketol-isomerase	PGI	5.3.1.9	de Vries and Meijer (1976)
Glyceraldehyde-3-phosphate dehydrogenase	GAD	1.2.1.12	de Vries *et al.* (1980)

* Exclusively for cytological material.

† By using Naphthyl-AS-MX phosphate and fast red violet LB. In order to differentiate between thermo- and fixation-labile and stable iso-enzymes, the reactions were repeated three times as prescribed, after prolonged fixation for 40 min on formol-macrodox and after preheating to 65°C for 5 min before incubation.

applied, freezing methods, histochemical reactions and histological stains are mentioned in Table 1 and/or in previous communications.

Tissue specimens intended for biochemical assays on homogenates were finely cut in a Petri dish and frozen in the same way in liquid nitrogen. For routine histological examination 6 μm sections were cut and stained with Harris' haematoxylin and eosin PAS and oil red-O. The method of von Kossa (1901) and alizarin red-S stain were used for the demonstration of calcium deposits. The fine needle aspiration biopsies and tissue prints were spread directly on the slides and membranes, or collected in RPMI-1640 or in Medium H-199, (both supplied by Flow Labs., U.K.) prior to the incorporation in polyacrylic membranes. The effusions were first centrifuged at 2000 *g* for 30 sec. The cells deposited were spread directly onto the membranes and the glass slides or mixed with an acrylic polymerate. Routine cytological examination was done on slides stained with modified Papanicolaou and Giemsa stains as performed in our laboratory.

Histochemistry

Cytological preparations and tissue sections cut at 6 μm on a cryostat at −26°C were used either fixed or unfixed according to prescriptions of the techniques employed. The sections were then incubated for the demonstration of the activity of the enzymes given in Table 1. The scheme of demonstration of enzyme activities was applied as completely as possible to all specimens. Some variations or omissions were inevitable but these were limited. They did not therefore influence the overall results. The histochemical techniques (Table 1) were performed as described in the original references, after modifications as mentioned in the text or as performed in our laboratory. The specificity of enzyme reactions was tested with control reactions as recommended in the literature covering the techniques

applied. The demonstration of oxidoreductases was rendered independent of the activity or the presence of auxiliary enzymes in the system. This was achieved by the use of specific enzyme inhibitors such as cyanides, Natrium azides or amytal to block the electron transfer to the cytochromes. Artificial electron carriers such as menadion and/or Phenazine methosulphate were then used to transport the electrons from the oxidized substrates to Nitro-BT via NAD^+. Such, and other, adaptations of the methods proved to be necessary for the study of different metabolic activities in the cells concerned (Meijer, 1978).

Biochemistry

For the biochemical study 82 tissue specimens, representing all types of mammary lesions involved and 62 specimens of adenocarcinomas of other organs, were weighed and finely cut in a frozen state. About 0.5–1.0 g of each sample was subjected to homogenization according to Korsten and Persijn (1972) using a microdisembrator (Braun, Melsungen, W.G.). The homogenated material was then mixed with four volumes of 10 mmol l^{-1} Tris buffer, pH 7.4 according to Mulder and Verhaar (1979) and centrifuged at 100.000 *g* for 60 min at 2°C. The protein content was then determined in the supernatant according to Lowry *et al.* (1951). The final dilution of the supernatant was then adjusted to a protein content of 2 g l^{-1}. The activity of GPDH was determined according to Kornberg and Horecker (1955), ICDH:$NADP^+$ according to Kornberg (1955) and that of LDH using a standard kit delivered by Merck-diagnostica since 1972 for automated systems. The determination of the different isoenzymes of LDH was performed according to Brendel *et al.* (1973). The activity of the total ALP was determined according to Morgenster *et al.* (1965). The determination of the different isoenzymes of ALP was performed by electrophoresis on agarose-gel according to Sunblad *et al.* (1973). The technique is a modification of the original method described by Wieme (1965).

RESULTS

1. *Histochemical and cytochemical results*

All specimens were evaluated individually. Similar cases were then grouped and the results were correlated together. Where any enzyme showed a patchy distribution within the same specimen (a common finding especially when dealing with dehydrogenases), the extent of variation of the activity was taken into account. A mean value of the findings was given when the variation in activity was minor or insignificant. The results concerning the mammary lesions are given in Table 2. The constancy of characters of the mammary cells described here, either benign or malignant, in cytological or tissue specimens, at the primary site or in metastatic lesions and irrespective of the technique of collection used, was a very important result, which eventually made the combination of the findings in a table form possible and representative. An increase in activity of some enzymes normally active in benign or ordinary epithelium was clearly seen in the more active benign cells such as the so called apocrine cells and in malignant epithelium. This increase, however, did not prove to be of a great diagnostic value in the differentiation between malignant and benign conditions but is of biological significance. The most striking finding was the constant and marked activity of alkaline phosphatase in the myoepithelium, normally present as an outer cell layer in benign glands and ducts and in all non infiltrating lesions (some schools of thought consider solid or intraductal epitheliosis as a carcinoma *in situ* or non-infiltrating carcinoma). At least 96% of mammary carcinomas, being an expression of infiltrating and proliferating epithelium devoid of any myoepithelial component, were

Table 2. The histochemically demonstrable activity of some enzymes examined in benign and malignant mammary lesions

	Benign			Malignant
	EP.	MP.	AP.	EP.
ACP	3+	2+	4+	3:4+
ALP	–	3+	–	–
Gluc	3+	2+	4+	3:>4+
LDH	3+	2+	4+	>4+
GPOX	4+	3+	4+	>4+
NADH	3+	3+	3+	4+
NADPH	3+	3+	3+	3+
PFK	2+	2+	2+	2+
PGM	3+	3+	3+	>4+
PGI	3+	3+	4+	>4+
GPDH	3+	3+	4+	⩾4+
PGDH	3+	2+	4+	⩾4+
NE	–:+	–	+:2+	–(80%): 2+(20%)
AS	–	–	–	–
MDH:NAD^+	2+	2+	2+	2+
MDH:$NADP^+$	2+	2+	2+	3+
ICDH:NAD^+	2+	2+	2+	2+
ICDH:$NADP^+$	3+	2+	4+	>4+
ATP.Ca	–:+	–:+	–:+	–:2+
ATP.Mg	–:+	–:+	–:+	–:+
AMP	–:+	–:+	–:+	–:+
GAD	3+	2+	3+	3+

The benign lesions include sclerosing adenosis, adenosins, ductal papillomas and other forms of cystic disease of the mamma as well as fibroadenoma and gynaecomasty. The malignant lesions include all variants of adenocarcinoma at primary sites or in metastases. The epithelium of the non-infiltrating intraductal carcinoma was similar to that of other carcinomas except that the ducts often show an outer cell layer of myoepithelium. The relative intensity of staining was considered to correspond to the degree of activity: – absent, + weak or slight, 2+ moderate, 3+ strong and 4+ tremendous or very strong. EP designates epithelial cells, MP Myoepithelium and AP Apocrine (metaplastic) cells.

found therefore devoid of any activity of ALP (see Fig. 1.). This finding could be used to differentiate between infiltrating carcinomas and benign lesions which histologically may mimic certain types of mammary carcinomas, especially benign sclerosing adenosis which seems to be a product of proliferation mainly of myoepithelium rich in ALP. The myoepithelial ALP was always found to be thermostable and stable to fixation. Only 10% of the mammary carcinomas, irrespective of the histological subtyping, showed a stromal activity of ALP, a phenomenon which we have been unable to explain to our satisfaction.

Adenocarcinomas arising in organs other than the breast shared some general characteristics with the mammary carcinomas, which can be summarized as follows. All adenocarcinomas, including mammary cancers, show a general increase in activity of enzymes such as GAD, ICDH:$NADP^+$, PGDH, GPDH, PGM, PGI and to a lesser extent GPOX, when compared to the activity of the same enzymes in benign tissue samples from the same organs. The change in the activity of enzymes such as PFK, ICDH:NAD^+, MDH:NAD^+, NADH and NADPH was insignificant or very limited. The activity of Gluc was clearly but variably increased in many tumour samples. This corresponded more with the type of differentiation of the tumour cells than with the degree of dedifferentiation or the organ of origin. Cancers producing more mucopolysaccharides or arising from cells related to steroid metabolism contained more activity of Gluc than other adenocarcinomas or benign samples from the same organs. The activity of LDH was also variably increased in the majority of the adenocarcinomas. Only a few adenocarcinomas showed a decrease in the activity of LDH. This decrease was mostly observed in colon or rectum carcinomas (about 20% of these tumours). The slightly or moderately differentiated cancer cells tended to show decrease rather than increase in the activity of LDH. No specific diagnostic value for the determination of the organ of origin could be attached to the change in activity of LDH which was found to be moderately or strongly active in the majority of adenocarcinomas.

Almost all the above mentioned enzymes especially the dehydrogenases and to a lesser extent Gluc often showed a patchy distribution within the same tumour sample and even in the same section.

The activity of NE was variable from nil in a few adenocarcinomas to a tremendous increase in the activity in malignant cells of other tumours of the same organ when compared to the benign cells. The

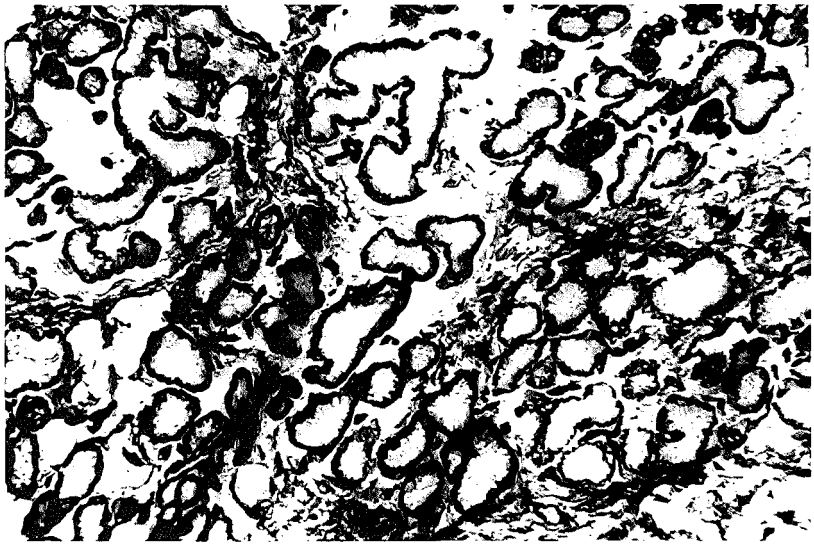

Fig. 1. Sections of benign mammary tissue examined for the activity of ALP. The outer cell layer is strongly active myo-epithelium, while the inner cell layer of epithelium is devoid of any activity of ALP. The same can be seen in fibroadenoma, cystosacroma phyllodes and in ductal papilloma.

degree of increase was more significant in tumours of organs in which this enzyme is commonly found. Such an increase was demonstrable in 95% of endometrial carcinomas, 90% of ovarian carcinomas (excluding the cystadeno carcinoma papilliferum which almost always showed a minor activity of NE as well as of ALP), 80% of renal carcinomas, about 50% of gastric, lung and prostate carcinomas and less than 30% of colon and breast carcinomas.

The activity of ACP was frequently found to be increased in tumour cells which contain a relatively high concentration of lysosomes, a general characteristic of glandular cells.

The evaluation of the activity of MDH:$NADP^+$ was rather difficult to evaluate. The activity was definitely higher in malignant cells than in benign cells provided that it was present in the benign epithelium of the same organ. We formed the impression that this rise in activity was more related to the increase in the concentration of the available $NADP^+$ molecules than to an increase in the activity of the enzyme itself.

Aryl-sulphatase was almost exclusively active and in a high level of activity in at least 96% colon–rectal cancers, and to a lesser extent but in an equal percentage of villous adenopapillomas. Less than 1% of other carcinomas in other organs demonstrated activity of this enzyme. Where this was demonstrated it was rather slight. Alkaline phosphatase was rarely encountered in malignant epithelium of adenocarcinomas except for those arising in the endometrium and ovary. Practically all carcinomas of the endometrium and the endometroid carcinomas of the ovary contained a highly active ALP. Only 4% of mammary carcinomas, less than 8% of lung carcinomas and 15% of gastric carcinomas showed a demonstrable activity of this enzyme. When this was present it was rather apical in the involved glandular cells or it was located at the cell membrane site. In contrast to the malignant epithelium, the stromal cells of more than 30% of stomach carcinomas and of about 20% of other cancers showed a high activity of this enzyme. In the case of mammary carcinoma the ALP was present in the stroma cells of only 10% of the cases. A good differentiation between ovarian and endometrial ALP was almost always possible. Endometrial ALP is composed mainly of thermolabile and fixation labile fraction(s) in contrast to the relatively stable fractions of ovarian ALP.

AMP, ATP–Mg and to a lesser extent ATP–Ca were variably but significantly active in the stromal cells of more than 50% of all cancers examined. No correlation was found between the stromal reaction and the slight positivity for these enzymes, when present, in the epithelial component. The AMP reaction was positive, but at a very low degree in the epithelial cells of 30% of colon carcinomas and less than 20% of stomach and lung adenocarcinomas. Its presence in cancer cells of other organs was much less significant. The activity of ATP–Ca in malignant epithelium was somewhat higher than that of ATP–Mg. Both were slightly more active than AMP, and both followed the same pattern of distribution as AMP in the malignant epithelium. This positivity was often associated with a histologically demonstrable positivity for calcium salts as shown by von Kossa and alizarin-S stains. It was also found where a positive oil red-O stain for lipoid deposits was present. This dubious positivity of the enzyme reactions was then tested by reliability tests to exclude formation of non-enzymatic, mainly calcium sulphide, complexes at the assumed sites of enzymatic activity. In these cases the conclusion was reached that a certain activity of ATP–Ca was present in association with a high concentration of lipoid deposits or calcium salts. This observation may suggest a possible increase in ATP-synthesis in the malignant cells, especially when the pentose phosphate pathway was strongly active.

The results from the cytological material were similar to those from the histological material. However, some restrictions must be considered. The examination of cytological material stained for many enzyme reactions described needs great experience to avoid misinterpretations. The identification of the malignant cells is often difficult. False positivity in unexperienced hands is possible. For example, patients with widespread metastases, especially to bones and with a high serum level of ALP, may show a rim of "positive" reactions for ALP on the outer surface of the cell membrane of the malignant cells in, for example, pleural effusions containing cancer cells of mammary origin. In spite of the statement of Engström (1964) that ALP is mainly active at cell membrane sites, such positivity can be due to ALP of external origin and allows this to be classified as a false positive. The identification of and differentiation between myoepithelium and epithelium in, for example, a mammary needle aspirate or between epithelial and other cells in a pleural effusion, is important for recording correct data. Such faults may often result in misleading conclusions, especially when dealing with cytological material, as will be discussed later.

The poor cohesion of cells collected from tissue

effusions, such as ascites, to the glass slides owing to a high surface tension or a high lipoid content in these fluids or cells concentrates necessitated the development of appropriate enzyme cytochemical techniques specially designed to suit the needs of cytology material. The use of fat solvents or detergents may lead to disastrous or erroneous results. The introduction of the polyacrylic membrane technique used for the demonstration of many enzymes studied in this project (Elias *et al.*, 1984a, 1984b) has proved valuable for obtaining reliable results.

2. *Biochemical results*

The differences in activity of enzymes biochemically estimated in mammary lesions, seen in Fig. 2, show a five–nine fold increase in the activity of ICDH:NADP$^+$ and GPDH in malignant tumours when compared with their activity in benign conditions. The same applies to the activity of LDH which was raised four–six fold its activity in benign conditions. A benign tumour such as fibroadenoma of the human breast, being a proliferative or active tissue, usually shows almost twice the activity of LDH and ICDH:NAPD$^+$ in a resting mammary tissue. Physiological adenosis shows an equally high activity of these enzymes. The rise in activity of acid phosphatase in malignant mammary tissues was variable from two to three fold its activity in benign conditions.

The homogenates of adenocarcinomas of other organs showed variable but still comparable measurable concentrations of ICDH:NAPD$^+$ as seen in Fig. 3. The highest concentration of this enzyme was noted in endometrial carcinomas. This finding is quite interesting. According to previous experience with the endometrium in different phases of the cycle and in benign conditions (Elias *et al.*, 1983), the concentration of ICDH:NADP$^+$ is at its highest in the progestagenic controlled conditions, i.e. secretory phase and pregnancy and at its lowest during the proliferative phase of the cycle, in atrophic and non-active or drug-suppressed endometria. A subsidiary hint is worth mentioning here that the malignant endometrial samples examined and showing a high concentration of Gluc and ICDH:NADP$^+$ were exactly the same samples which contained positive receptors for progestagen and oestrogen examined in a parallel study (Elias *et al.*, in preparation).

The high activity of LDH in all tumour specimens of different organs is unmistakable when compared with, for example, its normal activity in benign

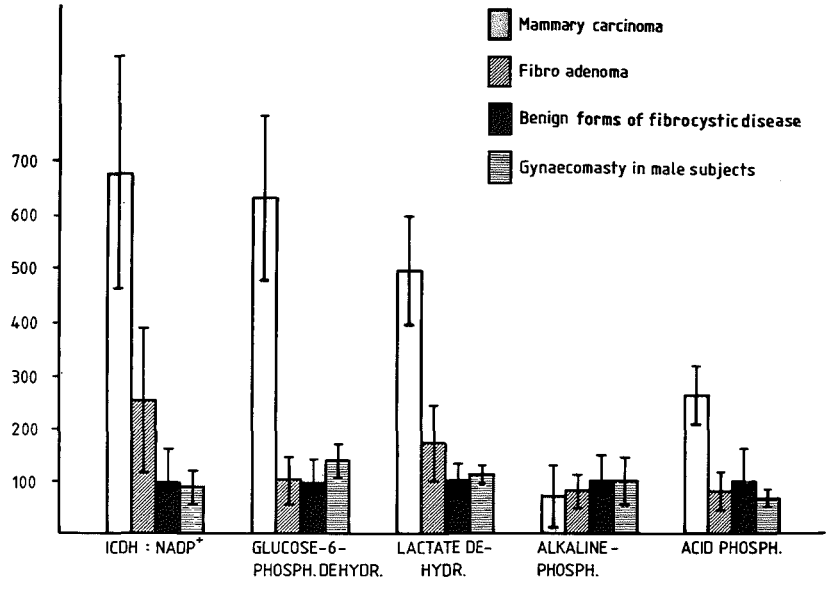

Fig. 2. The activity of biochemically estimated ICDH:NADP$^+$, GPDH, LDH, ALP and ACP in homogenates of benign and malignant mammary lesions expressed in percentual ratios.

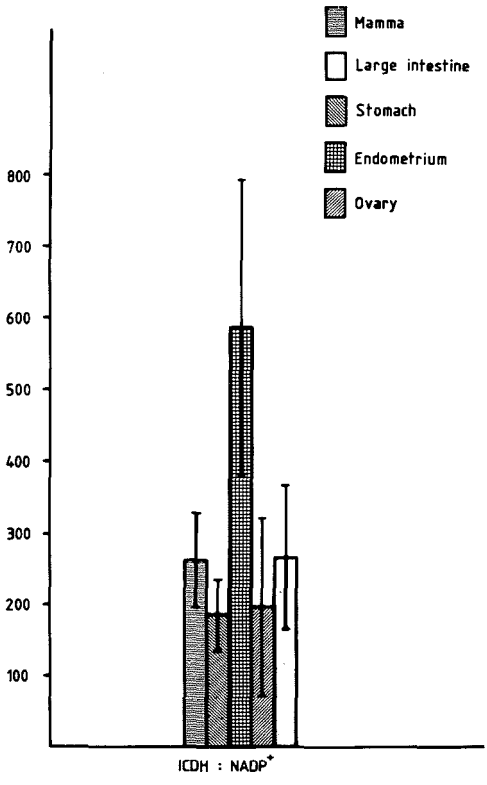

Fig. 3. The activity of ICDH:$NADP^+$ in adenocarcinomas of different organs, expressed in IU g^{-1} protein, l^{-1} in diluted tissue homogenates. The highest activity is observed in adenocarcinomas of endometrium and ovary, followed by mamma and colon, while gastric cancers show the lowest measurable activity of this enzyme.

endometrium or mamma (as seen in Fig. 4). Also the distribution of different fractions of isoenzymes of the LDH was clearly different (see Table 3) and showed more fluctuations of the standard deviation in homogenates of malignant tissues when compared to the normal serum distribution of these isoenzymes in normal subjects. The highest distribution in malignant homogenates was seen around fractions III, IV and V. The highest level of biochemically estimated ALP was found in confirmation to the histochemical observations, in the homogenates of endometrial and ovarian carcinomas, which showed 10–15 fold its activity in adenocarcinomas of other organs examined (see Fig. 5). This low activity of ALP in homogenates of other tumours examined is not in contradiction to its histochemical absence in the same samples. In such a case the activity is not explicitly epithelial in origin, as a large component of it can originate from blood vessels or stromal cells. Although endometrium and ovary carcinoma showed a higher activity of biochemically estimated ACP than adenocarcinomas of other organs, this was not as equally high as the increase in ALP. However, the high activity of ACP in benign endometrium may correspond to its high activity in malignant endometrium, which is not the case when speaking about ALP. The biochemical assays confirmed the histochemical observation that the ALP found in endometrium carcinoma differs in nature from the ALP in ovarian carcinoma. The ovarian ALP was found to be mainly thermostable, while the ALP in endometrial malignancy was found to contain a considerable fraction of thermolabile isoenzymes often defined as Reagan's, placental or carcinoplacental fraction, an observation which can be useful for the differentiation of the origin of the malignant cells examined (see Fig. 6).

DISCUSSION

One of the major problems in diagnostic pathology is the lack of uniformity in the classification of lesions arising in many organs especially the human breast. The most acceptable classification of mammary tumours are those presented by the A.F.I.P (U.S.A., 1968), W.H.O. (1982) and the scheme suggested by Azzopardi (1979). In order to avoid unnecessary complications and considering the complexity of the subject, the problems posed by differences in classification were

Table 3. Distribution of different isoenzymes of lactate dehydrogenase in homogenates of maligant cells examined, expressed in percentages

	I	II	III	IV	V
Mammary carcinoma	1 (±1)	4 (±2)	16 (±8)	40 (±7)	39 (±5)
Colorectal carcinoma	3 (±1)	7 (±3)	25 (±7)	45 (±7)	20 (±7)
Stomach carcinoma	5 (±2)	8 (±3)	28 (±9)	41 (±5)	18 (±12)
Endometrial carcinoma	4 (±3)	17 (±8)	25 (±5)	33 (±15)	21 (±8)
Pancreas carcinoma	2 (±1)	4 (±2)	18 (±3)	34 (±2)	42 (±5)

avoided as much as possible in tabulation and registration of the data. This was possible since the epithelial cells, either benign or malignant, besides showing general or specific changes characteristic of the malignant transformation, retained constant histochemical features similar to those of the cells of origin. Certain types of tumours such as colloid carcinoma of the breast and cystadenocarcinoma papilliferum of the ovary were excluded from the series, because of their peculiar clinical as well as histo-pathological and histochemical characteristics. Tumours such as the granulosa cell tumour of the ovary were beyond the scope of this communication.

It is an accepted practice in histochemistry to express the degree of activity of an enzyme as the relative density of colour deposits at the site of reaction of the enzyme concerned by means of microscopical evaluation. Theoretically such measurements are subject to a range of human errors. However the constancy of the application of standardized techniques under the most accurate conditions possible, as well as the multiplicity of measurements in a large series as presented in this communication, greatly reduces or removes the chance of significant errors of evaluation. Data obtained through biochemical assays on tissue homogenates also have their limitations. There can be no certainty with regard to the correct level of activity in the tumour cells themselves of the enzymes measured, since the homogenates contain all components present in the tissue including stromal cells, blood vessels and some blood cells, as

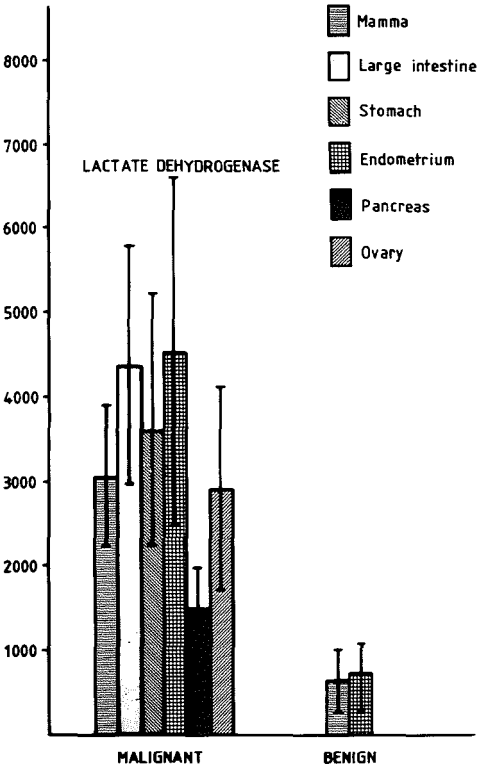

Fig. 4. The activity of LDH expressed in IU g^{-1} protein l^{-1} in diluted homogenates of adenocarcinomas of different organs. For comparison a control series of benign mammary and endometrial samples is shown.

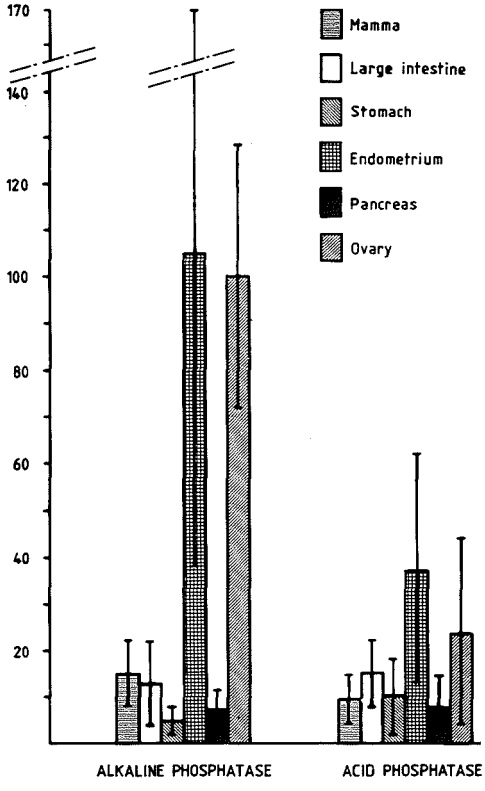

Fig. 5. The activity of ALP and ACP expressed in IU g^{-1} protein l^{-1} in diluted homogenates of adenocarcinomas of different organs. The activity of ALP is exceptionally high in adenocarcinomas of endometrium and ovary. The differentiation between both types of malignancy can be carried out by examination for different isoenzymes of ALP as further explained in the text.

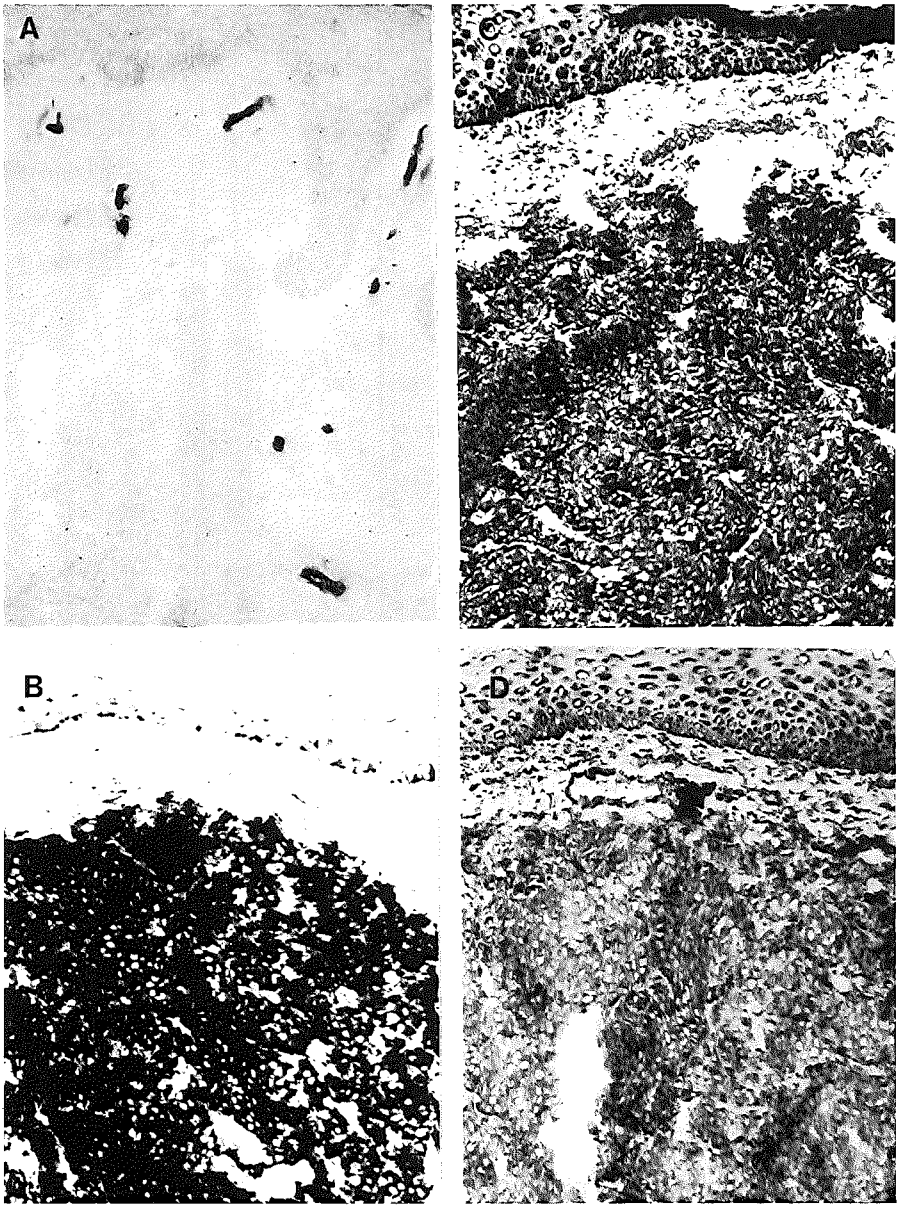

Fig. 6. A skin metastasis from breast carcinoma. The ALP is exclusively active in endothelium of blood vessels present (A). The activity of GPOX as seen in (B) shows a higher degree than that of NADH seen in (C). The MDH:$NADP^+$ shows a patchy but moderate to high activity, seen in (D) from the same tissue.

well as necrotic cell remnants and tissue cells belonging to the invaded organ. A relatively more accurate orientation may be realized by combining the histochemical studies with adjuvant biochemical assays performed on selected tumour tissue specimens which have been prepared as pure and representative as possible.

The use of reliable enzyme histochemical techniques is essential for correct localization and demonstration of the enzymes concerned. This is rather impossible, when enzymes are present in low concentration, are structurally loose or partially bound and when they are essentially demonstrable through the action of auxiliary enzymes. The answer has been found in the more sensitive and accurate semipermeable membrane techniques. The method involves elimination of the action of auxiliary enzymes by addition of selective inhibitors, completing the enzyme reaction by the addition of artificial electron carriers and preventing the diffusion of the enzymes or the enzyme reaction-products by using a porous dialysis membrane interposed between the gel-form incubation medium and the tissue sections or cellular smears. The membranes will allow ionic-exchange and passage of smaller molecules such as the substrate and will prevent the diffusion of the enzymes into the incubation medium. Since its introduction by Meijer and his co-workers in the seventies, the technique has proved useful with many hydrolases, oxidoreductases, transferases and isomerases as given in the individual references for the enzymes examined. The use of specially developed polyacrylic membranes incorporating the cells investigated proved to be necessary to suit the needs of and solve the problems encountered when dealing with cytology material. The experience in this field seems promising, at least for the enzymes published up till now (Noorden *et al.*, 1982; Elias *et al.*, 1984a, 1984b), especially for the cells collected from ascitic fluid, which often show low cohesion on the glass slides due to a high surface tension and presence of lipoid material in the cell concentrates.

Nevertheless the reliability of some enzyme reactions is still far from being complete. The histochemical techniques for the characterization of, for example, the different types of ATP are originally developed for muscle fibre research. Special reliability tests (Meijer and Vloedman, 1980) for differentiation between mitochondrial ATP and other types of ATP do not exclude other behaviour patterns of different types of ATP present in other types of tissues examined. Not only the reliability of the technique but also a correct interpretation of the observations is essential in the field of histochemistry. This demands a sound background and knowledge of the biochemical and biological significance of the findings.

The observations of Johansen and Theun (1981) claimed the presence of ALP activity in 56% of cytological preparations of carcinoma of the human breast and in all benign lesions. Such results are the highest we have encountered in the literature and can be considered to be the product of erroneous interpretation. The authors failed to differentiate between epithelium and myoepithelium in their study which also lacked any biochemical confirmation for the morphological observations. Koudstaal (1980) demonstrated the presence of ALP in only 4% of mammary carcinomas. This result, together with the activity in the stromal cells of about 10% of these cancers, corresponds with our own experience (a skin metastasis from a breast carcinoma is shown in Fig. 6). In the few cases where ALP was present in the malignant mammary epithelium, its activity was low. The biochemical analysis showed a very low or no demonstrable activity at all of ALP in the homogenates of such tumour specimens. As already mentioned in the results the correlation between the slight histochemical positivity for this enzyme, located mainly as an outer deposit on the cells, especially those collected from malignant pleural effusion and ascites, and the clinical data will often reveal a high serum level of ALP and/or widespread skeletal and liver metastases. The possibility of an extracellular origin of the activity must be considered. With mammary tumours, the histochemical study proved to be important for the identification of the myoepithelium which is normally present as an outer cell layer in all benign ducts and acinar or tubular structures, including solid epitheliosis or "non infiltrating duct carcinoma". Its presence in any mammary lesion which according to other criteria should be classified as infiltrating carcinoma, is to be considered accidental since careful study of serial sections will often show it to be a pre-existant component and not a product of malignant proliferation. Fibroadenoma and cystosarcoma phyllodes, for example, were found to preserve an outer cell layer of myoepithelium around the proliferating ducts. The same was observed in ductal papilloma. The histochemical identification of malignant lesions in the breast, especially the highly sclerotic tumours and the so-called scar carcinomas, proved to be straightforward since these lesions are epithelial and devoid of any myoepithelium. Sclerosing adenosis of the breast which may

histologically mimic malignancy could be identified by the very high activity of ALP present in the proliferating myoepithelium. The biochemical identification of mammary ALP showed that it is equivalent to the intestinal fraction. In accordance with the findings of Hampe (1980), the use of non-enzymatic techniques, such as the Wallart and Houette stain (1934), for the identification of myoepithelium fails to yield constant results, requires a high level of experience and gives no indication of the biological constitution or enzyme histochemical nature of the cells involved.

The application of the enzyme series described in this communication, especially when performed on cytological material, can be employed in almost any laboratory with histochemical experience. In contrast to mammary epithelium and malignant secretory epithelium of other organs examined, adenocarcinoma of the ovary and endometrium showed a high degree of activity of ALP when compared with benign conditions of the endometrium (Elias *et al.*, 1983). This finding was essential for the identification of an ovarian or endometrial origin of the malignant cells present in, for example, a metastatic lesion of malignant effusions. The differentiation between the two origins which is essential for a correct oncological approach to therapy was possible by the examination of the different isoenzymes of ALP. The endometrial tumours proved to contain a considerable amount of thermolabile and fixation labile fraction. This was identified and measured biochemically as Reagan's

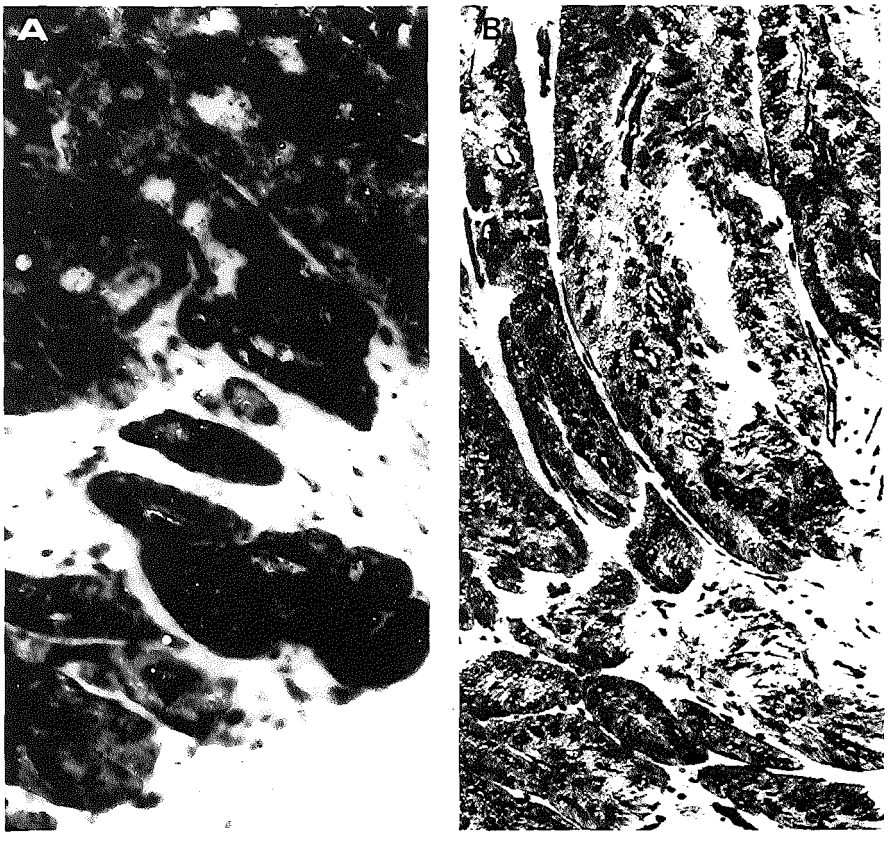

Fig. 7. In contrast to the stable isoenzymes of ovarian carcinoma, the high activity of ALP in endometrial carcinoma (A) can be reduced greatly by fixation or pre-heating the sections to 65°C for 5 min prior to incubation (B). The decreased histochemical activity of ALP corresponds to a biochemically measurable thermolabile fraction of ALP in endometrial carcinoma.

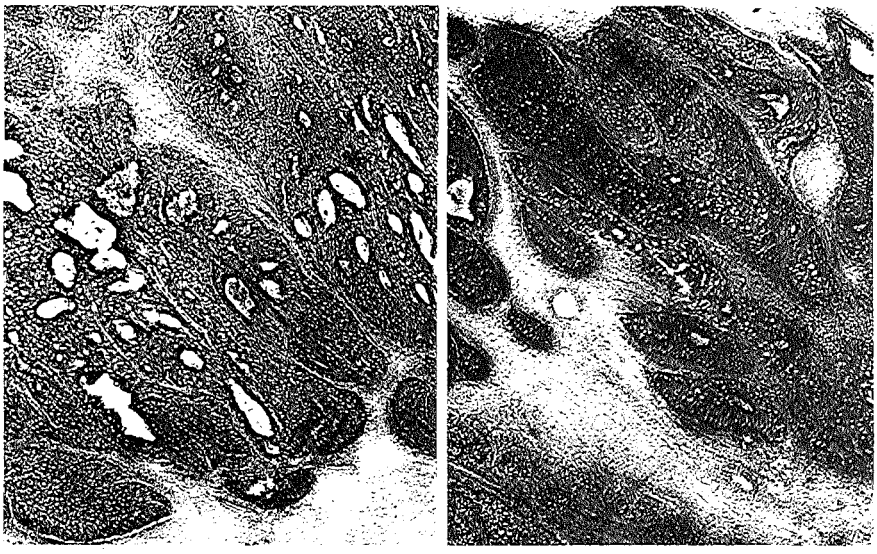

Fig. 8. The tremendous activity of the oxidative enzymes of the pentose phosphate pathway can be seen in these tissue sections examined for the activity of PGDH (left) and GPDH (right), in adenocarcinoma of the endometrium. Benign epithelium of the endometrium shows a minor activity of these enzymes except under the effect of progesterone, such as during pregnancy or the secretory phase of a normal cycle.

or carcino–placental fraction. Meanwhile the greatest component of ovarian ALP was thermostable and biochemically resembling the liver, intestinal or skeletal fraction. In some literature, mainly of Japanese origin, certain isoenzymes of alkaline phosphatase are often described in up to 29 of 75 gastric cancers (Takagi *et al.*, 1981). We have gained the impression that these are exceptionally high figures which we are not aware to be in material of European origin.

The biological significance of all enzymes examined in this study is well documented in the biochemical literature. The point which we like to stress in this concept is the role of both enzymes examined which belong to the oxidative part of the pentose phosphate pathway. Both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase show a tremendous increase in activity in all proliferating cells especially after the malignant transformation and especially in glandular malignancies.

The greater the proliferation, the greater is the activity of this pathway (see Fig. 8). This was confirmed biochemically. A relatively high activity of GPDH and PGDH will be associated with benign proliferative conditions such as lactation, pregnancy or physiological adenosis. In the highly active apocrine cells, the activity of both enzymes was clearly greater than in the ordinary epithelium. In cases of malignancy, the activity was certainly higher than in all benign conditions investigated. It is reasonable to assume that GPDH is the rate limiting enzyme of the pentose phosphate pathway and that forward reactions of both GPDH and PGDH are strongly favoured in the conditions examined in this study. The investigation for these enzymes is recommended to give a reliable impression of the capacity of the pentose phosphate pathway in the cells concerned. The pathway is of primary importance as a source of ribose and deoxyribose necessary for the rebuilding or synthesis of nucleic acids and many nucleotide co-enzymes (Henderson, 1972). Moreover the pathway plays a role in the production of triose phosphates necessary for energy metabolism. Its activity is necessary to bypass the rate limiting steps of the glycolysis (Kapucinski and Williams, 1981). Another important function of both oxidative enzymes of the pentose phosphate pathway is the production of NADPH necessary for many synthetic processes such as fatty acid and steroid synthesis (Eggleston and Krebs, 1974; Horecker, 1968). The regenerated

NADPH is of great importance in detoxifying the glutathion system (Autor, 1982; Freeman and Crapo, 1982) and also detoxifying singlet oxygen (Bodaness, 1982). Such observations lead to the assumption that a high capacity of pentose phosphate pathway is very useful or even a prerequisite for the rapidly growing tissues especially cancer cells (Meijer and Elias, 1985). Any increase in the activity of this pathway can indicate an increased demand for energy supply at a time when the major oxidative pathways are inadequate to supply it sufficiently. The reactivation of this pathway does not replace the major pathways such as glycolysis, tricarboxylic acid cycle or the respiratory chain, but it supports them as a subsidiary adjuvant pathway which becomes activated when needed. Previous studies on human muscle fibres (Meijer and Elias, 1976) demonstrated its presence in all muscle fibres but in a higher preponderance in the anaerobic type II fibres and in human cardiac muscle fibres, especially the fibres of the conducting system (Elias *et al.*, 1980b). The activity of the enzymes of this pathway, especially GPDH more than PGDH, was found to show an instant and dramatic increase in conditions such as myocardial ischemia (Elias *et al.*, 1982), neuromuscular diseases and myopathies (Meijer and Elias, 1977, 1984; Meijer *et al.*, 1977; Elias and Meijer, 1981, 1983) and in malignancies (Elias *et al.*, 1980a, 1981). The multiple approach to the functioning of this pathway demonstrated its capacity to readily increase in states of activity.

An increased activity of LDH, PGDH, PGM α-Glycerol phosphate dehydrogenase and PFK, as well as deviation in the ratio of their relative concentration in homogenates of human breast cancers in comparison to their activity and concentration in non-malignant mammary material, has been described by Desphande *et al.* (1977). Their results correspond roughly to our findings. However, our experience is that the increase in activity of GPDH is often more than the increase in activity of PGDH in the tumour cells, in contrast to the observations of Desphande. This contradiction might be explained by a non-epithelial origin of the biochemically estimated enzymes especially the PGDH, which is more present in many cells in a high concentration, especially in the erythrocytes or other blood cells.

A study of the enzymes active in the metabolism of DNA, collagen, amino acids and glucose in homogenates of foetal and neoplastic tissues of colon and lung performed by Herzfeld and Greengard (1980) confirmed our observations that the hexokinases and GPDH become more active in the majority of malignant cells examined. They found that this increase is associated with a rise in the activity of enzymes, such as thymidine kinase, peptidyl proline hydroxylase, phosphoserine phosphatase, ornithine transcarbamoyltransferase, γ-glutamyl transpeptidase and ornithine ketoacid aminotransferase in foetal tissues. The first five enzymes were found to be activated in cancer cells too. Activated biphasic kinetics of GPDH at the same time as highly raised linear kinetics of NADP in biochemically examined homogenates of malignant lesions were described by Ringler and Hill in 1974.

As early as 1951, Potter indicated a probable increase in an incomplete oxidation of glucose resulting in production of useful components to rebuild DNA and proteins necessary for the proliferating cancer cells. He suggested a forthcoming importance of the Embden–Meyerhof scheme of rapid conversion of glucose to lactic acid in tumour cells through anaerobic phosphorylated intermediates and also of increased products of the citric acid cycle. His predictions proved through the subsequent works to be realistic. The increased ICDH:$NADP^+$ and the tremendously activated enzymes of the pentose phosphate pathway in our experience may confirm directly or indirectly his view. The maze of anastomoses and interrelations in metabolism of proteins, carbohydrates, lipids and nucleic acids has been earlier emphasized by Zamecnik (1950) who described a nitrogen trap with an increased nitrogen input in cancer cells. The high ratio of activity of ATPase/citric acid cycle in cancer cells suggests an increased phosphorylation and rebuild of ATP molecules.

Previous experience with the human endometrium showed that the progestagenic controlled conditions including the secretory phase of the cycle are characterized by a tremendous increase in the activity of the pentose phosphate pathway associated with an increase in the activity of ICDH:$NADP^+$ (Elias *et al.*, 1983). The same mechanism is apparently involved in practically all adenocarcinomas examined. Both enzyme systems are involved in the regeneration of NADP necessary for many metabolic activities in the secretory cells either in benign or malignant conditions. The moderate increase in, or relatively constant degree of, activity of many enzymes belonging to the major oxidative and glycolytic pathways, such as GPOX and PFK and of other enzymes which were active

but not increased in most of the cancer cells examined, did not prove to be of diagnostic value. The observation is, however, of biological significance. This knowledge is valuable because it shows that a cancer cell can also resort to normal metabolic pathways to attain its energy requirements. This will occur alongside the already described activation of the subsidiary pathways and the deviated mechanism of glycolysis. The quantitative alteration in the biochemically measurable activity of different enzymes is in itself an interesting observation. PFK, the key enzyme in glycolysis, does not increase as much as PGI and PGM in adenocarcinoma cells. This might be regarded as an indication of a balanced or optimal functioning of glycolysis in secretory cells. The highly increased activity of PGI and PGM in the adenocarcinoma cells under these conditions suggests an alteration allowing an increased rate of glycolysis at a lower level to and without further involvement of the already optimally functioning PFK.

The degree of activity, presence or absence of enzymes serving a specific metabolic role is an indication of the presence or absence of this particular activity in the cancer cells examined. The pattern of activity can then be understood in the light of the nature and the secretory function of the glandular type of epithelium involved. This could explain the distribution of some glucosidases such as Gluc in mammary epithelium. Gluc has a higher activity in apocrine cells than in the ordinary mammary epithelium. Its activity is still higher in some adenocarcinoma cells. This might be related not only to change in glycolysis, but also to the role of such enzymes in the production of mucopolysaccharides and/or steroid metabolism (Wilkinson, 1976), possibly involved in the tumour cells.

From the results it is possible to assume that the malignant transformation is often associated with a certain increase in the activity of NE, provided that the enzyme has an assumed relation to or function in the cell of origin. Considering their function, the presence of a reasonable activity of the non-specific esterases (EC 3.1.1.2 and 3.1.1.1) could be expected in the more proliferative cells of, for example, pancreas, stomach, kidney and endometrium. However, the increase in their activity after malignant transformation is not homogeneous. Endometrial carcinomas in particular showed a tremendous increase in their activity when compared with adenocarcinomas of other organs. This increase is not apparently related to the secretory function of malignant endometrial cells. The benign secretory or progesterone-controlled endometria show in general less activity of NE than the proliferative oestrogen controlled phase or drug suppressed endometria (Elias *et al.*, 1983). The lowest degree of activity of NE and the lowest percentage of tumours which showed their presence were found in the mammary cancers. This type of observation can assist in the diagnosis but is not itself of great or defined significance.

The activity of AS, however, was found to be of great diagnostic value. It was found to be highly active, constantly present and almost exclusively characteristic in the majority of colo-rectal cancers and also in the proliferative non-invasive lesions such as the villous adeno-papillomas of the colon which are considered by some schools of thought to be a premalignant lesion. We therefore recommend the investigation of this enzyme as a tumour marker for this type of malignancy, also in metastases or cytological material. The AS is almost always absent in normal colonic epithelium. In case of adenopapilloma the activity was rather high but less intense than in the infiltrating carcinomas of the same organ. The increase of activity was roughly defined "to correspond to the degree of dedifferentiation" in such tumours.

The demonstration of the activity of ATP–Ca must be regarded with some circumspection. Its activity does not necessarily imply the involvement of a myosin-like component in the cancer cells described. This activity might be related to the increase in synthesis or degradation of ATP resulting from the degradation or building up of vital cellular components. However, a correlation between the histochemically demonstrated positivity for this enzyme, together with positive staining reactions for calcium deposits and positive fat stains, does not exclude a non-specific non-enzymatic deposition of a sulphide complex at these sites, at least partially.

One important finding, which was constantly observed, was the patchy distribution of many enzymes, especially the dehydrogenases or hydrolases, in different fields of the same sections or different parts of the same tumour (see Fig. 9). Although we can offer no convincing explanation for this phenomenon, it does suggest that the cancer cells in the same tumour are not homologous or that they do not behave in exactly the same manner. A non-predictable pattern of characteristics and behaviour can be assumed in the malignant cells which have lost the normal genetic control. The

question then arises about the monoclonality of the tumour cells in the same tumour mass.

As can be seen from the present study, and subsequent presentations, the field of enzyme histochemistry has to prove valuable for metabolic studies on living cells, especially for the study of malignant transformation. It may also serve as a diagnostic measure, at least in the identification of the origin or type of tumour cells; a factor which greatly influences the oncological approach to the treatment of cancer patients, especially when the tumour has metastasized beyond the reach of surgical cure. The relative constancy of characters of many cancer cells, specific for the organ of origin, may help to confirm the advantages of using simple approaches for collecting material adequate for diagnostic purposes, such as fine-needle aspiration punctures instead of or prior to more complicated surgical procedures. The presence or absence of any enzyme in a cancer cell must be regarded as a simple reflection of an altered, or preserved, ultrastructural integrity when compared with a corresponding benign cell. The alteration of activity must be then regarded as an expression of increased, decreased or at least altered activity of that organelle where the enzyme is normally located, e.g. lysosome, mitochondrion, cytoplasma or cell membrane. A final point which confirms the ultimate conclusion drawn by many workers in cancer enzymology is that a cancer specific enzyme does not yet exist. One cannot concentrate exclusively on one criterion or one characteristic of the malignant cells. All parameters must be considered, and the pathologist must continue to regard every individual lesion as a whole, evaluating all available data before arriving at a justified diagnosis.

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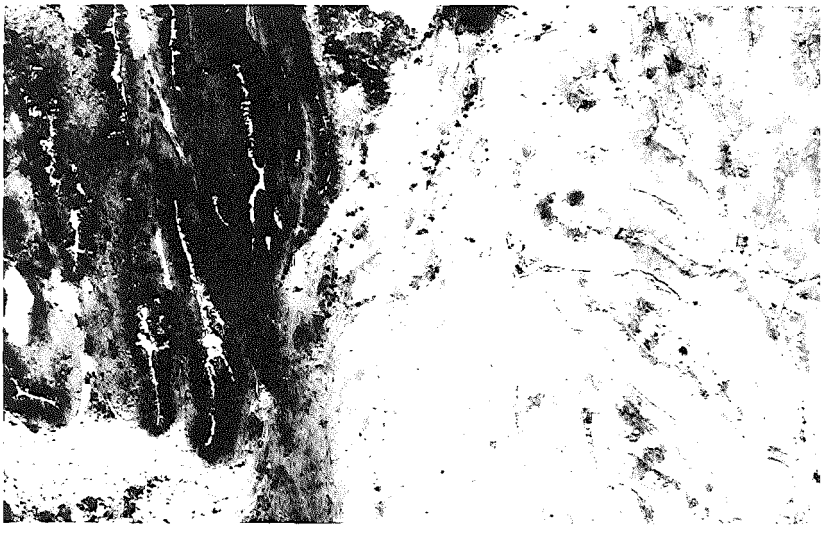

Fig. 9. The patchy distribution of many enzymes within the same tumour tissue is dramatically illustrated in this field of adenocarcinoma of colon, examined for the activity of β-Glucuronidase.

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CHAPTER 5

FINAL REMARKS

This thesis is mainly based on histochemical observations and biochemical data the greater part of which was published in the communications comprised. As seen in previous chapters the investigations were selected, designed and performed in a way sequenced to approach different facets of the same problem. They were applied to a variety of human tissue cells and when necessary those of experimental animals. Our material included specimens from apparently healthy and diseased skeletal muscles, human heart muscle fibres (proper and conducting) either apparently healthy or pathologically affected, human endometrium under different patho-physiological conditions including proliferative diseases and also diseased cells of other organs especially mammary glands under different conditions mainly malignant.

In many cases the activity and distribution of about 20 enzymes playing a role in aerobic and anaerobic major metabolic pathways were examined. The different diagnostic applications and metabolic implications are discussed in details in the communications concerned. The main observation stressed in this dissertation is the instant and extensive increase in the activity of the oxidative enzymes of the pentose phosphate pathway GPDH and PGDH under pathological conditions.

The biochemical investigation for the activity of some nonoxidative enzymes of the pentose phosphate pathway appeared necessary to get a better insight into the metabolic consequences of the increase in activity of GPDH and PGDH. Therefore the activity of TK, TA, RPE and RPI was studied in apparently healthy and diseased muscle fibres and other tissue specimens including proliferative lesions. The observations revealed a significantly high activity of these enzymes in

diseased cells. In another study the effect of protein synthesis inhibitors actinomycin D and cycloheximide on the observed increase in activity of GPDH and PGDH was investigated in muscle fibres. The findings showed that the increase in activity of GPDH and PGDH requires RNA and protein synthesis.

The observations concerning GPDH and PGDH in diseased skeletal and heart muscle fibres appeared not to be associated with any significant increase in activity of both other NADPH regenerating enzymes examined ICDH:$NADP^+$ and MDH:$NADP^+$. On the contrary, the latter appeared to decrease in activity in the pathologically altered fibres. In endometrium and other tissue cells investigated the activity of ICDH:$NADP^+$ showed a parallel increase to that of GPDH and PGDH. In the meanwhile no observable change in activity of the fourth NADPH regenerating enzyme studied MDH:$NADP^+$ could be defined in any of the conditions and cells examined.

The pentose phosphate pathway fulfills some important functions in metabolism such as the formation of ribose-5-phosphate necessary for nucleic acid synthesis, the synthesis of glycolytic ATP by which phosphofructokinase the rate limiting enzyme of the glycolysis is bypassed and the generation of NADPH necessary for many reductive biosynthetic reactions, (figures 1 & 2). Other intermediatees of the pentose phosphate pathway may be required for biosynthesis like sedoheptulose-7-phosphate for incorporation in cell wall glycolipids (Eidels & Osborn, 1971). It is acceptable that any increase in the demand for metabolites synthesized or generated through the pentose phosphate pathway will stimulate the activity of the pathway. This stimulation induces the new synthesis of enzymes involved, specifically GPDH and PGDH, (4.7). According to this assumption the high activity of the pentose phosphate pathway enzymes demonstrable in proliferating cancer cells which need much energy and resources for their growth becomes understandable.

The results of our combined histochemical and biochemical investigations points out certain correlation between the activity of GPDH & PGDH to one side and that of TA, TK, RPI and RPE to the other side. Also it is apparent that there is a parallel positive correlation between the activity of GPDH and that of PGDH.

Electron paramagnetic resonance studies showed a marked decrease of free radicals in tumour tissues as stated by Hockman (1981). On the other hand, antioxidants accumulate in high quantities in tumour cells. The antioxidants can then neutralize the free radicals. According to Duchesne (1977) the antioxidants are responsible for the disappearance of free radicals in cancer cells. The tight coupling between GSH/GSSG ratios (figure 3) and the activity of GPDH and PGDH (Krebs & Eggleston, 1979; May, 1981; Brigelius, 1983; Brigelius & Schult, 1984) may give another explanation for the relatively low concentrations of free radicals in tumour tissues characterized by high activity of GPDH, PGDH and ICDH:$NADP^+$. Furthermore, Kurak and Corwen (1982) stated that antioxidants are effective against tumour progression as well as in the initial steps of carcinogenesis. All together the last words can not yet be spoken. Other known enzyme systems such as NADPH-cytochrome P-450 reductase which is stimulated by NADPH, is exactly known to form free radicals (Aust & Svinsen, 1982). A mechanism of NADPH driven lipid peroxidation has been described by Baird et al., (1980).

The patchy distribution of many enzymes experienced in our investigations especially GPOX, PGDH, GPDH and many others in different cell populations of the same tumour is an indication of the polyclonality of cancer cells and that biochemical investigations alone done on tissue homogenates will never be able to deliver complete or precise information about distribution and activity of enzymes concerned. An increased awareness of the importance of tissue saving enzyme histochemical investigations as a diagnostic and research mean must be emphasized. The use of histochemical demonstration of a higher activity of GPDH and PGDH is advised as a reliable diagnostic measure not only for diagnosis of malignant transformation but also in all conditions explained throughout our investigations beginning with muscle diseases. In contrast to previously believed, the pentose phosphate pathway represented by GPDH and PGDH is active in muscle fibres especially type II anaerobic fibres and plays an important role in muscle metabolism, especially in disease. The same applies to cardiac metabolism too. In case of pathological change, histochemical examination for the activity of both enzymes is strongly recommended as one of the important and

early to detect changes of a diagnostic value. The increase of the activity of GPDH and PGDH is an expression of neo-synthesis and not a reactivation of a dormant capacity. This increase either considered as a defence mechanism against life threatening injury to the cells or as an alternative measure to satisfy an increased demand for energy, is significant and correlative with enzymatic fluctuations of other known enzymes in the same pathway or related enzyme systems.

The burden of future is demanding. The exact role of the free radicals in cancer cells, their formation, concentration and fate in malignant cells has to be studied in detail. The advancing observations and knowledge around the presence, activity and functions of the pentose phosphate pathway in conditions investigated here must be further correlated with other pathological or patho-physiological conditions. Application of similar investigations to, for example, other forms of malignancies especially soft tissue tumours, carcinomas with a squamous cell differentiation and melanogenic lesions either benign or malignant has been planned or is actually in advance prior to future publications. We are aware of the fact that further research is still necessary. The lack of technical facilities, financial means and man power will always remain, especially under the present economic circumstances and at least in our own situation a handicap to extending the research in this subject towards far horizons.

The importance of other technological approaches such as quantitative histochemistry and/or the role of electron microscopy in histochemical research can not be denied. The objective measurement through quantitative histochemical technology can be superior to the subjective qualitative estimations for comparing enzyme activities in situ. In practice, this is not always applicable. Many of the developed enzyme histochemical techniques and enzyme reactions used in our investigations are not suitable for quantitative measurements. It is difficult or it has not been available for us to realize enzyme histochemical methods in which the mean absorbance or fluorescence of the specific final reaction product is in linear relation to the thickness of the tissue sections, incubation time, substrate concentration and enzyme

concentration in situ. Such difficulties are more complicated when dealing with rate limiting enzymes which determine and control the flux in the metabolic pathways. At present, the quantitative histochemical techniques available run short in this field (Meijer, 1988). The combination between qualitative histochemical methods and biochemical estimations has been considered more meaningful for our investigations. Electron microscopic investigations may localize the reaction products at the resolution level of the electron microscope used. The electron microscopic procedures available at present do not yield up enough to allow the type of investigations applied or required in the studies presented.

A final remark is that a cancer specific enzyme is not (yet) known. All parameters "available" must be taken in consideration by the diagnostic pathologist to reach a justified diagnosis.

SUMMARY

This thesis is based on a series of metabolic studies conducted on material obtained from human and experimental animal tissues and cells. The studies are essentially enzyme histochemical complemented when thought necessary with biochemical investigations performed on tissue homogenates of the same material. The human material comprises skeletal muscle biopsies from patients with and without apparent diseases of the neuromuscular system, cardiac muscle specimens obtained through autopsy performances, surgical specimens and cytology material from different organs removed for diagnostic purposes. Skeletal muscle specimens from rats were prepared after the performance of experimental models conducted to induce the desired myopathy after the administration of myotoxic drugs in or without combination with protein synthesis inhibitors, and from rabbits with vitamin E deficiency. The examination for the activity of sometimes more than 20 enzymes belonging to different metabolic pathways, essentially oxidoreductases, hydrolases, transferases and isomerases, was found necessary for a reliable correlation. The results of each experience have already been published or are about to be published separately.

The problem of the demonstration of the activity of both **glucose-6-phosphate dehydrogenase** and **6-phosphogluconate dehydrogenase** which belong to the oxidative part of the **pentose phosphate pathway** is considered as a linking factor among the different sequential studies. The value of using enzyme histochemical technology as a reliable method for the study of the biological and patho-physiological conditions of cells and tissues concerned has become obvious since the early phases of the project. In this way a classification of the skeletal muscle

fibres into: **Type I** (aerobic), **Type II** (anaerobic) and **IMF** or intermediate muscle fibres according to their metabolic pattern was established. The demonstration of the activity of both **NADPH** regenerating enzymes: glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, present esssentially in Type II fibres, was possible under **normal conditions.** This was found to increase dramatically in many **pathological** conditions, especially in Type II fibres and in many cases in all fibres even before the appearance of observable morphological changes. The increased activity was described to be the result of a **neogenesis** of the enzymes concerned and not that of reactivation of already existing proformers.

The metabolic pattern of the working myocardial fibres of the **human heart** and those of its conducting system has been presented and showed also a tremendous increase of activity of these NADPH regenerating enzymes under pathological conditions such as myocardial ischemia and in inflammatory conditions. The phenomenon has proved again to be of a great diagnostic value.

Other tissues in dynamic metabolic change such as human **endometrium** showed a significant fluctuation in the activity of enzymes concerned varying with the biological or metabolic state of the cells. A discussion of the metabolic significance of enzymatic activities investigated for the study of **proliferative lesions** essentially malignant, has been presented after the application of the same technology. The main stress in discussing the observations in the included publications was laid on correlating the findings with some aspects of **energy metabolism.** In the general discussion and final remarks an additional or possible correlation with the phenomenon of presence of **free radicals** in the damaged and/or pathologically altered cells was suggested.

The use of reliable enzyme histochemical technology is still advisable not only to get a **better insight** in the biological processes in the living cells but also to widen the scope of **diagnostic facilities** available to the practising pathologist.

SAMENVATTING

Dit proefschrift is gebaseerd op een serie onderzoekingen die op menselijk en dierlijk materiaal verricht zijn. Het betreft voornamelijk een enzymhistochemisch onderzoek gekombineerd, waneer nodig geacht, met een aanvullend biochemisch onderzoek naar de aktiviteit van enkele enzymen in homogenaten van hetzelfde materiaal. Het menselijk materiaal betrof: biopsiëen van skeletspierweefsel van patienten met of zonder duidelijke neuromusculaire afwijkingen, hartspierweefsel inclusief het prikkelgeleidingssysteem verkregen tijdens obducties en diagnostische weefselmonsters alsook cytologisch materiaal uit verschillende organen. Voor het dierexperimenteel onderzoek werden biopsieën van skeletspieren onderzocht en wel van ratten behandeld met myotoxische drugs gekombineerd al dan niet met eiwitsyntheseremmers en van vitamine E deficiënte konijnen. Voor een betrouwbare correlatie was het histochemisch onderzoek naar de aktiviteit en lokalisatie van soms ruim 20 enzymen: oxidoreductasen, hydrolasen, transferasen en isomerasen toegepast. De bevindingen zijn grotendeels reeds afzonderlijk gepubliceerd in de, in dit proefschrift bijeengebrachte artikelen, of worden nog apart gepubliceerd. Bij de bespreking van de resultaten is voornamelijk aandacht besteed aan datgene wat gemeenschappelijk is in al die studies, c.q. de aktiviteit en lokalisatie van beide oxidatieve enzymen van de **pentose fosfaat cyclus: glucose-6-fosfaat dehydrogenase** en **6-fosfoguconaat dehydrogenase.**

Vanaf het begin van dit onderzoek is gebleken dat de toepassing van enzymehistochemische technieken zijn waarde toont voor de studie van biologische of pathofysiologische toestanden in de betrokken cellen. Op deze wijze konden wij de humane spiervezels indelen naar de daarin overwegende metabolische aktiviteit in: **Type I** aeroob, **Type II** anaeroob en **IMF** ofwel vezels met intermediar metabolisch patroon. Beide NADPH-regenererende enzymen: GPDH en PGDH zijn duidelijk

aanwezig maar in een geringe koncentratie in normale skeletspiervezels met name in Type II. Onder pathologische omstandigheden neemt deze aktiviteit toe. Deze stijging werd toegeschreven aan een neogenese van eiwitten.
Eveneens in humane harten zonder waarneembare afwijkingen is de aktiviteit van beide enzymem aantoonbaar in het myocard en in Purkinje vezels van het prikkelgeleidingssysteem en neemt sterk toe onder onderzochte pathologische omstandigheden, zoals ischemie en/of myocarditis. Dit fenomeen werd geacht van diagnostische belang te zijn.
Afhankelijk van de biologische status van het humane endometrium trad een duidelijke fluktuatie van de aktiviteit van de onderzochte NADPH-regenererende enzymen in de cellen op. Tenslotte toonden de proliferatieve aandoeningen, met name maligne tumoren, een sterk gepronunceerde aktiviteit van GPDH en PGDH. In de publikaties werd de aandacht gevestigd op de korrelatie tussen onze waarnemingen en sommige aspekten van de stofwisseling. In de slotbeschouwingen is vermeld dat de resultaten wijzen op een mogelijke korrelatie tussen de toename van aktiviteit van beide enzymen en de produktie of koncentratie van vrije radikalen in de pathologisch veranderde cellen.
Tenslotte is er op gewezen dat het toepassen van betrouwbare enzymhistochemische technieken is niet alleen van belang om inzicht in de status van de cellen te verkrijgen maar ook als een diagnostisch middel voor de patholoog-anatoom.

RESUME

Cette thèse repose sur une série d'études métaboliques menées sur matériaux obtenus à partir de tissus et cellules d'origine humaine et animale. Ces études sont essentiellement des études **d'histochimie enzymatique** completées et confirmées lorsque cela fut jugé nécessaire par des **essais biochimiques** exécutés sur des cytosoles de mêmes matériaux. Les tissus humains comprennent des biopsies de muscles volontaires de sujets avec ou sans apparentes maladies du système neuromusculaire, des spécimens du muscle cardiaque obtenus par autopsies, des spécimens chirurgicaux et des matériaux cytologiques de différents organes prélevés pour le diagnostique. Des spécimens de muscles volontaires de lapins ou de rats ont étés prélevés après l'accomplissement de modèles expérimentaux conduits pour amener la myopathie désirée, par exemple après l'administration de drogues myotoxiques, avec ou sans combinaison d'inhibiteurs de synthèse de protéines ou dans le cas d'une déficience en vitamine E. L'étude de l'activité de parfois plus de 20 enzymes appartenant à des voies métaboliques différentes, surtout des oxidoréductases, hydrolases, transférases et isomérases, s'avéra nécessaire pour établir une corrélation fiable. Les résultats de chacune de ces expériences ont déjà été publiés ou sont sur le point de l'être.
On considère que montrer l'activité des enzymes oxydatifs: **glucose-6-phosphate déhydrogénase** et **6-phosphogluconate déhydrogénase** de la voie du pentose phosphate, établit un lien entre les différentes études incluses dans cette thèse. L'intérêt d'utiliser la combinaison des techniques histochimiques et biochimiques comme méthode fiable pour éclairer les états biologiques, physiologiques ou patho-physiologiques des cellules et des

tissus en question est devenu évident à la suite des premiers essais. Grâce à cette approximation on a pu classer les fibres musculaires volontaires en: **Type I** aérobique, **Type II** anaérobique et un troisième type qui a été intitulé **I.M.F.** caractérisé par un métabolisme intermédiaire. Contrairement à ce que l'on pensait jusque là, l'activité à la fois du GPDH et du PGDH dans les conditions physiologique normales a été démontrée en particulier dans les fibres de Type II. En état pathologique on s'est apercu que cette activité **augmentait considérablement et instantanément,** surtout dans le Type II et parfois aussi dans les autres types bien avant l'apparition de changements morphologiques. Cette augmentation ne provenait pas de l'activation des éléments existants, mais résultait d'une nouvelle **synthèse.** Certaines des activités métaboliques des **fibres musculaires cardiaques** propres et des fibres du système conducteur cardiaque sous conditions normales ont été mises en relief. Là aussi on a constaté une augmentation considérable d'activité de ces enzyme regénératifs de la **NADPH** dans les conditions pathologiques surtout **l'ischémie** cardiaque. Ce phénomène à nouveau s'est revelé avoir une grande valeur pour le diagnostic. D'autres tissus qui eux sont sujets à un changement métabolique dynamique - tel **l'endomètre** humain - ont montré une **fluctuation** significative de l'activité de plusieurs des enzymes concernés **en fonction** de l'état biologique cellulaire.

Une discussion sur la signification métabolique des activités enzymatiques examinées dans le cas de **lésions prolifératives,** essentiellement malignes, a été présentée après avoir appliqué la même technologie. Dans les articles ci-inclus on a mis l'accent sur le **métabolisme d'énergie.** De plus une corrélation possible entre l'activité des ces enzymes et la présence de **radicaux libres d'oxygène** a été évoquée dans la discussion générale.

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ABBREVIATIONS

The constant use of abbreviations throughout the text has been avoided cosequentiallly for reason of clarity.
The enzymatic abbreviations used are given in Table 1. and Table 2. as well as in papers concerned.
Less common abbreviations are explained or listed duely.
The most appropriate to mention abbreviations include:

ATP	Adenosine triphosphate.
DNA	Deoxyribonucleic acid.
DPPD	N,N'-dimethyl-p-phenylenediamine or N,N'-dimethyl-p-diamino benzidine.
FIDP	2,6-dichlorophenolindolphenol.
NAD^+/NADH	oxidized and reduced forms of B-Nicotinamide-adenine dinucleotide.
$NADP^+$/NADPH	oxidized and reduced forms of B-Nicotinamide-adenine dinucleotide phosphate.
Nitro-BT	Nitro blue tetrazolium chloride or (2,2'-di-p-Nitrophenyl-5,5'-diphenyl-3,3'-3,3'-dimethoxy-4,4'-biphenyline).
RNA	Ribonucleic acid.
Vit.E	a-tocopherol

For technical reasons the profix **"a-"** or **"B-"** was used to indicate **"alpha-"** or **"Beta-"** respectively.

CURRICULUM VITAE

Ezzat Adly ELIAS Yacob, geboren 3 Januari 1942 te Zawiet El-Masloub, provincie Beny-Swif in het zuiden van Egypte, behorend bij de Koptische Orthodoxe Kerk, heeft het arts-diploma verworven aan de Ain Shams Universiteit te Cairo in juni 1968. Sinds september 1968 woonachtig in Nederland en inmiddels houder van de Nederlandse nationaliteit.

Zijn opleiding tot patholoog-anatoom is begonnen in februari 1969 aan de Vrije Universiteit te Amsterdam onder leiding van Prof. dr. R. Donner en voltooid in het Academisch Ziekenhuis Wilhelmina Gasthuis, Universiteit van Amsterdam in 1973 onder (bege-)leiding van Prof. dr. C. A. Wagenvoort.

Gedurende de specialisten opleiding aan beide instellingen en daarna bekleedde hij verschillende functies: o.a. arts-assistent, wetenschappelijk-medewerker, hoofdassistent en tenslotte wetenschappelijk-hoofdmedewerker aan de Universiteit van Amsterdam. Sinds april 1976 is hij werkzaam in dienstverband bij het Laboratorium voor de Volksgezondheid in Friesland.

Zijn persoonlijk werkterrein beslaat het zuidelijke deel van de provincie, hoofdzakelijk het ziekenhuis De Tjongerschans te Heerenveen.

De promovendus is getrouwd met Drs Francoise Tresy, kunst-historica, geboren te Frankrijk. Zij hebben twee dochters: Nophert, geboren te Amsterdam (1970) en Nephthys, geboren te Veenwouden (1977).

Als nevenactiviteit is betrokkene lid van vele, nationale en internationale, organisaties en werkgroepen die zich veelal met de klinische cytologie, pathologische anatomie, kindergeneeskunde, gynaecologie, oncologie, spierziekten en vooral histochemie bezig houden.

Het is zijn stellige overtuiging dat ook een klinisch georienteerde patholoog-anatoom naast de diagnostiek de taak heeft zich voortdurend bezig te houden met verbetering van de diagnostische mogelijkheden o.a. door toegepaste research en grondige kontakten met de klinici en in voorkomende gevallen ook de patient.