ENZYMHISTOCHEMISCHE ONDERZOEKINGEN VAN DUNNE DARM EPITHEELCELLEN VAN DE RAT

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM,
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. P.W. KLEIN
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INHOUD

APPENDICES :

I. W.G.J. Iemhoff, J.W.O. van den Berg, A.M. de Pijper, W.C. Hülsmann: Metabolic aspects of isolated cells from rat small intestinal epithelium. Biochem. Biophys. Acta. 215, 229-241 (1970)

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III. A.M. de Pijper, W.C. Hülsmann: The influence of in vivo administered chloramphenicol and oxytetracycline on some mitochondrial enzymes of rat-small--intestinal epithelium: histochemical data. Histochemie 33, 181-190 (1973)

IV. A.M. de Pijper: Histochemical studies of fructose--1,6-diphosphatase in various tissues of the rat. Histochemie 37, 197-206 (1973)

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LIJST VAN AFKORTINGEN

ADP	Adenosine-5'-difosfaat
AMP	Adenosine-5'-monofosfaat
ATP	Adenosine-5'-trifosfaat
DNA	Desoxyribonucleinezuur
DNP	2,4-Dinitrofenol
EDTA	Ethyleendiamine-tetra-acetaat
FDP	Fructose-1,6-difosfaat
FAD	Flavine-adenine-dinucleotide
NAD^+	Nicotinamide-adenine dinucleotide (geoxydeerd)
NADH	Nicotinamide-adenine dinucleotide (gereduceerd)
$NADP^+$	Nicotinamide-adenine dinucleotide fosfaat (geoxydeerd)
NADPH	Nicotinamide-adenine dinucleotide fosfaat (gereduceerd)
Nitro-BT	Nitro-blauw tetrazolium

ENZYMNOMENCLATUUR

De nummers en de systematische namen zijn gegeven volgens de aanbevelingen van de Enzym Commissie van de International Union of Biochemistry in 1972.

Enzym Commissie nummers	Geadviseerde naam	Systematische naam
1.1.1.8.	glycerol-3-fosfaat dehydrogenase	sn-glycerol-3-fosfaat: NAD^+ 2-oxydoreductase
1.1.1.37.	malaat dehydrogenase (NAD^+)	L-malaat: NAD^+ oxydoreductase
1.1.1.40.	malaat dehydrogenase (decarboxylerend) ($NADP^+$)	L-malaat: $NADP^+$ oxydoreductase (oxaloacetaat decarboxylerend)
1.1.1.41.	isocitraat dehydrogenase (NAD^+)	threo-D_S-isocitraat: NAD^+ oxydoreductase (decarboxylerend)
1.1.1.42.	isocitraat dehydrogenase ($NADP^+$)	threo-D_S-isocitraat: $NADP^+$ oxydoreductase (decarboxylerend)
1.1.1.49.	glucose-6-fosfaat dehydrogenase	D-glucose-6-fosfaat: $NADP^+$ 1-oxydoreductase

1.1.99.5.	glycerol-3-fosfaat dehydrogenase	sn-glycerol-3-fosfaat: (acceptor) oxydoreductase
1.2.1.12.	glyceraldehyde-3--fosfaat dehydrogenase	D-glyceraldehyde-3-fosfaat: NAD^+ oxydoreductase (fosforylerend)
1.2.3.2.	xanthine oxydase	xanthine: zuurstof oxydoreductase
1.3.99.1.	succinaat dehydrogenase	succinaat: (acceptor) oxydoreductase
1.6.99.1.?	NADPH dehydrogenase (NADPH: Nitro-BT oxydoreductase of NADPH diaforase	NADPH: (acceptor) oxydoreductase
1.6.99.3.?	NADH dehydrogenase (NADH: Nitro-BT oxydoreductase of NADH diaforase)	NADH: (acceptor) oxydoreductase
1.9.3.1.	cytochroom-c-oxydase (cytochroom aa_3)	ferrocytochroom c: zuurstof oxydoreductase
2.4.2.1.	purine nucleoside fosforylase	purine-nucleoside: orthofosfaat ribosyltransferase
2.7.1.1.	hexokinase	ATP: D-hexose 6-fosfotransferase
2.7.1.11.	6-fosfofructokinase	ATP: D-fructose-6-fosfaat 1-fosfotransferase
3.1.3.1.	alkalische fosfatase	orthofosfaat-mono-ester fosfohydrolase (alkalisch optimum)

3.1.3.2.	zure fosfatase	orthofosfaat-mono-ester fosfohydrolase (zuur optimum)
3.1.3.5.	5'-nucleotidase	5'-ribonucleotide fosfohydrolase
3.1.3.9.	glucose-6-fosfatase	D-glucose-6-fosfaat fosfohydrolase
3.1.3.11.	fructose-1,6-difosfatase (hexosedifosfatase)	D-fructose-1,6-bifosfaat 1-fosfohydrolase
3.2.2.7.	adenosine nucleosidase	adenosine ribohydrolase
3.5.4.4.	adenosine deaminase	adenosine aminohydrolase
4.1.2.13.	fructose-difosfaat aldolase	D-fructose-1,6-bifosfaat D--glyceraldehyde-3-fosfaatlyase

Litt: Comprehensive Biochemistry, enzyme nomenclature, vol-13, ed. M. Florkin en E.H. Stotz, 3^{o} druk, Elsevier Scientific Publishing Company; Amsterdam, London, New York, 1973.

HOOFDSTUK 1

INLEIDING

Voor het bestuderen van de correlatie tussen structuur en functie enerzijds en metabolisme en functie anderzijds is vaak de dunne darm gekozen als studieobject. In verband met de absorberende functie is een sterke vergroting van het inwendig darmoppervlak aanwezig, dat wordt gevormd door de plicae circularis, de villi en de microvilli (Palay en Karlin, 1959; Padykula, 1961 en 1962; Pearse en Riecken, 1967; Trier, 1967). De villi zijn opgebouwd uit uitstulpingen van de lamina propria (een bindweefsellaag waarin bloed- en lymfevaten zijn gelegen), die aan de lumenzijde worden begrensd door een éénlagig epitheelweefsel. De rest van het darmweefsel wordt door deze gesloten epitheellaag van de darminhoud gescheiden. Alle stoffen, die door de darm geabsorbeerd worden, moeten deze barrière passeren. De vingervormige uitsteeksels van de apicale celwand van de epitheelcel, die in direct contact staat met het voedsel, vormen samen de borstelzoom. Door deze microvilli wordt het darmoppervlak zeer sterk vergroot (Trier, 1967; Crane, 1969). Op de borstelzoom is een goed ontwikkelde mucopolysaccharide-rijke "cellcoat" aanwezig, die de glycocalix wordt genoemd (Ito, 1969; Johnson, 1969).

Omdat in het darmepitheel de delende, de differentiërende en de functionerende volwassen cellen in verschillende compartimenten gelocaliseerd zijn, is dit weefsel geschikt om hieraan op betrouwbare wijze celkinetika te bestuderen (Quastler en Sherman, 1959). De epitheelcellen ontstaan in de onderste helft van de crypten van Lieberkühn (Leblond en Stevens, 1948), die als instulpingen in de lamina propria rondom de villi zijn gelegen. In deze prolifererende zône is dan ook een sterk verhoogde DNA-synthese aan te tonen (Lesher e.a., 1964; Cairnie e.a., 1965). Uit experimenten met ^{3}H-thymidine-

Sjöstrand (1968) en volgens Harrison en Webster (1969) (appendix I). De preparaten zijn met elkaar vergeleken, waarbij de aandacht is gericht op het behoud van de morfologische en de metabole eigenschappen. Het behoud van de metabole eigenschappen is biochemisch o.a. nagegaan door bepaling van de glycolyse-snelheid in de verschillende darmcelpreparaten en vervolgens de gevonden waarden te vergelijken met die van de intacte omgestulpte darm, geïncubeerd onder vergelijkbare omstandigheden. Hierdoor is na te gaan of de cellen door de isolatieprocedure veel enzymen en/of co-factoren hebben verloren. De cellen die volgens de Harrison en Webster methode waren geïsoleerd vertonen een glycolyse-snelheid, die van dezelfde grootte-orde is als die van de intacte darm. Ook de efficiëntie van de fosforylering gekoppeld aan de oxydatie van ademhalingssubstraten (P:O verhouding) is hoger in mitochondriënpreparaten afkomstig van cellen, die volgens de methode van Harrison en Webster zijn geïsoleerd. Deze resultaten wijzen op een beter behoud van de metabole eigenschappen bij isolatie met deze methode (Iemhoff e.a., 1969; Hülsmann e.a., in druk). Het behoud van de morfologische kenmerken is nagegaan door middel van electronenmicroscopie. De structuren van de cellen, geïsoleerd volgens de methode van Harrison en Webster, vertonen sterke overeenkomst met de structuren van het in situ gefixeerde darmweefsel (van Dongen, persoonlijke mededeling) (vgl. ook Fig. 2A, B en C van appendix I).

Omdat met de methode van Harrison en Webster bovendien de villus- en de cryptcelpopulaties afzonderlijk kunnen worden geïsoleerd, is deze methode in het verdere biochemische onderzoek steeds toegepast.

Steeds bestaat echter het probleem dat de verkregen celpreparaten zijn samengesteld uit verschillende celtypen, die in het epitheelweefsel voorkomen, zoals hoofdcellen, slijmbekercellen, panethcellen en enterochromaffinecellen (Singh, 1967, 1971a en 1971b; Toner, 1968). Ook de uit de preparaten geïsoleerde fracties van celorganellen zijn daardoor heterogeen van samenstelling, zodat de biochemische activiteiten van een gemeten proces het resultaat is van de activiteiten in verschillende celtypes.

Door toepassing van microchemische technieken, waarbij slechts enkele cellen tegelijk worden bestudeerd, is nadere informatie te verkrijgen over het metabolisme van een gespecificeerd weefselgedeelte (Lowry, 1953). Nordström en medewerkers hebben een kwantitatief onderzoek gedaan naar de differentiatie van enkele enzymen, die direct bij de voedselabsorptie zijn betrokken. Doordat afwisselend transversale coupes van de darm voor biochemisch en morfologisch onderzoek werden gebruikt, konden zij de activiteit in een be-

paald gedeelte van de villus bepalen (vgl. ook Imondi, 1969). Aangezien niet alle villi even lang en evenwijdig gericht zijn, is echter geen uitsluitsel te geven over de celpositie. Bovendien liggen de overgangszônes van crypt naar villus niet op gelijke hoogte. Wanneer echter microdissectie wordt toegepast op gevriesdroogde coupes, is op microchemische wijze enzymactiviteit in gespecificeerde delen van het epitheelweefsel kwantitatief aan te tonen (Galjaard e.a., 1970).

Een andere techniek, waarbij informaties over de enzymactiviteit in situ kan worden verkregen, is de toepassing van histochemische kleuringen op weefselcoupes (Shnitka, 1960; Jervis, 1963; Pearse en Riecken, 1967; Fortin-Mangana e.a., 1970; Michael en Hodges, 1973). Met deze methode is kwantitatief onderzoek echter beperkt, doordat er meestal slechts gedurende korte tijd een lineair verband bestaat tussen de hoeveelheid product (neerslag), tijdens de reactie gevormd en de incubatietijd (vgl. onderzoek aan modelsystemen door van Duyn, 1970). Kwantitatieve bepalingen na toepassing van cytochemische kleuringen zijn echter wel gedaan, door de hoeveelheid neerslag te meten door middel van microdensitometrie of spectrofotometrie (Cabrini e.a., 1970; Butcher, 1972). Bij het in dit proefschrift besproken enzymhistochemisch onderzoek, waarbij de localisatie van de enzymen centraal staat, is geen kwantificering gepoogd. Wel werden de kwalitatieve resultaten zoveel mogelijk vergeleken met de numerieke waarden, die biochemisch onderzoek van geïsoleerde fracties van villus- en cryptepitheelcellen in ons laboratorium opleverde.

Uit vroeger onderzoek was reeds gebleken dat in darmweefsel, in vergelijking met andere weefsels, een hoge glycolyse-snelheid is aan te tonen (Sherratt en Hübscher, 1963; Clark en Sherratt, 1967; Shakespeare, 1969). Een hoge glycose-snelheid is mogelijk bij een hoge ([AMP] + [ADP]) / [ATP] - verhouding, waardoor o.a. de activiteit van fosfofructokinase gestimuleerd wordt. Dit enzym blijkt, althans in andere weefsels dan darm, de snelheidsbeperkende stap van de glycolyse te katalyseren (Passonneau en Lowry, 1962). Omdat in darmcelpreparaten evenals in de intacte uitgeprepareerde darm een hoge ([AMP] + [ADP]) / [ATP] - verhouding gevonden werd, is een onderzoek gedaan naar het mitochondriale fosforyleringsproces, dat in vivo AMP en ADP snel in ATP moet kunnen omzetten (zie appendix I en III). De efficiëntie van de oxydatieve fosforylering en de activiteit van sommige enzymen, die betrokken zijn bij de ademhalingsketen en de dehydrogenering van substraten werden derhalve onderzocht. De koppeling van de ademhaling aan de fosforylering van ADP tot ATP is histochemisch nagegaan door de snelheid van hydrolyse van

toegevoegd Mg-ATP te vergelijken in afwezigheid en aanwezigheid van een ontkoppelaar van de oxydatieve fosforylering, zoals 2,4-dinitrofenol (DNP). Daarnaast zijn succinaat (barnsteenzuur) dehydrogenase en glycerol-3-fosfaat dehydrogenase histochemisch aangetoond (zie appendix II).

Over de vraag in hoeverre de mitochodriale ademhalingsketen in situ een rol speelt bij de ATP-productie, zijn experimenten gedaan met remmers van de mitochondriale eiwitsynthese (zie appendix III). Vooral de remming van de synthese van cytochroom-c-oxydase kon histochemisch gemakkelijk worden gelocaliseerd.

Uit experimenten met gistcellen, regenererende levercellen en hartcellen is gebleken, dat deze antibiotica vooral invloed hebben op de mitochondriale synthese van de cytochromen aa_3, b en c_1 (Clark-Walker en Linnane, 1966 en 1967; Kroon en Jansen, 1968; Firkin en Linnane, 1968 en 1969; de Vries en Kroon, 1970). Op de mitochondriale eiwitsynthese van de darm is eveneens een dergelijk effect aan te tonen (Gijzel e.a., 1972; de Jonge en Hülsmann, 1973).

Een ander aspect van het metabolisme, de gluconeogenese, werd eveneens aan een histochemisch onderzoek onderworpen. Dit proces is te zien als de omkering van de glycolyse: uit pyruvaat wordt glucose of glycogeen gevormd. De hierbij betrokken enzymen zijn de reversibele enzymreacties van de glycolyse alsmede de vrijwel irreversibele, speciaal bij de gluconeogenese betrokken enzymreacties: pyruvaatcarboxylase, fosfoenolpyruvaatcarboxykinase, fructose-1,6-difosfatase (FDP-ase) en glucose-6-fosfatase. Uit biochemische experimenten (van Tol, niet gepubliceerde waarneming) was gebleken, dat FDP-ase activiteit in geïsoleerde epitheelcellen inderdaad is aan te tonen. Ginsburg en Hers (1960) hadden reeds gevonden dat in rattedarm het enzym glucose-6-fosfatase ontbreekt. Dus wat is de rol van FDP-ase ? Temeer daar in de hoofdcellen van het epitheelweefsel geen significante gluconeogenese uit pyruvaat, lactaat of alanine aantoonbaar is (Lamers e.a., 1974). Is het misschien zo, dat de FDP-ase activiteit berust op een contaminatie van epitheelcellen met lymfocyten of spiercellen uit de lamina propria of terug te voeren is op gluconeogenese in de slijmbekercellen, terwijl in de hoofdcellen dit proces ontbreekt? Met andere woorden histochemie van FDP-ase zou over deze celheterogeniteit veel informatie kunnen leveren (zie appendix IV).

Bij dit onderzoek naar de localisatie van FDP-ase in darmweefsel is gebleken, dat de hoge activiteit van alkalische fosfatase het localisatie-beeld van de reactie sterk kan verstoren. Gebleken is dat alkalische fosfatase niet alleen het substraat FDP en het product fructose-6-fosfaat kan afbreken, maar

ook andere fosfaatesters zoals een specifieke remmer van FDP-ase het adenylzuur (AMP) en de co-factor $NADP^+$.

Omdat de afbraak van de co-factor $NADP^+$ in darmweefsel invloed kan hebben op histochemische bepalingen is tenslotte de localisatie van een aantal $NADP^+$-afhankelijke enzymen, in af- en aanwezigheid van een competitief substraat voor alkalische fosfatase getest (zie appendix V).

Bij deze histochemische onderzoekingen naar de localisatie van enzymen in de rattedarm is gebleken dat de betrouwbaarheid vaak te wensen overlaat en dat daarnaast het vergelijkend biochemisch onderzoek vereist is om uiteindelijk de gewenste informatie over enzymtopografie te verkrijgen.

HOOFDSTUK 2

ENZYMHISTOCHEMISCHE ONDERZOEKINGEN VAN DUNNE DARM EPITHEELCELLEN VAN DE RAT

2.1. OXYDATIEVE FOSFORYLERING EN HISTOCHEMIE.

In mitochondriënpreparaten van cellen, die volgens de methode van Harrison en Webster zijn geïsoleerd, blijkt dat gekoppeld met de oxydatie er wel degelijk fosforylering van ADP optreedt, maar dat de stimulatie van de ademhaling door toevoeging van ADP (ademhalingscontrôle) echter gering is (appendix I). Een dergelijke geringe afhankelijkheid van de snelheid van ademhaling van fosfaat en fosfaatacceptor zou op een isolatie-artefact kunnen berusten. Ook de latentie van de hydrolyse van ATP, toegevoegd aan deze mitochondriënpreparaten, getest door de stimulerende invloed van een ontkoppelaar van de oxydatieve fosforylering te onderzoeken, bleek gering (appendix I).

Aanwijzingen voor een intacte foforylering zijn echter verkregen uit histochemische experimenten (appendix III, fig. 5a en 5b). De mitochondriale Mg^{2+}-afhankelijke ATP-ase activiteit bleek slechts aan te tonen in aanwezigheid van de ontkoppelaar 2,4-dinitrofenol. Het reactieproduct anorganisch fosfaat wordt hierbij gelocaliseerd op de plaatsen waar veel mitochondriën aanwezig zijn. Doordat de spontane ATP-ase activiteit onder in situ omstandigheden niet van betekenis is, lijkt het inderdaad mogelijk dat bij isolatie van mitochondriën uit homogenaten van rattedarmepitheel, er beschadiging optreedt, waardoor de latentie van de biochemisch gemeten ATP-ase verloren gaat. Uit biochemische experimenten is ook gebleken, dat er een hogere ademhalingscontrôle-index (dit is de mate van stimulatie van de ademhaling door toevoeging van ADP) aantoonbaar is, wanneer de metingen worden gedaan in een Mg^{2+}-vrij su-

crosemedium en in de aanwezigheid van de Mg^{2+}-chelator EDTA (de Jonge, 1973).

Uit deze histochemische en de genoemde biochemische experimenten (appendix I; de Jonge, 1973) is gebleken, dat er wel degelijk een goede koppeling van de oxydatieve fosforylering in darmmitochondriën van de rat aantoonbaar is en dat de aanvankelijk gevonden slechte koppeling van de oxydatieve fosforylering (Stanbury, 1961; Hübscher en Sherratt, 1962; Clark en Sherratt, 1967) aan isolatie-artefacten te wijten is.

2.2. LOCALISATIE VAN MITOCHONDRIAAL GLYCEROL-3-FOSFAAT DEHYDROGENASE EN SUCCINAAT DEHYDROGENASE.

Nadat gebleken was dat er een goede koppeling van de oxydatieve fosforylering in de epitheelcellen is aan te tonen, zijn een aantal deelprocessen, die betrokken zijn bij de fosforylering van ADP tot ATP, eveneens onderzocht.

Door vergelijking van villus- en cryptmitochondriën is tevens na te gaan of er differentiatie van mitochondriale enzymen optreedt. Dit probleem zou door biochemisch en enzymhistochemisch onderzoek naar de acitivteit en de localisatie van de mitochondriale enzymen succinaat dehydrogenase en glycerol-3-fosfaat dehydrogenase gemakkelijk geverifiëerd kunnen worden. Zowel mitochondriaal glycerol-3-fosfaat dehydrogenase als succinaat dehydrogenase zijn histochemisch met de Nitro-BT-methode bepaald:

$$\text{glycerol-3-fosfaat} + \tfrac{1}{2}\,\text{Nitro-BT}^{2+} \longrightarrow \text{dihydroxy-acetonfosfaat} + \tfrac{1}{2}\,\text{Nitro-BTH}_2 + H^+$$

$$\text{succinaat} + \tfrac{1}{2}\,\text{Nitro-BT}^{2+} \longrightarrow \text{fumaarzuur} + \tfrac{1}{2}\,\text{Nitro-BTH}_2 + H^+$$

Bij deze bepalingen behoeven geen co-factoren te worden toegevoegd, omdat de reductie-äquivalenten van de dehydrogenasen direct (eventueel via co-enzym Q) op Nitro-BT kunnen worden overgedragen.

Glycerol-3-fosfaat dehydrogenase wordt vooral aangetroffen in de epitheelcellen van de villus, zowel aan de basale als aan de apicale zijde van de kern. Succinaat dehydrogenase blijkt zowel in de crypt- als in de villusepitheelcellen gelocaliseerd te zijn. In het bovenste derde deel van de villus is de activiteit van succinaat dehydrogenase voornamelijk aan de basale zijde van de kern aan te treffen, terwijl op het overige gedeelte van de villus aan

weerszijden van de kern reactieproduct wordt gevormd. Bij beide histochemische kleuringen zijn ook sprongsgewijze toenamen van de activiteiten van beide enzymen op de overgang van crypt naar villus geobserveerd. Deze sprongsgewijze toenamen zouden kunnen wijzen op een vermeerdering van het aantal mitochondriën in deze zône, waardoor gedeeltelijk het activiteitsverschil tussen crypt en villus kan worden verklaard.

Het histochemisch onderzoek (appendix II) leek in overeenstemming met de eerder gedane biochemische observaties van relatief grote activiteitsverschillen tussen crypt en villus (appendix II; Iemhoff en Hülsmann, 1971). Uit de biochemische experimenten is verder gebleken, dat er o.a. voor succinaat dehydrogenase en glycerol-3-fosfaat dehydrogenase verschillende verhoudingen van activiteiten tussen villus en crypt bestaan (appendix II, tabel I). Bij latere experimenten is echter gebleken dat de hier weergegeven verhoudingen aan de hoge kant zijn, hoewel het verschil in activiteit van glycerol-3-fosfaat dehydrogenase tussen crypt en villus ook bij deze bepalingen groter is dan van succinaat dehydrogenase (Iemhoff en Hulsmann, 1971). Een deel van de verschillen blijkt een gevolg te zijn van een later gedane observatie (de Jonge en Hülsmann, 1973), dat door de geringe eiwitopbrengsten de gebruikte cryptfracties meer verdund waren en dit mogelijk zou hebben bijgedragen tot relatief snelle denaturatie van deze fracties en zodoende tot de soms zo grote verschillen in crypt/villus-activiteitsverhoudingen. Ook wanneer de cryptmitochondriën labieler zouden zijn dan die van de villus, bestaat de mogelijkheid dat hierdoor de activiteitsverhouding kan worden beïnvloed. De activiteit van succinaat dehydrogenase lijkt in de cellen van de top van de villus, vooral aan de lumenzijde, weer af te nemen. Indien dit juist zou zijn en niet zou berusten op een verdringing van mitochondriën naar de basale zijde van de cel, door bijvoorbeeld apicale vetophoping (González-Licea, 1971) dan zou de activiteitstoename op de overgang van crypt naar villus biochemisch onderschat kunnen worden, omdat biochemisch een gemiddelde activiteit van alle villuscellen wordt bepaald. De mogelijkheid bestaat, dat bij de eerder vermelde biochemische experimenten, tijdens het schoontrillen van de darm een aantal cellen van de top is weggewassen, waardoor hogere verhoudingen werden gemeten van villus/crypt-activiteit.

De hogere activiteit van glycerol-3-fosfaat dehydrogenase in de villus kan van betekenis zijn bij de resynthese van triglyceriden in de cel. Hoewel monoglyceriden de grootste bijdrage leveren tot de resynthese van triglyceriden, kan ook glycerol-3-fosfaat een bouwstof zijn (Kern en Borgström, 1965;

Clark en Sherratt, 1967; Higgins en Barrnett, 1971). Daarnaast kan glycerol-3--fosfaat dehydrogenase een rol spelen bij de extra-mitochondriale oxydatie van NADH (Bücher en Klingenberg, 1958) en/of bij de verwijdering van eventueel op te hopen glycerol-3-fosfaat (sequestratie van anorganisch fosfaat binnen de cel kan voor het metabolisme gevaarlijk zijn).

Uit deze histochemische en biochemische waarnemingen lijkt tevens, dat op de overgang van crypt naar villus, mitochondriale enzymsynthese of activatie zou plaats vinden. Voor een aantal andere, niet mitochondriale, enzymen was dit reeds aangetoond (Nordström e.a., 1968; Imondi e.a. 1969a en b en 1970; Galjaard, 1970).

2.3. HISTOCHEMISCH ONDERZOEK VAN DE INVLOED VAN MITOCHONDRIALE EIWITSYNTHESEREMMERS OP CYTOCHROOM-C-OXYDASE ACTIVITEIT.

Een ander proces, dat betrokken is bij de fosforylering van ADP tot ATP is de ademhalingsketen zelf. Om de bijdrage van de mitochondriën tot het metabolisme van de darm te onderzoeken werden o.a. ratten voorbehandeld met chlooramfenicol of oxytetracycline, remmers van de mitochondriale eiwitsynthese. Deze antibiotica remmen o.a. de synthese van cytochroom-c-oxydase (Kroon en Jansen, 1968; Firkin en Linnane, 1968). Deze enzymactiviteit is histochemisch te localiseren met de methode van Burstone (1959). Eveneens is de invloed van de antibiotica op de ATP-ase activiteit in aan- en afwezigheid van 2,4-dinitrofenol onderzocht. Zowel in de darm van de contrôle-rat als in die van een gedurende 48 uur met antibiotica voorbehandelde rat blijkt histochemisch vrijwel geen spontane ATP-ase activiteit aantoonbaar. Uit biochemische experimenten is ook gebleken dat na 48 uur behandeling, wanneer de activiteit van cytochroom-c-oxydase is gedaald tot 30% van de oorspronkelijke activiteit, nog steeds een goede koppeling van de fosforylering aan oxydatie aantoonbaar is (de Jonge, 1973). Ondanks de sterk verminderde cytochroomvoorraad blijken de mitochondriën nog steeds in staat te zijn de ATP-concentratie van de epitheelcellen op peil te houden (de Jonge, 1973).

Zoals boven reeds is aangegeven, is de localisatie van de mitochondriale cytochroom-c-oxydase synthese nagegaan. Voor dit onderzoek werden ratten gebruikt, die gedurende 12, 24 of 48 uur voorbehandeld waren met chlooramphenicol of oxytetracycline. De activiteit in de contrôle-darm is vergeleken met de activiteit in de darm van de behandelde rat.

Evenals voor de enzymen succinaat dehydrogenase en glycerol-3--fosfaat dehydrogenase is ook voor cytochroom-c-oxydase een sprongsgewijze toename van activiteit te zien op de overgang van crypt naar villus. De invloed van de antibiotica is in de histochemische experimenten na 15 uur behandeling, behalve aan een verlaagde cryptactiviteit, zichtbaar aan het ontbreken van de activiteitstoename op de overgang van crypt naar villus. Dit wijst op een netto synthese van cytochroom-c-oxydase op deze plaats. Omdat tijdens de behandeling de epitheelcellen van de basis van de villus naar een hogere celpositie zijn opgeschoven is nu een activiteitstoename hoger op de villus zichtbaar.

Dat de enzymsynthese niet alleen is beperkt tot de delende celpopulatie en de cellen op de overgang van crypt naar villus, blijkt uit de histochemische waarnemingen, waar na een behandeling van 15 uur, ook in de top van de villus minder activiteit van cytochroom-c-oxydase wordt gevonden in vergelijking met een onbehandelde contrôle. Dit blijkt ook uit biochemische bepalingen van de activiteitsverhoudingen tussen villus en crypt van cytochroom-c-oxydase na 12, 24 en 48 uur voorbehandeling, waarvoor vergelijkbare waarden zijn gevonden (de Jonge en Hülsmann, 1973). Dit wijst op een remming van enzymsynthese zowel in crypt als villus. Gijzel e.a. (1972) isoleerden naast elkaar fracties van het bovenste en het onderste deel van de villus en toonden na 16 uur behandeling al een afname van cytochroom-c-oxydase in de bovenste fractie aan, waaruit eveneens blijkt dat ook in de villus cytochroom-c-oxydase synthese mogelijk is.

Errata

In fig. 2a-d (appendix III) zijn de activiteiten van cytochroom-c-oxydase na verschillende behandelingsperioden weergegeven. Deze opnamen zijn gemaakt met een automatische camera, waarbij de openingstijd van de sluiter wordt bepaald door de hoeveelheid licht, die op een ingebouwde fotometer valt. Hierdoor worden intensiteitsverschillen tussen preparaten met een hoge en een lage activiteit genivelleerd. Dit resulteerde in foto's, die in tegenstelling tot de licht-microscopische beelden vergelijkbare activiteiten weergeven. Bij de andere experimenten werden de opnametijden gelijk gehouden, waardoor de verschillen in enzymactiviteiten in de juiste verhouding worden weergegeven.

2.4. VERGELIJKEND HISTOCHEMISCH ONDERZOEK VAN FRUCTOSE-1,6-DIFOSFAAT 1-FOSFATASE.

De motivering van histochemisch onderzoek naar de localisatie van fructose-1,6-difosfaat 1-fosfatase, één van de sleutelenzymen van het gluconeogeneseproces, werd reeds in de inleiding uiteengezet. Andere processen waarbij dit enzym (FDP-ase) een rol kan spelen zijn de complete oxydatie van hexosemonofosfaat met behulp van pentose-fosfaat-shunt enzymen, alsmede de regulatie van de glycolyse (Opie en Newsholme, 1967; Newsholme en Crabtree, 1970). Omdat uit biochemisch onderzoek (van Tol, ongepubliceerde waarnemingen) is gebleken, dat de activiteit van FDP-ase in de preparaten van geïsoleerde darmcellen laag is in verhouding tot die van leverhomogenaten, werd de mogelijkheid niet uitgesloten geacht dat de gemeten enzymactiviteit te wijten was aan activiteit van weefselbestanddelen, anders dan de hoofdcellen van het dunne darm epitheel (zie inleiding). Om hierover uitsluitsel te geven is een histochemisch onderzoek gedaan naar de localisatie van FDP-ase in darmweefsel.

FDP-ase in de darm is een enzym, dat, evenals in andere weefsels, in de oplosbare fractie van de cel is gelocaliseerd (van Tol, niet gepubliceerde waarneming). Hierdoor kan het enzym gemakkelijk tijdens de incubatie in het incubatiemedium weglekken. Door de membraanmethode van Meijer (1972) toe te passen, kan dit weglekken worden voorkomen, doordat bij deze methode de coupe van het incubatiemedium wordt gescheiden door een semi-permeabele membraan. Deze methode is toegepast bij de histochemische kleuring door middel van Nitro-BT-reductie.

Fructose-1,6-difosfaat afhankelijke fosfaatbepaling.

FDP-afhankelijke fosfaatvorming in darmweefsel werd door Dempsey en zijn medewerkers in 1949 histochemisch aangetoond, voornamelijk in de borstelzoomzône van het epitheelweefsel. Hierbij werd de methode van Gomori (1939) voor de detectie van anorganisch fosfaat gebruikt. De mogelijkheid werd niet uitgesloten geacht, dat een groot deel van het gevormde reactieproduct afkomstig zou kunnen zijn van de in de borstelzoom aanwezige hoge activiteit van alkalische fosfatase. Daarom leek het noodzakelijk de specificiteit van de reactie te testen, door bij de histochemische kleuring de incubatie uit te voeren in af- en aanwezigheid van de specifieke remmer van FDP-ase, het adenylzuur (AMP).

FDP-ase activiteit is bepaald volgens de methode van Wachstein en Meisel (1957), waarbij het bij de enzymreactie vrijkomende anorganische fosfaat met loodionen (i.p.v. met cobaltionen volgens de methode van Gomori) wordt neergeslagen als loodfosfaat (appendix IV). Na de incubatie wordt dit neerslag omgezet in loodsulfide. Omdat FDP-ase door loodionen wordt geremd (Underwood en Newsholme, 1965), zijn deze vervangen door mangaanionen. Het gevormde mangaanfosfaat wordt vervolgens omgezet in loodfosfaat en tenslotte in loodsulfide.

Vanwege de lage Km-waarde voor FDP-ase in darmcelhomogenaten (4 µM: van Tol, niet gepubliceerde waarneming) is aanvankelijk de substraatconcentratie tijdens de incubatie laag gehouden. De activiteit is dan echter gering. Bovendien blijkt in de blanco-reactie, waarbij geen FDP aan het incubatiemedium is toegevoegd, reeds een neerslag (mogelijk door fosfaat in de coupe) op te treden. Bij een verhoging van de FDP-concentratie, boven 10 maal de Km-waarde, blijkt nog steeds meer reactieproduct te worden gevormd. Dit moet worden toegeschreven aan andere dan FDP-specifieke fosfatase activiteiten. AMP, een specifieke remmer van FDP-ase blijkt bovendien geen vermindering, maar juist een toename van de fosfaatvorming te geven. AMP zelf is een substraat voor andere fosfatase reacties (zoals alkalische fosfatase, zure fosfatase of 5'-nucleotidase) en kan om deze reden niet als specifieke remmer bij deze bepaling worden gebruikt. Bij de FDP-ase kleuring volgens de methode van Wachstein en Meisel (1957) is EDTA toegevoegd aan het incubatiemedium (bij overmaat Mn^{2+}). Uit biochemische bepalingen is gebleken, dat de activiteit van alkalische fosfatase onderdrukt werd door binding van het voor de reactie noodzakelijke Zn^{2+}. Toevoeging van EDTA geeft echter in de histochemische bepaling, in een concentratie van 5 mM, nog steeds onvoldoende remming van alkalische fosfatase activiteit.

Door bovengenoemde factoren is het in de darm niet mogelijk, op deze wijze, FDP-specifieke hydrolase activiteit aan te tonen.

Fructose-1,6-difosfaat afhankelijke Nitro-BT-reductie in darmweefsel.

Een andere methode om FDP-ase activiteit aan te tonen, is de methode waarbij gebruik gemaakt wordt van de volgreacties, die door respectievelijk glucose-6-fosfaat isomerase en glucose-6-fosfaat dehydrogenase worden gekatalyseerd (Olson en Marquardt, 1972);

d-fructose-1,6-difosfaat + H_2O ⟶ D-fructose-6-fosfaat + Pi
(fructose-1,6-difosfaat 1-fosfatase)

d-fructose-6-fosfaat ⟶ D-glucose-6-fosfaat
(glucose-6-fosfaat isomerase)

d-glucose-6-fosfaat + $NADP^+$ ⟶ D-glucose-δ-lacton 6-fosfaat + NADPH + H^+
(glucose-6-fosfaat dehydrogenase)

NADPH + ½ Nitro-BT^{2+} ⟶ NAD^+ + ½ Nitro-BTH_2
(NADPH:Nitro-BT oxydoreductase).

Aangezien bij de membraanmethode geen enzymen kunnen worden toegevoegd, kan deze methode pas worden gebruikt wanneer glucose-6-fosfaat isomerase en glucose-6-fosfaat dehydrogenase in relatieve overmaat in het weefsel zelf aanwezig zijn. Dit werd geverifiëerd door in plaats van FDP, fructose-6-fosfaat, als substraat aan te bieden en de formazaanproductie na te gaan.

Bij de FDP-ase bepaling moet bovendien rekening worden gehouden met een mogelijke afbraak van de co-factor $NADP^+$, waarbij NAD^+ wordt gevormd. NAD^+ is slechts in katalytische hoeveelheden nodig om de glyceraldehyde-3-fosfaat dehydrogenase reactie te doen verlopen, waardoor eveneens een FDP-afhankelijke formazaanproductie mogelijk wordt. Deze reacties zijn de volgende:

fructose-1,6-difosfaat ⟶ glyceraldehyde-3-fosfaat + dihydroxyacetonfosfaat
(fructose-1,6-difosfaat aldolase)

glyceraldehyde-3-fosfaat + NAD^+ + Pi ⟶ 1,3-difosfoglyceraat + NADH + H^+
(glyceraldehyde-3-fosfaat dehydrogenase; te remmen met monojoodazijnzuur)

NADH + ½ Nitro-BT^{2+} ⟶ NAD^+ + ½ Nitro-BTH_2.
(NADH:Nitro-BT oxydoreductase)

of $NADH + H^+ +$ dihydroxy-acetonfosfaat $\longrightarrow$ glycerol-3-fosfaat + NAD^+
(glycerol-3-fosfaat dehydrogenase)

glycerol-3-fosfaat + $\frac{1}{2}$ Nitro-BT^{2+} $\longrightarrow$ dihydroxy-acetonfosfaat + $\frac{1}{2}$ Nitro-BTH_2 + H^+
(glycerol-3-fosfaat:Nitro-BT oxydoreductase).

De NADH-vorming leidt eveneens tot reductie van Nitro-BT.

Deze processen zijn dus mogelijk wanneer $NADP^+$ wordt gehydrolyseerd [$NADP^+ + H_2O \longrightarrow NAD^+ + Pi$ (fosfatase)], of wanneer voldoende endogeen NAD^+ aanwezig is.

Door de hoge activiteiten van alkalische en zure fosfatase in darmepitheel zal hydrolyse van $NADP^+$ tijdens de incubatie op kunnen treden. Omdat in darmcelpreparaten een hoge glycolyse-snelheid is te meten (vgl. appendix I), zal bovengenoemd oxydatieproces hier eveneens kunnen leiden tot Nitro-BT-reductie.

Ook de FDP-afhankelijke formazaanproductie moet specifiek door AMP kunnen worden geremd. Echter bij toepassing van de Nitro-BT-methode blijkt in darmweefsel een AMP-afhankelijke formazaanvorming op te treden. Deze wordt waarschijnlijk veroorzaakt door afbraak van AMP tot adenosine door alkalische of zure fosfatase en/of 5'-nucleotidase. Adenosine kan verder worden geoxydeerd tot urinezuur door adenosine deaminase en xanthine oxydase, die in darmcellen een vrij hoge activiteit hebben, hetgeen eveneens tot formazaanvorming leidt. De volgende reacties spelen hierbij een rol:

AMP + H_2O $\longrightarrow$ adenosine + Pi
(5'-nucleotidase)

adenosine + H_2O $\longrightarrow$ inosine + NH_3
(adenosine deaminase)

inosine + Pi $\longrightarrow$ hypoxanthine + D-ribose 1-fosfaat
(purine nucleoside fosforylase)

hypoxanthine + H_2O + O_2 + FAD $\longrightarrow$ xanthine + H_2O_2 + $FADH_2$
(xanthine oxydase)

xanthine + H_2O + O_2 + FAD $\longrightarrow$ urinezuur + H_2O_2 + $FADH_2$
(xanthine oxydase)

Omdat dit FAD eveneens in staat is om Nitro-BT te reduceren is het niet mogelijk om op deze wijze, met behulp van AMP-remming, de specificiteit van FDP-ase te testen in weefsels (zoals darmweefsel), waarin FDP-ase in vergelijking met deze AMP-afbrekende reacties een lage activiteit heeft.

In de lever wordt AMP zeker ook in urinezuur omgezet, zodat xanthine oxydase een andere bijdrage tot de blanco Nitro-BT-reductie kan leveren. Sackler (1966) vond bij biochemische bepalingen in de lever echter een 5 maal zo lage activiteit dan in de darm. Omdat xanthine oxydase in het cytoplasma is gelocaliseerd, is de kans op weglekken tijdens de incubatie erg groot. Dit kan de reden zijn geweest, dat Sackler histochemisch geen activiteit van xanthine oxydase in lever kon aantonen. Met de membraanmethode moet echter toch rekening met deze enzymactiviteit worden gehouden. Wanneer deze AMP-afhankelijke formazaanvorming niet in ogenschouw zou worden genomen, zou de remming van FDP-ase door AMP kunnen worden onderschat.

In lever waar lagere alkalische fosfatase en xanthine oxydase activiteiten aanwezig zijn en bovendien een lagere verhouding is van aldolase/FDP-ase activiteiten, blijkt de FDP-afhankelijke formazaanproductie wel specifiek door AMP te kunnen worden geremd (vgl. Fig. 2a en b, appendix IV).

In hart en rode spier, waarin biochemisch vrijwel geen FDP-ase activiteit kan worden aangetoond (Opie en Newsholme, 1967), blijkt AMP de vorming van formazaan niet specifiek te kunnen remmen. In deze weefsels bestaat een hogere verhouding tussen de activiteit van aldolase en FDP-ase. Omdat AMP hier kan worden vervangen door een willekeurige fosfaatester berust in deze weefsels het effect van AMP niet op remming van de FDP-ase, maar op competitieve remming van AMP op de afbraak van $NADP^+$ tot NAD^+ door fosfatasen (zoals alkalische fosfatase en zure fosfatase). 5'-Nucleotidase blijkt geen effect op deze splitsing te hebben (Meijer, persoonlijke mededeling).

Conclusie

In darmweefsel moet de activiteit van FDP-ase specifiek worden getest, omdat immers FDP eveneens kan worden afgebroken door de hier aanwezige hoge fosfatase activiteiten. Dit brengt problemen met zich mee, omdat AMP zowel bij de fosfaatkleuring, als bij de Nitro-BT-methode zelf, een substraat is voor de reacties, die tot vorming van neerslag leiden. In darmweefsel is het daarom niet mogelijk om met bovengenoemde methoden FDP-ase activiteit specifiek aan te tonen. In lever is, door de lagere activiteit van alka-

lische fosfatase en de hogere FDP-ase activiteit, met de Nitro-BT-methode wel specifieke FDP-afhankelijke formazaanproductie aantoonbaar. Door de relatief hoge activiteit van aldolase in spierweefsel, moet aan het incubatiemedium monojoodazijnzuur worden toegevoegd. Hierdoor wordt de activiteit van glyceraldehyde-3-fosfaat dehydrogenase sterk geremd. FDP-ase activiteit blijkt op bovengenoemde methoden in spierweefsel echter niet aantoonbaar.

2.5. LOCALISATIE VAN EEN AANTAL $NADP^+$-AFHANKELIJKE ENZYMEN OF ENZYMSYSTEMEN.

In de inleiding is de motivering van dit onderzoek reeds kort weergegeven. Gekozen werd een onderzoek naar de localisatie van een aantal enzymen, die direct of indirect bij het koolhydraat-metabolisme betrokken zijn, zoals glucose-6-fosfaat dehydrogenase, hexokinase en malaat dehydrogenase (decarboxylerend) ($NADP^+$). Bij de localisatie van de genoemde oxydoreductasen en bij hexokinase, waarbij het product glucose-6-fosfaat door middel van glucose-6-fosfaat dehydrogenase wordt gedetecteerd, is de Nitro-BT-reductiemethode toegepast. Bij de localisatie van enzymen, waarvan NAD^+ of $NADP^+$ als co-factor wordt gereduceerd, wordt meestal van de diaforase-reactie gebruik gemaakt, waarbij de reductie-equivalenten door respectievelijk NADH:Nitro-BT oxydoreductase en NADPH:Nitro-BT oxydoreductase worden overgedragen op Nitro-BT:

$$NAD(P)H + \tfrac{1}{2}\ \text{Nitro-BT}^{2+} \longrightarrow NAD(P)^+ + \tfrac{1}{2}\ \text{Nitro-BTH}_2.$$

Als diaforasen kunnen diverse flavoproteinen, zoals NADH:cytochroom-c oxydoreductase en NADPH:cytochroom-c oxydoreductase, fungeren. Zie verder appendix V.*

Uit biochemische experimenten is gebleken, dat alkalische fosfatase (bereid uit kalfsdarm) ook in staat is om NADPH af te breken tot NADH. Een dergelijke reactie is eveneens mogelijk bij een histochemische kleuring, omdat in de coupe ook alkalische fosfatase aanwezig is. Bij de histochemische kleuring zal nu niet alleen de localisatie van NADPH- maar ook die van NADH:Nitro-BT oxydoreductase worden aangetoond. Hierdoor wordt de indruk verkregen, dat de localisatie van NADPH-diaforase minder beperkt is, dan in wezen het geval is. Door toevoeging van een competitieve remmer van alkalische fos-

* In het artikel werd uiteengezet dat acyl-CoA dehydrogenase, ademhalingsketen NADH dehydrogenase en flavoproteinen betrokken bij de citroenzuurcyclus etc. ook NADH-diaforasen zijn.

fatase kan de afbraak van NADPH worden onderdrukt, zodat in aanwezigheid van de remmer voornamelijk de localisatie van NADPH:Nitro-BT oxydoreductase zal worden weergegeven (vgl. Fig. 1a en b, appendix V). Deze activiteit blijkt hoofdzakelijk aan de lumenzijde van de villus onder de bostelzoom gelocaliseerd te zijn. De activiteit van NADPH:Nitro-BT oxydoreductase is laag t.o.v. de som van NADH:Nitro-BT oxydoreductase activiteiten.

Wanneer geen rekening wordt gehouden met de beperkte localisatie van NADPH-diaforase, kan voor de localisatie van enzymen, waarbij van deze reactie specifiek als volgreactie gebruik wordt gemaakt, de indruk worden verkregen, dat de localisatie van het bestudeerde enzym samenvalt met die van NADPH-diaforase. Dit blijkt bijvoorbeeld uit de histochemische kleuring op hexokinase en glucose-6-fosfaat dehydrogenase.

Localisatie van hexokinase en glucose-6-fosfaat dehydrogenase

Indien naast de co-factor $NADP^+$ een substraat (bijvoorbeeld glucose of glucose-6-fosfaat) wordt toegevoegd, zodat langs directe weg slechts NADPH en niet NADH kan worden gevormd, zal het betrokken enzym alleen Nitro-BT-diformazaan doen ontstaan waar NADPH:Nitro-BT oxydoreductase voorkomt. Glucose-6-fosfaat dehydrogenase en hexokinase worden dan ook slechts aangetoond vlak onder de borstelzoom (van den Berg en Hülsmann, 1972), aangezien hier NADPH:Nitro-BT oxydoreductase is gelocaliseerd (appendix V). Door de formazaanvorming onafhankelijk van deze reacties te laten verlopen, zoals bij het gebruik van fenazinemethosulfaat als plaatsvervangende electronenoverdrager, is de juiste localisatie van glucose-6-fosfaat dehydrogenase te bepalen in darmweefsel (vgl. Fig. 2a-d, appendix V). Uit dit onderzoek volgt dat glucose-6-fosfaat dehydrogenase zowel in de villus als in de crypt is gelocaliseerd en niet beperkt is tot de zône van de borstelzoom. De van glucose-6-fosfaat-dehydrogenase-afhankelijke hexokinase-kleuring laat zien dat ook hexokinase zowel in de villus als in de crypt is gelocaliseerd.

Localisatie van malaat dehydrogenase (decarboxylerend) ($NADP^+$)

Tijdens de incubatie kan alkalische fosfatase toegevoegd $NADP^+$ ook afbreken tot NAD^+, hetgeen eveneens aanleiding kan geven tot verkeerde interpretaties van de verkregen resultaten. Dit kan voorkomen bij reacties waarbij

een substraat is betrokken, dat zowel door de co-factor $NADP^+$ als NAD^+ kan worden geoxydeerd, zoals malaat. Voor de juiste localisatie van de $NADP^+$-afhankelijke reactie moet de omzetting van $NADP^+$ in NAD^+ door toevoeging van een overmaat van een andere fosfaatester competitief worden geremd (vgl. Fig. 3a en b, appendix V). In afwezigheid van een competitieve remmer lijken de NAD^+- en de $NADP^+$-afhankelijke malaat dehydrogenasen ongeveer even actief te zijn. In aanwezigheid van een competitieve remmer echter blijkt de $NADP^+$-afhankelijke reactie veel minder actief dan de NAD^+-afhankelijke malaat dehydrogenase reactie. Bij biochemische bepaling van deze enzymen in de darm van de rat, blijkt inderdaad het NAD^+-afhankelijke enzym een ongeveer 11 maal zo grote activiteit dan het $NADP^+$-afhankelijke decarboxylerende malaat dehydrogenase te vertonen (Hülsmann, niet gepubliceerde waarneming).

Conclusie

Bij een histochemische reactie, waarbij een fosfaatester is betrokken, moet derhalve, zeker in de darm, met de aanwezigheid van hoge, niet substraat-specifieke fosfatasen activiteiten, rekening worden gehouden met een mogelijke afbraak van deze ester. Hiermede rekeninghoudend blijkt uit de experimenten dat de enzymen, hexokinase, glucose-6-fosfaat dehydrogenase en malaat dehydrogenase (decarboxylerend) ($NADP^+$) zowel in de crypt als in de villus zijn gelocaliseerd.

Onder in vivo omstandigheden zal alkalische fosfatase van de borstelzoom geen belangrijke rol spelen bij de afbraak van $NADP^+$ of NADPH. Het enzym NADPH:Nitro-BT oxydoreductase is volgens Pearse (1972) te vergelijken met NADPH:cytochroom-c oxydoreductase. Uit biochemisch onderzoek is gebleken dat NADPH:cytochroom-c oxydoreductase aanwezig is in de microsomale fractie van de cel, die hoofdzakelijk bestaat uit fragmenten van het endoplasmatisch reticulum. Uit cytologische waarnemingen (o.a. Toner, 1968) is verder bekend, dat het endoplasmatisch reticulum voornamelijk gelocaliseerd is aan de apicale kant van de villusepitheelcel. Wanneer bovengenoemde enzymen (NADPH:Nitro-BT oxydoreductase en NADPH:cytochroom-c oxydoreductase) inderdaad identiek zouden zijn, kan dit betekenen dat in de villus NADPH beter kan worden geoxydeerd dan in de crypt, waardoor ook de oxydatieprocessen waarbij deze co-factor betrokken is in het apicale gedeelte van de villusepitheelcellen goed zullen kunnen verlopen.

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af- en aanwezigheid van de ontkoppelaar van de oxydatieve fosforylering, dinitrofenol. Het bleek (appendix III), dat in de darm dinitrofenol de Mg^{2+}-ATP-ase sterk stimuleert, zodat verwacht mag worden, dat in situ de oxydatieve fosforylering goed gekoppeld is.

Hoofdstuk 2.2 (appendix II) vergelijkt o.a. de enzymhistochemische activiteit van de mitochondriale enzymen glycerol-3-fosfaat dehydrogenase en succinaat dehydrogenase (barnsteenzuur dehydrogenase) in crypten en villi. De villus/crypt-activiteitsverhouding blijkt voor glycerol-3-fosfaat dehydrogenase groter te zijn dan voor succinaat dehydrogenase. Voor beide enzymen is echter enzymhistochemisch een toename van activiteit aan te tonen op de overgang van crypt naar villus, waaruit blijkt, dat op deze plaats van deze mitochondriale enzymen een netto verschil in synthese of activatie optreedt.

Voor de nadere bestudering van de rol, die de ademhalingsketen speelt bij de energielevering en voor de bestudering van de mitochondriale eiwitsynthese, zijn naast biochemische bepalingen, enzymhistochemische experimenten gedaan met proefdieren, die met oxytetracycline of chlooramfenicol werden behandeld (Hoofdstuk 2.3., appendix III). Deze antibiotica remmen de mitochondriale synthese van de cytochromen aa_3, b en c_1. Uit vergelijkende experimenten met contrôle en korte tijd behandelde dieren (15-48 uur) blijkt, dat bij 80% remming van cytochroom-c-oxydase synthese nog voldoende cytochroomvoorraad aanwezig is, om de koppeling van de oxydatieve ademhaling aan het fosforyleringsproces te handhaven. Tevens blijkt uit de experimenten met mitochrondiale eiwitsyntheseremmers, dat er behalve in de delende cellen in de crypten, ook synthese plaats vindt in de functionerende cellen van de villi. In de cellen aan de top van de villus is immers, reeds na 15 uur behandeling, afname van cytochroom-c-oxydase activiteit waar te nemen.

Naast de bovengenoemde mitochondriale enzymen is ook onderzoek gedaan naar de localisatie van het cytoplasmatische enzym fructose-1,6-difosfaat 1-fosfatase (FDP-ase) (Hoofdstuk 2.4., appendix IV). In homogenaten van epitheelweefsel, afkomstig van de rattedarm, werd van dit enzym activiteit gemeten. De functie van dit enzym in de rattedarm is niet duidelijk, omdat het proces waarbij dit enzym (althans in andere weefsels) een belangrijke rol speelt (de gluconeogenese) in de hoofdcellen van darmweefsel mogelijk niet belangrijk is, doordat glycogeen in deze cellen niet of nauwelijks aantoonbaar is. De mogelijkheid werd daarom niet uitgesloten, dat de gemeten activiteit afkomstig is van andere cellen dan van de hoofdcellen van het epitheelweefsel, of van een contaminatie van de epitheelcellen met andere cellen, zoals spiercellen en

lymfocyten. Om hierover uitsluitsel te geven is een enzymhistochemisch onderzoek naar de localisatie van dit enzym gedaan. Bij voorkeur wordt de methode gebruikt, waarbij het product fructose-6-fosfaat enzymatisch wordt omgezet in glucose-6-fosfaat, dat via de glucose-6-fosfaat dehydrogenase reactie Nitro-BT in formazaan kan omzetten.

Vanwege de hoge activiteit van alkalische fosfatase in de darm, werd het noodzakelijk geacht de specificiteit van de enzymreactie te testen. Een specifieke remmer van FDP-ase is het adenosine-5'-monofosfaat (AMP). In de lever is de activiteit van FDP-ase volgens biochemische experimenten veel hoger dan in de darm, terwijl de activiteit van alkalische fosfatase in de lever lager is dan in de darm. In leverweefsel blijkt dan ook langs histochemische weg, dat FDP-ase wel degelijk te remmen is door toevoeging van AMP. Dit is voor darmweefsel echter niet aan te tonen, omdat de hoge alkalische fosfatase activiteit niet alleen het substraat FDP, doch ook de remmer AMP hydrolyseert. In spierweefsel werd de histochemische reactie ook getest, omdat in dit weefsel weinig alkalische fosfatase voorkomt en FDP-ase, langs biochemische weg bepaald, zeer gevoelig is voor AMP-remming. Desalniettemin blijkt bij histochemie van dit weefsel de FDP-afhankelijke reactie te worden gestoord door de aanwezigheid van sterke fructose-difosfaat aldolase en glyceraldehyde-3-fosfaat dehydrogenase activiteiten. Slechts een geringe afbraak van de toegevoegde co-factor nicotinamide-adenine dinucleotide fosfaat ($NADP^+$) blijkt slechts nodig om, langs de door aldolase gestimuleerde weg, gereduceerd nicotinamide-adenine dinucleotide (NADH) te vormen, zodat op alternatieve wijze Nitro-BTdiformazaan wordt gevormd. In spierweefsel wordt deze laatste vorming dan ook sterk geremd door toevoeging van monojoodazijnzuur, dat een bekende remmer van glyceraldehyde-3-fosfaat dehydrogenase is. Toevoeging van een competitieve remmer voor de niet substraat-specifieke fosfatase reactie geeft een vergelijkbaar beeld te zien.

Omdat de hoge activiteit van alkalische fosfatase in darmweefsel de FDP-ase bepaling stoorde, is ook de invloed van alkalische fosfatase op andere enzymhistochemische bepalingen, waarbij eveneens fosfaatesters zijn betrokken, nagegaan. Deze zijn: NADPH:Nitro-BT oxydoreductase, hexokinase, glucose-6-fosfaat dehydrogenase en malaat dehydrogenase (decarboxylerend) ($NADP^+$), (Hoofdstuk 2.5., appendix V). Toevoeging van een overmaat van een substraat voor alkalische fosfatase, zoals β-glycerolfosfaat of p-nitrofenylfosfaat, aan het incubatiemedium kan de afbraak van andere fosfaatesters, die direct betrokken zijn bij de enzymreactie (nicotinamide-adenine dinucleotide

substraten of co-factoren), competitief remmen.

De enzymhistochemische bepaling van hexokinase is gedaan met behulp van de volgreactie, die gestimuleerd wordt door glucose-6-fosfaat dehydrogenase, waarbij $NADP^+$ wordt gereduceerd. Oxydatie van deze beperkte hoeveelheid NADPH door NADPH:Nitro-BT oxydoreductase kan leiden tot Nitro-BT-reductie. Aanvankelijk werd alleen reactieproduct gevonden aan de lumenzijde van de epitheelcellen van de villus. Dit beeld is te verwachten wanneer hexokinase of glucose-6-fosfaat dehydrogenase gelocaliseerd is in het gebied vlak onder de borstelzoom van de villus, óf wanneer de localisatie van NADPH:Nitro-BT oxydoreductase is beperkt tot dit deel van het epitheelweefsel. Toevoeging van een plaatsvervangende electronenoverdrager, zoals fenazinemethosulfaat, waardoor Nitro-BT zonder de NADPH:Nitro-BT oxydoreductase reactie kan worden gereduceerd, geeft echter een meer uitgebreide localisatie en een hogere activiteit te zien van hexokinase (Hoofdstuk 2.5., appendix V). Hieruit blijkt, dat de beperkte localisatie, die gevonden wordt in afwezigheid van fenazinemethosulfaat, wordt veroorzaakt door de beperkte localisatie van NADPH:Nitro-BT oxydoreductase. Wanneer NADPH niet in een lage concentratie geregenereerd wordt, zoals in de hexokinase bepaling, maar als substraat in overvloed direct aanwezig is, is de localisatie van het gevormde formazaan echter niet beperkt tot het gebied onder de borstelzoom van de villus. Bij deze hoge concentraties van NADPH kan voldoende NADH worden gevormd door alkalische fosfatase, zodat naast de NADPH- de NADH:Nitro-BT oxydoreductase reactie gaat verlopen. De localisatie van NADH:Nitro-BT oxydoreductase is namelijk niet beperkt tot de lumenzijde van de villuscellen, maar komt verspreid over de villus- en cryptcellen aan de lumen- en aan de basiszijde van de cel voor. In afwezigheid van een competitieve remmer voor de alkalische fosfatase reactie kunnen dus de NADPH- en de NADH:Nitro-BT oxydoreductase reacties beide verlopen, waardoor een verkeerde indruk van de localisatie wordt verkregen (Hoofdstuk 2.5., appendix V).

Wanneer het $NADP^+$-specifieke decarboxylerende malaat dehydrogenase histochemisch in de darm wordt bestudeerd, wordt de illusie verkregen, dat het enzym in vergelijking met het NAD^+-specifieke malaat dehydrogenase redelijk actief is (Hoofdstuk 2.5., appendix V). Bij biochemische bepalingen echter blijkt het eerste enzym een activiteit te hebben, die een grootte-orde lager ligt dan die van het laatste enzym. Ook dit verschil blijkt nu weer op een enzymhistochemisch artefact te berusten. Immers alkalische fosfatase kan $NADP^+$ afbreken tot NAD^+, zodat dan beide enzymreacties zullen verlopen. Bij gelijk-

tijdige aanwezigheid van een overmaat p-nitrofenylfosfaat of β-glycerolfosfaat, wordt $NADP^+$ gespaard en blijkt inderdaad, dat malaat dehydrogenase (decarboxylerend) ($NADP^+$) weinig actief is in vergelijking met malaat dehydrogenase (NAD^+).

Uit deze histochemische studies is gebleken, dat de enzymhistochemie zeker kan bijdragen tot de localisatie van metabole processen. Ook is echter gebleken, dat met de enzymhistochemische methode gemakkelijk artefacten zijn te introduceren, zodat het in het algemeen wenselijk is om naast de histochemische kleuringen biochemische bepalingen te verrichten. Wanneer de resultaten van beide onderzoek methoden met elkaar in overeenstemming zijn, is het mogelijk om nadere informatie te krijgen over het verloop van metabole processen in bepaalde weefsel- of celtypen.

SUMMARY

The study of metabolism in intestinal absorptive cells is of current biochemical interest. In our laboratory energy metabolism per se as well as certain aspects of the resorptive process are under study for the past 5 years. The intestinal absorptive cell is also cell-biologically of great interest. All sorts of developmental stages occur simultaneously: proliferating and maturating cells in the crypts and functional cells on the villi. The biochemical aspects of differentiation can be studied thanks to the possibility to isolate intact villous and crypt cells separately by the mechanical method of Harrison and Webster (Chapter I, appendix I). We were able to confirm that DNA synthesis occurs almost exclusively in the crypt cells, that brush border activities such as invertase occur almost exclusively in the villi and that certain enzymes involved in the generation of adenosine-5'-triphosphate (ATP) are almost equally present in villi and crypts. Some developmental aspects could easily be verified enzyme histochemically. Enzyme histochemistry can be an important aid in the study of enzyme topography, so that this method was followed and, when possible, compared with biochemical data obtained in our laboratory with isolated crypt- and villous cells, or subcellular fractions the reof. Enzyme histochemistry often proved to be disappointing, partly due to its semiquantitive nature but mainly due to undesirable sidereactions and locazation of reactionproducts in areas of the cell distinct from those where the ratelimiting enzyme should be present. It seemed to us of importance to report as well the positive as the negative results in this thesis.

In chapter 2.1 (appendices I and III) a literature survey mentions the absence of a tight coupling between oxidation and phosphorylation in rat small intestinal mitochondria. Since the degree of tightness of coupling may be expressed as the degree of latency of the mitochondrial Mg^{2+}-ATPase this cri-

terium was tested enzyme histochemically by comparing the ATPase activity in the absence and in the presence of an uncoupler of oxidative phosphorylation such as 2,4-dinitrophenol. It appeared (appendix III) that in rat small intestine 2,4-dinitrophenol sharply stimulates the Mg^{2+}-ATPase, so that in situ oxidative phosphorylation may be expected to be tightly coupled.

In chapter 2.2. (appendix II) an enzyme histochemical study of the mitochondrial enzymes glycerol-3-phosphate dehydrogenase and succinic dehydrogenase is described. For glycerol-3-phosphate dehydrogenase the activity in the villi appears to be much higher than in the crypts. The villus/crypt activity ratio for succinate dehydrogenase, however, is much lower. The increase in activity of both enzymes at the transition of crypt to villus suggests that at this transition place there is an increased synthesis or an activation of these mitochondrial enzymes. Differences in enzyme synthesis in this transitional zone can lead to different villus/crypt activity ratios. It is also possible that new mitochondria are assembled at the transition between villus and crypt and that the mitochondria of the villus have a higher glycerol-3-phosphate dehydrogenase/succinate dehydrogenase ratio than those of the crypt. Therefore experimental animals were treated with oxytetracycline or chloramphenicol (chapter 2.3., appendix III). These antibiotics inhibit the mitochondrial synthesis of the cytochromes aa_3, b, and c_1. Comparison between controls and short time treated animals (15–48 hours) shows, that synthesis not only exists in the dividing cells of the crypts but also in the functioning cells of the villi. Even in the cells of the top of the villus a decrease of cytochrome aa_3 synthesis is observed after short term treatment. Prolonged antibiotic treatment (96 hours) resulted not only in an overall depression of activity of the cytochrome aa_3 but also in secundary effects such as shortening of the villi and more readily release of cells at the villus tops during the preparation of the tissue for enzyme histochemistry.

Another histochemical study was dedicated to the study of the localization of the cytoplasmic enzyme fructose-1,6-diphosphatase (FDPase) (chapter 2.4., appendix IV). In homogenates of epithelial tissue, originating from the rat intestine enzyme activity was observed. The function of this enzyme in the intestine of the rat is not clear, because this enzyme is a key enzyme in gluconeogenesis, a process not occurring in the chief cells of intestinal tissue. In these cells, in which glycogen is generally absent, gluconeogenesis from pyruvate, lactate, alanin or fructose is virtually absent. Therefore the possibility was not excluded that the FDPase activity is originated from other cells but the

chief cells of the epithelial tissue, or from contamination of the epithelial cells with other cells present in the intestine. A decisive answer was hoped to be obtained from an enzyme histochemical study of the localisation of this enzyme. On account of the high activity of alkaline phosphatase in the intestine it was considered necessary to test the specificity of the enzyme reaction. Since in biochemical assays FDPase may be inhibited by low concentrations of adenosine-5'-monophosphate (AMP) this property was tested in the histochemical assay. However, no inhibition was found. Two methods were followed: a) in which the phosphate liberated from the substrate FDP is determined and b) in which the product fructose-6-phosphate is allowed to generate reducing equivalents to reduce added Nitro-BT to the corresponding formazan. In the first method the addition of AMP increased the amount of Pi formed and in the second method AMP increased the amount of formazan formed. The latter is probably due to the stepwise conversion of AMP to hypoxanthine, which provides reducing equivalents in the xanthine oxidase reaction. In the liver the activities of alkaline phosphatase and xanthine oxidase are lower than in the intestine, so that in the liver the specificity of FDPase could be tested. In muscle tissue the FDP-dependent Nitro-BT reduction appeared to be strongly disturbed by the presence of the high FDP-aldolase and glyceraldehyde-3-phosphate dehydrogenase activities. It was found that even a slow rate of dephosphorylation of the added cofactor nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to nicotinamide-adenine nucleotide (NAD^+) suffices to generate enough reduced nicotinamide-adenine dinucleotide (NADH) in the glyceraldehyde-3-phosphate dehydrogenase reaction to disturb the "b method" for FDPase determination. In agreement with this is the strong inhibition of formazanformation in muscle by the addition of monoiodoacetic acid, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase. Addition of a competitive phosphatase inhibitor, to avoid breakdown of $NADP^+$, led to a similar result.

Because the high activity of alkaline phosphatase in intestinal tissue not only disturbs the FDPase determination, but probably also other enzyme histochemical determinations, in which other phosphate esters are involved, a number of other reactions were screened. These are NADPH:Nitro-BT oxidoreductase, hexokinase, glucose-6-phosphate dehydrogenase and malate dehydrogenase (decarboxylating) ($NADP^+$) (chapter 2.5., appendix V). Addition of an excess of a substrate for alkaline phosphatase, such as β-glycerolphosphate or p-nitrophenylphosphate, to the incubation medium can inhibit the breakdown of the nicotinamide-adenine nucleotides used competitively.

The enzyme histochemical determination of hexokinase is carried out by determining the glucose-6-phosphate formed. This is accomplished by coupling the hexokinase reaction with the glucose-6-phosphate dehydrogenase reaction in which NADPH is formed, so that Nitro-BT may be reduced. In the first experiments whereby $NADP^+$ is reduced the formazan was localized at the lumenal side of the epithelial cells of the villus. This picture is to be expected if hexokinase or glucose-6-phosphate dehydrogenase would be localized in the area immediately under the brush border of the villus, or if the localization of NADPH:Nitro-BT oxidoreductase would be limited to this part of the epithelial tissue. The addition of an artificial electroncarrier, such as phenazinemethosulphate, so that Nitro-BT can be reduced without the intervention of NADPH: Nitro-BT oxidoreductase, however, showed a more diffuse localization over the cell and a higher activity of the overall process (chapter 2.5., appendix V), indicating that the picture in the earlier experiments was due to the localization of the bulk of the NADPH:Nitro-BT oxidoreductase activity in the apical part of the cell. When NADPH is not present in a low steadystate concentration, as in the hexokinase determination, but is added in substrate amounts to the incubation medium, the localization of the formazan formed is not confined to the area just under the brush border of the villus. At these high concentrations of NADPH enough NADH can be formed by phosphatase activity so that in addition to the NADPH- the NADH:Nitro-BT oxidoreductase reaction can proceed. The localization of NADH:Nitro-BT oxidoreductase apparently is not limited to the lumenal side of the villus cells, but is also found in other parts of the villus cells as well in the crypt cells. In the presence of excess of an additional substrate for the phosphatase reaction such as β-glycerolphosphate or p-nitrophenylphosphate, when the conversion of NADPH to NADH is competitively inhibited, the localization of NADPH:Nitro-BT oxidoreductase is again confined to the apical portion of the villous cells (chapter 2.5., appendix V).

When the $NADP^+$ specific decarboxylating malate dehydrogenase reaction is histochemically studied in the intestine, the illusion is obtained that the enzyme, compared with the NAD^+ specific malate dehydrogenase, is rather active (chapter 2.5., appendix V). From biochemical determinations, however, the former enzyme appeared to have an activity of one magnitude lower than that of the latter enzyme. Also here the difference appeared to be due to an enzyme histochemical artifact. Again alkaline phosphatase converts part of the $NADP^+$ to NAD^+ so that both enzyme reactions can proceed. When an excess of p-nitrophenylphosphate or β-glycerolphosphate is added as well, less NAD^+

is formed from $NADP^+$ and then indeed it is found that malate dehydrogenase (decarboxylating) ($NADP^+$) is little active in comparison to malate dehydrogenase (NAD^+).

From these histochemical studies it appeared that the enzyme histochemistry in a number of cases can certainly contribute to localization studies and produces patterns in harmony with biochemical findings. However, it is also clear that artifacts are easily obtained so that in general it is advisable to run biochemical determinations as well.

CURRICULUM VITAE

In 1959 werd op het Zandvliet Lyceum te 's-Gravenhage door mij het examen HBS-B afgelegd. In hetzelfde jaar volgde inschrijving aan de Rijksuniversiteit te Leiden met als studierichting biologie.

In 1964 legde ik het candidaatsexamen af. Voor het doctoraal-programma werd als hoofdvak plantenanatomie gekozen (Prof. Dr. W.K.H. Karstens), waarvan het onderzoek werd verricht op het Laboratorium voor Bloembollen Onderzoek te Lisse. Als bijvakken werden gekozen systematische dierkunde (Prof. Dr. L.D. Brongersma) en experimentele plantkunde (Prof. Dr. A. Quispel). Tijdens de studie vervulde ik van september 1962 tot september 1968 een assistentschap op de afdeling Plantenanatomie van de Rijksuniversiteit te Leiden en gedurende de periode van augustus 1967 tot augustus 1968 was ik verbonden als lerares biologie voor enkele lesuren aan het Fioretti College te Lisse. In 1968 behaalde ik het doctoraalexamen.

In 1969 volgde een aanstelling als wetenschappelijk medewerkster aan de Medische Faculteit te Rotterdam, op de afdeling Biochemie I, waar onder leiding van Prof. Dr. W.C. Hülsmann het histochemisch gedeelte van het in dit proefschrift beschreven onderzoek is verricht. Op de afdeling Celbiologie van deze faculteit, die onder leiding staat van Prof. Dr. H. Galjaard, is het electronen-microscopisch onderzoek uitgevoerd.

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METABOLIC ASPECTS OF ISOLATED CELLS FROM RAT SMALL INTESTINAL EPITHELIUM

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SUMMARY

1. Epithelial cells from the small intestine of the rat were isolated according to mechanical procedures as described by SJÖSTRAND[1] and HARRISON AND WEBSTER[2,3], respectively. The two methods were compared with regard to yield and integrity of the cells obtained.

2. While the cells in the suspension prepared by the method of SJÖSTRAND showed signs of appreciable morphological and biochemical damage, the cells harvested according to the principle of HARRISON AND WEBSTER appeared essentially intact and displayed a glycolytic activity, in the absence of added cofactors, of the same order as intestinal mucosa *in situ*.

3. The cells isolated by the second method were homogenized and the homogenate subjected to differential centrifugation. A mitochondrial preparation was obtained which showed intact oxidative phosphorylation. The stimulation of respiration by the addition of ADP was small. The mitochondrial preparation had ATPase activity, stimulated by Mg^{2+} or 2,4-dinitrophenol.

4. Analysis of adenine nucleotide concentrations in intestinal mucosa revealed a high AMP+ADP to ATP concentration ratio. This finding could explain the high rate of aerobic glycolysis of rat small intestinal epithelium.

INTRODUCTION

In the past few years several methods have been described for the isolation of cells from small intestinal epithelium, methods based on incubation of the intestine in media containing trypsin and pancreatin[4,5], lysozyme[6], hyaluronidase[7] or citrate[8].

Recently two procedures of a different type, based on the application of mechanical forces, were introduced. SJÖSTRAND[1] removed the mucosa by applying pressure to the surface of a rotating everted intestine. The harvested epithelium was subsequently broken up into isolated cells and mucus was removed by dispersion in large volumes of medium, made hypertonic by the addition of sucrose. The method of HARRISON AND WEBSTER[2,3] is based on low-amplitude high-frequency vibration of the everted intestine in saline, fortified with 5 mM EDTA. By this method sheets

of cells leave the exposed villi and can be harvested by centrifugation. After subsequent dilatation of the intestine by insufflation of gas into the interior of the everted intestine, crypt cells can also be released. This fractionated removal has the advantage that crypt and villous epithelium can be studied independently.

By the method of SJÖSTRAND the mucosa is dislodged rapidly and gently, but crypt and villous cells are harvested together. In order to separate these types of cells it would be necessary to break up the obtained sheets of mucosa into isolated cells by prolonged stirring. Subsequent metabolic studies, reported in this paper, indicate, however, that such isolated cells are heavily damaged. In the procedure of HARRISON AND WEBSTER the mucosal cells are exposed to high-frequency vibration, which on the one hand could damage membranes, but on the other hand offers the advantage that the intestinal epithelium is removed in fractions, with a separation of cells from villi and crypts.

MATERIALS AND METHODS

All chemicals were analytical grade. Enzymes and cofactors were obtained from Boehringer and Co, Mannheim, Germany. Radioactive materials were purchased from the Radiochemical Centre, Amersham, Great Britain. Bovine serum albumin was Fraction V from Sigma Chemical Co, St. Louis, Mo., U.S.A.; the albumin was defatted by charcoal treatment[9] and dialysed.

Animals

Male rats of approx. 200 g were used. Because of frequent heavy infections with the protozoa *Trichomonas* or *Giardia* (see also ref. 10), elimination of these parasites by oral treatment with metronidazolum (Flagyl) was first attempted. This treatment, however, did not eliminate the contamination of the crypts with the protozoa. Therefore, specified pathogen-free wistar rats were purchased from TNO, Zeist, The Netherlands. This strain is derived from initially germ-free animals, in which the intestines have subsequently been inoculated with staphylococcus albus, enterococcus, several bacilli and fusiform bacteria. Protozoa were never observed. Until used, these animals were kept in closed sterilized cages (with an air filter of glass wool) containing sterilized food. Except when indicated, the drinking water contained 5% glucose. The experiments shown in this article were carried out with these animals.

Assays

Protein was determined by the biuret reaction after solubilization of membrane-bound protein by treatment with deoxycholate[11] and/or by sonication.

Lactate was determined, in principle according to the procedure of HOHORST[12], after deproteinization with $HClO_4$ (final concn. 4%) and neutralization with KOH.

CO_2 was trapped in hyamine, which was introduced at the end of the experiment in a glass cup connected to the rubber cap closing the reaction vessel, prior to the addition of $HClO_4$ to terminate the reaction. The glass cup was cleaned of condensed water and transferred to a counting vial.

For measurement of thymidine incorporation, the reaction medium, after addition of ice-cold trichloroacetic acid at a final concentration of 5%, was transferred to tubes containing 5% trichloroacetic acid and centrifuged. The precipitates

were washed 3 times with 5% trichloroacetic acid and once with ethanol–diethyl ether (2:1, v/v). The precipitated material was suspended in hyamine (1 M in methanol) and counted.

Counting was performed in a liquid scintillation counter; the counting efficiency was determined by the channels ratio method. The counting vials contained 10 ml toluene with 5 g/l 2,5-diphenyloxazole and 0.3 g/l 1,4-bis-(5-phenyloxazolyl-2)benzene.

ATP was determined in $HClO_4$-deproteinized samples, neutralized with KOH. The medium contained: 0.25 M Tris–HCl buffer (pH 8.0), 0.8 mM glucose, 0.17 mM $NADP^+$, 1 mM $MgCl_2$, 0.7 munit glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 3.5 munits phosphoglucose isomerase (EC 5.3.1.9). The reaction was started by the addition of 5.6 munits hexokinase (EC 2.7.1.1). The final volume was 3 ml. The reaction was followed in an Aminco-Chance dual-wavelength spectrophotometer (wavelengths: 340 and 380 nm). AMP and ADP were also determined with this instrument following the enzymic methods described by ADAM[13].

Inorganic phosphate was determined according to the method of SUMNER[14].

Alkaline phosphatase (EC 3.1.3.1) was determined with *p*-nitrophenyl phosphate as the substrate, as described in ref. 15, with the exception that 1 mM $MgCl_2$ and 5 mM $ZnCl_2$ were added to the incubation medium for optimal activity[3].

Cytochrome *c* oxidase (EC 1.9.3.1) was measured according to the method of SOTTOCASA *et al.*[16] in an oxygraph with a Clark oxygen electrode.

Invertase (EC 3.2.1.26) was determined according to DAHLQVIST[17].

Mitochondrial respiration and phosphorylation were studied in differential manometers, as described by HÜLSMANN *et al.*[18].

Electron microscopic studies

The cell suspension was fixed in 1.5% glutaraldehyde in 300 mosM phosphate buffer (pH 7) and postfixed in 1% OsO_4 in 300 mosM phosphate buffer (pH 7). The Millipore filtration method of BAUDHIN *et al.*[19] was followed.

Preparation of the cell suspensions

Method A (according to SJÖSTRAND[1]). The animals were stunned and bled. The small intestine was removed and the lumen was flushed with 150 ml ice-cold saline. Further work was done at 0–4°. The gut was everted and 15-cm portions applied on a metal rod that was connected to a stirring motor. The procedure of SJÖSTRAND was followed, except that Ficoll was used instead of Dextran. The final pellet was taken up in Ca^{2+}-free Krebs–Ringer phosphate buffer or, when shown, in Ca^{2+}-free Krebs–Ringer phosphate buffer to which 25 mM $NaHCO_3$, gassed with a mixture of CO_2–O_2 (5:95, v/v), was added. The entire isolation procedure lasted 90–120 min.

Method B (according to HARRISON AND WEBSTER[2,3]). As described by these authors, the intestine was everted on metal rods that were attached to a base piece and exposed to vibration of 100 Hz by a Vibromixer (Type E1, Chemap AG, Männedorf, Switzerland). In order to facilitate the insufflation of the everted intestine after removal of the villous epithelium, the metal base piece and the rods were constructed in such a way that the gas could pass through the base piece, along the rods, and escape between the tied ends of the gut. The cells were sedimented from the isolation media by centrifugation at $1000 \times g$, washed once by resuspension and centrifugation and taken up in Ca^{2+}-free Krebs–Ringer phosphate buffer, with or without bicar-

bonate. This procedure lasted 60 min. With both methods the cells showed a marked tendency to aggregate on standing; however they were easily resuspended by passing through a plastic pipette. All isolation and incubation procedures were carried out with plastic materials.

Isolation of mitochondria

Cells isolated according to Method B were taken up in 20 ml of ice-cold medium, containing 50 mM Tris–HCl buffer, 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 1 mM EDTA and 0.8 mg/ml bovine serum albumin; the final pH was 7.3. The cells were homogenized for 60 sec in a Polytron homogenizer (type 20 ST, Kinematica, Lüzern, Switzerland) set at Position 5. The homogenate was passed through glass wool and centrifuged for 5 min at $1000 \times g$. Usually only a very small pellet was seen. On top of the medium a viscous mass floated which still contained particles, since if this was discarded, considerably less mitochondria were finally obtained. Therefore this floating material was taken up in the medium and rehomogenized by hand in a Teflon–glass Potter–Elvehjem homogenizer. This resuspended material, together with the rest of the $1000 \times g$ supernatant, was centrifuged again for 5 min at $1000 \times g$. The supernatant was subsequently centrifuged for 10 min at $12000 \times g$. A brownish pellet was obtained without a fluffy layer but with an irregular whitish border, possibly indicating contamination with microsomes. The pellet was finally suspended in the isolation medium and homogenized in a small Potter–Elvehjem homogenizer. Protein was determined and corrected for the amount of albumin present.

RESULTS

Morphological aspects of the cells

With Methods A and B to prepare intestinal cells, the epithelium was selectively removed from the underlying connective tissue. That the crypt cells were also harvested is shown in Figs. 1A and 1B. The isolated material consisted of sheets of epithelial cells. In Method A these sheets were further broken up into isolated cells by stirring the sheets two times for 30 min. In both methods the final suspension was only scarcely contaminated with connective tissue cells, naked villi, nuclei, isolated brush borders and bacteria. With the specified pathogen-free rats used, protozoa were never observed. Fig. 2A gives a representative illustration of the cells isolated by Method A. These cells were swollen, contained a swollen endoplasmic reticulum and appeared to contain vacuoli. Many mitochondria were damaged. The nuclear membrane had partly disappeared. That these mitochondria were also functionally damaged was demonstrated by isolating the mitochondria from a homogenate by differential centrifugation and testing for oxidative phosphorylation. P:O ratios not exceeding 1.5 with glutamate as the respiratory substrate were obtained (not shown). By contrast the cells isolated by Method B did not have a swollen appearance and the cellular architecture appeared grossly intact (Figs. 2B and 2C). As will be shown later the mitochondria isolated from these cells gave normal values of phosphorylation efficiency.

Fig. 2A shows an older villous cell with long microvilli and Fig. 2B shows young villous cells with relatively small microvilli. After stretching of the intestine by insufflation, whole crypts were removed. The crypt cells had sparse and short

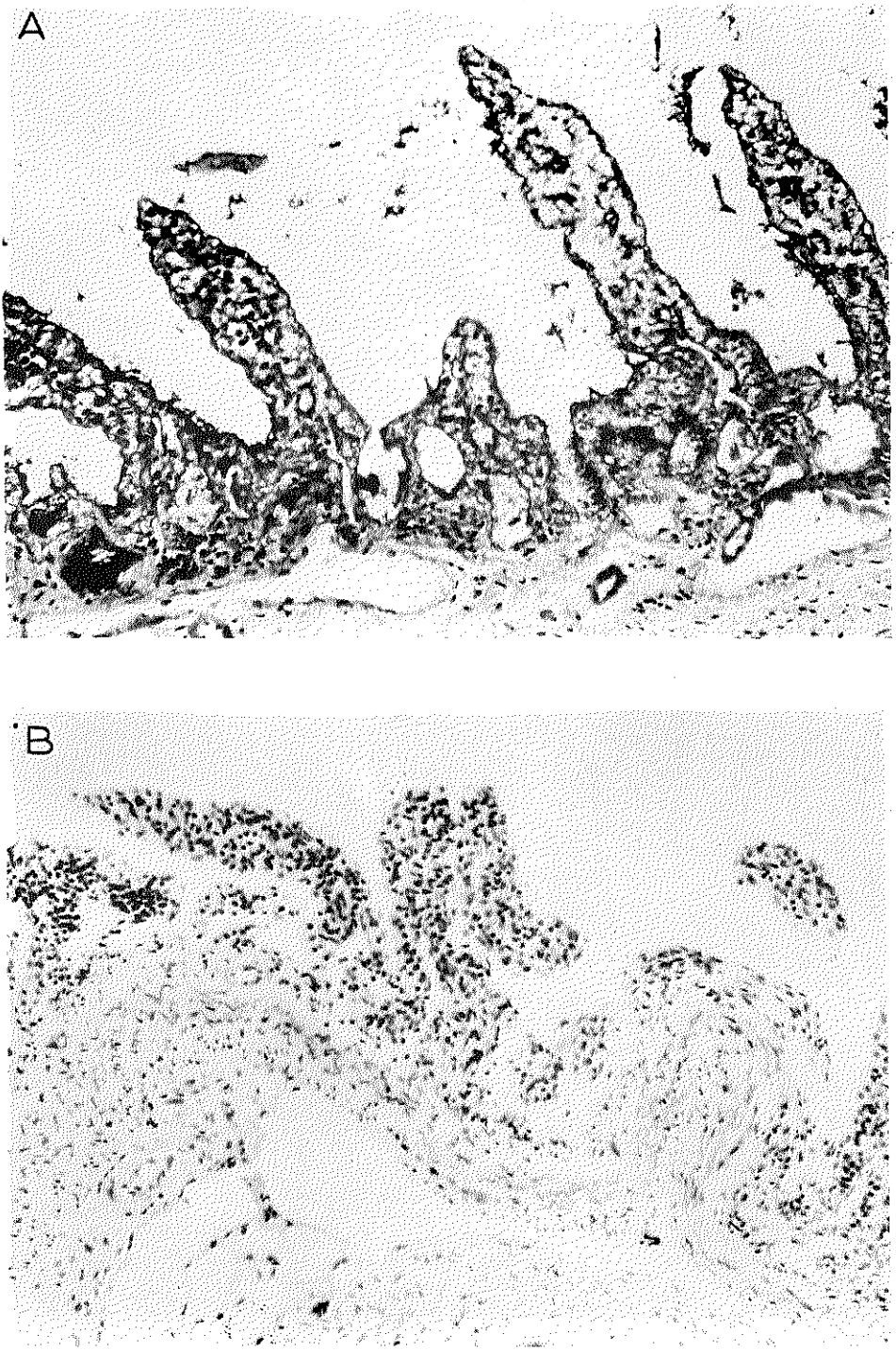
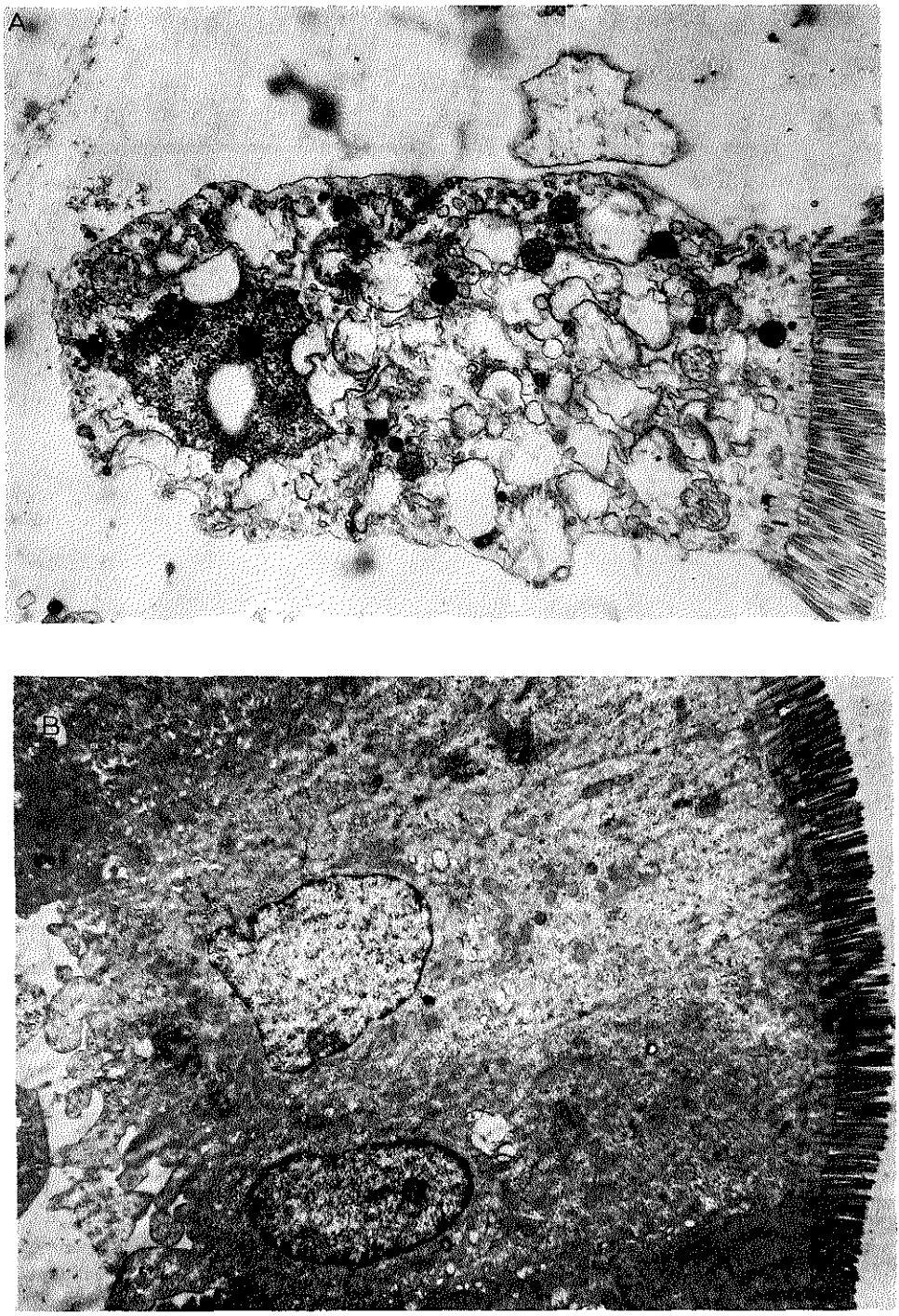


Fig. 1. Histology of intestine after removal of epithelium. A. Method A; B. Method B.


A
B

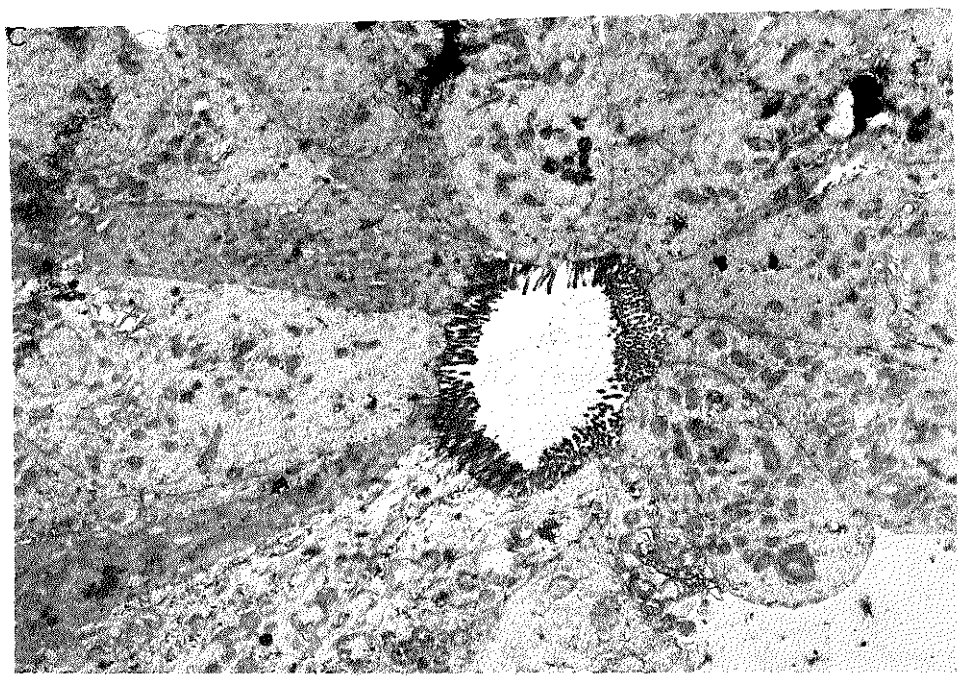


Fig. 2. Electron micrographs of epithelial cells. A. A villous cell isolated according to Method A. B. Villous cells isolated according to Method B. C. Crypt cells isolated according to Method B.

microvilli (Fig. 2C). These morphological details, in connection with biochemical studies to be mentioned below (Fig. 3), assured that a reasonably good separation of these types of cells had been obtained and made further breaking up of the villous sheets and of the crypts unnecessary.

Biochemical aspects of the cells

HARRISON AND WEBSTER[2,3] demonstrated the differential removal of the intestinal epithelium from the lamina propria of the villi. Their evidence for the gradual removal of the cells, in the sense that the cells at the top of the villi are dislodged first and those from the crypts last, is based on the decline of the specific activities of several brush border enzymes in the subsequent fractions, in contrast to the specific activities of mitochondrial or microsomal enzymes which did not change. Moreover they showed that 10 h after the intraperitoneal injection of [^{3}H]thymidine the specific radioactivity increased gradually in the harvested fractions, with a maximum in the last fraction where the crypt cells were expected. However, 72 h after the injection the highest specific radioactivity was observed in the villous cell fractions, which is in accordance with the known high turnover rate of small intestinal epithelium. We found incorporation of [^{3}H]thymidine *in vitro* only in the cells collected after maximal dilatation of the gut (Fig. 3). The rate of incorporation was 0.026 pmole/mg protein per min as calculated from an experiment lasting 20 min; on prolonged incubation the activity declined. It is unlikely that bacteria are the cause of the thymidine incorporation for the following three reasons: With Method A, the same order of DNA synthesis was observed whether normal rats or germ-free animals were used (specified

pathogen-free rats were not available at that time). Secondly, if heavy bacterial contamination were present, it is not clear why thymidine incorporation would only be present in the crypt cell fraction isolated according to Method B. Thirdly, a declining rate of incorporation was always found, instead of a gradual increase, as would be expected with bacterial overgrowth. From these findings we concluded that indeed the method of HARRISON AND WEBSTER allows differential isolation of intestinal cells. Other confirmatory data are shown in Fig. 3, demonstrating the distribution of two brush border enzymes and one mitochondrial activity in the harvested fractions.

Table I shows that the cells harvested according to Method B manifest the same glycolytic activity as the intestinal mucosa *in situ*, whereas the preparation by

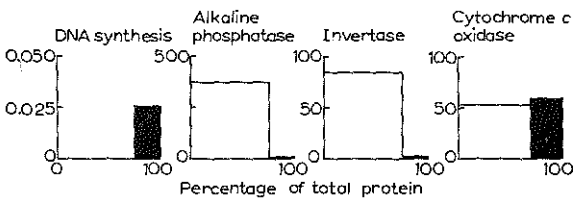


Fig. 3. Enzyme activities in villous and crypt cell fractions. Distribution of some activities in the cellular material harvested before (white) and after (black) dilatation of the small intestine. Cells were isolated according to Method B and taken up in Ca^{2+}-free Krebs–Ringer phosphate–bicarbonate buffer (DNA synthesis) or 0.15 M NaCl (other enzyme activities). The activities of alkaline phosphatase, invertase and cytochrome *c* oxidase were determined in samples after sonication for 5 sec at 20 kHz in order to obtain a more homogeneous material. Alkaline phosphatase and invertase activities were not appreciably altered by sonication. It was not investigated whether cytochrome *c* oxidase activity was increased by longer sonication. All activities were measured at 37°. The values for cytochrome *c* oxidase were taken from a different experiment in which the same procedure was followed. DNA synthesis was determined in intact cells as [^{3}H]thymidine incorporation. 2.0 ml of the cell suspension (containing 26.4 mg cellular protein for the villous cells and 7.4 mg for the crypt cells) in Krebs–Ringer phosphate–bicarbonate buffer were incubated in the presence of 12.5 μmoles glucose, 22 mg bovine serum albumin and 44 nmoles [^{3}H]thymidine, 125 μC/μmole. The reaction was terminated after 0, 20 or 40 min by the addition of trichloroacetic acid. Further details are described under MATERIALS AND METHODS. The specific activities (for DNA synthesis, pmoles thymidine incorporation per mg protein per min; for the other enzyme activities, nmoles per mg protein per min) are plotted against the percentage of total protein in each fraction.

TABLE I

AEROBIC GLYCOLYTIC ACTIVITY OF RAT SMALL INTESTINAL EPITHELIUM: COMPARISON OF EPITHELIUM *in situ* AND ISOLATED CELLS

In Expt. 1 the lumen of 60 cm of small intestine was flushed with cold saline, everted and mounted on the four metal rods of the device used in Method B as described in MATERIALS AND METHODS. After vibration for 1 min in cold saline, the rods holding the intestine were slowly rotated in fresh Ca^{2+}-free Krebs–Ringer phosphate–bicarbonate buffer, containing 10 mM glucose. In Expts. 2 and 3 the cells were incubated in the same solution. In Expt. 3 the cells were isolated in 0.15 M NaCl–0.005 M EDTA (pH 7.4), made hypertonic (450 mosM) with sucrose. In Expts. 2 and 3 the incubation vessels were agitated in a metabolic shaker. All experiments were performed at 37°. Incubation time was 30 min. All animals were glucose-fed. Representative values of four different experiments are given.

Expt.	*Lactate production (µmoles/min)*	
	Per mg protein	*Per 60 cm intestine*
(1) Everted intestine	—	2.1
(2) Isolated cells, Method A	0.002	0.25
(3) Isolated cells, Method B	0.021	2.7

Method A results in a dramatic loss of this activity. For this reason we abandoned Method A and concentrated further work on Method B cells. Moreover, in order to separate cells of the villous compartment from the crypt compartment, in Method A an extra step is involved (such as the feeding of corn oil to the animal prior to sacrifice, allowing the accumulation of lipid in the villous cells, causing them to float on centrifugation).

The influence of the nutritional status, of the tonicity of the isolation medium and of various additions to the incubation medium upon the metabolism of the Method B cells is shown in Table II. As expected from earlier studies on the dependence of intestinal glycolytic enzyme activity on feeding conditions[20,21], the nutri-

TABLE II

SOME FACTORS INFLUENCING METABOLISH OF ISOLATED SMALL INTESTINAL EPITHELIUM CELLS (METHOD B)

Cells were isolated according to Method B and incubated in Ca^{2+}-free Krebs–Ringer phosphate buffer with or without bicarbonate, containing 10 mM [6-^{14}C]glucose (25 μC/mmole). The experiments were carried out at 37° for 30 min in a metabolic shaker. When shown, NAD^+ and ADP were added in final concentrations of 1 mM and bovine serum albumin in 10 mg/ml. In all experiments the combined cell-fractions were used, except in Expt. 2 where (in 2A) cells were harvested before dilatation of the everted gut and (in 2B) cells were obtained after dilatation. Only the CO_2 originating from the 6-position of glucose is measured.

Expt.	*Tonicity of isolation medium*	*Nutritional status*	*Additions*	*Lactate production (nmoles per mg protein per min)*	*CO_2 production (nmoles per mg protein per min)*
1	Isotonic	Fed	—	10.2	—
			NAD^+, ADP	8.2	—
			NAD^+, ADP, bovine serum albumin	10.4	—
2A	Isotonic	Fed	—	8.7	0.59
2B			—	2.7	0.18
3	Isotonic	Fasted	Bovine serum albumin	9.1	0.14
	Isotonic	Fed	Bovine serum albumin	11.5	0.51
	Isotonic	Glucose fed	Bovine serum albumin	22.0	0.93
4	Hypertonic	Glucose fed	NAD^+, ADP, bovine serum albumin	21.3	—
	Hypertonic (−EDTA)	Glucose fed	NAD^+, ADP, bovine serum albumin	24.0	—

tional status turned out to be an important variable. The results obtained with the glucose-fed animals were fully reproducible. In order to avoid variable results as much as possible, glucose-feeding of the animals was included in the standard procedure. When the isolation medium was made hypertonic with sucrose (450 mosM), the rate of glycolysis, as judged by lactate production, was not influenced. With Method B, omission of EDTA from the isolation medium decreased the amount of cellular protein harvested to less than half. The cells did not suffer from EDTA addition as judged by the rate of glycolysis. Addition of NAD^+, ADP or bovine serum albumin to the incubation media did not stimulate lactate production. In two experiments with glucose-fed animals a lower glycolytic activity was observed in crypt, as compared with villous, cells. This phenomenon requires further investigation.

Experiments in which [6-^{14}C]glucose was used (sucrose was then omitted from the isolation medium to avoid unknown dilution of the intracellular radioactive glucose pool) showed that between 7 and 11 times as many molecules of glucose are

converted to lactate then to CO_2 originating from the 6-position (*cf.* Table II). The highly active aerobic glycolysis of intestinal epithelium cells is well known and confirmed in these experiments with isolated cells. In order for phosphofructokinase EC 2.7.1.11) to be active, a high ratio of [AMP] *plus* [ADP] to [ATP] in the cytoplasm is required[22]. Indeed, it can be seen from Table III that when the intestinal

TABLE III

RATIO OF ([AMP]+[ADP])/[ATP] IN RAT SMALL INTESTINAL MUCOSA

In Expt. 1, 15 cm of small intestine were everted on a metal rod and incubated during 15 min at 37° in Ca^{2+}-free Krebs–Ringer phosphate–bicarbonate buffer with 20 mM glucose. The tissue was rapidly fixated by immersion in isopentane at −160° (in a vessel placed in liquid N_2). The rod with the intestine was subsequently placed in a reaction vessel containing 4% $HClO_4$, and the mucosa was scraped off. In Expt. 2 the cell suspension, after incubation for 15 min in the above-mentioned medium, was injected into the isopentane at −160°; the cell suspension froze instantly and was subsequently placed in 4% $HClO_4$. The concentrations of adenine nucleotides were determined, after melting and centrifugation, in samples neutralized with KOH. The AMP concentrations are expressed arbitrarily as 100.

	Expt. 1: Everted intestine (average of two experiments)	*Expt. 2: Isolated cells (Method B)*
[AMP]	100	100
[ADP]	28	300
[ATP]	11	67
([AMP])+([ADP])/[ATP]	12	6

epithelium is rapidly fixed by immersion of the everted gut in isopentane at −160°, a considerably higher concentration of AMP *plus* ADP is found when compared to that of ATP. The ratio [AMP] *plus* [ADP] to [ATP] in isolated cells, where the metabolism was stopped by injection of the cell suspension in isopentane at −160°, was found to be of the same order. These results led us to a preliminary investigation of mitochondria isolated from cells obtained by Method B.

Behaviour of mitochondria from isolated intestinal cells

Cells were homogenized in a Polytron homogenizer (see MATERIALS AND METHODS), since it was found that the use of the conventional Potter–Elvehjem homogenizer failed to break up a sufficient number of cells. Table IV shows that the mitochondria obtained by differential centrifugation (see MATERIALS AND METHODS for details) catalyse oxidative phosphorylation with a number of substrates. The mitochondrial preparation requires further purification since it is likely from the low Q_{O_2}'s that the preparation contains a relatively large amount of extraneous protein. Only from cells, isolated in the hypertonic media could mitochondria be isolated which showed some respiratory control (see Table IV), while the mitochondria from cells isolated in isotonic media generally showed oxidative phosphorylation but never respiratory control. This is different from previous results with mitochondria from the rat small intestine (*cf.* refs. 23–25) in which oxidative phosphorylation was absent. It can be seen from Table V that the mitochondrial preparation shows ATPase activity which can be stimulated by Mg^{2+} or 2,4-dinitrophenol. The ATPase activity in the presence of 2,4-dinitrophenol is partly oligomycin and atractyloside resistant.

These results can be explained by loose coupling of oxidative phosphorylation (*cf.* ref. 26) or by extramitochondrial ATPase contaminating the mitochondria. These observations require further investigation.

TABLE IV

OXIDATIVE PHOSPHORYLATION AND RESPIRATORY CONTROL OF MITOCHONDRIA ISOLATED FROM RAT SMALL INTESTINAL EPITHELIUM

Mitochondria were prepared from the total cell yield, as described under MATERIALS AND METHODS, and incubated in Warburg vessels at 37° in a medium containing 22.5 μmoles potassium phosphate, 20 μmoles glucose, 1 μmole NAD^+, 0.03 μmole cytochrome *c*, 25 μmoles Tris–HCl buffer, 50 μmoles KCl, 2.5 μmoles $MgCl_2$, 0.5 μmoles ATP, 0.5 μmole EDTA and 0.4 mg/ml bovine serum albumin; where indicated, 1.2 μmole sodium malate and 12 μmoles sodium glutamate, sodium pyruvate or sodium succinate were present, except in the blanks, to which 18 μmoles NaCl were added instead. Final volume 1.0 ml, final pH, 7.4. After 30 min of incubation 1.4 units hexokinase was added. For further details see MATERIALS AND METHODS. In Expt. 1 a Q_{O_2} (37°) of 53 was calculated.

Expt.	*Isolation medium of the cells*	*Respiratory substrate(s)*	*P/O ratio*	*Respiratory control index*
1	Isotonic	Glutamate	3.3	1.0
2	Isotonic	Glutamate	2.9	1.0
		Glutamate, malate	3.2	1.0
		Succinate	1.9	0.9
3	Hypertonic	Glutamate, malate	2,6	1.5
		Pyruvate, malate	3.5	1.2

TABLE V

ATPASE ACTIVITY OF MITOCHONDRIA ISOLATED FROM THE RAT SMALL INTESTINE

Mitochondria were prepared as described in Table IV, Expt. 3. About 0.05 mg protein was incubated at 20° in 1.1 ml of a medium containing 20 μmoles Tris–HCl buffer, 40 μmoles KCl, 0.4 mg bovine serum albumin, 1.9 μmole ATP, 100 μmoles sucrose and other additions as shown. The reaction was started by the addition of the mitochondria and after 15 min stopped with 1 ml 10% trichloroacetic acid. After centrifugation inorganic phosphate was determined in the supernatant. The pH of the incubation medium was 7.4.

Conditions	*P_i production (arbitrary units)*	
	Expt. 1	*Expt. 2*
2 mM $MgCl_2$, 3.4 mM EDTA	100	100
2 mM $MgCl_2$, 0.4 mM EDTA	428	160
2 mM $MgCl_2$, 3.4 mM EDTA, 0.045 mM 2,4-dinitrophenol	257	200
2 mM $MgCl_2$, 3.4 mM EDTA, 10 μg/ml oligomycin, 0.045 mM 2,4-dinitrophenol	—	120
2 mM $MgCl_2$, 3.4 mM EDTA, 50 μg/ml atractyloside, 0.045 mM 2,4-dinitrophenol	—	130

DISCUSSION

High aerobic glycolysis is a striking characteristic of small intestinal epithelium. This was observed 30 years ago by DICKENS AND WEIL-MALHERBE[27]. WILSON AND WISEMAN[28], using distended sacs of everted rat small intestine, observed an aerobic lactate production of 14 μl per mg dry wt. per h at 37°; this corresponds to 5 μmoles/min per 60 cm of small intestine (dry wt. approx. 0.5 g). These authors found that in ileum anaerobiosis stimulated the rate of lactate production, but that in the upper jejunum this Pasteur effect was absent. We observed in the aerobic mucosa *in situ* a lactate production of 2.1 μmoles/min per 60 cm at 37°. The higher figure found by WILSON AND WISEMAN can probably be explained by the greater exposition of the mucosal surface in the distended sacs and by other experimental differences. In addition we found (not shown) that 0.1 mM 2,4-dinitrophenol stimulated aerobic glycolysis of small intestinal mucosa 1.5–2-fold.

In the preparation of rat small intestinal cells, isolated by incubation of the gut with trypsin and pancreatin, STERN AND REILLY[5] observed a lactate production of 0.006 μmoles/min per mg protein at 30°; this activity increased 1.5 times with anaerobiosis. Our measurement of aerobic glycolysis, using Method B cells, is 0.021 μmoles lactate/min per mg protein at 37°.

STANBURY[23] reported that succinate was not appreciably oxidized by isolated mitochondria from the rat, when compared with the mitochondria isolated from the guinea pig intestinal mucosa. SHERRAT AND HÜBSCHER[25] describe for isolated intestinal guinea pig mitochondria proper oxidative phosphorylation but defective mitochondrial activity for other species, including the rat. In the present paper it is shown that oxidative phosphorylation is present also in the rat. However, oxidative phosphorylation was not tightly coupled (Table IV). Whether the absence of tight coupling and the presence of spontaneous ATPase activity (Table V) is due to an intrinsic property of these mitochondria or to an artifact of the isolation must still be investigated. A first approach to this problem led us to determine the levels of the various adenine nucleotides in intact intestinal mucosa. In the presence of glucose we observed relatively low ATP levels (Table III). This contributes to the high rate of anaerobic glycolysis.

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Unequal rates of development of mitochondrial enzymes in rat small intestinal epithelium

Recent work from this laboratory[1] showed that mitochondria, isolated from rat small intestinal epithelium, catalyze oxidative phosphorylation with succinate, pyruvate and glutamate as respiratory substrates. A subsequent investigation, in which other substrates were used, showed that glycerol 1-phosphate and β-hydroxybutyrate were also oxidized. Differential removal of the epithelial cells from villi and crypts, by the method of HARRISON AND WEBSTER[2], allowed the separate isolation of mitochondria from villous and crypt epithelium. After collection of the cells by centrifugation, a homogenate was prepared in a medium containing 0.25 M sucrose, 20 mM Tris–HCl (pH 7.4) and 1 mM EDTA. A Polytron homogenizer (Type 20 ST, Kinematica, Luzern, Switzerland) was used (75 sec in position 5). The homogenate was centrifuged at $1000 \times g$ (5 min) to remove nuclei, intact cells and brush borders. Then the supernatant was spun at $14000 \times g$ (10 min). The sediment from the second centrifugation was resuspended in isolation medium, and recentrifuged at $8500 \times g$ (10 min) to sediment the mitochondria.

It can be seen from Table I that several mitochondrial enzyme activities are different in the villi as compared with the crypts. The rate of succinate oxidation in villous mitochondria is twice as great as in crypt mitochondria, that of glycerol 1-phosphate oxidation 4 times as great. A comparison of the rates of oxidation of β-hydroxybutyrate and of acetoacetate also reveals differences between villi and crypts: whereas the rate of oxidation of β-hydroxybutyrate is virtually the same, that of

TABLE I

MITOCHONDRIAL ENZYME ACTIVITIES IN RAT SMALL INTESTINAL EPITHELIUM

The oxidation of glycerol 1-phosphate and succinate was measured polarographically at 37° in a medium containing 230 mM sucrose, 18 mM Tris–HCl (pH 7.4), 0.9 mM EDTA, 5 mM $MgCl_2$, 0.012 mM cytochrome *c*, 12 mM glycerol 1-phosphate or succinate and approx. 0.3 mg protein in a final volume of 1.7 ml. For the determination of the rate of oxidation of β-hydroxybutyrate and acetoacetate, β-hydroxy [3-^{14}C]butyrate (0.094 mC/mmole) was used as a substrate. Approx. 3 mg of mitochondrial protein were agitated at 37° in a medium containing 5 mM potassium phosphate, 43 mM Tris–HCl (pH 7.4), 180 mM sucrose, 0.5 mM EDTA, 6 mg/ml bovine serum albumin, 0.02 mM cytochrome *c*, 1 mM NAD^+, 1.3 mM DL-malate, 2.5 mM ATP and 10 mM β-hydroxy [3-^{14}C]butyrate, in a final volume of 1 ml. The reactions were stopped with $HClO_4$ (final concn. 4%); acetoacetate was determined in the deproteinized samples, neutralized with KOH, using NADH and β-hydroxybutyrate dehydrogenase. The CO_2 evolved during the reactions was trapped in hyamine (1 M in methanol) that was introduced prior to the termination of the incubations, in glass cups connected to the rubber caps closing the reaction vessels. The cups were cleared of condensed water, transferred to counting vials containing 10 ml toluene with 5 g/l 2,5-diphenyloxazole and 0.3 g/l 1,4-bis-(5-phenyloxazolyl-2)benzene, and counted. Acetoacetate oxidation is calculated from the production of $^{14}CO_2$ from β-hydroxy [3-^{14}C]butyrate, whereas β-hydroxybutyrate oxidation is expressed as the sum of the acetoacetate accumulated and acetoacetate oxidized. The results are expressed as average values (± S.D.). n = number of experiments.

Substrate	*Enzyme activity (nmoles substrate oxidized per mg protein per min)*	
	Crypt mitochondria	*Villous mitochondria*
Glycerol 1-phosphate (n = 5)	12 ± 4	52 ± 13
Succinate (n = 5)	96 ± 30	223 ± 69
Acetoacetate (n = 2)	0.2	2.5
β-Hydroxybutyrate (n = 2)	8.7	6.1

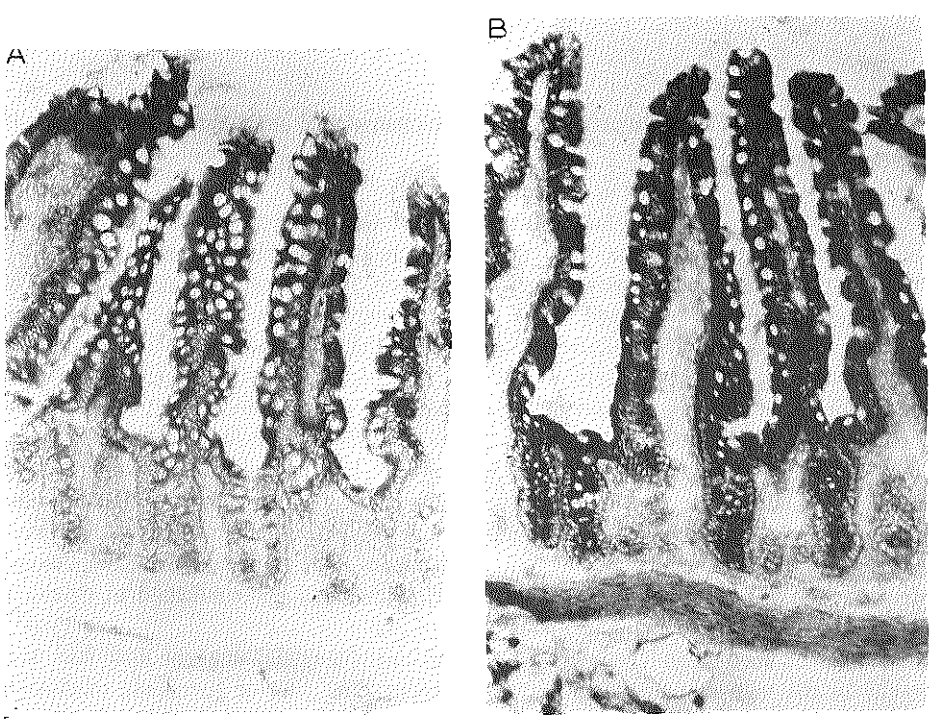

Fig. 1. Histochemical demonstration of mitochondrial glycerol 1-phosphate dehydrogenase and succinate dehydrogenase activities in jejunum of a rat fed with 5% glucose. Cryostat sections (8 μ thick) were incubated at 37° with about 0.3 ml of a medium containing 41.6 mM sodium phosphate buffer (pH 7.4), 0.5 mM nitroblue tetrazolium, 0.13 mM $CaCl_2$, 0.25 mM $AlCl_3$, 25 mM $NaHCO_3$ and 48 mM disodium DL-glycerol 1-phosphate or 41.6 mM disodium succinate. The incubation with glycerol 1-phosphate (A) was carried out for 60 min and that with succinate (B) for 30 min. The reactions were stopped by submersion of the sections in water, immediately followed by fixation in formaldehyde vapour for 10 min.

acetoacetate oxidation is 12 times greater in the villous mitochondria than in the crypt mitochondria.

A complementary histochemical examination of mitochondrial glycerol-1-phosphate dehydrogenase (EC 1.1.99.5) and succinate dehydrogenase (EC 1.3.99.1) activities (Fig. 1) also reveals differential rates of development. In the crypts a virtual absence of glycerol-1-phosphate dehydrogenase was observed, compared with the villi. The succinate dehydrogenase was observed both in crypts and in villi, with a slightly higher activity in the latter.

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The Influence of *in vivo* Administered Chloramphenicol and Oxytetracycline on some Mitochondrial Enzymes of Rat-Small-Intestinal Epithelium: Histochemical Data

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Summary. The inhibition of rat small intestinal cytochrome *c* oxydase by *in vivo* administration of chloramphenicol and oxytetracycline has been demonstrated histochemically. Since the dwelling time of newly formed epithelial cells in the crypts of jejunum is 10–14 h, it was surprising to find inhibition of cytochrome *c* oxydase after 12 h treatment not only in the crypts but also in the villus. These experiments led to the conclusion that cytochrome *c* oxidase is continuously synthesized, and probably also degraded in the villous cells. Coupling of oxidative phosphorylation is still present after 48 h antibiotic treatment, when the effect on cytochrome *c* oxydase appeared to be maximal, as judged by the persistance of 2,4-dinitrophenol-stimulated adenosinetriphosphatase. Finally, prolonged ($\geqslant$ 48 h) antibiotic treatment often led to retraction of connective tissue (which supports the villous epithelial cells), resulting in loosening and loss of cells from the tops of the villi.

Introduction

In parallel biochemical and histochemical studies an attempt was undertaken to determine the importance of extramitochondrial energy generation by *in vivo* treatment of rats with high doses of inhibitors of mitochondrial protein synthesis. Chloramphenicol (CAP) or Oxytetracycline (OTC) were used to inhibit the synthesis of some components of the mitochondrial respiratory chain. The biochemical studies (De Jonge and Hülsmann, 1972) showed a marked depression of the synthesis of the cytochromes a, a_3, b, and c_1 by the above mentioned drugs, administered intramuscularly for 1–2 days (OTC: every 8 h 100 mg/kg body weight; CAP: every 4 h 280 mg/kg body weight), confirming studies with other tissues (Firkin and Linnane, 1969; De Vries and Kroon, 1970). Intestinal epithelium is a good target for these drugs in animal studies, since the turnover of intestinal epithelial cells is only 36–48 h. In the intestinal crypts the cell cycle of the principle cells is only 10–14 h (Cairnie *et al.*, 1965). The histochemical studies, to be presented below, were carried out to study the sequence of events along crypts and villi more precisely, since the biochemical methods followed only allowed the separation of sheets of cells from villi and of crypts (comp. Iemhoff *et al.*, 1970). The histochemical studies and earlier studies from our laboratory (Hülsmann *et al.*, 1970; Iemhoff and Hülsmann, 1971) revealed the possibility that in the transitional zone, between crypt and villus, differentiation may require extra synthesis of mitochondrial proteins.

Materials and Methods

Tissue specimen of the intestine were initially taken from fixed parts of as well duodenum, jejunum as of ileum. Later, only pieces of the jejunum were used when it was found that the antibiotic treatment affected the enzymes of the duodenum and ileum in a similar manner, ileum responding more slowly. Moreover, the jejunum contributed quantitatively most to the homogenates employed in the parallel biochemical studies (De Jonge and Hülsmann, 1972). In all experiments specific pathogen-free Wistar rats, weighing 200–230 g, were utilized in order to eliminate contamination of the intestine with protozoa (Iemhoff *et al.*, 1970). The animals were killed by cervical fracture and subsequent bleeding. The small intestine was removed and rinsed with cold saline. Samples (1–2 cm) were taken from fixed parts of the proximal duodenum, jejunum (30 cm from the pylorus) and the ileum (distal part) and frozen in isopentane at liquid nitrogen temp. Cross sections of 6 μ were made with a microtome (International Equipment Co., model CTF) at −20° C. Pieces of intestine from control and treated rats were mounted together in order to allow direct comparison.

Hematoxylin-eosin was used for morphological identification. The histochemical enzyme assays were carried out either by adding the incubation mixture on top of the tissue ("orthodox method"), or by applying the method of Meijer (1972). The latter is based on reaction between the incubation mixture in agar and the tissue, which are separated by a semipermeable membrane (dialysis tubing). This method has the drawback of less intimate contact between enzymes and substrates by diffusion limitation, but the advantage of not diluting enzymes and tissue cofactors by the direct application of the incubation medium. The method of Meijer cannot be used if the addition of enzymes or of other high molecular substances to the enzymes tested is required, because of the permeability barrier. Cytochrome *c* oxydase was determined by the method of Burstone (1959) either by applying the incubation mixture, fortified with 0.5 mg cytochrome *c* (Boehringer and Sons, Mannheim, W. Germany) per ml, directly to the tissue for 20 min at 4° C or by applying the method of Meijer (absence of added cytochrome *c*) for 45 min at 37° C. Mg^{2+}-dependent adenosinetriphosphatase (ATP-ase) was determined by the direct application of the incubation medium, in the absence or presence of 1 mM 2,4-dinitrophenol (DNP), to the tissue; the method of Wachstein and Meisel (1957) was followed. The determination of lactate dehydrogenase was carried out by applying the incubation method of Meijer (compare also McMillan, 1967) in the presence of 0.5 mM phenazinemethosulphate and the presence or absence of 1 mM KCN. The incubation period was 45 min at 37° C. The composition of the incubation media is given in the legends to the figures.

Results

Antibiotic Treatment and Morphology

When rats were pretreated for less than 120 h with CAP or less than 48 h with OTC, in the amounts mentioned in the Introduction, leading to plasma levels of at least 10 μg/ml (De Jonge and Hülsmann, 1972), no morphological alterations were observed. When treatments with the antibiotics were continued beyond the periods mentioned, retraction of connective tissue was often observed. This then resulted in shortening of the villi, loosening of epithelial cells from the underlying connective tissue and finally to the loss of cells, especially from the tops of the villi of duodenum and jejunum (Figs. 1a and b). Even beyond 120 h treatment with either of the antibiotics, cell divisions in the crypts were found to be present.

Influence of CAP and OTC Treatment on Cytochrome c Oxydase

Cytochrome *c* oxydase activity decreased gradually by treatment with both antibiotics. A maximum decrease was obtained after 48 h treatment, which is

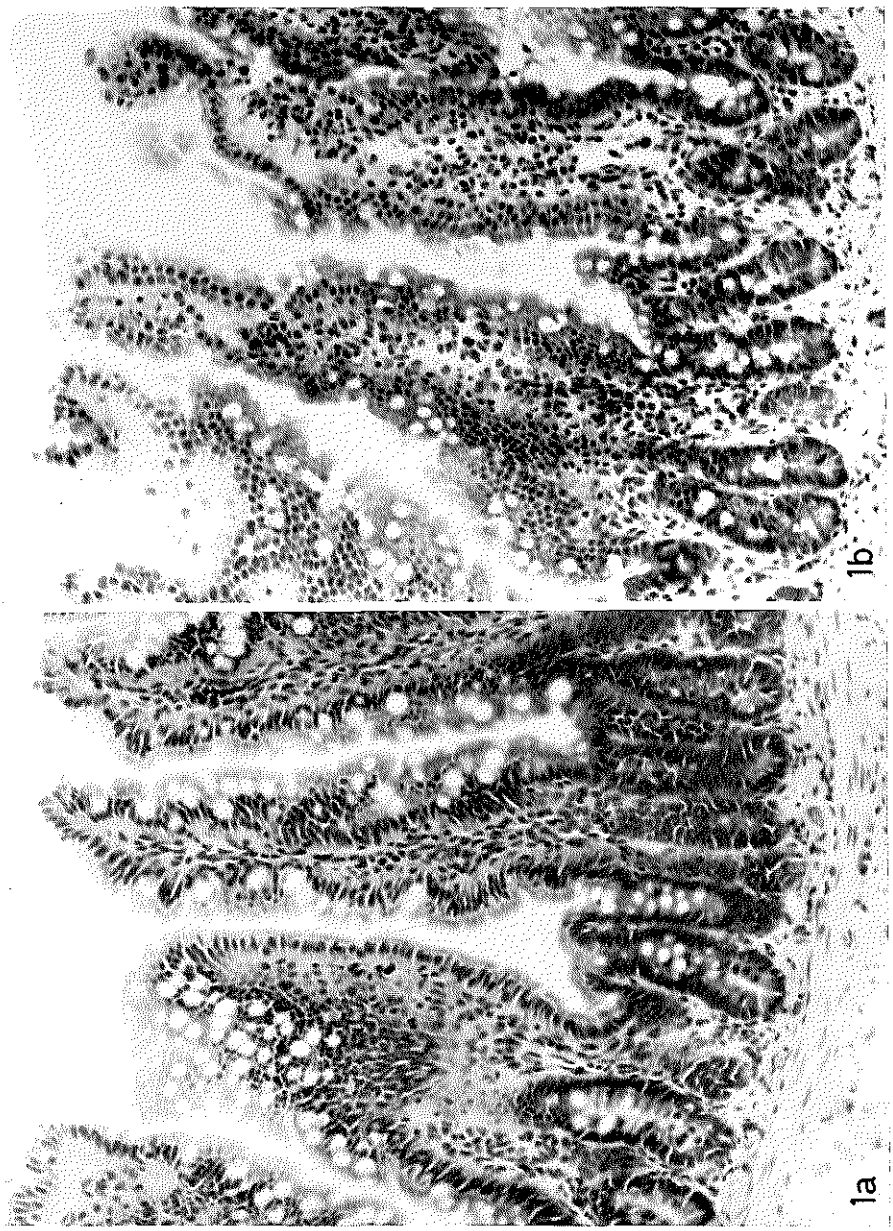


Fig. 1a and b. Loosening of epithelial cells from the tops of the villi by pretreatment of rats with oxytetracycline for 48 h. (a) Haematoxylin-eosin staining of jejunum of a control animal and (b) the same of a treated animal

in agreement with the biochemical data (De Jonge and Hülsmann, 1972). The histochemical data are shown in Fig. 2. Figs. 2a and d compare control and 48 h OTC pretreatment. 12 or 24 h treatment resulted already in an overall decrease of cytochrome *c* oxydase activity, as may be concluded by comparing Figs. 2b

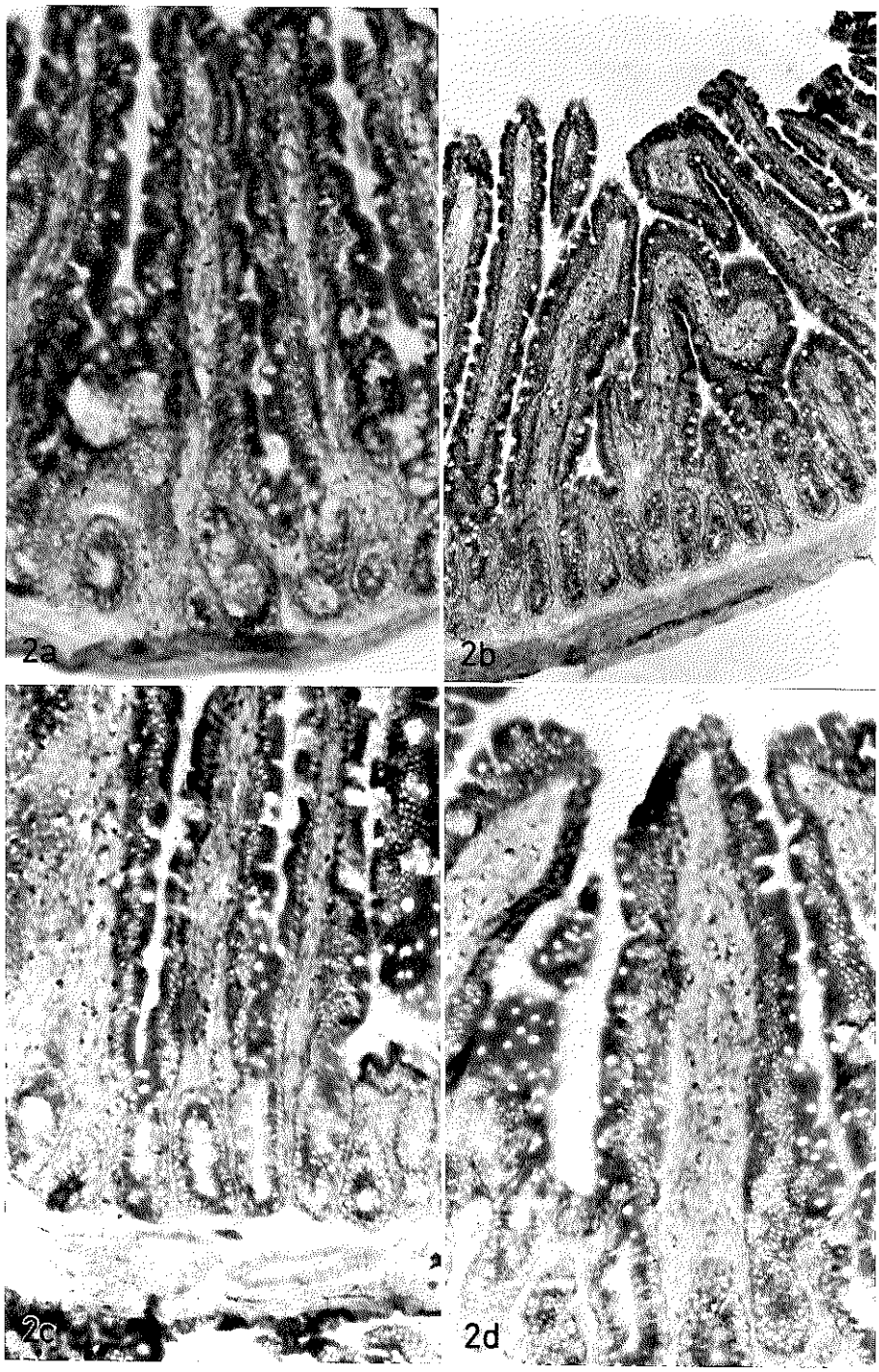


Fig. 2a—d

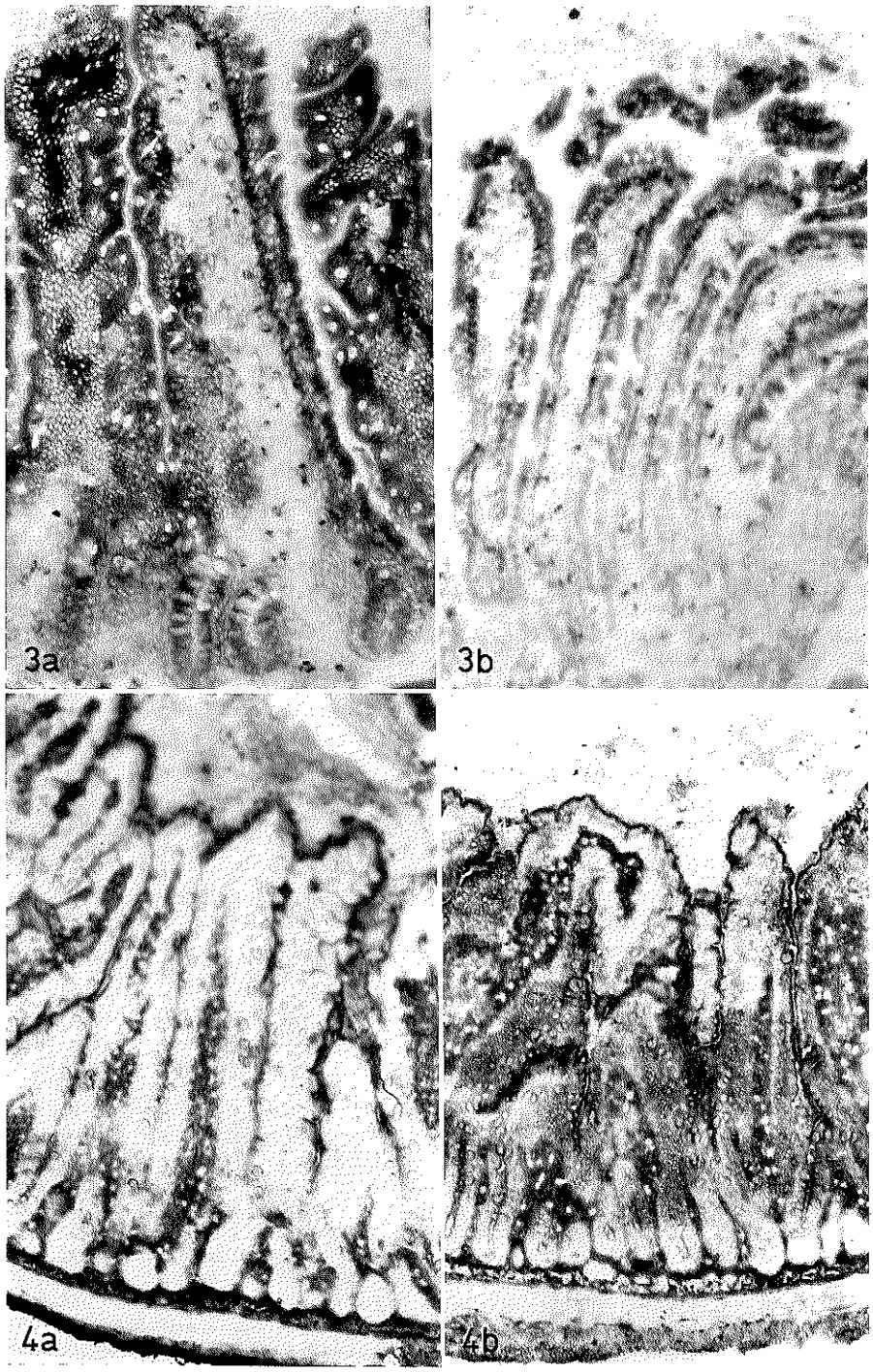
and c with Fig. 2a. In these experiments cytochrome *c* was added to the incubation medium, which was directly applied to the tissue sections. In the absence of added cytochrome *c* the rate of the reaction becomes extremely slow (data not shown), indicating that cytochrome *c* leaks out of the mitochondria into the incubation mixture.

The effect of short term pretreatment with the antibiotics is more clearly observed when the method of Meijer (1972) is followed, omitting the addition of cytochrome *c*. Cytochrome *c* is synthesized extramitochondrially (Sherman and Stewart, 1971) and its level is therefore not affected by the drug treatment. The cytochrome *c* oxydase reaction in this method is rather slow, necessitating incubation at a higher temperature (37° C) and for a longer time (45 min) to obtain sufficient reaction product. It can be seen from Figs. 3a and b that after 15 h CAP treatment, the medication being started intravenously with 60 mg CAP, the entire crypt and the lower part of the villus has almost no cytochrome *c* oxydase activity. Considerable activity only remains in the top of the villus.

That 12–15 h treatment with the antibiotics causes a decrease of cytochrome *c* oxydase activity not only in the crypt, but also in the lower and middle part of the villus was, considering a dwelling time of newly formed cells in the crypt of 10–14 h (Cairnie *et al.*, 1965), rather unexpected. Apparently, cytochrome *c* oxydase is not only formed when the cells devide, but also in the higher part of the crypt and probably even in part of the villus, since otherwise 15 h treatment would not have affected the villous activity. This conclusion is supported by the biochemical finding (De Jonge and Hülsmann, 1972) that 12, 24 or 48 h treatment, although lowering the cytochrome *c* oxydase activity, does not affect the villous/crypt activity ratio. The slower reaction in the Meijer method (Fig. 3) has the advantage over the very fast reaction which occurs when incubation is carried out in the "orthodox manner" (Fig. 2) that differences in reaction rates are more easily detected. In the former method then, 21 h treatment resulted in a strong reduction of the activity at the top of the villus (not shown), which also indicates that cytochrome *c* oxydase has a more rapid turnover than the rat jejunal epithelium cells (36–48 h).

The rapid *de novo* synthesis of cytochrome *c* oxydase in the transition zone between crypt and villus is particularly apparent from the following experiment. When lactate dehydrogenase activity is measured according to Meijer (1972), in a medium containing lactate, buffer, NAD^+, phenazinemethosulphate, nitrobluetetrazoleum and agar, a clear activity is observed in pieces of jejunum from both control and treated rats, when 1 mM KCN is added to inhibit the respiratory chain (results not shown). This is not surprising since lactate dehydrogenase is synthesized extramitochondrially. In the absence of KCN, however, a clearcut

Fig. 2a—d. Loss of cytochrome *c* oxydase activity from jejunum by pretreatment of rats with OTC for 0 h (a), 12 h (b), 24 h (c) or 48 h (d). The incubation medium, added on top of the sections, consisted of: 60 mM Tris-HCl (pH 7.6), 1.4 mM p-amino-p-methoxydiphenyl amine, 1.6 mM p-amino diphenylamine, 130 mM dimethylformamide and 38 mM cytochrome *c*. Incubation was carried out for 20 min at 4° C



Figs. 3 and 4

difference exists (Figs. 4a and b). Apparently, cytochrome *c* oxydase competes with nitrobluetetrazoleum for reduced phenazinemethosulphate. Therefore, reduced cytochrome *c* oxydase activity (*in vitro* KCN addition or inhibited synthesis of cytochrome *c* oxydase by pretreatment with the antibiotics) results in a higher rate of the formazan formation.

Coupling of the Oxidative Phosphorylation

When freshly prepared slices from freshly frozen tissue are incubated with ATP according to Wachstein and Meisel (1957), ATP hydrolysis is observed. This can be stimulated by the addition of DNP, an uncoupler of oxidative phosphorylation. In the absence of DNP a positive reaction is only observed in the brush-border region of the cell, where alkaline phosphatase is localized (Goldfisher *et al.*, 1964). DNP addition causes the appearance of reactionproduct within the cell, where the mitochondria are to found (Figs. 5a and b). DNP-stimulated ATP-ase is seen whether control- or 48 h antibiotic treated animals are used, indicating unimpaired respiratory chain phosphorylation, whether the animals are treated or not. These results are in agreement with the biochemical data, in which oxidative phosphorylation was measured in isolated mitochondria or in homogenates (De Jonge, 1972).

Discussion

In this paper we reported the influence of CAP and OTC on the structure of the intestine and on the activity of cytochrome *c* oxydase in the epithelial cells. Although the influence of both antibiotics on cytochrome *c* oxydase is identical, the effects of the two antibiotics on the morphology is quantitatively different. OTC affects the morphology of duodenum and jejunum already after 48 h treatment, whereas CAP treatment only results in morphological alterations after 120 h treatment. Although duodenum and jejunum are readily affected morphologically by OTC, the structure of ileum (as well as the cytochrome *c* oxydase activity) is rather resistant, as judged by light microscopy. Perhaps this discrepancy is caused by a longer turnover time of the epithelial cells in ileum when compared with more proximal portions of rat small intestine (Cairnie *et al.*, 1965).

Fig. 3a and b. Loss of cytochrome *c* oxydase activity from jejunum by brief treatment (15 hrs) of rats with CAP. (a) is control and (b) is jejunum of treated rat. The incubation medium, applied as described by Meijer (1972) consisted of 60 mM Tris-HCl (pH 7.6), 1.4 mM p-amino-p-methoxy diphenylamine, 1.6 mM p-amino diphenylamine, 130 mM dimethyl formaide and 1% agar. The incubation time was 45 min at 37° C

Fig. 4a and b. Lactate dehydrogenase-dependent nitrobluetetrazoleum reduction in jejunum of control (a) and 15 hrs. OTC (b) treated rats. The reaction was carried out in principle as described by McMillan (1967). The incubation medium (containing 120 mM lactate, 1 mM NAD^+, 6 mM $MgCl_2$, 0.75 mM nitrobluetetrazoleum, 0.5 mM phenazinemethosulphate, 87.5 mM sodium phosphate buffer of pH 7.4 and 1% agar) was separated from the tissue as described by Meijer (1972). The reaction time was 45 min and the temp. was 37° C

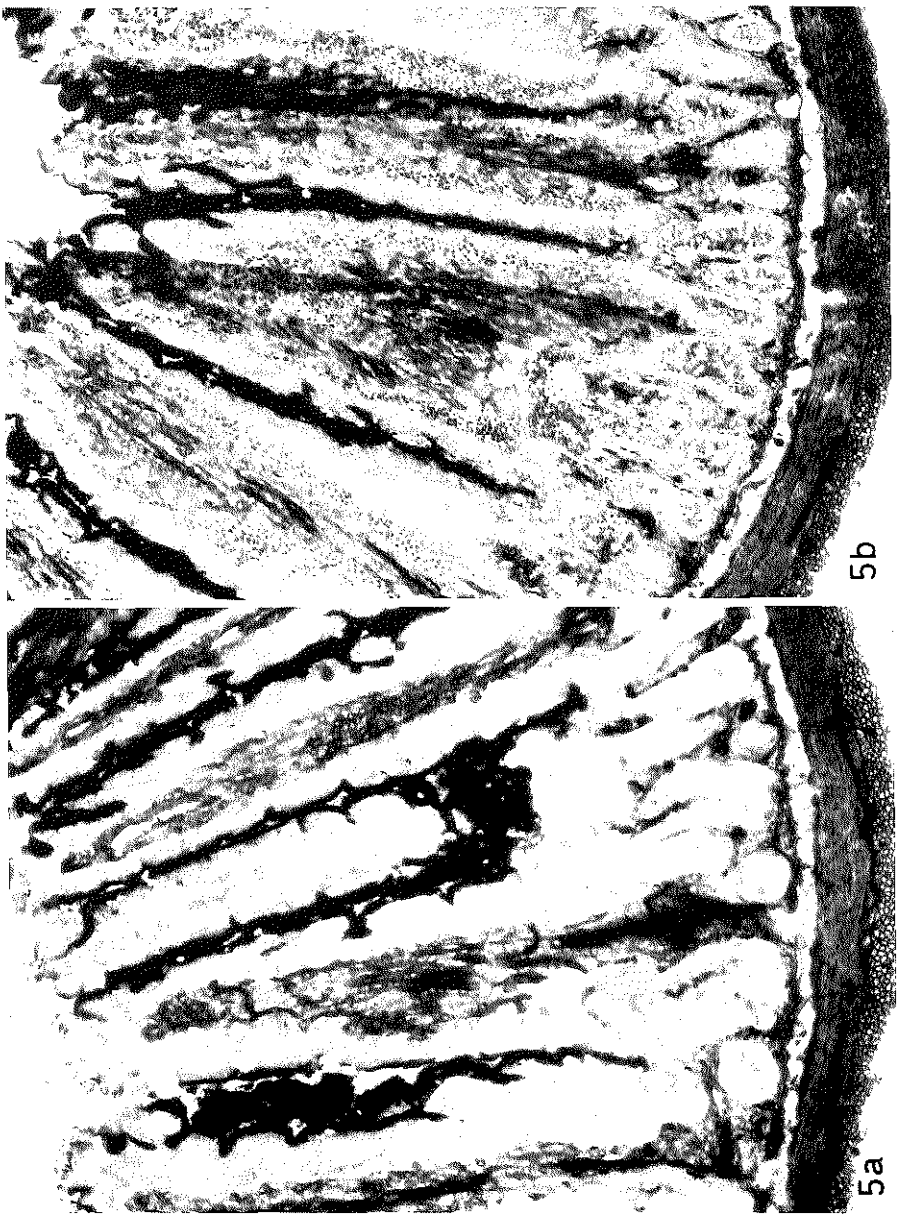


Fig. 5a and b. Stimulation of adenosinetriphosphatase activity by 2,4-dinitrophenol in jejunum. The rat was 48 h pretreated with OTC. The reaction was carried out essentially as described by Wachstein and Meisel (1957). The basic incubation medium contained: 0.8 mM ATP, 80 mM Tris-maleate buffer (pH 7.2), 4 mM $Pb(NO_3)_2$ and 5 mM $MgSO_4$. The incubation time was 45 min at 37° C. (a) Incubation without 2,4-dinitrophenol and (b) incubation with 1 mM 2,4-dinitrophenol

It was rather unexpected that the treatment with both antibiotics for 12–15 h not only influenced the activity of cytochrome *c* oxydase in the crypts but also in the villus. That celldivisions in the crypts are accompanied by the synthesis of mitochondria seems unquestionable. Mitochondria or mitochondrial components are apparently also synthesized in the villus (compare also: Hülsmann

et al., 1970; Iemhoff and Hülsmann, 1971 and De Jonge and Hülsmann, 1972). Antibiotic treatment for only 7 h did not result in detectable alterations (expts. not shown). After 15 h, however, a clearcut effect was seen (Figs. 3 and 4). In fact, in the mid villus region a boundery may often be observed. In the upper half, the cytochrome *c* oxydase activity is higher than in the lower half. After 21 h treatment the cytochrome *c* oxydase activity had almost vanished and the boundery reached the top of the villus (not shown). When it is considered that for the cells to move from the lower part of the villus to the top at least 24 h are required, we might still expect cytochrome *c* oxydase activity at the top. Comparing sections from control and treated animals we always observed less activity in the top area of the villus even though antibiotic treatment was only carried out for 15 h. This then indicates that there is also turnover of cytochrome *c* oxydase in the higher part of the villus. Experiments of Webster and Harrison (1969) showed that the cytochrome *c* oxydase activity remains rather constant in various villous fractions, which could suggest that the net synthesis of cytochrome *c* oxydase stops in the lower part of the villus.

From the experiments as illustrated in Fig. 5 the conclusion may be drawn that oxidative phosphorylation in rat small intestinal mitochondria is coupled. That tight coupling of oxidative phosphorylation occurs was initially difficult to demonstrate biochemically (comp. Iemhoff *et al.*, 1970). It is clear from the present results that fragments of brush border, which contaminate isolated mitochondria, with alkaline phosphatase activity, have a non-specific ATP-ase activity. This activity requires bivalent metal ions and is therefore inhibited by omitting Mg^{2+} and adding EDTA to the incubation medium (results not shown). These very same precautions also led to the demonstration of tight coupling of oxidative phosphorylation in homogenates and mitochondrial preparations from rat small intestine (De Jonge, 1972). The histochemical and biochemical experiments are therefore also in this respect in agreement. That the coupling of oxidative phosphorylation is not abolished by the antibiotic treatment suggests that mitochondrial protein synthesis is not essential in the synthesis of the enzymes involved in the phosphorylation process, provided that the treatment is sufficient enough to inhibit mitochondrial protein synthesis to a high enough degree.

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Histochemical Studies of Fructose-1,6-Diphosphatase in Various Tissues of the Rat

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Summary. Two methods to determine fructose-1,6-diphosphatase activity histochemically were tested on liver, intestine, skeletal muscle and heart of rats. Using lead ions to precipitate inorganic phosphate, according to Wachstein and Meisel, the addition of the specific inhibitor adenosine monophosphate caused an increase of phosphate precipitation. Therefore this method is often not suitable. A coupled assay, used to detect fructose-6-phosphate formed after conversion to glucose-6-phosphate (which in its turn may reduce tetrazolium dyes in the glucose-6-phosphate dehydrogenase reaction), was found to be satisfactory in liver to demonstrate specific fructose-1,6-diphosphatase activity, since adenosine monophosphate was strongly inhibitory. In intestine acid- and alkaline phosphatases, however, were found to interfere. In the latter organ, added adenosine monophosphate itself strongly stimulates formazan formation, which is probably due to high xanthine oxidase activity.

In muscle, where a high aldolase activity is present, monoiodoacetate must be included in the incubation medium. Since fructose-1,6-diphosphatase activity in muscle is low compared with that of liver, the results obtained with muscle are often difficult to interpret.

Introduction

For gluconeogenesis from pyruvate or lactate the presence of four gluconeogenic key enzymes, in addition to enzymes of glycolysis and appropriate cofactors, is required. These key enzymes are pyruvate carboxylase (EC 6.4.1.1), phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose-1,6-diphosphatase (EC 3.1.3.11) and glucose-6-phosphatase (EC 3.1.3.9). According to the available techniques the latter two enzymes should be detectable histochemically in organs with gluconeogenic properties. Indeed, glucose-6-phosphatase has been demonstrated in liver and kidney cortex, employing methods that detect the liberation of inorganic phosphate. The success of demonstrating glucose-6-phosphatase activity is partly due to the insoluble character of this enzyme, which is localized in the endoplasmic reticulum of liver and kidney. Since fructose-1,6-diphosphatase (FDP-ase) is localized in the soluble portion of the cell, care must be taken that attempts to demonstrate the enzyme do not fail because of leakage of the enzyme from the sections during incubation. A few methods are available to cope with this leakage problem. For instance incubation in gels may limit diffusion or the use of relatively thick sections or separation of the tissue from the incubation medium as described more recently (Meijer, 1972).

Although hydrolysis of added fructose-1,6-diphosphate (FDP) has been demonstrated histochemically earlier (comp. Pearse, 1968; Dempsey *et al.*, 1949) we were not convinced that the reactions studied really reflected FDP-ase activity specifically, since for instance acid- or alkaline phosphatases may also hydrolyse added FDP. Therefore in the present investigation use has been made of specific properties of FDP-ase, such as substrate specificity (McGilvery, 1955), the low K_m for FDP, inhibition of the enzyme adenosine monophosphate (AMP) (Krebs and Woodford, 1965; Opie and Newsholme, 1967; Salas *et al.*, 1964), occurrence in e.g. liver, intestinal epithelium and white muscle fibres, but not in heart or red muscle fibers (comp. for instance Opie and Newsholme, 1967; Stifel *et al.*, 1969; Olsen and Marquardt, 1972).

Materials and Methods

For all experiments male Wistar rats were used. They were killed by cervical fracture and subsequent bleeding. Tissue specimens were taken from the liver, gastrocnemius muscle, the heart and from a fixed part of the jejunum, and as soon as possible frozen in isopentane cooled with liquid nitrogen. Frozen sections of 8 μ or 16 μ were made with a microtome (International Equipment Co., model CTF) at $-16°$ C.

Two methods of determination were used. Method I in principle according to Wachstein and Meisel (1957) in which the phosphate formed was detected and Method II, in which the fructose-6-phosphate formed was measured in an indirect way. In both methods followed the substrate concentration used was $\leqq 1$ mM.

Method I. The sections were prefixed in some instances in formalin vapour for 2 min, then in all instances rinsed with 0.1 M Tris buffer (pH = 7.8) and incubated in the absence or presence of 1 mM AMP. The fixation with formalin vapour did not influence FDP-dependent activity significantly. Substrate dependency was checked by omitting FDP from the incubation medium or by replacing the substrate by other organic phosphate compounds, such as fructose-6-phosphate, fructose-1-phosphate or β-glycerolphosphate. The phosphate liberated was precipitated by the addition of Mn^{2+}. The activity of alkaline phosphatase was depressed by the presence of 5 mM EDTA (6 mM $MnCl_2$ present). Although in biochemical experiments this concentration seems to be enough, this is not the case in histochemical experiments. The reaction is stopped by removing the incubation fluid from the sections, followed by rinsing with 0.1 M Tris buffer (pH = 7.0). A saturated solution of lead-nitrate (at pH of 7.0) is used to convert the manganese phosphate into lead phosphate. Subsequently, phosphate is removed by the addition of ammonium sulfide, so that a black precipitate of lead sulfide is formed. The composition of the incubation medium used is given in the legend to Fig. 1.

Method II. This method employs the detection of the fructose-6-phosphate formed by its conversion by phosphoglucose isomerase into glucose-6-phosphate and the subsequent oxidation to 6-phosphogluconate with $NADP^+$ and glucose-6-phosphate dehydrogenase. The NADPH formed is reoxidized with phenazine methosulphate and nitroblue tetrazoleum, so that finally a formazan can be detected. The membrane method of Meijer (1972) is used, in which the tissue section is separated from the incubation medium with a semipermeable membrane. Therefore the addition of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase to the incubation medium has no sense. The enzymic interconversions therefore depend on excesses of endogenous phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. The latter is tested by replacing the substrate FDP by fructose-6-phosphate. The specificity of the reaction is checked by adding the inhibitor AMP to the incubation medium. All incubations were carried out in the presence of 3 mM KCN to prevent the oxidation of reduced phenazine methosulphate by the respiratory chain (De Pijper and Hülsmann, 1973). Mn^{2+} was added as the divalent cation that stimulates FDP-ase activity in low concentrations (comp. Kirtley and Dix, 1971). The composition of the incubation medium used is given in the legend to Fig. 2.

Results

Method I: Detection of Inorganic Phosphate Formed by the Enzymic Hydrolysis of Fructose-1,6-Diphosphate

In general phosphate formed during the enzymatic hydrolysis in histochemical sections is bound by cobalt or lead ions present in the incubation medium (comp. Wachstein and Meisel, 1957) in relatively high concentrations. Although cobalt

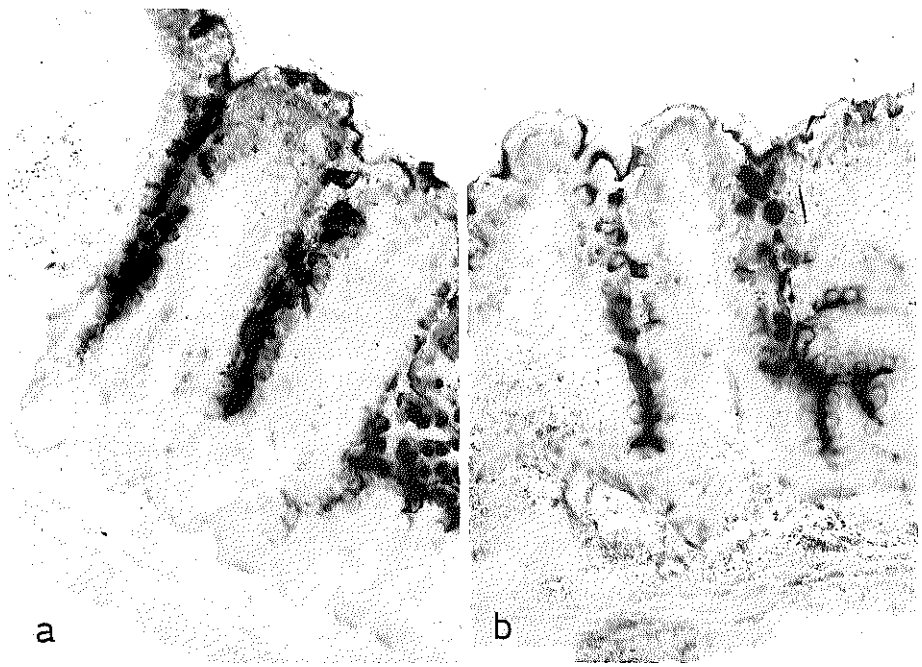

Fig. 1 a and b. FDP dependent precipitation of PbS in jejunum in the absence and in the presence of AMP respectively. The incubation medium consisted of 93 mM Tris-HCl buffer (pH = 7.8), 1.5 mM FDP, 15 mM $MnCl_2$ and (only in experiment b) 1 mM AMP. The incubation was carried out for 30 min at 37° C on 16 μ sections

ions under certain conditions may be used instead of Mg^{2+} or Mn^{2+} to stimulate FDP-ase (Kirtley and Dix, 1971) concentrations of cobalt (or lead) ions required to precipitate inorganic phosphate, inhibit the enzyme completely. Therefore Mg^{2+} and Mn^{2+} were tested for their ability to bind inorganic phosphate. Of these only Mn^{2+} was found suitable at a pH of 7.8. At that pH interfering activities of acid- and alkaline phosphatases were small, compared with the rate of FDP hydrolysis and at pH 7.8 manganese phosphate was rather insoluble and relatively little FDP precipitated by manganese (data not shown). After the incubation was completed, rinsing was also performed at pH 7.0. Post-fixation of the phosphate precipitate was carried out as described under Methods. Indeed, under those conditions a FDP dependent precipitate is formed after 30 min incubation of rat jejunal sections at 37° C, as is shown in Fig. 1. The addition of the specific inhibitor AMP, however, does not result in a decrease of the amount of precipitate. When

FDP was replaced by β-glycerolphosphate the same result was obtained. This then indicates that this technique does not allow the detection of specific FDP-ase in rat small intestinal epithelium. It was often observed that the addition of AMP, instead of decreased, increased the amount of phosphate precipitated formed. This was particularly clear in light areas such as connective tissue and muscularis mucosae (Fig. 1 b). When the reaction was applied to sections of skeletal muscle, which contains practically no acid- or alkaline phosphatase, no reaction was seen at all. Only in the presence of AMP, precipitates around the blood vessels were clearly visible, most probably due to the enzyme 5′-nucleotidase (EC 3.1.3.5).

Method II: Detection of Fructose-6-Phosphate Formed by the Enzymic Hydrolysis of Fructose-1,6-Diphosphate

As has been mentioned under methods, the glucose-6-phosphate formed finally reduces $NADP^+$, which is reoxidized by nitroblue tetrazoleum, in the presence of phenazine methosulphate. Since this method employs the enzymes phosphoglucose isomerase and glucose-6-phosphate dehydrogenase in addition to be determined, it is necessary to determine the reaction rate with fructose-6-phosphate as the substrate, to see whether the former two enzymes are not rate-limiting. (As was mentioned earlier, the membrane-method followed does not allow the addition of these enzymes to the tissue section). It was found that both in liver and intestine the rate of formazan formation with fructose-6-phosphate as the substrate exceeds by far that with FDP. The latter reaction in liver is shown in Fig. 2. The activity shown in Fig. 2 a represents specific FDP-ase activity, since the allosteric inhibitor AMP indeed strongly inhibits the reaction (Fig. 2 b). The reactions in the absence of the substrate are shown in Fig. 2 c and d, the latter representing the pattern obtained with AMP present.

That in the presence of AMP and absence of FDP little formazan is formed, holds for liver but certainly not for rat small intestine. This is shown in Fig. 3 a. It was subsequently demonstrated that this effect of AMP on nitroblue tetrazoleum reduction was not specific for the phosphorylated compound, since adenosine, inosine, hypoxanthine and xanthine gave similar results. Apparently, dephosphorylation and deamination of AMP (Brady and Donovan, 1965) in rat small intestine is very active, as well as xanthine oxidase (EC 1.2.3.2; Sackler, 1966). Fig. 3 b shows the result that is obtained when AMP is replaced by hypoxanthine. Virtually no activity is descernable in the crypts, brushborders or nuclei. Because of the high rate of degradation of AMP in rat small intestine, this compound cannot be used as an inhibitor of FDP-ase in the present histochemical study. Hence

Fig. 2 a—d. FDP dependent nitrobluetetrazolium reduction in liver (a) and the inhibition with AMP (b). The reaction without FDP is shown in (c) (AMP absent) and (d) (AMP present). The incubation medium consisted of 0.64 mM $NADP^+$, 1.5 mM $MnCl_2$, 1.5 mM nitrobluetetrazolium, 3 mM KCN, 3 mM monoiodoacetate, 0.1 mM phenazine methosulphate, 66 mM triethanolamine buffer, 1.2 % agar and where shown 0.5 mM FDP and/or 1 mM AMP. The final pH was 7.8, the incubation time 30 min and reaction temperature 37° C. Sections of 8 μ were used. The membrane method of Meijer was followed

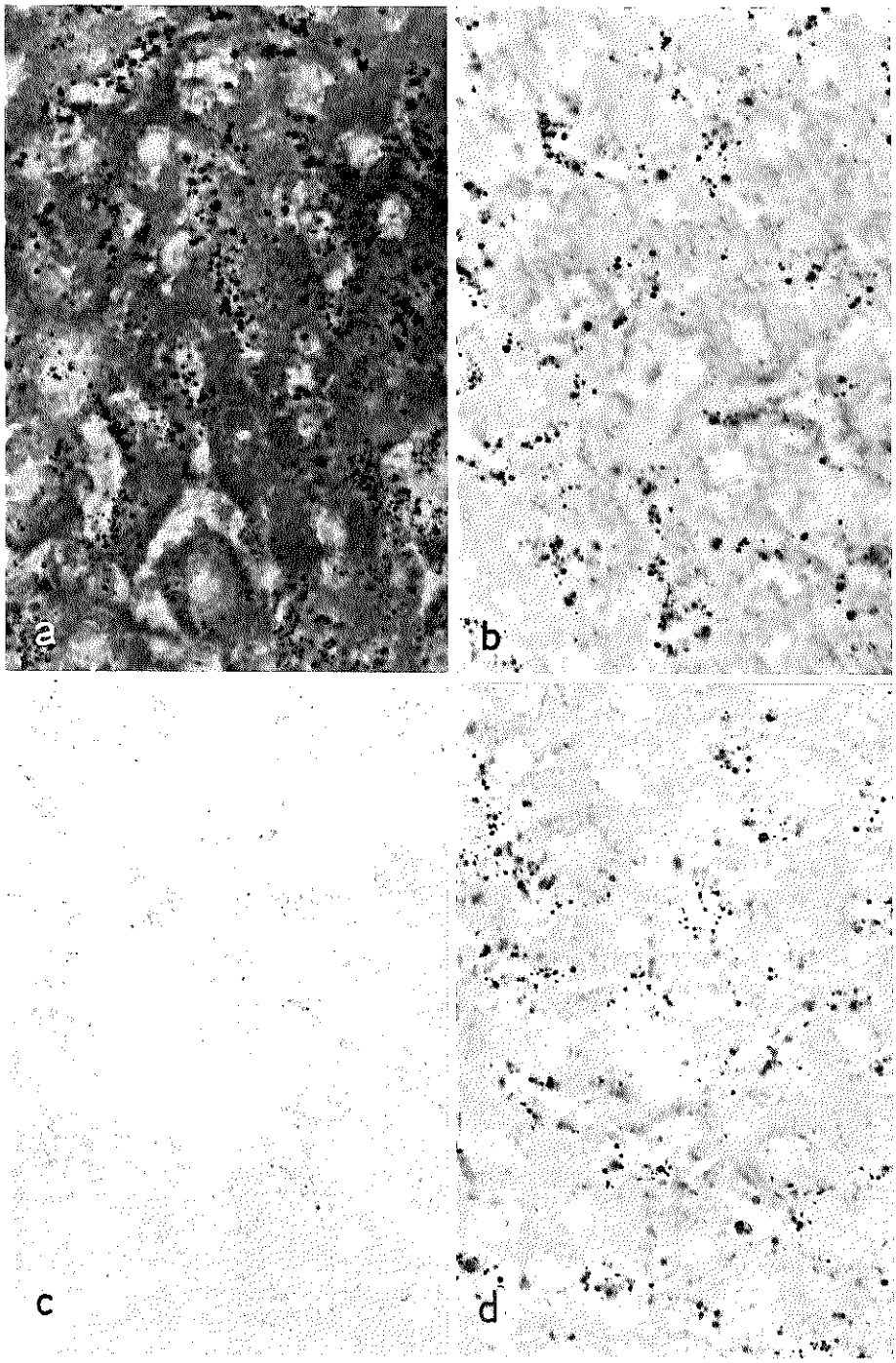

Fig. 2a—d

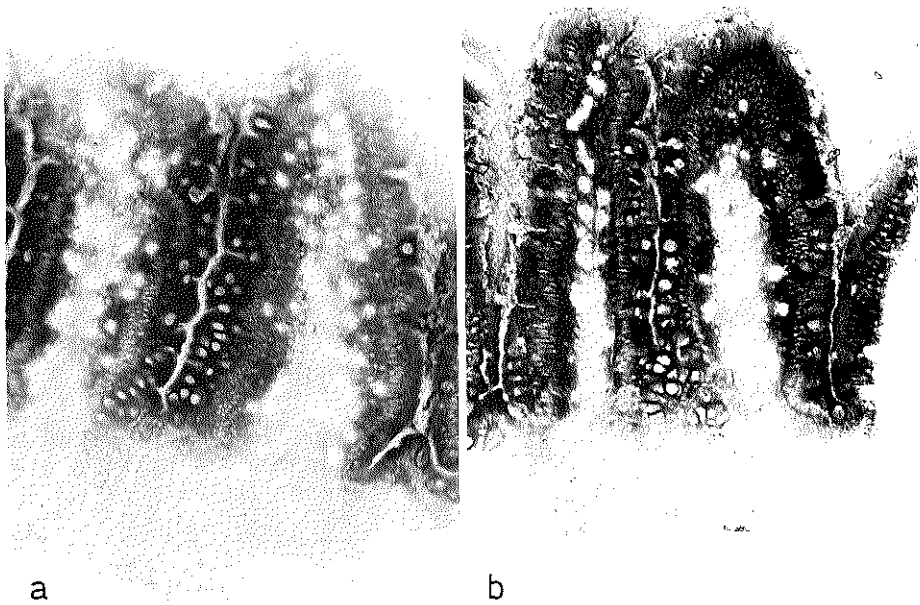

Fig. 3a and b. AMP dependent reduction of nitrobluetetrazolium in rat jejunum (a) and (b) the same for hypoxanthine. The incubation medium consisted of 2 mM AMP or hypoxanthine, 1.5 mM $MnCl_2$, 3 mM KCN, 1.5 mM nitrobluetetrazolium, 133 mM triethanolamine buffer (pH 7.5), 0.1 mM phenazine methosulphate and 1.2 % agar. The membrane method of Meijer was followed. Incubation was carried out for 20 min at 37° C on sections of 8 μ

FDP-ase, although present in rat small intestinal mucosa (Stifel *et al.*, 1969), is not demonstrable by method II. That the use of a specific inhibitor is absolutely required for the detection of FDP-ase is clear, when it is considered that in intestine non-specific alkaline- and acid phosphatases, leading to fructose-6-phosphate formation, are highly active.

An important side reaction that may occur when FDP is used as the substrate, is the aldolase (EC 4.1.2.13) reaction. Especially in muscle, where aldolase activity is high and FDP-ase activity is low (comp. Opie and Newsholme, 1967), as well as glucose-6-phosphate dehydrogenase activity. Therefore, the measurement of fructose-1,6-diphosphatase activity may not be measured by method II, as will be shown below. Initial experiments showed in muscle a high rate of the FDP dependent reduction of nitroblue tetrazoleum, as measured by method II. In heart, where FDP-ase is known to be absent (Opie and Newsholme, 1967), the activity was equally present which led us to investigate whether the activity could be blocked by AMP. Indeed, AMP inhibited the reaction considerably (not shown). A possibility which had not been excluded was aldolase activity, followed by glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), which might lead to nitroblue tetrazolium reduction if part of the $NADP^+$, present in the incubation medium, would have been converted to NAD^+ by non-specific phosphatase

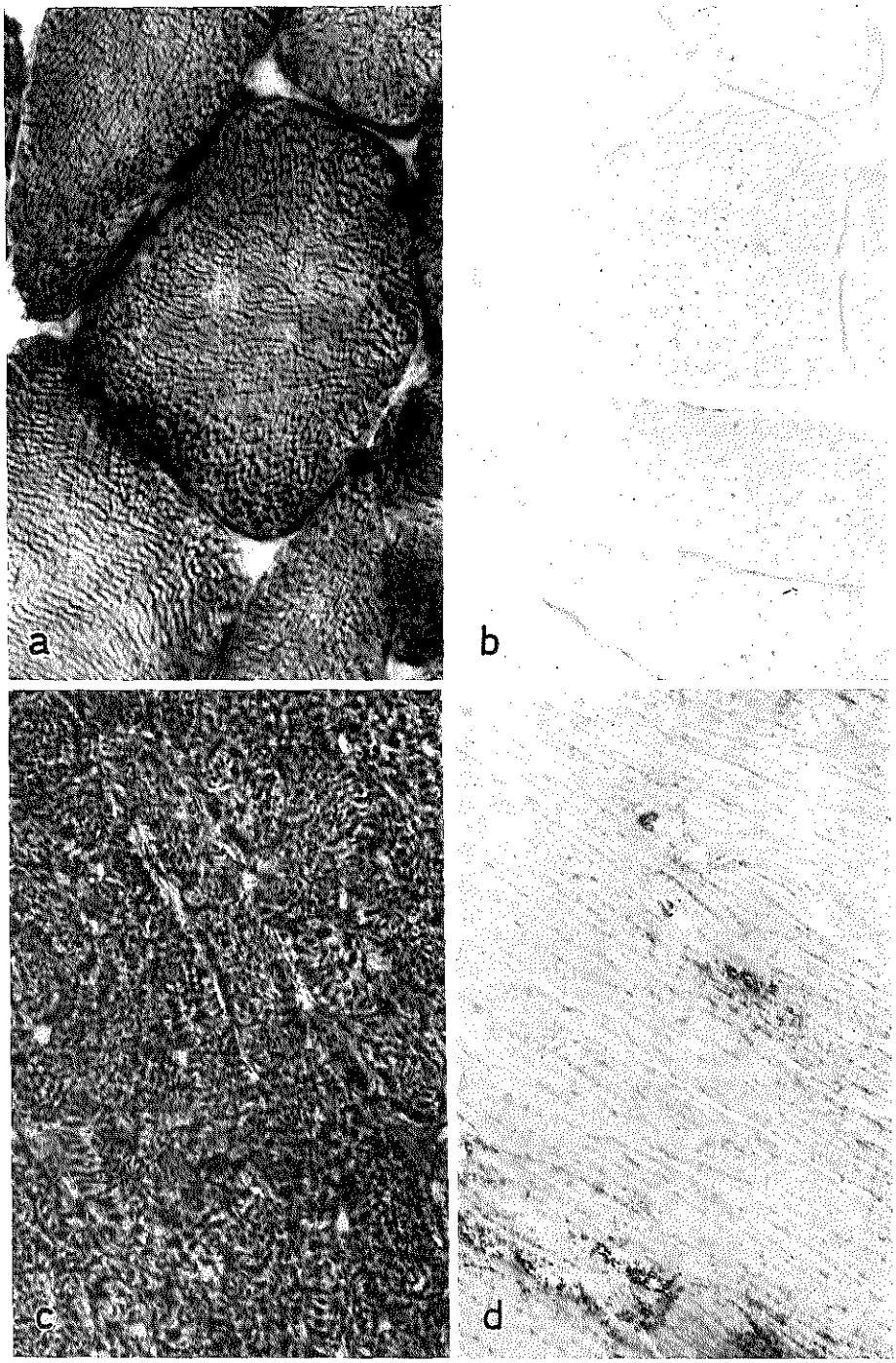

Fig. 4a—d. FDP dependent reduction of nitrobluetetrazolium in gastrocnemius and heart muscle of the rat in the absence or presence of 3 mM monoiodoacetate. The incubation medium is described in Fig. 2. The incubation time was 45 min at 37° C on sections of 8 μ. (a) gastrocnemius muscle, monoiodoacetate absent, (b) gastrocnemius muscle, monoiodoacetate present, (c) heart muscle, monoiodoacetate absent and (d) heart muscle, monoiodoacetate present

activity. Such a phosphatase activity could then also act on AMP as substrate, so that it would spare $NADP^+$. The following reactions might then be involved:

1) Fructose-1,6-phosphate aldolase, leading to synthesis of glyceraldehyde-3-phosphate.

2) Non-specific phosphatase, leading to the synthesis of catalytic amounts of NAD^+ from $NADP^+$.

3) Glyceraldehyde-3-phosphate dehydrogenase, leading to the generation of NADH (and reduction of nitroblue tetrazolium in the presence of the dye and phenazine methosulphate).

To test this hypothesis $NADP^+$ was first replaced by NAD^+ and indeed a strong reaction ensued, which could not be inhibited by the addition of AMP (not shown). In another series of experiments the effect of the addition of monoiodoacetate, which inhibits glyceraldehyde-3-phosphate dehydrogenase, to the $NADP^+$ containing medium (method II) was studied. These experiments are illustrated in Fig. 4. Fig., 4a and b demonstrate the FDP dependent reaction in sections of the gastrocnemius muscle of the rat in the absence and presence of monoiodoacetate respectively and Fig. 4c and d demonstrate the same in rat heart.

The results obtained so far clearly demonstrate that of the tissues tested histochemically for the presence of FDP-ase, intestine and muscle (both with relatively low activities as determined biochemically) gave false positive results. Only in liver, where the enzyme has a much higher activity, the enzyme may be demonstrated histochemically. The undesirable side reactions are relatively little important. In a later experiment (not shown) the pattern observed in Fig. 2 was not altered by the addition of monoiodoacetate.

Discussion

The presently obtained results clearly indicate that the histochemical determination of FDP-ase is full of pitfalls. In the rat the enzyme is relatively active only in liver and kidney, organs with high gluconeogenic properties. Some other tissues, such as intestinal mucosa and skeletal muscle (especially the white fibers) have an activity which, as measured biochemically, is one order of magnitude lower than in the gluconeogenic organs. This together with the side reactions which may occur in these tissues, makes it rather unlikely to demonstrate the enzyme in intestine and skeletal muscle histochemically. In liver we were more successful in demonstrating the enzyme. Since the enzyme is localized in the cytosol and therefore could easily leak away from the tissue slices during incubation, the membrane technique as described by Meijer (1972) was followed. One of the side reactions in liver that can interfere with the assay is the aldolase reaction (comp. Results section), followed by the glyceraldehyde-3-phosphate dehydrogenase reaction. It was found, however, that in liver the pattern obtained was not influenced by the addition of monoiodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase. In determining the specificity of the FDP-ase reaction in liver, advantage was taken of the (allosteric) inhibition of the enzyme by AMP. The enzyme is not inhibited by other nucleoside monophosphates, such as for instance inosinemonophosphate. Indeed in experiments not shown, inosine monophosphate in contrast

to adenosine monophosphate did not inhibit the reaction in liver. Larger magnification of results as shown in Fig. 2a, clearly demonstrate that the enzyme activity is mainly present in the parenchymal cells, suggest only low, or absent activity in Kupffer cells or other non-parenchymal cells. The same has been found for another gluconeogenic enzyme glucose-6-phosphatase (Lentz, 1971). Hence, it can be concluded that gluconeogenesis is confined to the parenchymal cells in liver. This conclusion was already earlier reached by Van Berkel *et al.* (1972), who separated parenchymal cells from non-parenchymal cells and demonstrated biochemically that the Liver-type of pyruvate kinase, which can be inhibited during conditions favourable for gluconeogenesis, is confined to parenchymal cells, whereas the Muscle-type, which is not shut off under physiological conditions, is confined to the non-parenchymal cells.

In Fig. 3 it was shown that in jejunal mucosa of the rat AMP or hypoxanthine were potent electron donors. It was mentioned that adenosine, inosine and xanthine gave similar results and that xanthine oxidase was probably responsible for the reduction of nitroblue tetrazolium. Since the enzyme activity was present in the villi, and not in the crypt, we investigated whether the passage of food was required for the enzymatic activity. This was found not to be the case, since in a 3-weeks old subcutaneously localized Thiry-Vella fistula (the operation was carried out by Dr. H. de Ruiter, Department of Celbiology I, Rotterdam Medical School, who is greatly acknowledged) of a mature rat, the activity was identical to the residual jejunum, which was exposed to food.

Therefore the appearance of the enzyme activity in the villus is not due to food induction. Because of this potent activity in rat small intestine, the histochemical demonstration of oxidation processes in which AMP may be formed (for instance during fatty acid activation) may severely be hindered.

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Pitfalls in Histochemical Localization Studies of NADPH Generating Enzymes or Enzyme Systems in Rat Small Intestine

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Summary. In the presence of excess β-glycerophosphate or p-nitrophenylphosphate NADPH diaphorase of the epithelial cell in rat small intestine has its highest activity in the villi at the basis of the microvilli, where smooth endoplasmic reticulum of the cells is present. In the absence of β-glycerophosphate or p-nitrophenylphosphate the diaphorase activity is much higher and broader localized in crypts and villi. The increased activity is due to the conversion of NADPH to NADH by non-specific (mostly alkaline-) phosphatase activity, so that both NADPH and NADH diaphorase activities are measured.

When NADPH is generated by a specific $NADP^+$-linked dehydrogenase, such as glucose-6-phosphate dehydrogenase, and nitroblue tetrazolium is present to trap the reducing equivalents formed, the histochemical localization of the dehydrogenase is identical to that of NADPH diaphorase, although the dehydrogenase may be present in another cell compartment (glucose-6-phosphate dehydrogenase for instance has a cytosolic distribution). Therefore the localization of a dehydrogenase may be falsely interpreted histochemically, according to the diaphorase reaction involved. A different localization may be obtained when the diaphorase reaction is circumvented by the addition of an alternative hydrogen carrier, such as phenazine methosulphate. Also in coupled dehydrogenase assays this may be observed. The hexokinase reaction, coupled to the glucose-6-phosphate dehydrogenase reaction in the presence of nitroblue tetrazolium and the absence of phenazine methosulphate, has a distribution identical to NADPH diaphorase. In the presence of phenazine methosulphate the enzyme has an almost ubiquitous distribution in the small intestinal epithelial cell. When a substrate may react both with $NADP^+$- and NAD^+-linked dehydrogenases, such as L-malate: NADP oxidoreductase (decarboxylating) and L-malate: NAD oxidoreductase respectively, the high activity of intestinal alkaline phosphatase may influence the histochemical distribution by converting part of $NADP^+$ to NAD^+. The addition of another phosphate ester, such a β-glycerophosphate or p-nitrophenylphosphate, may therefore influence the observed distribution.

Introduction

For the histochemical determination of $NAD(P)^+$ linked dehydrogenases, use is generally made of the nitroblue tetrazolium (NBT) method, which is based on the ability of NBT to reoxidize the reduced nicotinamide adenine dinucleotides in the presence of a flavoprotein (Farber *et al.*, 1956) or an artificial catalyst such as phenazine methosulphate (PMS) (Fahimi and Amarasingham, 1964).

$$NAD(P)H + H^+ + NBT \xrightarrow[PMS]{\text{flavoprotein}} NAD(P)^+ + NBT \text{ diformazan.}$$

PMS as the catalyst is presently favoured since addition of PMS makes the

overall formation of insoluble NBT diformazan independent of the localization of flavoproteins. A disadvantage of the use of PMS in the histochemical study of insoluble or insolubilized NAD(P)H generating enzymes or enzym systems is that $PMSH_2$, formed as an intermediate, diffuses away from the site(s) of NAD(P)H formation and therefore contributes to unsharp localizations. For that reason a number of histochemical NBT-assays still employ the flavoprotein-mediated reduction of the dye. This implies that the localization of the $NAD(P)^+$ linked dehydrogenases studied may be identical to the localization of the flavoprotein required for the ultimate reduction of NBT, thus creating false localizations. Moreover, NBT diformazan has a strongly lipophylic character and will therefore tend to adhere to membranes. Recently it was presented as a lipoprotein stain (Segal *et al.*, 1973). In our laboratory (Van den Berg and Hülsmann, 1971) for instance it was therefore believed that the bulk of rat small intestinal hexokinase was localized in the terminal web region of the cell, which agreed with the biochemical detection of hexokinase in isolated brush border preparations. A subsequent biochemical distribution study, however (Hülsmann *et al.*, 1973), learned that although a small amount of the hexokinase may be present in the brush border region of the cell, at least the bulk is present in the cytosolic and mitochondrial fractions. This could be confirmed histochemically by including PMS in the incubation media, as will be demonstrated in the present paper.

There are plenty of examples of NADH generating enzymes that histochemically seem to be localized at the site of NADH-diaphorase (for instance the localization of lactate dehydrogenase in mitochondria). A parallel biochemical distribution study then often reveals the fallacy. It may be of interest to note that NADPH diaphorase in many tissues has a localization distinct from NADH diaphorase. Whereas NADPH flavoproteins are mainly localized in the extramitochondrial compartment [NADPH-cytochrome-c reductase (EC 1.6.2.3) can be used as a marker enzyme for small intestinal microsomes (Clark, 1969)], the bulk of the NADH flavoproteins is mainly found in the mitochondria. The mitochondria contain in the innermembrane/matrix compartment the flavoproteins involved in the respiratory chain, in the citric acid cycle and in the β-oxidation of fatty acids. The outer membrane contains the flavoprotein that catalyzes rotenone insensitive NADH-dependent reduction of cytochrome *c*.

Indeed, in agreement with these biochemical localization studies, the earlier histochemical studies of Nachlas *et al.* (1958a, b) have shown that the localization of NADH diaphorase is often restricted to the mitochondria, whereas the NADPH diaphorase is distributed in the cytoplasma. It is clear that when NADPH can be converted to NADH in the tissue section, that the overall diaphorase reaction may increase and that the localization may be as well extra as intramitochondrial. Since in the brush border of the intestinal epithelial cells alkaline phosphatase is very active, the villi with well developed brush borders especially may have the extra activity, although the crypts may also have some extra activity due to the generation of NADH by villous alkaline phosphatase in the incubation medium. The present paper will show that the inclusion of a phosphate ester other than NADPH will strongly influence the activity and the localization of the diaphorase reaction, probably by competing with NADPH for phosphatase

action. It will also be shown that NADPH generating enzyme systems for their histochemical localization may strongly suffer from the operation of non-specific phosphatase(s) since $NADP^+$ may easily be converted to NAD^+, leading in some instances to NADH formation, so that localization and activity of the reaction under study may be falsely interpreted. The use of a competitive inhibitor of NADP(H) phosphatases such as β-glycerophosphate or p-nitrophenylphosphate therefore is recommended in the histochemical assay of $NAD(P)^+$ linked reactions in tissue with high phosphatase activity.

Materials and Methods

In all experiments male Wistar rats of 200 ± 20 g were used. After killing the animal by cervical fracture and subsequent bleeding, the intestine was removed and as soon as possible pieces of 1 cm were frozen in isopentane cooled with liquid nitrogen. Frozen sections of 10 μ were made with a microtome (Int. Equipment Co. Model CTF) at $-16°$ C. The sections were incubated at 37° C with or without prefixation in cold aceton of $-20°$ C. A few drops of incubation fluid were added directly on the sections. Substrate dependency was checked by omitting the substrate from the incubation medium. Specificity of the reaction for $NADP^+$ was checked by omitting the cofactor or by replacing this cofactor by NAD^+. In all cases the reaction was stopped by replacing the incubation fluid by a mixture containing 1 part 40% formalin, 8 parts 6% macrodex in 0.9% NaCl and 1 part 1% $CaCl_2$.

NADPH diaphorase was determined, according to Scarpelli (1958), by incubation of the tissue sections with NADPH as is mentioned in Fig. 1. Hexokinase and glucose-6-phosphate dehydrogenase were determined, according to Meijer (1967) in the absence or presence of PMS, as shown in Fig. 2. Malic enzyme [L-malate: NADP oxidoreductase (decarboxylating), EC 1.1.40] was determined as shown in Fig. 3.

Results

NADPH Diaphorase Activity and Its Localization in Rat Small Intestine

NADPH diaphorase was determined histochemically, in principle according to Scarpelli *et al.* (1958), in rat small intestinal epithelium. It was also tested, whether saturation of phosphatases by the addition of excess p-nitrophenylphosphate influenced the reaction. It can be seen from Fig. 1 a that in the absence of p-nitrophenylphosphate the reaction has an almost ubiquitous distribution. The addition of p-nitrophenylphosphate, however, strongly inhibits the reaction and alters the distribution (Fig. 1 b) in that now the crypts become relatively inactive. The villi also loose considerable activity with the exception of the area between the nucleus and the microvilli. A similar effect was obtained when p-nitrophenylphosphate was replaced by β-glycerophosphate (not shown). Apparently, a phosphate ester other than NADPH, inhibits the activity. Hence it is conceivable that *e.g.* alkaline phosphatase localized in the brush border region of the villous cell converts part of the NADPH to NADH (this reaction was verified biochemically, using purified calf intestinal alkaline phosphatase; not shown). The main activity of NADPH diaphorase is apparently present in the apical region of the villous cell, where indeed smooth endoplasmic reticulum is well represented. NADH diaphorase on the other hand is more active, and is distributed throughout the cell (mainly in mitochondria—comp. the Introduction).

Fig. 1 a and b. NADPH diaphorase activity in duodenum in the absence (Fig. 1 a) and in the presence (Fig. 1 b) of 16 mM p-nitrophenylphosphate. The incubation medium contained: 0.84 mM NADPH, 1.4 mM NBT, 153 mM Triethylamino-HCl (TEA). The incubation was carried out [after (10 min) prefixation of the tissue slices in cold aceton] for 30 min at pH 7.4

Glucose-6-Phosphate Dehydrogenase (EC 1.1.1.49) Determination in the Presence or Absence of Phenazine Methosulphate

It can be seen from Fig. 2a that in the absence of PMS the glucose-6-phosphate dependent formation of reduced nitrobluetetrazolium is localized in the brush border region of the epithelial cells of the villi. A similar result was obtained when glucose-6-phosphate was generated in the hexokinase reaction (Van den Berg and Hülsmann, 1971) or when NADPH was added in substrate amounts in the presence of p-nitrophenylphosphate or β-glycerophosphate (previous section). Apparently, this localization corresponds to that of NADPH diaphorase. Since biochemical studies have shown that a large part of hexokinase is cytosolic in nature, as well as most, if not all, of the glucose-6-phosphate dehydrogenase, it must be concluded that in the absence of PMS these enzyme localizations follow that of the rate limiting step NADPH diaphorase. Indeed, when this reaction is circumvented by the addition of PMS, the glucose-6-phosphate dehydrogenase (Fig. 2b) and to a lesser extent the hexokinase reaction, coupled to the former (Fig. 2d), increases in activity and shows a distribution not only in the brush border region, but also in other parts of the villous cells, while the activity now also appears in the crypts. The latter also correlates with biochemical measurements of isolated crypts, whether obtained by the method of Harrison and Webster (1969) or by microdissection of freeze-dried material (H. R. de Jonge and N. de Both, unpublished). The glucose-6-phosphate dehydrogenase reaction in the absence of PMS (Fig. 2a) appeared not to be influenced by p-nitrophenylphosphate (not shown). For this reaction it is imperative whether part of the added $NADP^+$ is converted by phosphatase(s) to NAD^+, since glucose-6-phosphate can only reduce $NADP^+$ and not NAD^+. This may be different when glucose-6-phosphate is replaced by malate, as will be shown in the next section.

Malic Enzyme and Malate Dehydrogenase

Malic enzyme [L-malate: NADP oxidoreductase (decarboxylating)] which is specific for $NADP^+$ as a cofactor and which requires Mg^{2+} or Mn^{2+} for activity can be measured in analogy to glucose-6-phosphate dehydrogenase, since both enzyme reactions generate NADPH. However, in the case that malate is the substrate, the NAD^+ formed from $NADP^+$ by phosphatase action may also react with NAD^+-specific malate dehydrogenase (EC 1.1.1.37). Indeed, comparison of Fig. 3a and b shows that competitive inhibition of NADPH phosphatase by the addition of p-nitrophenylphosphate causes a considerable inhibition of the rate of NBT reduction in the epithelial cells. Therefore when the malic enzyme reaction is carried out in the absence of p-nitrophenylphosphate, the illusion is obtained that malic enzyme is a rather active enzyme in the epithelial cell. That this is not the case was also demonstrated biochemically by comparing the forward reactions catalysed by malic enzyme and malate dehydrogenases in homogenized isolated epithelial cells. These results (not shown) indicated that $NADP^+$-specific malic enzyme has an activity one order of magnitude lower than NAD^+-specific malate dehydrogenases. In the presence of p-nitrophenylphosphate (Fig. 3b) the activity as tested histochemically is low and the activity in the muscular layer is more pronounced than in the epithelial layer. An analogous but less clearly

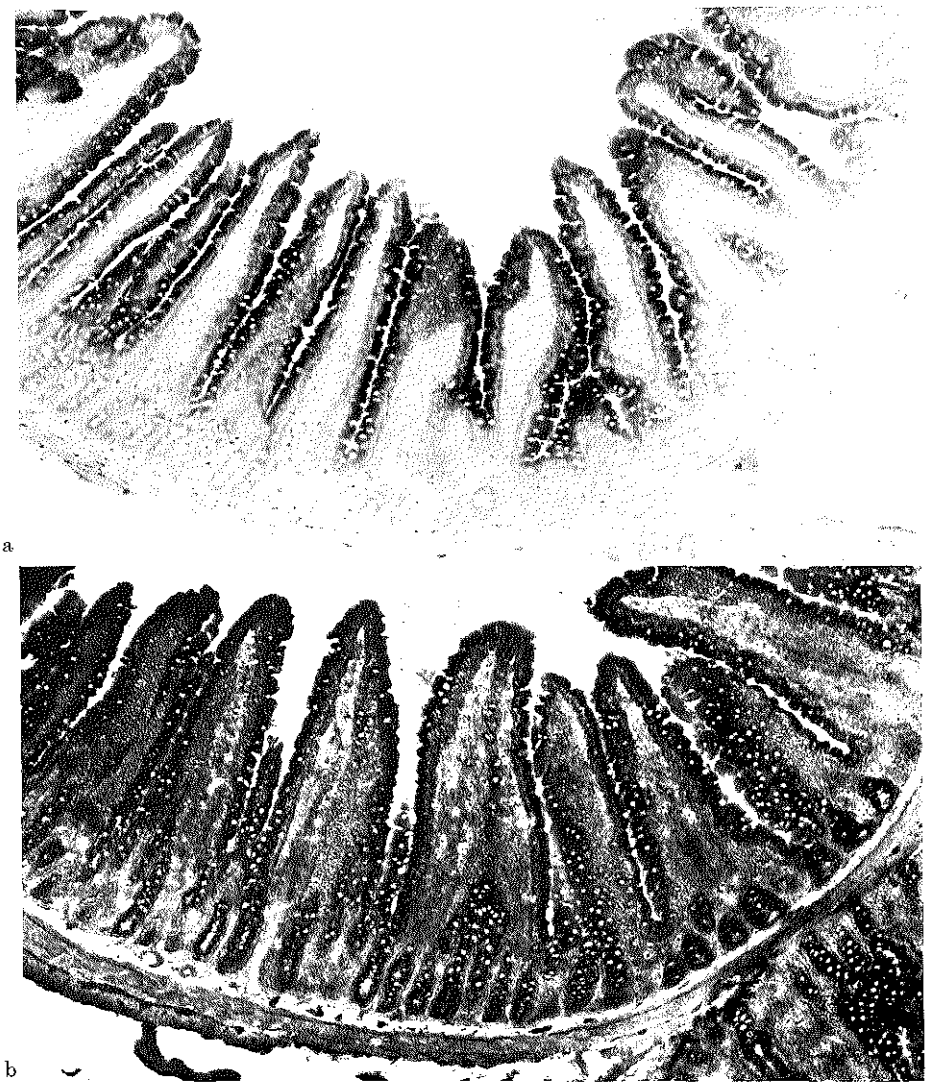

Fig. 2a and b. Glucose-6-phosphate dehydrogenase (Figs. 2a and b) and hexokinase (Fig. 2c and d: activities in rat small intestine. The incubations were carried out according to Meijer. Fig. 2a and c: incubation in the absence and Fig. 2b and d in the presence of 0.1 mM phenazine methosulfate. The incubation medium is consisted either 23 mM glucose-6-phosphate (glucose-6-phosphate determination) or 28 mM glucose and 1.5 mM ATP (hexokinase determination) and furthermore 0.5 mM $NADP^+$, 16 mM $MgCl_2$, 0.5 mM NBT, 1.8 mM NaN_3 and 160 mM TEA. Gelatin was present in a final concentration of 1%. The incubation was carried out [after (10 min) prefixation of the tissue slices in cold aceton] for 50 min at pH 7.4

expressed situation was obtained when malate was replaced by isocitrate (not shown) since also with this substrate a $NADP^+$-dependent decarboxylating enzyme (EC 1.1.1.42) exists besides a NAD^+-dependent isocitrate dehydrogenase (EC 1.1.1.41).

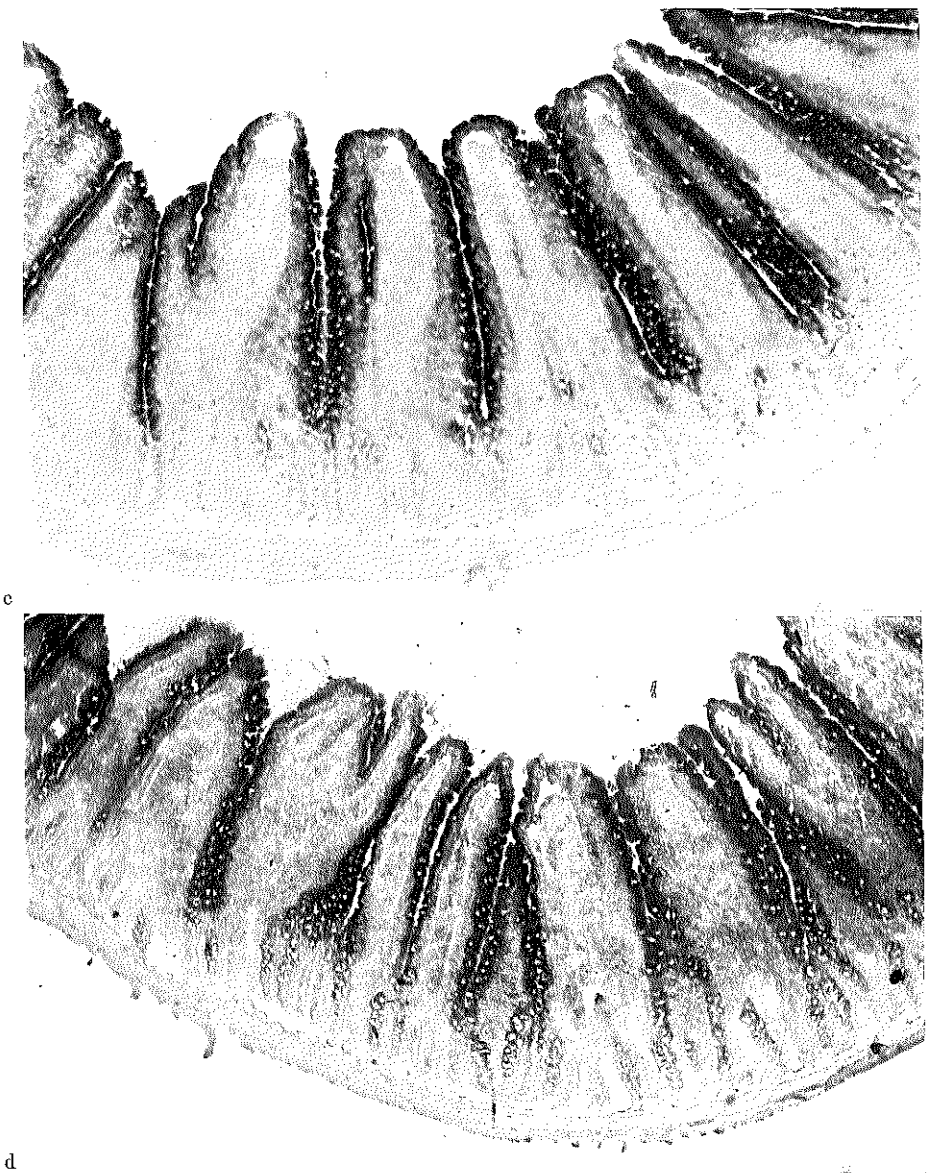

Fig. 2c and d

Discussion

NADPH Diaphorase Activity in Intestine

When NADPH diaphorase activity is measured in intestine, according to the method of Scarpelli *et al.* (1958), a localization pattern comparable to that of NADH diaphorase is observed after prolonged incubation. Short term incubation (2 min), however, shows a clear difference between NADPH and NADH added

Fig. 3a and b. Malic enzyme determination in the absence (Fig. 3a) or presence (Fig. 3b) of 16 mM p-nitrophenylphosphate of 16 mM β-glycerophosphate. The incubation medium contained: 11 mM L-malate, 0.5 mM $NADP^+$, 9.8 mM NaN_3, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1.5 mM NBT, 0.1 mM PMS and 70 mM TEA buffer. The incubation was carried out [after 10 min) prefixation of the tissue slices in cold aceton] for 45 min at pH 7.4.

as the substrates (not shown). The NADPH-dependent reaction is relatively slow and confined to the apical portion of the villous cell, whereas the NADH-dependent reaction is much more active and is seen all over the villous cell. Moreover, the NADPH dependent reaction is virtually absent in the crypt in contrast to the reaction with NADH. Different factors can be responsible for the latter.In the first place it is possible that a large part of NADPH diaphorase in the crypt is solubilized and is removed during incubation from the tissue section. Another contribution may be the increase of the endoplasmatic reticulum at the basis of the villus (Toner, 1968). Nachlas *et al.* described already in 1958 that this diaphorase is possibly the same as NADPH-cytochrome *c* reductase, a marker enzyme for the microsomal fraction (Clark *et al.*, 1969), which contributes to the jump of NADPH diaphorase activity between crypt and villus.

Since phosphatase activity causes the formation of NADH from NADPH in the incubation medium, it is to be expected that both in villus and crypt the mitochondrial NADH diaphorase contributes to the formation of formazan. Indeed, when during incubation with NADPH p-nitrophenylphosphate is also added to the incubation medium, to compete with NADPH for the phosphatase, the mitochondrial localization disappears. Inhibition by the addition of p-nitrophenylphosphate may partly be due to the formation of the uncoupler of oxidative phosphorylation, p-nitrophenol, which in large amounts may inhibit part of the mitochondrial repiratory chain (Hülsmann, 1958). To investigate this possible contribution of p-nitrophenylphosphate inhibition of NBT reduction the compound was replaced by β-glycerophosphate. The result was virtually the same, indicating that saturation of phosphatase(s) is the most likely explanation. From these results then it may be concluded that histochemically NADPH diaphorase of rat small intestinal epithelium is mainly detectable in the villi in the supranuclear zone, containing the bulk of endoplasmic reticulum.

Glucose-6-Phosphate Dehydrogenase and Hexokinase Determined in the Absence or in the Presence of p-Nitrophenylphosphate

In the absence of p-nitrophenylphosphate (or β-glycerophosphate) the localization of glucose-6-phosphate dehydrogenase reaction (or of the hexokinase reaction, when glucose-6-phosphate is replaced by ATP and glucose) is that of NADPH diaphorase. When part of the $NADP^+$ added to the section is converted to NAD^+ by phosphatase activity, the pattern is not influenced since glucose-6-phosphate dehydrogenase is strictly $NADP^+$ specific and the NADPH formed is rapidly oxidized, not allowing sufficient accumulation of NADH to saturate NADH diaphorase(s). When the NADPH diaphorase reaction is circumvented by the addition of PMS, the distribution of the formazan formed no longer follows that of NADPH diaphorase, but corresponds to the distribution of the enzymes of rat small intestinal epithelium, found in biochemical studies.

$NADP^+$ and NAD^+ Dependent Malate Dehydrogenases

Whereas for the activity of glucose-6-phosphate dehydrogenase the addition of p-nitrophenylphosphate (or β-glycerophosphate) had no effect, the activity of "malic enzyme" was severely influenced. The reason for the latter finding is that

dephosphorylation of part of the $NADP^+$ to NAD^+, brings malate dehydrogenases also into action. These NAD^+ dependent enzymes have a double localization in most cells: in the mitochondrial matrix and in the cytosol. The same principle was observed when malate was replaced by isocitrate. The inhibition of NBT reduction by the addition of p-nitrophenylphosphate then, however, was much less. Plaut and Sung (1954) already found that the NAD^+ dependent activity was much less than the $NADP^+$ dependent activity, so that the contribution of the NAD^+ linked enzyme to the overall isocitrate dependent reduction of nicotinamide adenine nucleotides is much less.

Considering the present results care must be taken that data based on NADPH diaphorase-dependent histochemical reactions in intestine are not influenced by conversions of NADP(H) by the highly active alkaline phosphatase of small intestine. In other tissues alkaline phosphatase is generally much less active, but acid phosphatase may interfere for the same reasons. In general, the addition of PMS to circumvent diaphorases may be recommended, although still care should be taken that conversion of $NADP^+$ to NAD^+ by phosphatase action does not interfere in cases, where the substrate used may react with different enzymes, specific for $NADP^+$ and NAD^+.

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