BBA 65950

INHIBITION OF MAMMALIAN URIDINEDIPHOSPHOGLUCOSE 4-EPIMERASE BY THE DITHIOTHREITOL-STIMULATED FORMATION OF NADH

THOMAS A. TEDESCO AND WILLIAM J. MELLMAN

Departments of Medical Genetics and Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pa. (U.S.A.)

(Received April 10th, 1969)

SUMMARY

1. Human fibroblast lysates preincubated with dithiothreitol showed inhibition of UDPGlc 4-epimerase (EC 5.1.3.2) activity at endogenous levels of NAD^+. This effect is probably due to NADH that is generated by the dithiothreitol stimulation of an NAD^+-reducing system. Inhibition was reversed by the addition of (at least) 0.2 mM NAD^+. A concentration-dependent inhibition of epimerase was also observed by NADH.

2. Several components of human fibroblasts and erythrocytes separable by gel electrophoresis are capable of NAD^+ but not of $NADP^+$ reduction in the absence of other added substrates. Another separate component is capable of NAD^+ and $NADP^+$ reduction only in the presence of dithiothreitol. These systems appear enzymatic in nature.

INTRODUCTION

UDPGlc 4-epimerase (EC 5.1.3.2) catalyzes the reversible conversion of UDPGlc to UDPGal. The dependence of epimerase activity on NAD^+ (refs. 1, 2) and its inhibition by NADH[3,4] have been documented. More specially, the effect of particular ratios of NAD^+/NADH on epimerase activity have been demonstrated with L and HeLa cell cultures[3]. It has also been reported that incubation of human fibroblast preparations with dithiothreitol caused inhibition of UDPGlc consumption at endogenous levels of NAD^+ (ref. 5).

This report will show that the inhibition of epimerase produced by dithiothreitol is due to stimulation of an NAD^+-reducing system endogenous to human fibroblast preparations.

METHODS AND MATERIALS

Maintenance and preparation of human diploid cell cultures

Human diploid fibroblast cultures were derived from skin biopsies and were

cultivated as monolayers by the method of HAYFLICK AND MOORHEAD[6]. Protein was determined by the method of LOWRY *et al.*[7]. Cells were harvested, and DNA was determined as previously described[8]. Fibroblast lysates were prepared by suspending $10 \cdot 10^6$–$20 \cdot 10^6$ cells/ml water. The suspensions were rapidly freeze-thawed 5 times and were centrifuged at 20 000 $\times$ *g* for 15 min. All centrifugations were performed at 4°. The supernatants were used in experimental procedures. Several lysates were homogenized according to KOBAYASHI *et al.*[9] for the preparation of mitochondria-free lysates, except that proteolytic treatment with nagase was omitted and that centrifugation was at 20 000 $\times g$. In the particular experiments indicated, lyophilized whole fibroblasts which had been stored below 0° were reconstituted at 20 or 40 mg dry wt./ml water and were centrifuged at 20 000 $\times g$ just before use.

Human erythrocyte preparations

Washed packed red cells were lysed by the addition of an equal volume of cold water followed by freezing and thawing. Hemolysates were mixed with an equal volume of 50% DEAE-cellulose suspension which had been previously equilibrated with 0.005 M phosphate buffer (pH 7.0). The mixture was stirred for 1 h at 4° and was poured onto a chromatographic column. Hemoglobin was removed from the column with 5 mM phosphate buffer (pH 7.0) until 280 mμ absorption of the eluate was below 0.05. The cellular components capable of NAD^+ and $NADP^+$ reduction were eluted from the column by supplementing the phosphate buffer with 50 mM $(NH_4)_2SO_4$. Crystalline $(NH_4)_2SO_4$ was added to the eluate to a final concentration of 20 g/100 ml, and the precipitate was centrifuged and discarded. A precipitate obtained (20–42 g/100 ml) was collected by centrifugation, was redissolved and was lyophilized. The dry powder which contained NAD^+- and $NADP^+$-reducing activity was stored below 0°. This material, which will be referred to as "semipurified erythrocyte preparation", was reconstituted at 20 or 40 mg dry wt./ml water and was centrifuged at 20 000 $\times g$ just before use.

Epimerase activity (spectrophotometric)

Spectrophotometric determinations were performed with either a Hitachi Perkin–Elmer Model 139 or a Gilford-converted Beckman. Epimerase activity in both directions was assayed by a modification of the two-step procedure described by MAXWELL[10]. The reaction system in a final volume of 0.25 ml contained 0.1 M glycylglycine (pH 8.7), fibroblast lysate equivalent to 10^6 cells, 20 mM dithiothreitol where indicated (33 mM in the preincubation system before the addition of substrate) varying concentrations of NAD^+, NADH and NADPH as indicated in particular experiments and 1 mM UDPGlc or UDPGal. Either UDPGlc or UDPGal was added to start reactions at 37°, and reactions were stopped by placing tubes in a boiling-water bath and by adding 0.05 ml of 0.35 M HCl. After boiling for 5 min, tubes were cooled, and UDPGlc formation or UDPGlc disappearance was measured with UDPGlc dehydrogenase as previously described[5].

In the experiments involving preincubation with dithiothreitol, 0.05 ml of 0.5 M glycylglycine (pH 8.7), 0.05 ml of lysate (10^6 cells) and 0.05 ml of 0.1 M dithiothreitol in a volume of 0.15 ml were incubated at 37° before the addition of UDPGlc or UDPGal.

In the experiments involving treatment of fibroblast lysate with NAD dinucleo-

tidase, 0.05 ml of fibroblast lysate equivalent to 10^6 cells was mixed with 0.05 ml of NAD dinucleotidase (0.1 unit). After 5 min at 37°, the mixture was used for epimerase assay as described above.

Epimerase activity (fluorimetric)

Epimerase activity was measured fluorimetrically at 27° by coupling the reaction with UDPGlc dehydrogenase in the presence of 1 mM NAD^+. Increasing fluorescence was recorded as a measure of UDPGlc formation. The reaction system in a final 0.5-ml vol. contained 0.1 M glycylglycine (pH 8.7), fibroblast lysate equivalent to $0.2 \cdot 10^6$ cells, 1 mM NAD^+, 100 units of UDPGlc dehydrogenase and 1 mM UDPGal. The reaction was started by the addition of UDPGal. The effect of 1–20 mM dithiothreitol was tested in this reaction. Control reactions were identical except that UDPGal was deleted.

Fluorimetric determinations were performed with an Eppendorff fluorimeter containing a 313 + 366 nm excitation filter and a 400–3000 nm output filter. The fluorimeter was equipped with a zero suppression scale expander unit (registrier-adapter 2134) and a Honeywell recorder.

UDPGlc dehydrogenase activity (fluorimetric)

UDPGlc dehydrogenase was assayed at 27° by recording increasing fluorescence as a measure of NADH formation. The reaction system in a final 0.5-ml vol. contained 0.1 M glycylglycine (pH 8.7), fibroblast lysate equivalent to $0.2 \cdot 10^6$ cells, 1 mM NAD^+ and 1 mM UDPGlc. UDPGlc was added to start the reaction.

Measurement of NAD^+ reduction (fluorimetric)

The reaction system used to measure the conversion of NAD^+ to NADH in the absence of other added substrates in a final 0.5-ml vol. contained 0.1 M glycylglycine (pH 8.7), fibroblast lysate equivalent to 10^6 cells or 1 mg dry wt. of semipurified erythrocyte preparation and varying concentrations of NAD^+ from 0.2 to 1.0 mM. Reactions were started by the addition of NAD^+, and the increase in fluorescence was recorded as a measure of NADH formation at 27°.

$NADP^+$ was tested in this system by substituting $NADP^+$ for NAD^+ at equimolar concentrations.

Measurement of dithiothreitol-dependent NAD^+ and $NADP^+$ reduction (fluorimetric)

The reaction system in a final 0.5-ml vol. contained 0.1 M glycylglycine (pH 8.7), lyophilized whole fibroblasts or semipurified erythrocyte preparation in the amounts indicated in individual experiments, 10 mM dithiothreitol and 1 mM NAD^+ or $NADP^+$. Reactions were started with either dithiothreitol or coenzyme, and the increase in fluorescence was recorded at 27°.

In experiments to determine the effect of pH, 0.2 M Tris–HCl was substituted for glycylglycine buffer at pH's 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5.

K_m values for dithiothreitol, comparing erythrocyte and fibroblast preparations, were obtained by varying the dithiothreitol concentration between 0.01 and 10 mM.

The effect of 10 mM dithioerythritol, reduced glutathione, cysteine, mercaptoethanol, 2,3-dimercaptopropanol, erythritol and xylitol was tested. Each was substituted for dithiothreitol in the reaction using $NADP^+$ as coenzyme.

The effect of 1 mM Cu, Mn, Mg, Zn and $CaCl_2$ and of 1 mM Na_2EDTA was each tested in the reaction with 10 mM dithiothreitol and 1 mM $NADP^+$.

Vertical polyacrylamide gel electrophoresis

Electrophoresis was performed using 6.6% polyacrylamide gel in a continuous buffer system with 0.1 M Tris–borate buffer (pH 9.0). The vertical polyacrylamide gel electrophoresis apparatus was from E.C. Apparatus Co. Semipurified erythrocyte and lyophilized whole fibroblasts preparations were reconstituted at 40 mg dry wt./ml water and were centrifuged at 20 000 × *g* for 15 min. 0.05 ml of the supernatants were electrophoresed at 4° for 16 h with 100 V (30 mA) applied across the gel. Reducing activity in the gel was located with longwave ultraviolet light after the gels had been divided into strips and had been incubated at 27° in 0.2 M glycylglycine buffer (pH 8.7), containing 2 mM NAD^+ or $NADP^+$ in combination with 10 mM dithiothreitol or other substrates tested.

RESULTS

Epimerase activity (spectrophotometric)

The influence of substrate concentration on human fibroblast epimerase activity was tested by the two-step method at endogenous levels of NAD^+ and supplemented with 1 mM NAD^+. At endogenous NAD^+, the K_m for UDPGlc was 0.3 mM and that for UDPGal was 0.12 mM. When supplemented with 1 mM NAD^+, the K_m for UDPGlc was 0.21 mM and that for UDPGal was 0.27 mM. After treatment of lysates with NAD nucleosidase no epimerase activity could be detected.

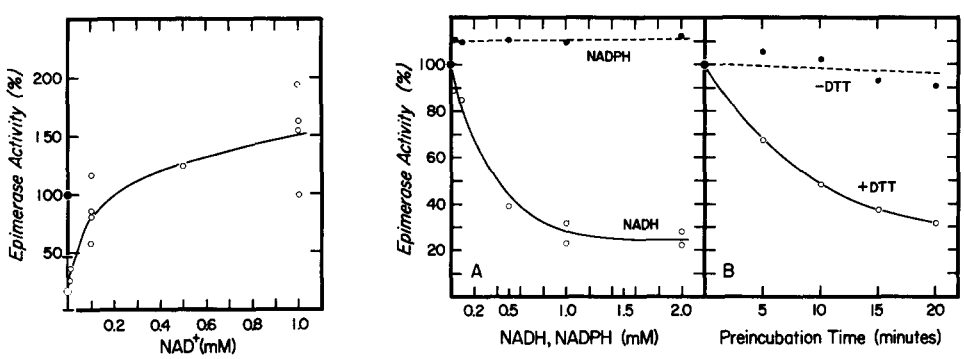


Fig. 1. The effect of adding NAD^+ to dithiothreitol-inactivated epimerase systems in fibroblast lysates. Lysates were preincubated with 33 mM dithiothreitol for 20 min at pH 8.7. Epimerase activity (UDPGlc to UDPGal) was assayed by the two-step assay. Activity without dithiothreitol is arbitrarily at 100% (solid dot). At endogenous levels of NAD^+ the range for dithiothreitol inhibition is 52–100% with a mean of 81% (large circle), based on 40 lysates. Upon adding increasing levels of NAD^+ to duplicate systems after dithiothreitol inactivation, reactivation of epimerase is observed. The line represents the mean of four separate experiments.

Fig. 2. The effect of increasing concentrations of NADH and NADPH on the epimerase activity of fibroblast lysates compared with the inhibitory effect of dithiothreitol (DTT) as a function of preincubation time at endogenous levels of NAD^+. A. Replicate tubes containing buffered lysate were treated with increasing concentrations of NADH and of NADPH before starting the reactions with UDPGlc. B. Replicate tubes containing buffered lysate preincubated for the times indicated in the presence and absence of 33 mM dithiothreitol before starting the reactions with UDPGlc.

Preincubation of fibroblast lysates buffered at pH 8.7 with 33 mM dithiothreitol for 20 min before the addition of epimerase substrate caused 52–100% inhibition in both directions. This inhibition could not be demonstrated if reaction systems were supplemented with 1 mM NAD^+. In 40 lysates tested, UDPGlc consumption after preincubation with dithiothreitol showed a mean inhibition of 81% with only endogenous NAD^+ present. As seen in Fig. 1, epimerase activity inhibited by dithiothreitol could be restored by supplementing inactivated systems with NAD^+, and the NAD^+ response was concentration dependent. The mean specific activity for epimerase (UDPGlc to UDPGal) at endogenous levels of NAD^+ was 40 nmoles/min per mg DNA* (n = 28, range 13 – 88), and that for epimerase (UDPGal to UDPGlc) was 35 nmoles/min per mg DNA* (n = 18, range 13 – 88).

Epimerase activity (fluorimetric)

The presence of 20 mM dithiothreitol in the fluorimetric rate reaction for epimerase activity had no effect. It is evident from the data in Fig. 1 and from coupled rate reaction studies that assay procedures which contain more than 0.2 mM NAD^+ are not inhibitable by dithiothreitol and that dithiothreitol has no direct effect on epimerase. The mean specific activity of epimerase in the coupled fluorimetric assay with 1 mM supplemental NAD^+ was 580 nmoles/min per mg DNA ($n = 7$), range 240 – 1050).

Comparison of epimerase inhibition by dithiothreitol and by NADH

UDPGlc consumption by epimerase was assayed by the two-step method after varying periods of preincubation in the presence and absence of dithiothreitol. Fig. 2B shows that dithiothreitol inactivation of epimerase is a function of preincubation time. Similar assays without preincubation were performed with increasing concentrations of NADH and NADPH. Fig. 2A demonstrates that NADH exerts a concentration-dependent inhibition on epimerase, while NADPH in equimolar concentrations has no effect.

This combined evidence suggested the hypothesis that preincubation of fibroblast preparations with dithiothreitol-stimulated generation of NADH from endogenous NAD^+ and that inhibition of epimerase by dithiothreitol is mediated by this reaction.

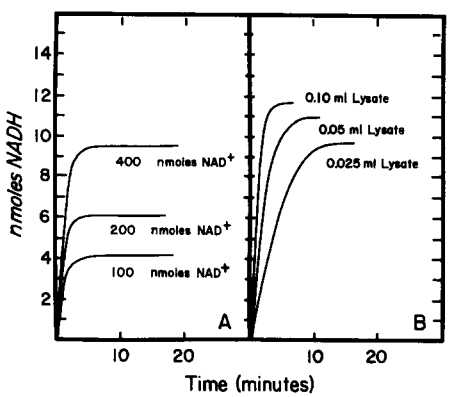
UDPGlc dehydrogenase activity

In order to eliminate the possibility that endogenous UDPGlc dehydrogenase activity was introducing significant error in epimerase assays, 5 lysates were tested in the fluorimetric rate reaction assay for both epimerase and UDPGlc dehydrogenase using equivalent amounts of lysate. It was found that UDPGlc dehydrogenase activity was not detectable at endogenous levels of NAD^+ under the conditions of our assay. UDPGlc dehydrogenase was barely detectable when 0.2 mM NAD^+ was added. In the presence of 1 mM NAD^+, the measurable UDPGlc dehydrogenase activity was 14–34% of the epimerase activity (mean 26.7%).

NAD^+ reduction by fibroblast and erythrocyte preparations

The addition of NAD^+ to fibroblast lysates buffered at pH 8.7 resulted in a rapid

* 10^6 fibroblasts = 13 μg DNA = 300 μg total cell protein.



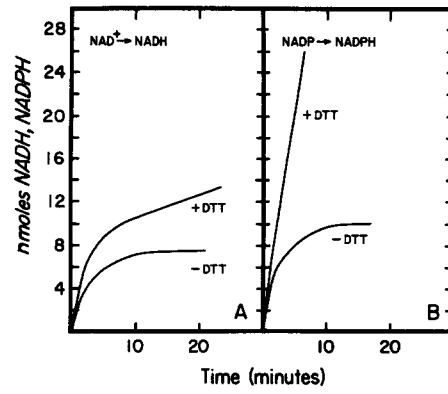


Fig. 3. Fluorimetric determination of NAD^+-reducing activity. A. NADH formation after the addition of 0.1, 0.2 or 0.4 μmole NAD^+ to cuvettes containing 0.1 M glycylglycine buffer (pH 8.7) and fibroblast lysate equivalent to 10^6 cells, in a final reaction volume of 0.5 ml. B. NAD^+ reduction after the addition of 0.4 μmole NAD^+ to cuvettes containing 0.1 M glycylglycine buffer (pH 8.7): cell culture lysate equivalent to $0.5 \cdot 10^6$, $1.0 \cdot 10^6$ or $2.0 \cdot 10^6$ cells (0.025-, 0.05-, and 0.1-ml lysates) in a final reaction volume of 0.5 ml.

Fig. 4. The effect of dithiothreitol on the conversion of NAD^+ to NADH and $NADP^+$ to NADPH by fibroblast lysates. The assay system contained 0.1 M glycylglycine buffer (pH 8.7); fibroblast lysate equivalent to $0.4 \cdot 10^6$ cells; 20 mM dithiothreitol where indicated in a final volume of 0.5 ml. Reactions were started by adding 0.5 μmole NAD^+ or $NADP^+$. A. NAD^+ to NADH reactions and the effect of dithiothreitol stimulation. B. The same reactions for $NADP^+$ to NADPH.

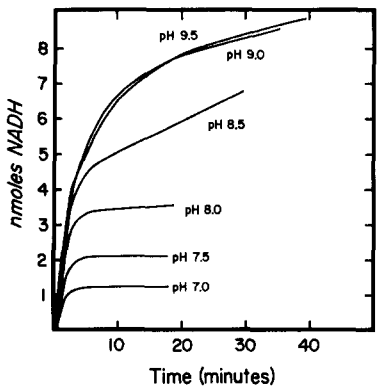
increase in fluorescence that quickly reached a maximal level. This level represented the reduction of 2–5% of the total NAD^+ in the cuvette, approx. 10 nmoles NADH per mg fibroblast DNA. Fig. 3A shows that the maximal level of this response is proportional to the amount of NAD^+ in the cuvette. If a second aliquot of NAD^+ is added to a system which has reached its maximal level, the reaction is repeated suggesting that the initial reaction was not due to exhaustion of endogenous substrates for NAD^+-linked reactions. Fig. 3B shows that the rate of this reaction is proportional to lysate concentration. Similar activity was observed with semipurified erythrocyte preparations.

When $NADP^+$ is substituted for NAD^+ in fibroblast systems, a reaction similar to those shown in Fig. 3A occurs. This reaction is independent of $NADP^+$ concentration and does not show proportionality between rate of reaction and lysate concentration. The maximal level of fluorescence obtained with $NADP^+$ is proportional to lysate concentration suggesting the utilization of endogenous substrates for $NADP^+$-linked reactions. Electrophoresed material did not show any activity with $NADP^+$ (see below) supporting the suggestion that endogenous substrates are needed for this reaction.

Semipurified erythrocyte preparations had no detectable activity with $NADP^+$ alone.

Dithiothreitol-stimulated NAD^+ and $NADP^+$ reduction

The addition of dithiothreitol to both NAD^+- and $NADP^+$-containing systems caused a linear increase in fluorescence. Fig. 4 shows the effect of dithiothreitol on NAD^+ and $NADP^+$ reduction for a fibroblast lysate. Similar reactions were observed



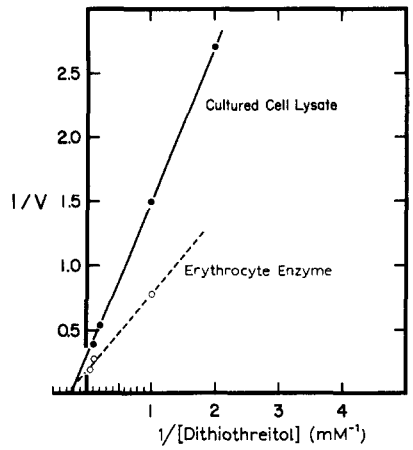


Fig. 5. The effect of pH on $NAD^+ \rightarrow NADH$ reactions in the presence of dithiothreitol. Reaction systems contained 0.2 M Tris–HCl buffer at the pH's indicated, fibroblast lysate equivalent to 10^6 cells; and 10 mM dithiothreitol in a final volume of 0.5 ml. Reactions were started with 0.2 μmole NAD^+. The effect of dithiothreitol on the rate of NADH formation is maximal at pH 8.5.

Fig. 6. Lineweaver–Burk plots comparing the effect of dithiothreitol concentration on the rate of $NADP^+ \rightarrow NADPH$ activity for a lyophilized whole fibroblast and a semipurified erythrocyte preparation. Reaction systems contained 0.1 M glycylglycine buffer (pH 8.7), 1 mg dry wt. of lyophilized whole fibroblast or 1 mg dry wt. of semipurified erythrocyte preparation, 1 mM $NADP^+$ in a final vol. of 0.5 ml. Varying concentrations of dithiothreitol were added to start the reactions. 1/[dithiothreitol] (mM^{-1}) final concentration was plotted *vs.* $1/v$ (expressed as 1/the number of recorder scale divisions per 10 min $\times$ 10). The points obtained at 1/[dithiothreitol] (mM^{-1}) = 10 are not shown in the plot.

for a semipurified erythrocyte preparation. These activities were found in the supernatant fraction after fibroblasts were homogenized and centrifuged at 20 000 $\times g$.

The mean activity measured in 8 fibroblast lysates for dithiothreitol-stimulated NAD^+ reduction was 11.1 nmoles NADH per min per mg. DNA (range: 3.8–26.8), and

TABLE 1

RELATIVE REDUCTION OF NAD^+ AND $NADP^+$ BY ERYTHROCYTE AND FIBROBLAST PREPARATIONS WITH DITHIOTHREITOL AND WITH OTHER THIOLS AND ALCOHOLS

Substrate (10 mM)	*Cofactor (1 mM)*	*Relative activity* (%)*	
		Erythrocyte	*Fibroblast*
Dithiothreitol	$NADP^+$	100	100
Dithiothreitol	NAD^+	130	27
Dithioerythreitol	$NADP^+$	42	81
GSH	$NADP^+$	173	3.9
Mercaptoethanol	$NADP^+$	15.4	6.9
2,3-Dimercaptopropanol	$NADP^+$	4.8	4.9
Cysteine	$NADP^+$	0	2.9
Erythreitol	$NADP^+$	2	4
Xylitol	$NADP^+$	2	0

* Activities related to that with dithiothreitol and $NADP^+$.

the mean activity of 4 lysates for dithiothreitol-stimulated $NADP^+$ reduction was 62.7 nmoles NADPH per min per mg DNA (range: 24–101).

None of these activities were present in heat-inactivated lysates, and dithiothreitol had no direct effect on either NAD^+ or $NADP^+$ reduction in the absence of lysates.

The effect of pH on the reactions converting NAD^+ to NADH in the presence of dithiothreitol is shown in Fig. 5 for a fibroblast lysate. Maximal levels of NADH increased with increasing pH, but the initial rate of reaction did not appear to change significantly. A dithiothreitol-stimulated response was evident at pH 8.5. Dithiothreitol-stimulated $NADP^+$ reduction of fibroblast lysate was maximal at pH 8.0. At pH's 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5, the relative activities were 26, 65, 100, 73, 60 and 53%, respectively.

The effect of dithiothreitol concentration on the rate of $NADP^+$ reduction was tested with a fibroblast lysate and a semiperified erythrocyte preparation. Fig. 6 shows Lineweaver–Burk plots comparing the two cell types. At 1 mM $NADP^+$ the K_m for dithiothreitol was 4.0 mM for both the erythrocyte and fibroblast preparation.

Cu^{2+} and Zn^{2+} completely inhibited NAD^+ and $NADP^+$ reduction in the presence of dithiothreitol, and no reactivation was observed with the addition of EDTA to inactivated systems. Ca^{2+}, Mn^{2+}, Mg^{2+} or EDTA alone had no effect.

Other thiols and alcohols that were tested for their effect on $NADP^+$ reduction are listed in Table I with their relative activities compared to the activity of dithiothreitol. Erythrocyte and fibroblast preparations were compared as well as the relative activities of NAD^+ and $NADP^+$ with dithiothreitol. As seen in Table I, dithioerythritol

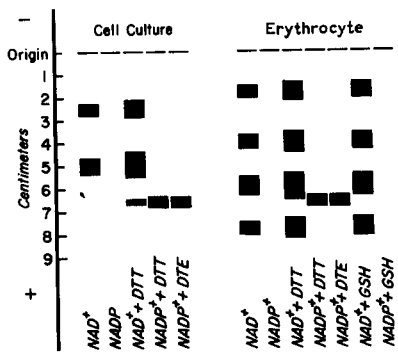


Fig. 7. Vertical polyacrylamide gel electrophoresis comparing lyophilized whole fibroblast and semipurified erythrocyte preparations. 6.6% polyacrylamide gel in 0.1 M Tris–borate (pH 9.0) was used in a continuous buffer system. Semipurified erythrocyte and lyophilized whole fibroblast preparations were reconstituted at 40 mg dry wt./ml water and were centrifuged at 20 000 × *g* for 15 min. 0.05 ml of the supernatants was used in each slot for electrophoresis. Preparations were electrophoresed at 4° for 16 h with 100 V (30 mA) applied across the gel. Gels were then divided into strips corresponding to the width of each slot and the length of the gel. Each strip was individually incubated in a test tube with its specific staining media. Various staining media in 0.2 M glycylglycine buffer (pH 8.7) contained 2 mM NAD^+ or $NADP^+$ alone or in combination with 20 mM dithiothreitol (DTT), dithioerythritol (DTE) or GSH as indicated in the illustration at the bottom of each strip. After the bands had developed (2–4 h) the strips incubated under different conditions were reassembled so that individual bands could be compared in terms of intensity of fluorescence and distance they had migrated toward the anode. The bands were located by their fluorescence with longwave ultraviolet light.

shows significant $NADP^+$ reducing activity with both erythrocyte and fibroblast preparations. Reduced glutathione (GSH) has significant activity only in the erythrocyte preparation.

Polyacrylamide gel electrophoresis

Reconstituted lyophilized whole fibroblast and semipurified erythrocyte preparations were electrophoresed in 6.6% gel. After incubation in 0.2 M glycylglycine buffer (pH 8.7) containing 2.0 mM NAD, 2 fluorescent bands appeared in the lyophilized whole fibroblast channels and 4 bands appeared in the semipurified erythrocyte channels. (This semipurified erythrocyte preparation represents pooled erythrocytes from four different individuals.) When incubated in 2.0 mM $NADP^+$, no fluorescence was observed in either preparation. Dithiothreitol alone gave no fluorescence, but dithiothreitol in the presence of $NADP^+$ gave a single band in both semipurified erythrocyte and lyophilized whole fibroblast preparations (see Fig. 7). In the lyophilized whole fibroblast channels a band appeared in the dithiothreitol + NAD^+ staining mix with the same mobility as the dithiothreitol + $NADP^+$ band. The dithiothreitol-dependent band was less intense with NAD^+ than with $NADP^+$. The 4 semipurified erythrocyte bands that appeared with NAD^+ alone obscured any dithiothreitol-dependent band that might be present. Dithioerythritol when substituted for dithiothreitol gave the same band (see Fig. 7). GSH, which caused significant $NADP^+$ reduction in the fluorescence assay with semipurified erythrocyte preparations, produced no obvious bands in the gel. Fig. 7 illustrates the fluorescent bands observed due to reducing activity in the gel and compares semipurified erythrocyte and lyophilised whole fibroblast preparations.

DISCUSSION

The dependence of uridinediphosphogalactose 4-epimerase on NAD^+ and its inhibition by NADH was demonstrated with lysates of cultured human diploid cells. Inhibition of epimerase activity was also shown to be a function of preincubation time in the presence of dithiothreitol at endogenous levels of NAD^+. Since high levels of dithiothreitol had no effect on epimerase activity in the presence of supplemental NAD^+, it is most probably that dithiothreitol has no direct effect on the epimerase enzyme.

Endogenous levels of NAD^+ in human fibroblast lysates were estimated at 1–10 μM in preparations containing $20 \cdot 10^6$–$50 \cdot 10^6$ cells/ml lysate, and NAD nucleosidase actively was not detectable*.

In order to link together the observations that (1) dithiothreitol preincubation, and (2) NADH concentration both inhibit epimerase activity, it was necessary to first identify NAD^+-reducing systems in fibroblast cultures. Such systems were indeed found to be present in fibroblast lysates. As supportive evidence for the presence of endogenous NAD^+-reducing systems, it was found that intact fibroblasts grown on glass cover slips, then washed and air dried, showed formazan staining after incubation with NAD^+ alone in the presence of nitro blue tetrazolium*, while incubation with

* W. J. MELLMAN AND T. A. TEDESCO, unpublished observations.

$NADP^+$ under similar conditions did not produce staining. Having established the presence of such systems, their response to dithiothreitol was investigated.

Dithiothreitol, since its description by CLELAND[11], has become a common sulfhydryl group protector in enzyme reactions and protein purification procedures and has recently been employed by VACQUIER AND MAZIA[12,13] to promote twinning of sand dollar and sea urchin embryos. Our observations show that dithiothreitol may play a more active role than sulfhydryl group protection in biological systems.

The present study indicates that dithiothreitol inhibition of epimerase activity is in fact mediated by dithiothreitol stimulation of NAD^+ reduction. Human erythrocyte and cultured diploid fibroblasts not only contain endogenous activities that reduce NAD^+ but also a discrete activity, separable by gel electrophoresis, that reduces NAD^+ and $NADP^+$ only in the presence of dithiothreitol. Furthermore, since dithioerythritol serves as a substitute for dithiothreitol in this reaction while erythritol does not, it appears that free SH groups are necessary for this activity. Dithiothreitol was shown to be capable of saturation in this reaction and to obey Michaelis–Menten kinetics. Other thiols and alcohols tested were either inactive or considerably less active.

The activity observed with GSH in erythrocyte preparations is most likely a separate system, since no activity was observed with GSH in fibroblast lysates.

Although the reducing activities described in this paper appear to function as enzymatic systems, they cannot be clearly defined as enzymatic at this time; nor can any significance be placed on their presence as endogenous systems in human erythrocytes and cultured diploid fibroblasts.

The use of dithiothreitol in the UDPGlc consumption assay of α-D-galactose-1-phosphate uridyltransferase (EC 2.7.7.12) demonstrates a useful application of this system, since dithiothreitol not only provides the sulfhydryl requirement for transferase activity, but also inhibits UDPGlc consumption by epimerase[5]. Furthermore, the dithiothreitol-stimulated reduction of NAD^+ and of $NADP^+$ described here should be recognized by investigators as a possible source of error in a variety of enzyme reactions.

ACKNOWLEDGEMENTS

This investigation was supported by Grants FR-40, HD-00588 and HD-15545 from U.S. Public Health Service, Washington, D.C.

REFERENCES

1 E. S. MAXWELL, *J. Am. Chem. Soc.*, 78 (1956) 1074.
2 W. NG, G. DONNELL, J. HODGMAN AND W. BERGREN, *Nature*, 214 (1967) 283.
3 E. A. ROBINSON, H. M. KALCKAR AND H. TROEDSSON, *J. Biol. Chem.*, 241 (1966) 2737.
4 R. COHN AND S. SEGAL, *Biochim. Biophys. Acta*, 171 (1969) 333.
5 T. A. TEDESCO AND W. J. MELLMAN, in D. Y. Y. HSIA, *Galactosemia*, Thomas, Springfield, 1968, in the press.
6 L. HAYFLICK AND P. S. MOORHEAD, *Exptl. Cell Res.*, 25 (1961) 585.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 T. A. TEDESCO AND W. J. MELLMAN, *Exptl. Cell Res.*, 45 (1966) 230.

9 S. KOBAYASHI, B. HAGIHARA, M. MASUZUMI AND K. OKUNUKI, *Biochim. Biophys. Acta*, 113 (1966) 421.
10 E. S. MAXWELL, K. KURAHASHI AND H. M. KALCKAR, in S. P. COLOWICK AND N. A. KAPLAN, *Methods in Enzymology*, Vol. 5, Academic Press, New York, 1962, p. 174.
11 W. W. CLELAND, *Biochemistry*, 3 (1964) 480.
12 V. D. VACQUIER AND D. MAZIA, *Exptl. Cell Res.*, 52 (1968) 209.
13 V. D. VACQUIER AND D. MAZIA, *Exptl. Cell Res.*, 52 (1968) 459.