A New Hepatic Protein Inactivating Glucose 6-Phosphate Dehydrogenase

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(Received 10 April 1967)

Two NADP-cleaving enzymes, namely NADP glycohydrolase and NADP pyrophosphatase, are present in a rat liver extract that inactivates G6PD (glucose 6-phosphate dehydrogenase). The following results suggest that a third G6PD-inactivating protein is present in this extract. (1) Nicotinamide, which selectively inhibits NADP glycohydrolase, enhances the G6PD inactivation under conditions where G6PD activity in control experiments is rather stable. (2) DEAE-cellulose adsorbs the bulk of both NADP glycohydrolase and NADP pyrophosphatase, whereas most of the G6PD-inactivating ability is unadsorbed. (3) Out of 37 liver extracts that were prepared, two were found to lack NADP pyrophosphatase. After removal of NADP glycohydrolase from these extracts by centrifugation, they were still found to inactivate G6PD. (4) Deproteinization of DEAE-cellulose supernatants results in a complete loss of G6PD-inactivating ability; moreover, kinetic experiments performed with the extracts lacking pyrophosphatase strongly support the view that the inactivating protein is an enzyme, although its mechanism is not clear. (5) NADP protects G6PD from inactivation and also reactivates the enzyme completely, thus supporting the view of some action of the inactivating protein on the G6PD-bound NADP.

A preparation has been obtained from rat liver that inactivates the enzyme G6PD* under physiological conditions of temperature (37°) and pH (7·8) (De Flora, Mangiarotti & Lorenzoni, 1965; Mangiarotti, De Flora & Lorenzoni, 1965*a,b*). Results reported in the present paper demonstrate that this preparation contains: (1) NADP glycohydrolase (EC 3.2.2.6), which has been previously demonstrated to inactivate G6PD *in vitro* and probably *in vivo* (Carson, Schrier & Kellermeyer, 1959; Marks, Szeinberg & Banks, 1961; Fantoni, Leoncini, Calissano, Cartasegna & Fornaini, 1965); (2) NADP pyrophosphatase (EC 3.6.1.9), whose G6PD-inactivating action has already been described (Bonsignore, De Flora, Mangiarotti, Lorenzoni & Alemà, 1967); (3) a third factor, possessing properties of a protein, that inactivates G6PD. Although DEAE-cellulose adsorbed most of the NADP-cleaving enzymes, a complete separation from the third inactivating protein could not be achieved. However, two active liver preparations were obtained that were devoid of NADP glycohydrolase and NADP pyrophosphatase, thus confirming the presence of the inactivating protein and allowing a

preliminary investigation of its properties to be made.

MATERIALS AND METHODS

Glycylglycine, glucose 6-phosphate, EDTA, NADP, NMN, yeast G6PD (*Zwischenferment*) and alcohol dehydrogenase were commercial preparations, as already reported (Bonsignore *et al.* 1967). Benzoyl-DL-arginine *p*-nitroanilide hydrochloride was purchased from Mann Research Laboratories (New York, N.Y., U.S.A.) and trypsin (twice crystallized) from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Whatman DEAE-cellulose was supplied by W. and R. Balston Co. Ltd. (Maidstone, Kent) and Aquax from G. T. Gurr Ltd. (London, S.W. 6).

Rats were killed by exsanguination and the livers, after a rapid washing with cold 0·15 M-KCl, were homogenized in 0·15 M-KCl at 30% (w/v). Homogenates were centrifuged for 1 hr. at 26000*g* and the supernatants carefully collected ('extract').

The extracts were dialysed for 5 hr. at 2° against 100 vol. of 15 mM-KCl adjusted at pH 7·0 with NaHCO₃ ('dialysed extract'). Incubation of the dialysed extracts at 37° in the presence of 50 mM-glycylglycine buffer, pH 7·5, results in an increasing inactivation of G6PD, whose features have already been reported (Bonsignore *et al.* 1967); this extract ('G6PD-free liver extract') was investigated for inactivation of erythrocyte G6PD.

Human erythrocyte G6PD was partially purified as described by Marks *et al.* (1961), except that haemolysates

* Abbreviation: G6PD, glucose 6-phosphate dehydrogenase.

(acid-citrate-dextrose blood provided by the Genoa Blood Bank) were fractionated with $(\text{NH}_4)_2\text{SO}_4$ at a protein concentration of 65–70 mg./ml. and that the elution from calcium phosphate gel was performed with 30 mM-potassium phosphate buffer, pH 7.6, after a previous washing with 5 mM-potassium phosphate buffer, pH 7.6. This procedure results in a higher degree of purification and in a lower yield of the enzymic activity.

Before the purified enzyme [both the first and the second $(\text{NH}_4)_2\text{SO}_4$ fractions] was employed for inactivation assays, it was dialysed 3 hr. at 2° against 500 vol. of 15 mM-KCl adjusted to pH 7.0 with NaHCO_3 and containing 2 μM -NADP.

G6PD activity was assayed by the method of Kornberg & Horecker (1955) by following the reduction of NADP at 340 $\text{m}\mu$ in the presence of 75 mM-glycylglycine buffer, pH 7.5, 2 mM-glucose 6-phosphate and 0.5 mM-NADP, in a final volume of 1.0 ml., at 25°. One unit of G6PD activity was defined as the amount giving an increase of 1.0 E_{340} unit/min.

The total activity of the NADP-cleaving enzymes was assayed by following the rate of disappearance of free NADP in a system incubated at 37° and containing 30 mM-potassium phosphate buffer, pH 7.0, and 2 mM-NADP, in a final volume of 2 ml.; at different time-intervals NADP was assayed by means of yeast G6PD. Parallel experiments without the enzymes and with 0.1 M-nicotinamide were performed (nicotinamide is a selective inhibitor of NADP glycohydrolase). One unit of NADP-splitting activity was defined as the amount causing the disappearance of 0.1 μmole of NADP in 90 min. of incubation at 37°, in the assay system described above.

In other experiments the rate of disappearance of NADP was measured by the KCN method described by Colowick, Kaplan & Ciotti (1951). By this method we were able to differentiate NADP glycohydrolase, which causes a decrease of E_{325} , from NADP pyrophosphatase, which does not affect E_{325} .

NADP deaminase activity was assayed by the method of Nikiforuk & Colowick (1955). NADP phosphatase activity was assayed by determining both the release of P_i , measured by the method of Fiske & Subbarow (1925), and the release of NAD, measured by means of alcohol dehydrogenase.

The inactivation of erythrocyte G6PD was assayed by following the disappearance of G6PD activity in a system incubated at 37° and containing, in a final volume of 1 ml.: 50 mM-glycylglycine buffer, pH 7.5, 1.0 unit of dialysed partially purified G6PD and different amounts of the inactivating proteins. Control experiments were performed without the inactivating extracts and with bovine serum albumin to reach the same protein concentration as in the inactivation experiment. One unit of the inactivating enzymes was defined as the amount inactivating 0.1 unit of G6PD activity in 45 min. at 37° under the conditions described above.

Proteolytic activity was evaluated by different methods: trypsin-like activity was assayed by the method of Erlanger, Kokowsky & Cohen (1961); release of amino acids or small peptides was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) as described by Greenberg (1955) for assay of plant proteolytic enzymes, or by the ninhydrin method as modified by Rosen (1957): at various times samples of the incubation mixtures containing the inactivating proteins and erythrocyte G6PD were deproteinized at 0° with trichloroacetic acid (final concn. 3%,

w/v) and then centrifuged for 10 min. at 6000g, and the supernatants analysed for amino acids and small peptides by the methods described above.

RESULTS

Inactivation of G6PD by liver extracts. A typical pattern of the inactivation of erythrocyte G6PD by G6PD-free liver extracts is illustrated in Fig. 1. The features of this inactivation have been reported previously (Mangiarotti *et al.* 1965a,b; Bonsignore *et al.* 1967).

Presence of NADP glycohydrolase in the liver extracts. Assay of the NADP-cleaving enzymes performed by following the disappearance of free NADP as described in the Materials and Methods section showed that G6PD-free liver extracts exhibit a total NADP-splitting activity of approx. 0.35 unit/mg. The presence of 0.1 M-nicotinamide decreased the disappearance of NADP; moreover, the cyanide method showed a decrease of E_{325} , thus indicating a cleavage of the nicotinamide-ribose linkage. The NADP glycohydrolase activity of the inactivating extracts, assayed both by the cyanide method and by subtracting the decrease of free NADP in the presence of nicotinamide from that observed without nicotinamide, varied between 0.19 and 0.42 unit/mg. The G6PD-inactivating action of NADP glycohydrolase from different cell specimens has been reported by Carson *et al.* (1959),

Fig. 1. Inactivation of erythrocyte G6PD by liver extracts. Each ml. of incubation mixture contained 50 mM-glycylglycine buffer, pH 7.5, 1.0 unit of partially purified (110-fold) erythrocyte G6PD [$(\text{NH}_4)_2\text{SO}_4$ fraction I, previously dialysed as described in the Materials and Methods section], 0.08 ml. of G6PD-free liver extract (corresponding to 2.5 mg. of protein) and 2.5 mg. of bovine serum albumin (●). A control experiment without the liver extract and containing 5 mg. of bovine serum albumin was also performed (○). Incubation was at 37° in a water bath. At the times indicated, 0.05 ml. samples were assayed for G6PD activity.

Marks *et al.* (1961), Fantoni *et al.* (1965) and Bonsignore *et al.* (1967).

Other NADP-cleaving enzymes in the liver extracts. No phosphatase-catalysed or deaminase-catalysed cleavage of NADP by the G6PD-free liver extracts could be demonstrated; however, chromatographic evidence was obtained that these extracts contain NADP pyrophosphatase activity, accounting for the degradation of NADP in the presence of nicotinamide. The procedure was essentially that described by Martelli, Pallini & Ricci (1964) for liver NAD pyrophosphatase activity: 7 μ moles of free NADP were incubated at 37° in 60mm-tris-hydrochloric acid buffer, pH8.0, with 0.5ml. of a G6PD-free liver extract (corresponding to 150mg. wet wt. of tissue) in the presence of 0.1M-nicotinamide in a final volume of 1ml. After 6hr. of incubation, 6.1 μ moles of NADP (87% of the initial concentration) had disappeared; 0.05ml. of the incubation mixture was placed on Whatman no. 1 paper and two standards of NMN (40 μ g.) and of NADP (40 μ g.) were run in parallel. The descending solvent was 95% (v/v) ethanol-m-ammonium acetate (7:3, v/v) at pH5 and the spots were located by using the butan-2-one-ammonia reagent (Kodicek & Reddi, 1951) and viewing in ultraviolet light. Four different spots appeared from the chromatographed incubation mixture, one corresponding to the NADP standard, another to the NMN standard and the two other spots to unidentified compounds. Therefore an enzyme exists in the G6PD-free liver extracts that cleaves free NADP at the pyrophosphate group with release of NMN. NADP pyrophosphatase present in these extracts, assayed as described in the Materials and Methods section in the presence of 0.1M-nicotinamide or by subtracting from the total NADP-splitting activity that due to NADP glycohydrolase (calculated by the cyanide method), showed wide variations, being undetectable in some cases and reaching values of 0.17 unit/mg. in other cases.

Also, NADP pyrophosphatase, like NADP glycohydrolase, was shown to catalyse the inactivation of G6PD in a reconstructed system containing these partially purified enzymes (Bonsignore *et al.* 1967).

Effect of nicotinamide on G6PD inactivation. Nicotinamide, at a concentration of 50mM, markedly accelerates the inactivation of hepatic G6PD when dialysed liver extracts are incubated at 37° as described in the Materials and Methods section; moreover, 50mM-nicotinamide was also found to enhance the rate of inactivation of erythrocyte G6PD by the G6PD-free liver extracts (Fig. 2). This effect is partially due to an action directly exerted by nicotinamide itself on the molecule of G6PD, as Marks *et al.* (1961) have

Fig. 2. Effect of nicotinamide on G6PD inactivation by liver extracts. Each ml. of incubation mixture contained: ●, 50mM-glycylglycine buffer, pH7.5, 1.0 unit of partially purified (550-fold) erythrocyte G6PD [dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction II] and 0.1 ml. of G6PD-free liver extract (values reported were corrected for the variations of a control containing 3.4mg. of bovine serum albumin); O, the same + 50mM-nicotinamide [corrected for variations of a control (▲) containing bovine serum albumin and 50mM-nicotinamide]. Incubation was at 37° in a water bath; 0.05ml. samples were periodically assayed for G6PD activity.

pointed out and as the slight inactivation in the control with nicotinamide clearly indicates; however, the higher inactivation observed in the system containing nicotinamide and the extract suggests that nicotinamide also stimulates the inactivating action of the extracts. This conclusion was also supported by the fact that small amounts of NADP (about 10 μ M) can balance the inactivating effect of nicotinamide in the controls, without affecting the inactivation due to the extract plus nicotinamide (higher concentrations of NADP were needed to counteract this latter inactivation). Nicotinamide accelerates the G6PD inactivation by the liver extracts only during the early part of the reaction: after 15–20min. of incubation at 37° the rate of inactivation is markedly lower than that without nicotinamide. The early additional inactivation induced by nicotinamide, under conditions where the G6PD-inactivating action of NADP glycohydrolase of the extracts is completely inhibited (Bonsignore *et al.* 1967), could be due to an early activation of NADP pyrophosphatase by nicotinamide itself. However, this possibility is unlikely because the rate of disappearance of NADP incubated with the G6PD-free liver extract and 0.1M- or 50mM-nicotinamide (i.e. that hydrolysed by pyrophosphatase only) is represented by a straight line (Fig. 3).

These experiments provide a first indication that there exists in liver extracts a G6PD-inactivating agent that does not diffuse through a dialysis

membrane and that can be activated by nicotinamide, in contrast with NADP glycohydrolase (which is inhibited) and with NADP pyrophosphatase (which is not affected).

Fractionation of the G6PD-inactivating agents. To produce more satisfactory evidence for the G6PD-inactivating substance, attempts were made to purify it and to separate it from NADP glycohydrolase and NADP pyrophosphatase. The main difficulties were the facts that most electrolytes affect G6PD activity (Mangiarotti, Garré, De Flora & Bonsignore, 1965; Mangiarotti & Garré, 1965; Mangiarotti, Garré, Acquarone & Silengo, 1966) and that many of them, including ammonium sulphate, phosphate (above 10mM) and chloride (above 40mM), counteract the inactivation of G6PD by liver extracts. For example, ammonium sulphate fractionation, although precipitating the G6PD-inactivating proteins (Mangiarotti *et al.* 1965a; Bonsignore *et al.* 1967), does not result in any

Fig. 3. NADP hydrolysis by liver extracts in the presence of nicotinamide. Each ml. of the incubation mixture contained 60mM-potassium phosphate buffer, pH7.0, 2μmoles of NADP, 50mM-nicotinamide and 0.5ml. of G6PD-free liver extract. A control was also set up without the extract. Incubation was at 37°; at the times indicated samples were assayed for NADP by yeast G6PD.

purification of these proteins (including NADP glycohydrolase and NADP pyrophosphatase), which are themselves partially inactivated by such a procedure; attempts to fractionate extracts with acetone, ethanol and calcium phosphate gel were also unsuccessful, because these procedures do not lead to any separation of the G6PD-inactivating ability from NADP glycohydrolase and NADP pyrophosphatase. However, the following treatment with DEAE-cellulose gives an easy way of adsorbing both the NADP-splitting enzymes, while the bulk of the original G6PD-inactivating ability is unadsorbed. To 10ml. of a G6PD-free liver extract in 50mM-glycylglycine buffer, pH7.5, corresponding to 280mg. of protein, was added 37ml. of 5mM-potassium phosphate buffer, pH7.0, containing 1.4g. of washed DEAE-cellulose powder (previously washed with N-sodium hydroxide and then with distilled water, before being dried under vacuum). Then 37ml. of cold distilled water was immediately added to give a final phosphate concentration of 2.5mM and the mixture, after 30min. of gentle stirring, was centrifuged at 9800g. The carefully collected supernatant (49ml.), showing a protein concentration of 0.94mg./ml., was concentrated in 16hr. to 10ml. by means of finely powdered Aquax that had been put into a dialysis bag and dipped in the supernatant. (Attempts to concentrate the DEAE-cellulose supernatants by other means, e.g. by ultracentrifugation, freeze-drying or vacuum evaporation, led to a complete loss of the G6PD-inactivating ability.) Both the liver extract and the concentrated DEAE-cellulose supernatant were then assayed for G6PD-inactivating ability, NADP glycohydrolase activity, NADP pyrophosphatase activity and protein as described in the Materials and Methods section. Results shown in Table 1 indicate that the concentrated DEAE-cellulose supernatant contains about 70% of the inactivating ability present in the starting extract, whereas the DEAE-cellulose adsorbed 97% of the NADP glycohydrolase activity and 93% of the NADP pyrophosphatase activity. It may be

Table 1. Partial purification of the G6PD-inactivating ability by DEAE-cellulose fractionation

For details see the text.

	G6PD-inactivating ability					NADP glyco-	NADP	Ratio	Ratio
	Activity (a) (units/ml.)	Total activity (units)	Yield (%)	Protein (mg./ml.)	Sp. activity (units/mg.)	hydrolase activity (b) (units/ml.)	pyrophos- phatase activity (c) (units/ml.)		
Extract	43	430	100	28	1.53	12.2	8.1	3.52	5.3
Concentrated DEAE-cellulose supernatant	30	300	69.7	4.6	6.52	0.4	0.55	75	54.5

noted that both the NADP-cleaving enzymes of this extract showed unusually high specific activities.

Thus a considerable though not complete separation of the NADP-splitting enzymes from the G6PD-inactivating ability was achieved, and it was clear that the latter was due to some factor other than NADP glycohydrolase and NADP pyrophosphatase. The DEAE-cellulose procedure results in an apparent fourfold purification of the G6PD-inactivating ability; however, as the starting extracts contain the NADP-cleaving enzymes, most of which are adsorbed by DEAE-cellulose, the true purification of the factor not absorbed is higher than fourfold. From results obtained in other fractionations, estimated recoveries were 70–100% for the factor not adsorbed on DEAE-cellulose, 2–7% for NADP glycohydrolase activity and 5–20% for NADP pyrophosphatase activity.

Effect of deproteinization of DEAE-cellulose supernatant. When the DEAE-cellulose supernatants, after concentration with Aquax, were deproteinized (either by heating for 6 min. at 100° or by treatment with 10% trichloroacetic acid followed by readjustment of the pH to 7.0 with α -sodium hydroxide), the G6PD-inactivating ability was completely lost, suggesting that the third G6PD-inactivating substance is a protein.

Investigation on the mechanism of action of the third G6PD-inactivating protein. Both the liver extract and the concentrated DEAE-cellulose supernatant inactivate G6PD without any concomitant release of free amino acids or small peptides, assayed by the ninhydrin method or by the method of Greenberg (1955) as described in the Materials and Methods section. Moreover, neither the liver extract nor the DEAE-cellulose supernatant showed any trypsin-like or papain-like activity, as assayed by hydrolysis of benzoyl-DL-arginine *p*-nitroanilide by the method of Erlanger *et al.* (1961). The observed G6PD inactivation by DEAE-cellulose supernatants is unaffected by 1 mM- ϵ -aminohexanoic acid, which is known to prevent hydrolysis by proteolytic enzymes such as plasmin (Yoshida, 1966), trypsin, Pronase and kallikrein (Bertelli, Donati & Rossano, 1964). From the foregoing experiments it appears unlikely that the G6PD-inactivating protein is a protease.

Inactivation by liver preparations lacking NADP pyrophosphatase. Evidence for the G6PD-inactivating protein was strengthened and some of its properties could be investigated by working with some liver extracts completely lacking NADP pyrophosphatase activity. Of the 37 extracts we have prepared, two did not show any detectable NADP pyrophosphatase activity (with an average of three livers per extract). The extracts lacking NADP pyrophosphatase were centrifuged at

105 000 *g* for 30 min. in a Beckman Spinco model L-2 preparative ultracentrifuge with a T50 rotor to remove microsome-bound glycohydrolase, and both the NADP-cleaving activities were shown to be absent from the supernatants. Nevertheless inactivation of G6PD occurred in the presence of these supernatants, accounting for 27% of the original activity, in 50 mM-glycylglycine buffer, pH 7.5. With 0.15 ml. of the extract and 0.99 unit of G6PD/ml. the inactivation proceeded very rapidly at first, attaining almost linearly a value of 0.28 unit of G6PD inactivated/ml. in 12 min. and then stopping (Fig. 4, curve B). With 0.3 ml. of the supernatant (curve A) the inactivation occurred to the same extent (0.27 unit of G6PD/ml. of incubation mixture) but at a higher rate. The same amount of inactivation was also observed with another extract lacking NADP pyrophosphatase and prepared as before (Table 2); moreover, by lowering the amount of the inactivating supernatant, this inactivation was found to follow zero-order kinetics.

NADP, at a concentration of 1 mM, completely prevents inactivation of G6PD by the liver supernatants lacking NADP-cleaving activities and produces a slight activation. The effect of glucose 6-phosphate on this inactivation could not be clearly established because glucose 6-phosphate itself, at a concentration of 1 mM, partially inactivated the G6PD in control experiments (Bonsignore *et al.* 1967).

The specific inactivation of G6PD by the liver supernatants lacking NADP-cleaving activities is known to be completely reversed under the same conditions that partially reverse the inactivation by NADP glycohydrolase (Bonsignore *et al.* 1967). A sample (1.76 units) of partially purified erythrocyte

Fig. 4. G6PD inactivation by liver extracts lacking NADP pyrophosphatase. An erythrocyte G6PD preparation was employed that was 2200-fold purified [dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction II]. For other details see the text.

Table 2. *Inactivation of G6PD by liver extracts lacking NADP pyrophosphatase*

The same G6PD preparation was used as in Fig. 4 (0.98 unit/ml.). The reported values were corrected for variations of the controls, from which the extract had been omitted. For other details see the text.

Vol. of extract (ml.)	Incubation time (min.)	...	...	...	G6PD inactivated (unit/ml.)						
					4	8	12	16	20	24	28
0.05					0.050	0.11	0.17	0.24	0.28	0.27	0.27
0.10					0.080	0.16	0.23	0.27	0.28	0.30	0.29
0.15					0.085	0.18	0.25	0.29	0.29	0.27	0.28

G6PD (specific activity 1.2 units/mg.), dialysed as described in the Materials and Methods section, was incubated in 50 mM-tris-hydrochloric acid buffer, pH 8.0, with 0.3 ml. of the same inactivating supernatant as in Fig. 4, in a final volume of 2 ml. After 34 min. at 37°, 0.70 unit of G6PD was found to be inactivated, but no further inactivation occurred on further incubation at 37°. There was no inactivation in the control, containing 1.8 units of the same preparation of G6PD and 10.5 mg. of bovine serum albumin in 50 mM-tris-hydrochloric acid buffer, pH 8.0, in a final volume of 2 ml. These values correspond to an apparent equilibrium constant of 0.66 for the reaction active form $\rightleftharpoons$ inactive form of G6PD. To both the experimental and the control mixtures were added 1 mM-NADP, 0.5 mM-EDTA and 7 mM- β -mercaptoethanol; the two incubation mixtures (experimental and control) were kept for 45 min. at 37° and then for 12 hr. at 25° before being assayed for G6PD activity. This procedure leads to an almost complete (98%) reactivation of the inactivated G6PD. Neither EDTA nor β -mercaptoethanol alone was effective in reactivating G6PD. NADP alone led to a slight reactivation, restoring 25% of the lost activity. The specific inactivation catalysed by the soluble extracts lacking NADP-cleaving enzymes occurred at the same rate and to the same extent in the presence of ϵ -aminohexanoic acid, indicating that no proteolytic enzymes are involved in such an inactivation.

Moreover, the absorption spectrum of the inactivated G6PD did not show any difference from that of native G6PD; for these experiments a 4000-fold purified preparation of human erythrocyte G6PD was employed, obtained by modifying the method of Marks *et al.* (1961) as described in the Materials and Methods section.

DISCUSSION

Results reported in the present paper demonstrate that different G6PD-inactivating proteins are present in rat liver cells. These proteins are: NADP glycohydrolase, which was always found in the

G6PD-free liver extracts; NADP pyrophosphatase, which was present in most extracts; and a third factor, which does not show any action on free NADP. Evidence for the last inactivating agent was given by experiments with nicotinamide and especially by its partial dissociation from the NADP-splitting activities by means of DEAE-cellulose. This inactivating agent shows protein-like features; thus deproteinization leads to the complete loss of its activity. Unfortunately, DEAE-cellulose does not completely remove NADP pyrophosphatase (microsomal NADP glycohydrolase can be simply removed by centrifuging the extracts at 105 000g); thus interference by this inactivating enzyme could not be eliminated. The only experiment on the third G6PD-inactivating protein in the absence of other inactivating enzymes was performed with crude liver extracts lacking NADP pyrophosphatase. From these experiments it may be inferred that the G6PD-inactivating protein acts by an enzymic mechanism; in fact the rate of inactivation by such 'pyrophosphatase-lacking' extracts follows zero-order kinetics at 37° (no inactivation occurs at 25°) (Table 2). Moreover, an apparent equilibrium is reached between the active and the inactive form of G6PD, whose equilibrium constant was calculated to be 0.39 in glycylglycine buffer, pH 7.5, and 0.66 in tris-hydrochloric acid buffer, pH 8.0; the same values were found by starting with 1.5 and 2.0 units respectively of G6PD instead of the usual 1.0 unit, the initial rate of inactivation being the same in all three cases. The independence of the percentage inactivation with respect to G6PD concentration is more suggestive of an enzyme-catalysed equilibrium than of the formation of an inactive G6PD-inhibitor complex (Kern & Natale, 1958). Moreover, the observed independence of the inactivation rate with respect to the initial G6PD activity (above 1.0 unit) indicates that saturation is reached at lower values than 1.0 unit of G6PD, confirming what had been demonstrated for inactivation of G6PD by crude liver extracts (Mangiarotti *et al.* 1965b).

The inactivation, besides showing a rate that was proportional to the concentration of the inactivating factor (Bonsignore *et al.* 1967), was inhibited by NADP, which also shifted the apparent equilibrium between native G6PD and the inactive form. These results strongly suggest an enzymic mechanism; moreover, in some preliminary experiments no reactivation of G6PD could be observed by simply dissociating the inactive form of G6PD from the inactivating enzymes of a concentrated DEAE-cellulose supernatant; dissociation was performed by means of a small Sephadex G-100 column (0.7 cm. $\times$ 11 cm.) and elution was with distilled water to avoid activation or inhibition effects. The inactivating protein (still slightly contaminated by residual NADP-cleaving activities) was excluded by the resin, whereas G6PD was retained, being detectable in fractions 5–7 (each fraction had a volume of 1.5 ml.); the eluted G6PD activity accounted for 93% of that chromatographed. This result demonstrates that inactivation involves some structural modification of the G6PD molecule; if the inactivating protein acted as an inhibitor of G6PD, its dissociation from inactivated G6PD would result in reactivation, as is usually observed after dissociating an enzyme–non-protein inhibitor complex by dialysis.

With regard to the mechanism of action of the inactivating protein, no clear evidence emerged from the foregoing experiments; however, complete protection and reversal of this specific inactivation by NADP, and the observation that NADP also prevents the additional inactivation by nicotinamide, might be interpreted as suggesting some primary action on the 'structural' NADP. Further investigation of this point is hindered by the presence of contaminating NADP pyrophosphatase activity in the partially purified preparations.

The G6PD-inactivating factor, being located like NADP pyrophosphatase and G6PD itself in the soluble fraction of rat liver cell, may be involved in G6PD catabolism *in vivo*. Many experimental and pathological conditions have been described where G6PD activity in liver shows wide variations. Thus a decline of this activity occurs during starvation and diabetes (Glock & McLean, 1955) and a marked rise after re-feeding (Tepperman & Tepperman, 1958; Mangiarotti & Calabria, 1963). In particular, the increase of G6PD activity after re-feeding with a balanced normal diet occurs without any new synthesis of the enzyme (Weber, Banerjee & Bronstein, 1962), which on the other hand appears to be involved during re-feeding with a high-carbohydrate diet (Tepperman & Tepperman, 1963; Acquarone, Garré & Cancedda, 1966). These experiments suggest that hepatic G6PD is controlled, not only at the level of synthesis, but also by different factors that may involve: (a) inter-

conversion between catalytically different forms of G6PD; (b) NADP glycohydrolase (this may be of minor importance since it is located in microsomes); (c) NADP pyrophosphatase; (d) the G6PD-inactivating factor.

The fact that both NADP pyrophosphatase and the G6PD-inactivating factor described in this paper were found in the cytoplasm of nucleated cells (De Flora *et al.* 1965) and not in human erythrocytes (Mangiarotti *et al.* 1965a) would be consistent with the involvement of such enzymes in controlling the turnover of G6PD; in fact they appear to operate in those cells where some protein-synthesizing systems may balance inactivation of G6PD, counteracting exhaustion of this enzyme activity.

This work was supported by the Consiglio Nazionale delle Ricerche, Rome, Italy.

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