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BOVINE ADRENAL GLUCOSE-6-PHOSPHATE DEHYDROGENASE

III. CONTROL OF THE ACTIVITY BY ASCORBATE, SUBSTRATES, AND HYDROPHOBIC MOLECULES*

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SUMMARY

1. Earlier studies with bovine adrenal glucose-6-phosphate dehydrogenase demonstrated that the enzyme was highly susceptible to inactivation by a substance or substances present in the adrenal and removed during purification of the enzyme, and that the rate of inactivation was greatly accelerated by the presence of *n*-butanol at a concentration of 2%. The work reported here identified ascorbic acid as the principal inactivating factor whose action is potentiated by *n*-butanol. The highly purified enzyme is highly sensitive to concentrations of ascorbate several orders of magnitude lower than those found in the adrenal, and is protected from inactivation by $1 \cdot 10^{-3}$ M $NADP^+$ in the presence of 1% bovine serum albumin.

2. Formation of hydrogen peroxide and free radicals by ascorbate appears not to be an obligatory intermediate in the inactivation process. Potentiation of ascorbate inactivation by hydrophobic alcohols and ketones is attributed to hydrophobic interactions of these substances with the enzyme. Attempts to restore activity after inactivation by ascorbate have been unsuccessful.

3. The enzyme is also inactivated upon removal of $NADP^+$ by dialysis. A 3-fold reactivation may be accomplished by incubation in the presence of $NADP^+$ and β-mercaptoethanol.

INTRODUCTION

During the development of a purification procedure for bovine glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase)[1,2] it became clear that an agent present in the adrenal extract but removed during the purification process was exerting a profound effect in inactivating the enzyme. Furthermore, inactivation by this agent was greatly accelerated by butanol at a

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concentration of 2%. Subsequent work reported here shows that the principal inactivating factor of the adrenal, whose action is greatly potentiated by 2% butanol, is ascorbate. Our studies of the inactivation of the enzyme by ascorbate, protection of the enzyme from inactivation by ascorbate by substances present in the adrenal, the effect of hydrophobic alcohols and ketones, and exploratory studies into the mechanism of inactivation are reported here.

MATERIALS AND METHODS

Highly purified glucose-6-phosphate dehydrogenase was prepared from bovine adrenals according to the procedure of SQUIRE AND SYKES[1]. The activity of the enzyme was measured by the method of NOLTMANN *et al.*[3] at 25°. Protein concentrations were determined from spectrophotometric measurements at 280 nm and 260 nm, by means of KALCKAR's formula[4]. The rate of proteolysis was determined as described previously[2]. Ascorbic acid determinations were made according to a recent modification[5] of the method of ROE AND KUETHER[6].

The gel filtration experiments were performed on a 2.5 cm × 60 cm column packed with Sephadex G-100 and equilibrated with 0.02 M phosphate buffer, pH 6.3, containing 0.02% sodium azide as a preservative. The centrifugation preceding the gel filtration experiment was performed on a Beckman Model L-4 ultracentrifuge.

The chemicals used were reagent grade with the exceptions of 3-pentanone (Eastman, practical grade), and L-epinephrine (Baker, U.S.P grade). Crystallized bovine serum albumin was obtained from Armour Pharmaceutical Company. Sephadex G-100 medium (40–120 μm) was obtained from Pharmacia Fine Chemicals, Inc.

RESULTS

Identification of ascorbic acid as the inactivating factor potentiated by butanol

Previous results[2] suggested that adrenal proteases were primarily responsible for the inactivation of glucose-6-phosphate dehydrogenase. This concept has been abandoned as a consequence of the following experimental results: (a) The inactivating factor, potentiated by butanol is heat stable (95° for 10 min) and dialyzable. (b) On Sephadex G-25 gel filtration a substantial portion of the inactivating agent emerges from the column at approximately the total column volume. (c) The acetone-soluble fraction obtained by extracting the glands with an acetone–water solution (85:15, v/v) in the preparation of an acetone powder contains one of the highest concentrations of the inactivating factor of any of the fractions we have tested.

Various metabolites known to be present in the adrenal were tested with regard to enzyme inactivation as well as potentiation by *n*-butanol. These exploratory experiments were performed in a reaction medium containing 0.01 M acetate buffer, pH 5.5, 0.3 M $(NH_4)_2SO_4$ and an enzyme concentration of approximately $1 \cdot 10^{-6}$ M. At concentrations ranging from $0.33–79.0 \cdot 10^{-6}$ M L-epinephrine had no effect on the stability of the enzyme, in the presence or absence of 2% *n*-butanol. Cysteine had no effect on stability at concentrations less than $1 \cdot 10^{-3}$ M, but caused very rapid inactivation of the enzyme at a concentration of $7.5 \cdot 10^{-3}$ M, and butanol had no observable effect on the rate of inactivation in the presence of cysteine.

Results of studies on the effect of ascorbate on the stability of the enzyme, as

TABLE I

INACTIVATION OF ADRENAL GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY ASCORBATE AND *n*-BUTANOL AT pH 5.5

The enzyme, at a concentration of approx. $1 \cdot 10^{-8}$ M, was dissolved in 0.01 M acetate buffer, pH 5.5, containing 0.3 M $(NH_4)_2SO_4$. Incubations were performed at about 23°. The initial specific activity was 10 *u*/mg.

Enzyme purity	*Ascorbate (M)*	*n-Butanol (%)*	*Time (h) required for activity loss of*		
			20%	*50%*	*100%*
Crystalline	—	—	>48		
	$1 \cdot 10^{-11}$	—		28	
	$1 \cdot 10^{-9}$	—	0.1	25	
	$1 \cdot 10^{-9}$	2%	>0.05	0.12	
	$1 \cdot 10^{-1}$	—	0.10	18	
	$1 \cdot 10^{-6}$	—	0.03	0.8	
	$1 \cdot 10^{-5}$	—		0.05	
	$1 \cdot 10^{-4}$	—			<0.03
	$1 \cdot 10^{-3}$	—			<0.03
Homogenate Cow adrenals	$1 \cdot 10^{-4}$	—	approx. 48		
	$1 \cdot 10^{-3}$				

well as potentiation by 2% *n*-butanol are presented in Table I. The stability of the enzyme decreases rapidly with increasing ascorbate concentration. In the presence of *n*-butanol at a concentration of 2%, the half-life of the enzyme activity is reduced from 25 h to 7 min. Thus in a defined system we have now reproduced the 200-fold potentiation by *n*-butanol previously reported[2] from studies of a crude protein fraction from the adrenal. For reference, we have indicated in Table I that the ascorbate concentration of the adrenal homogenate at the beginning of our purification procedure[1] is about $1 \cdot 10^{-4}$ M, and the enzyme is highly stable under these conditions. Correction for dilution indicates an ascorbate concentration in the bovine adrenal of about $1 \cdot 10^{-3}$ M. Data in the literature [7-9] record bulk concentrations of ascorbate within the adrenal in excess of $1 \cdot 10^{-2}$ M. Possible compartmentation within the adrenal is a factor that must be considered in any discussion of regulatory controls*. Nevertheless we observe that in an adrenal extract where the ascorbate concentration is $1 \cdot 10^{-4}$ M, and structures that could provide compartmentation are absent, the enzyme is still quite stable. Obviously there must be within the adrenal, and in an adrenal extract, substances that protect the enzyme from concentrations of ascorbate 10^4 times the levels that produce rapid inactivation of the unprotected enzyme.

Studies similar to those reported in Table I have been repeated several times on various preparations of the crystalline enzyme. The rapid increase of inactivation with increasing ascorbate concentration is always observed, but the ascorbate concentration associated with a given rate of inactivation may differ by as much as a factor of 200. These variations in the rate of inactivation are attributed to variations in the amount of bound $NADP^+$. Dissolved preparations of the enzyme crystallized in the presence of $1 \cdot 10^{-3}$ M $NADP^+$ have a half-life of about 17 h in the presence of

* It is of interest, however, that the results of subcellular fractionation studies of the rat ovary reported by SULIMOVICI AND BOYD[8] suggest that over half of the ascorbate is in the cytosol fraction.

$1 \cdot 10^{-6}$ M ascorbate, but after 48 h of dialysis the initial activity decreases to 20–40 units/mg and the inactivation rates approximate the values reported in Table I.

A comparison of the rates of inactivation by ascorbate and dehydroascorbate has also been made. The enzyme is inactivated very rapidly by either reagent at a concentration of $1 \cdot 10^{-3}$ M but is much more rapidly inactivated by ascorbate than by dehydroascorbate at concentrations of $1 \cdot 10^{-4}$ or $1 \cdot 10^{-5}$ M.

Attempts to reactivate glucose-6-phosphate dehydrogenase inactivated by ascorbate

After the enzyme had been inactivated by ascorbate to 20–30% of its original activity, various metal ions and biological compounds were tested as possible reactivating agents. In these studies we tested $CaCl_2$, $MgCl_2$, riboflavin, cystine, reduced glutathione, $NADP^+$ and 3′,5′-cyclic AMP. With the exception of cystine all sub-

TABLE II

ANALYSIS OF FRACTIONS OBTAINED BY SEPHADEX GEL FILTRATION OF ADRENAL EXTRACTS ON SEPHADEX G-100 (Fig. 1)

Glucose-6-phosphate dehydrogenase at a concentration of approx. $4 \cdot 10^{-8}$ M was added to all samples for the stability study except samples 16–20 which contained the enzyme from the adrenal homogenate. Molecular weights were determined from v/v_0 values according to the method of SQUIRE[10].

Tube		v/v_0	*Molecular weight*	*Ascorbate concentration (M)*	*Stability (% of initial activity after 30 h)*
Number	*pH*				
16–20	6.7	1.17	130 000	not detected	66
22–35	6.8	1.49	63 000	$1.77 \cdot 10^{-7}$	100*
22–35	6.8	1.49	63 000	$5 \cdot 10^{-5}$**	68
41–51	6.8	2.72	300	$1.87 \cdot 10^{-4}$	3.8
52–58	7.0	3.40	—	$4.26 \cdot 10^{-7}$	38

* After 134 h, 27% of the activity remained.
** Additional ascorbate was added for the stability study.

stances were incubated with the enzyme at concentrations of $1 \cdot 10^{-3}$ M. Cystine was studied at $1 \cdot 10^{-4}$ M. Activity measurements were performed after 14, 24 and 48 h of incubation at 4°. All attempts to restore activity after inactivation by ascorbate were unsuccessful.

Protection of glucose-6-phosphate dehydrogenase from inactivation by ascorbate

The obvious existence within the adrenal of agents that protect the enzyme from inactivation by overwhelming concentrations of ascorbate was mentioned earlier. Several studies showed that substantial protection of the enzyme was provided by $NADP^+$, but even at concentrations as high as $1 \cdot 10^{-3}$ M, the enzyme was not stable in the presence of 0.03 M ascorbate. Thus the presence of other stabilizing agents within the adrenal is indicated and we next attempted to identify them. 70 g of adrenal cortices taken from six pairs of fresh glands, were homogenized in 140 ml of water for 30 sec in a blender, and mixed at 4° for 1 h. This mixture was then centrifuged at 106 000 × *g* for 1.5 h. 6.5 ml of the filtrate, having a protein

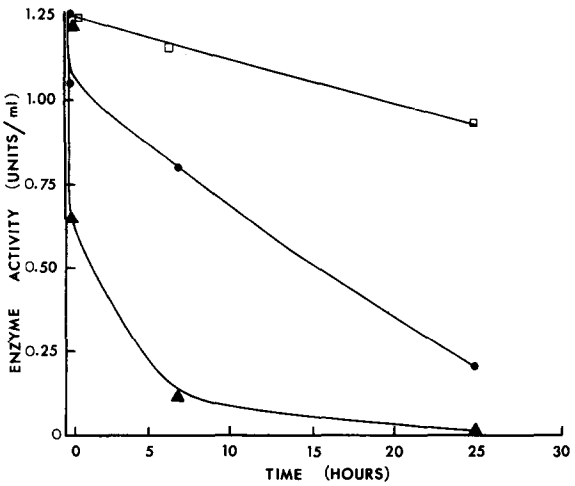

Fig. 1. Protection of glucose-6-phosphate dehydrogenase from ascorbate inactivation by Fraction II (tubes 22–35). Enzyme with a specific activity of 20.8 μ/mg was added to all samples, $3 \cdot 10^{-8}$ M. □—□, stability in Fraction II containing ascorbate at a concentration of $5 \cdot 10^{-5}$ M; ●—●, stability in phosphate buffer, pH 6.8; ▲—▲, stability in phosphate buffer containing ascorbate at a concentration of $5 \cdot 10^{-5}$ M. All experiments were done at pH 6.8.

concentration of 37 mg/ml and an enzyme concentration of 7.75 units/ml were then passed through a Sephadex G-100 column. Tubes of each of the four protein peaks obtained were pooled as follows: Tubes 16–20, peak 1; tubes 22–35, peak 2; tubes 41–51, peak 3, and tubes 52–58, peak 4. Ascorbic acid concentrations as well as the stability of added enzyme were determined in each of the four fractions. The results are shown in Table II. The protective effect of Fraction II (tubes 22–35) in the presence of $5 \cdot 10^{-5}$ M ascorbate is shown in Fig. 1. From the values of v/v_0 (elution volume/void volume) molecular weights can be estimated from the data compiled by SQUIRE[10]. From Table IV the molecular weight of the protecting agent is approximately 62 000. Hemoglobin and serum albumin have molecular weights close to this value and may be involved in protection of the enzyme.

The presence of a small amount of "protein-bound" ascorbate in adrenal homo-

TABLE III

REGULATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY BY CYSTEINE, $NADP^+$, AND BOVINE SERUM ALBUMIN

The buffer was 0.01 M phosphate, pH 7.2, containing 0.08 M KCl. Initial specific activity was 22 u/mg at a concentration of $6 \cdot 10^{-8}$ M.

Bovine serum albumin	*$NADP^+$ (M)*	*Cysteine (M)*	*Ascorbate (M)*	*Activity (% of initial)*	
				6 h	*24 h*
—	—	—	—	74	65
—	—	—	0.02	0	0
1%	—	—	0.02	84	71
1%	$1 \cdot 10^{-3}$	—	0.02	89	75
1%	$1 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	0.02	90	82

genates (tubes 22–35) has been shown earlier by FIDDICK AND HEATH[11] by gel filtration studies on rat adrenal homogenates.

The results of experiments designed to study the combined effects of bovine serum albumin, $NADP^+$ and cysteine in stabilization of the enzyme are recorded in Table III. As expected, in the presence of 0.02 M ascorbate, the enzyme had lost all its activity before 6 h. Stabilization by bovine serum albumin was substantial; in fact, the enzyme was more stable in the presence of 1% bovine serum albumin and 0.02 M ascorbate than it was in the control system in which neither had been added. Further stabilization results from the addition of $NADP^+$ or $NADP^+$ and cysteine to the bovine serum albumin. Thus, in defined systems we have demonstrated enzyme stability approximating that found in the adrenal even in the presence of $1 \cdot 10^{-2}$ M ascorbate.

Potentiation of enzyme inactivation by hydrophobic substances

We have seen that the rate of glucose-6-phosphate inactivation by ascorbate is increased by as much as 200-fold by the addition of *n*-butanol at a concentration of 2%. Two possible mechanisms for potentiation suggest themselves at once: (a) By hydrophobic interactions with the protein, butanol may render the enzyme more sensitive to inactivation, and (b) *n*-butanol may be converted to butyl hydroperoxide by peroxides generated by traces of ascorbate[12] and the hydroperoxide may be a more effective inactivating agent than hydrogen peroxide itself. If mechanism (a) is correct, one might expect a rough inverse correlation between water solubility and potentiation quite independent of the ease of formation of hydroperoxides. If mechanism (b) is correct, ease of hydroperoxide formation should be closely related to potentiation. Since ketones do not form peroxides under the conditions of these experiments, the results of incubations with hydrophobic ketones as well as alcohols, should permit us to distinguish between the two mechanisms under consideration.

TABLE IV

POTENTIATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE INACTIVATION BY ALCOHOLS AND KETONES

Incubations were performed in the presence of $1 \cdot 10^{-6}$ M ascorbate. The initial enzyme activity was 71 *u*/mg at a concentration of $0.6 \cdot 10^{-7}$ M. The incubations were performed in 2 mM phosphate, 13 mM KCl, pH 6.7.

Potentiator	*Solubility in water at 20° (g/100 ml)*	*Activity (% of initial)*		
		30 min	*4.5 h*	*16 h*
—	—	99	91	73
Ethanol	∞	99	87	65
tert.-Butanol	∞	99	87	62
n-Propanol	∞	92	51	9.4
2-Butanone	33	91	56	32
Isobutanol	9.5	95	74	69
n-Butanol	7.9	83	72	61
2-Pentanone	6.3	75	24	6.3
3-Pentanone	4.7	51	5.4	0
n-Pentanol	2.7	57	11	6.8
Isopentanol	2.7	82	43	32

Several alcohols and ketones were incubated separately with glucose-6-phosphate dehydrogenase and $1 \cdot 10^{-6}$ M ascorbic acid at room temperature. Activity measurements were made at 30 min, 4.5 h and at 16 h. The results are recorded in Table IV. Here it may be seen that the ketones, 2-butanone, 2-pentanone, and 3-pentanone, all increase the rate of inactivation; in fact, 3-pentanone had the largest potentiating effect of any of the substances tested. Since ketones do not form hydroperoxides under these conditions, inactivation by this mechanism is clearly ruled out and the hypothesis that inactivation is accelerated by conformational changes induced by hydrophobic substances gains support. Further support comes from the observation that substances of low water solubility tend to show a faster rate of inactivation than ethyl alcohol and *tert.*-butanol which are infinitely soluble in water. We note, however, that the inverse correlation between water solubility and rate of inactivation is not without exceptions.

Effects of hydrogen peroxide and free radicals

CHANCE[12] showed that the activity of catalase is decreased in the presence of ascorbic acid resulting from the formation of a complex between catalase and H_2O_2 generated by the oxidation of ascorbate. ORR[13] proposes that the free radicals $\cdot OH$ and $\cdot O_2H$ formed from the autoxidation of ascorbate are responsible for the inactivation of the enzyme by ascorbate. It was therefore of interest to study the effects of H_2O_2 and free radicals on the activity of the enzyme. Fenton's reagent ($FeSO_4 + H_2O_2$) was used to generate the $\cdot OH$ free radical. Four experiments were performed in parallel in a buffer pH 6.7, containing 1.7 mM phosphate and 13 mM KCl. The specific activity of the enzyme was 108 *u*/mg and the concentration was $9 \cdot 10^{-8}$ M. To one of the samples we added H_2O_2 ($1 \cdot 10^{-5}$ M), to a second H_2O_2 and $FeSO_4$, both at a concentration of $1 \cdot 10^{-5}$ M. The third, with no additions, served as a control. All samples showed a 10–20% decline in activity over a period of 22 h. Thus, under these experiments neither H_2O_2 nor Fenton's reagent had a substantial effect on the activity of the enzyme. The fourth sample, to which $FeSO_4$ was added, also $1 \cdot 10^{-5}$ M, lost 23% of its activity after 22 h suggesting a mild inactivating effect of Fe^{2+}.

Reactivation of dialyzed glucose-6-phosphate dehydrogenase

The enzyme loses activity upon removal of $NADP^+$ by dialysis, and, in contrast to our failure to reactivate enzyme that has been inactivated by ascorbate, reactivation of enzyme inactivated by dialysis has been proven possible. In a representative experiment a sample of 3 times crystallized enzyme with an initial specific activity of 92 units/mg was dialyzed against 0.1 M acetate buffer, pH 5.5, for 49 h at 4°. The activity following dialysis was 52 units/mg. The sample was diluted 4-fold with 0.01 M phosphate buffer, pH 7.1, and stored 6 weeks at 4° after which time the specific activity had decreased to 5.1 units/mg. Reactivation of the enzyme by β-mercaptoethanol and by $NADP^+$ and by a combination of the two reagents was then attempted at 25°. The results are presented in Fig. 2. Here it may be seen that $1 \cdot 10^{-2}$ M β-mercaptoethanol alone has little or no effect. $NADP^+$ at a concentration of $1 \cdot 10^{-3}$ M increases the activity by about 50% and, in combination, $NADP^+$ and β-mercaptoethanol triple the specific activity. The specific activity is approximately doubled by incubation in the presence of $1 \cdot 10^{-5}$ M $NADP^+$ in combination with $1 \cdot 10^{-2}$ M β-mercaptoethanol.

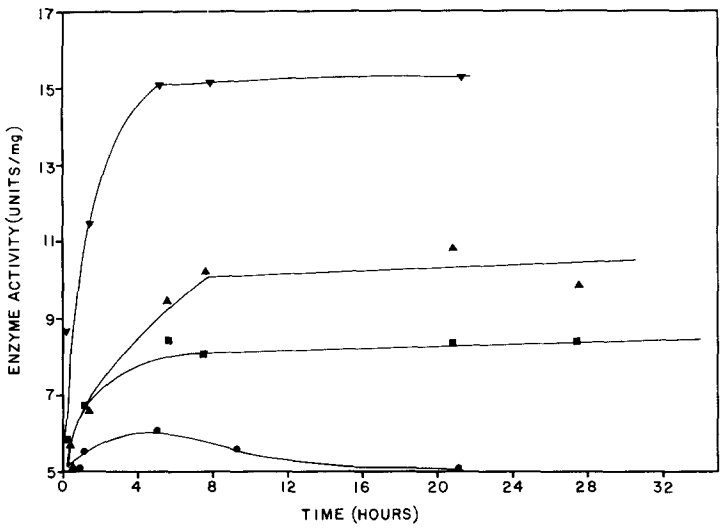

Fig. 2. Reactivation of glucose-6-phosphate dehydrogenase at 25° following dialysis against 0.1 M acetate, pH 5.5, for 49 h. The protein concentration was $7.1 \cdot 10^{-9}$ M. All experiments were performed in 0.04 M phosphate buffer containing 0.02% sodium azide, 0.04 M KCl, 0.5 mM EDTA and additives as follows: ●—●, 0.01 M β-mercaptoethanol; ■—■, 1.0 mM $NADP^+$; ▲—▲, 0.01 mM $NADP^+$ and 0.01 M β-mercaptoethanol; ▼—▼, 1.0 mM $NADP^+$ and 0.01 M β-mercaptoethanol.

DISCUSSION

The high sensitivity of an adrenal enzyme to concentrations of ascorbate several orders of magnitude lower than those found in the adrenal and the augmentation of this effect by hydrophobic substances, appear to be new information that may be of considerable interest. The possible use of ascorbate in the presence and absence of hydrophobic substances as a probe of structure–function relationships suggests itself immediately. These studies also suggest that the use of glucose-6-phosphate dehydrogenase as a NADPH-generating system in studies designed to elucidate mechanisms by which enzymes are controlled by ascorbate[8,14] may be hazardous unless the enzyme is protected from inactivation by ascorbate. In any case, the activity of the enzyme in the milieu under study should be measured and reported.

Our results show that the enzyme loses activity upon removal of $NADP^+$ by dialysis and can be reactivated by incubation with $NADP^+$ and β-mercaptoethanol. These results are qualitatively similar to those obtained with enzyme preparations obtained from human erythrocytes[15–17], the rat mammary gland[18], the bovine ovary[19] and the human placenta[20]. LEVY *et al.*[18] have remarked on the general instability of glucose-6-phosphate-dehydrogenases from various sources and the general observation that the enzyme is greatly stabilized by the coenzyme. The experiments describing the loss of activity on dialysis (RESULTS, *Reactivation of dialyzed glucose-6-phosphate dehydrogenase*) are representative of several from this laboratory, some of which have been most unfortunate. The time-dependent loss of activity of the apoenzyme followed by a reactivation process requiring about 4 h incubation (Fig. 2) with coenzyme and β-mercaptoethanol suggests that the inactivation–reactivation processes may

result from conformational changes in the enzyme. This would imply that the conformation of the enzyme in its active form may be determined not only by the amino acid sequence but by the presence of bound coenzyme as well.

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