Chromate Inhibition, in Erythrocytes, of Chemically Stimulated Increases in Glucose Oxidation via the Hexosemonophosphate Pathway (HMP)* (33533)

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From time to time it has been postulated that in erythrocytes the glutathione, glutathione reductase (GSSG-R) system plays an important intermediary role in certain chemically induced increases in glucose oxidation via the hexosemonophosphate pathway (HMP), e.g., those which occur in presence of hydrogen peroxide (1) or in association with methemoglobinemia (2). The present research was initiated on the premise that *specific* GSSG-R inhibitors, used at appropriate dosage, should inhibit only those chemically induced increases in erythrocyte HMP rate wherein the glutathione, GSSG-R system does play such a role. Sodium chromate (Na_2CrO_4), hereinafter referred to as chromate, was used because of the evidence presented by Koutras *et al.* (3, 4) that it specifically inhibits human erythrocyte GSSG-R. Use of 2,5-dinitrobenzoic acid (DNBA) has been predicated on the report by Buzard and Kopko (5) that this compound is a potent inhibitor of rat erythrocyte GSSG-R. It soon became clear that DNBA was much more selective than chromate in respect to the kinds of chemically induced increases in erythrocyte HMP rates which it would inhibit. This in turn led to experimentation to elucidate what reaction(s) chromate might inhibit in addition to that catalyzed by GSSG-R. The HMP stimulating compounds used (cf. 1, 2, 6–10) were selected with reference to expectation that they would or would not utilize the glutathione, GSSG-R system, as follows: To utilize system, hydrogen peroxide (H_2O_2) and oxidized glutathione (GSSG); not to utilize system, methylene blue, new methylene blue, and the oxidized sodium salt of nicotinamide adenine dinucleotide phosphate (NADP+); not readily predictable, L-cysteine (CHS), reduced glutathione (GSH), dehydroascorbic acid (DHA), ascorbic acid (AA). Since GSSG and NADP+ penetrate very poorly if at all into erythrocytes, these compounds were used only in hemolysate experiments.

Materials and Methods. Aqueous solutions were prepared in glass distilled water. ($^{14}C_1$)-D-glucose was obtained from Volk; other labeled substrates from Nuclear-Chicago. Specific activities were in the range 1–3 mCi/mmole. Compounds obtained from Sigma included disodium adenosine-5-triphosphate (ATP), GSSG, GSSG-R, heparin, type III yeast hexokinase, and NADP+. Hyamine, POPOP, and PPO came from Packard Instrument Co.; Na_2EDTA from Matheson, Coleman and Bell; DNBA from Eastman Organic Chemicals; anhydrous unlabeled D-glucose from Mallinkrodt; GSH from General Biochemicals; methylene blue and new methylene blue from Allied Chemicals; 5,5-dithiobis-2-nitrobenzoic acid (BIS) from Aldrich Chemical Co. Other chemicals used, all reagent grade, came from Fisher Scientific Co. Scintillation fluid contained 4 g of PPO and 0.5 g of POPOP/liter spectral quality toluence. Krebs–Ringer phosphate buffer of pH 7.4 was prepared by mixing: 0.9% NaCl, 100 vol; 1.15% KCl, 4 vol; 1.22% $CaCl_2$, 1.5 vol; 3.82% $MgSO_4 \cdot 7H_2O$, 1 vol, 0.1 *M* sodium phosphate buffer of pH 7.4, 21 vol. The white rats used, mostly males, were obtained from Laboratory Supply Co., Indianapolis, Ind., and at time of sacrifice had body weights between 250 and 350 g. Food and water were given *ad libitum*.

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Each rat provided erythrocytes for a single set of HMP rate estimations (cf. Tables). Each heparinized blood sample was centrifuged at 4° and 1500*g* for 20 min, after which the plasma and buffy coat were removed. The packed erythrocytes were washed three times using 0.9% NaCl and suspended to hematocrit of 25 ± 5% in the Krebs–Ringer. Hemolysates were prepared by subjecting such erythrocyte suspensions to ultrasonication at 4° using a Branson sonifier cell disruptor set to read 4–5 A. Smears exhibited some crystals but no intact cells.

Incubation and ^{14}C counting mixtures and procedures. The water bath incubators (Dubnoff; Warner-Chilcott) were set at 37° and 70 oscillations/min. Each incubation mixture per 50-ml Erlenmeyer incubation flask consisted of 0.8 ml of erythrocyte suspension or hemolysate prepared as described above, plus 1.2 ml of Krebs–Ringer containing the desired chemicals (final hematocrit or equivalent, 10 ± 2%). When the labeled substrate used with a hemolysate was glucose the incubation mixture also contained, per ml, 5 μmoles of ATP and 0.5 extra μmoles of $MgSO_4$. Each incubation flask was sealed with a serum cap from which was suspended a glass tube containing 0.5 ml of hyamine, to trap $^{14}CO_2$ formed. After 60 min incubation each hyamine tube was placed upside down in a counting vial containing 8 ml of scintillation fluid. The ^{14}C counts were obtained using an appropriately programmed Packard Tri-Carb model 314EX Liquid Scintillation Spectrometer. For each set of incubation flasks a FULL COUNT vial was also prepared, which contained 4 ml of 100% ethanol, 0.5 ml of hyamine, 8 ml of scintillation fluid and 0.4 ml of labeled substrate in incubation mixture. The counts so obtained were used to estimate the ratios, ^{14}C/mmole of glucose, needed to convert $^{14}CO_2$ counts to mmoles of glucose. In the earlier experiments, all done using substrates labeled at carbon 1 only, it was found impractical to estimate terminal erythrocyte NPSH and methemoglobin concentrations, and also include the acid addition, reincubation step to drive over dissolved $^{14}CO_2$. This step was included in the experiments done using uniformly labeled substrates [with which there is also no recycling error (11)]. In a direct comparison experiment, estimated rat erythrocyte HMP rates were over a wide range consistently about one-fourth lower for the combination, carbon 1 labeled substrate plus omission of the acid addition, reincubation step, than for the combination, uniformly labeled substrate plus inclusion of the acid addition, reincubation step.

Nonprotein sulfhydryl (NPSH) estimations. Extracts were prepared using ice-cold perchloric acid, Na_2EDTA solutions (final concentrations, 0.3 *M* perchloric acid; 10^{-3} *M* EDTA). The NPSH concentration per extract was estimated by the Bis method of Ellman (12).

Methemoglobin estimations were done as described by Evelyn and Malloy (13). In each of the experiments, the incubation flasks were set up in banks of four. Flasks (b) and (d) contained at desired concentration the selected GSSG-R inhibitor; flasks (c) and (d) contained at desired concentrations(s) the compound or combination of compounds used to stimulate the HMP rate; flask (a) was a control flask.

Results. Contrary to original expectation, the pattern of inhibitions of chemically stimulated increases in HMP rates of intact rat erythrocytes differed markedly for chromate and DNBA (cf. Table I). Chromate significantly inhibited all chemically stimulated increases in HMP rate tested (in the case of L-cysteine, which rapidly reduces hexavalent chromate to inactive trivalent chromate, this inhibition could be demonstrated only by allowing the chromate to penetrate the cells before adding the L-cysteine). In contrast, DNBA significantly inhibited only those increases in HMP rates of rat erythrocytes which were induced by L-cysteine, or by hydrogen peroxide plus the catalase poison azide. Table II presents data obtained in pilot type experiments using D-glucose-$^{14}C_1$, and mammalian erythrocytes of species origin other than the rat. In each of these experiments, and regardless of the species origin of the erythrocytes, or the kind of HMP stimu-

TABLE I. Effects of the Glutathione Reductase Inhibitors, Na_2CrO_4 and 2,5-Dinitrobenzoic Acid (DNBA) on the Action of Certain Chemicals to Stimulate Glucose Catabolism via the Hexosemonophosphate Pathway (HMP) in Unhemolyzed Rat Erythrocytes.

Substrate used for $^{14}CO_2$ production[a]	HMP stimulating compounds[b]	n[c]	Mean HMP rates ± SE (increase over those of control flasks, and as μmoles of glucose/ml of cells/hr)[d]		Probability at <.01 level that GSSG-R inhibitor signif. lowered HMP rate from that with stim. cpd. only[e]
			Stim. cpd. only	Stim. cpd. + GSSG-R poison	
	I. Experiments in which 0.3 *M* Na_2CrO_4 was the GSSG-R poison used				
G-($^{14}C_u$)	H_2O_2 + NaN_3	9	2.03 ± 0.06	0.55 ± 0.05	S
	Mb	9	5.85 ± 0.32	0.81 ± 0.03	S
G-($^{14}C_1$)	H_2O_2 + NaN_3	14	1.21 ± 0.08	0.63 ± 0.04	S
	Mb	45	3.60 ± 0.14	0.53 ± 0.05	S
	NMb	13	2.92 ± 0.19	0.58 ± 0.08	S
	DHA	18	0.32 ± 0.04	0.13 ± 0.03	S
	GSH	19	0.68 ± 0.09	0.24 ± 0.04	S
	CSH	15	1.96 ± 0.12	2.13 ± 0.16	NS
	CSH, preincubation	9	1.98 ± 0.10	0.15 ± 0.04	S
	II. Experiments in which 0.1 *M* DNBA was the GSSG-R poison used				
G-($^{14}C_u$)	H_2O_2 + NaN_3	9	2.03 ± 0.06	0.46 ± 0.04	S
	Mb	9	5.85 ± 0.32	6.06 ± 0.11	NS
G-($^{14}C_1$)	H_2O_2 + NaN_3	5	1.41 ± 0.15	0.51 ± 0.12	S
	Mb	15	3.55 ± 0.16	3.55 ± 0.16	NS
	NMb	5	3.12 ± 0.59	3.37 ± 0.43	NS
	DHA	7	0.29 ± 0.06	0.29 ± 0.06	NS
	GSH	11	0.42 ± 0.05	0.54 ± 0.04	NS
	CSH	10	1.98 ± 0.09	0.86 ± 0.07	S

[a] G-($^{14}C_u$) = D-glucose uniformly labeled with ^{14}C; at end of experiment acid was used to drive over dissolved $^{14}CO_2$; G-($^{14}C_1$) = D-glucose labeled at first carbon only; acid was not used to drive over dissolved $^{14}CO_2$. This resulted in about 25% underestimations of HMP rates (see text).

[b] H_2O_2 = 1 m*M* hydrogen peroxide; NaN_3 = 10 m*M* azide; Mb = 0.01 m*M* methylene blue; NMb = 0.01 m*M* new methylene blue; DHA = 3 m*M* dehydroascorbic acid; GSH = 10 m*M* reduced glutathione; CSH = 10 m*M* L-cysteine; CSH, preincubation indicates that cells were incubated with the Na_2CrO_4.

[c] n = no. of rat erythrocyte donors, and no. of increases in HMP rate used to estimate each mean ± SE, next two columns.

[d] HMP rates for control flasks and those in which Na_2CrO_4 only had been added were nearly identical, and of the order of 0.1 μmoles of glucose/ml of cells/hr, G-($^{14}C_u$) experiments, or 0.2 μmoles of glucose/ml of cells/hr, G-($^{14}C_1$) experiments. DNBA used alone induced slight (0.1–0.2 μmoles of glucose/ml of cells/hr) but statistically significant increases in HMP rates.

[e] S = significant at 0.01 level; NS = not significant.

lating compound used, those erythrocytes which were exposed to both an HMP stimulating compound and chromate exhibited appreciably smaller HMP rate than did erythrocytes exposed only to the HMP stimulating compound. Conversely, presence or absence of DNBA was apparently unimportant, except perhaps for the HMP stimulations obtained for canine and bovine origin erythrocytes, by combined use of hydrogen peroxide and azide. The disparity between chromate and DNBA inhibitory abilities *re* erythrocyte HMP rates suggests that chromate must owe some of its erythrocyte HMP inhibitory actions to inhibition of one or more reactions other than that catalyzed by

TABLE II. Pilot Tests on Chromate Inhibition of Chemically Stimulated Increases in HMP Rates of Erythrocytes Taken from Mammals Other than the Rat.

Species origin of the erythrocytes	HMP stimulating compounds[a]	HMP rates (single values, μmoles of glucose/ml of cells/hr) Control flask (a)	0.3 m*M* Na_2CrO_4 (b)	Stim. cpd. only (c)	Stim. cpd. + 0.3 m*M* Na_2CrO_4 (d)
I. Experiments in which Na_2CrO_4 (final concentration, 0.3 m*M*) was added to (b) and (d) flasks at same time HMP stimulating compound was added to (c) and (d) flasks					
Human	H_2O_2 + NaN_3	0.11	0.27	1.24	0.55
	DHA	0.11	0.27	1.24	0.78
	GSH	0.11	0.27	1.32	0.28
	Mb	0.11	0.27	2.18	0.37
Mouse	H_2O_2	0.22	0.51	3.01	2.26
	Mb	0.22	0.51	7.05	2.06
Hamster	H_2O_2	0.35	0.39	2.25	1.56
	Mb	0.35	0.39	5.75	2.08
Dog	H_2O_2 + NaN_3	0.15	0.18	0.62	0.39
	Mb	0.15	0.18	0.96	0.40
Guinea pig	H_2O_2 + NaN_3	0.22	0.27	0.85	0.34
	Mb	0.22	0.27	2.29	0.27
Rabbit	H_2O_2 + NaN_3	0.08	0.17	0.61	0.45
	Mb	0.08	0.17	1.13	0.25
II. Experiments in which Na_2CrO_4 (final conc, 0.3 m*M*) was added to (b) and (d) flasks 30 min before HMP stim. cpd. was added to (c) and (d) flasks					
Cat	CSH	0.13	0.37	0.71	0.38
	Mb	0.13	0.37	1.22	0.59
Bovine	H_2O_2	0.09	0.11	0.39	0.08
	AA	0.09	0.11	0.23	0.06
	DHA	0.09	0.11	0.23	0.16
	CSH	0.09	0.11	0.29	0.12
	Mb	0.09	0.11	0.73	0.32
Guinea pig	CSH	0.22	0.27	1.25	0.48
	Mb	0.22	0.27	2.29	0.40
III. Experiments in which DNBA (final conc, 0.1 m*M*) was added to (b) and (d) flasks at same time HMP stim. cpd. was added to (c) and (d) flasks					
Human	H_2O_2 + NaN_3	0.11	0.34	1.24	1.16
	Mb	0.11	0.34	2.18	2.10
Mouse	Mb	0.22	0.57	7.05	7.35
Dog	H_2O_2 + NaN_3	0.15	0.16	0.62	0.48
	Mb	0.15	0.16	0.96	1.07
Guinea pig	H_2O_2 + NaN_3	0.22	0.30	0.85	0.74
	Mb	0.22	0.30	2.29	2.36
Rabbit	Mb	0.08	0.17	1.13	1.07
Bovine	H_2O_2 + NaN_3	0.09	0.12	0.39	0.23
	Mb	0.09	0.12	0.73	0.75

[a] H_2O_2 = 1 m*M* hydrogen peroxide; NaN_3 = 10 m*M* azide; DHA = 3 m*M* dehydroascorbate; GSH = 10 m*M* reduced glutathione; Mb = 0.01 m*M* methylene blue; CSH = 10 m*M* L-cysteine; AA = 3 m*M* ascorbate.

TABLE III. Relation of Substrate Used, Glucose-($^{14}C_u$) or Glucose-6-phosphate-($^{14}C_u$), to Abilities of NaCrO and 2,5-Dinitrobenzoate (DNBA) to Inhibit Chemically Stimulated Increases in HMP Rates of Rat Erythrocyte Hemolysates.

Substrate used for $^{14}CO_2$ production[a]	HMP stimulating compounds[b]	n[c]	Mean HMP rates ± SE (increases over those of control flasks, and as μmoles of glucose/ml of cells/hr)		Probability at <.01 level that GSSG-R inhibitor signif. lowered HMP rate from that with stim. cpd.[d]
			Stim. cpd. only	Stim. cpd. + GSSG-R poison	
	I. Experiments in which 1 m*M* Na_2CrO_4 (Cr6) was the GSSG-R poison used				
G-($^{14}C_u$)	H_2O_2 + NaN_3	9	0.41 ± 0.03	0.25 ± 0.02	S
	Mb	9	1.93 ± 0.16	0.44 ± 0.02	S
	NADP (1.5 mmoles)	9	5.66 ± 0.15	0.87 ± 0.03	S
	NADP (5 mmoles)	9	4.68 ± 0.10	0.62 ± 0.03	S
G6P-($^{14}C_u$)	H_2O_2 + NaN_3	9	0.58 ± 0.03	0.58 ± 0.03	NS
	GSSG	9	0.42 ± 0.09	0.20 ± 0.03	NS
	Mb	9	7.58 ± 0.24	6.78 ± 0.15	NS
	NADP (1.5 mmoles)	9	4.15 ± 0.06	5.17 ± 0.08	NS
	NADP (5 mmoles)	9	22.67 ± 0.34	11.48 ± 0.31	S
	II. Experiments in which 0.1 *M* DNBA was the GSSG-R poison used				
G-($^{14}C_u$)	H_2O_2 + NaN_3	9	0.41 ± 0.03	0.27 ± 0.02	S
	Mb	8	5.60 ± 0.36	5.85 ± 0.42	NS
	NADP (1.5 mmoles)	7	3.58 ± 0.43	4.04 ± 0.40	NS
	NADP (5 mmoles)	12	3.04 ± 0.09	3.14 ± 0.22	NS
G6P-($^{14}C_u$)	H_2O_2 + NaN_3	9	0.48 ± 0.02	0.28 ± 0.02	S
	GSSG	9	0.42 ± 0.09	0.10 ± 0.03	S
	Mb	9	7.58 ± 0.24	6.99 ± 0.33	NS
	NADP (1.5 mmoles)	6	3.50 ± 0.50	3.47 ± 0.42	NS
	NADP (5 mmoles)	6	18.90 ± 3.44	17.60 ± 2.68	NS

[a] G-($^{14}C_u$) = uniformly ^{14}C labeled D-glucose; G6P-($^{14}C_u$) = uniformly ^{14}C labeled glucose-6-phosphate. At ends of experiments acid was added to drive over dissolved $^{14}CO_2$.

[b] H_2O_2 = 1 m*M* hydrogen peroxide; NaN_3 = 10 m*M* azide; GSSG = 1 m*M* oxidized glutathione; Mb = 0.01 m*M* methylene blue; NADP = oxidized form of nicotinamide adenine dinucleotide phosphate = TPN+.

[c] n = no. of rat erythrocyte donors, and no. of increases in HMP rate used to estimate each mean ± SE, next two columns.

[d] S = significant at 0.01 level; NS = not significant.

GSSG-R. An obvious possibility was the hexokinase stimulated reaction whereby glucose is phosphorylated and thus made ready for oxidation via the pentose pathway. Experiments were therefore carried out, data of which are shown in Table III, to determine what inhibitory actions chromate and DNBA might have against various chemically stimulated increases in rat erythrocyte GOPP rates, not only when using ^{14}C labeled glucose, but also similarly labeled glucose-6-phosphate. Since phosphorylated hexoses do not penetrate appreciably into erythrocytes, it was necessary to do these experiments using hemolysates, and when the substrate was labeled glucose to add sufficient ATP and Mg^{2+} to assure adequate phosphorylation of the glucose. Using labeled glucose as substrate, hemolysates gave findings similar to those previously obtained using intact erythrocytes: (I) chromate significantly inhibited all chemically stimulated increases in HMP rate tested; (II) DNBA significantly inhibited only that increase in

TABLE IV. Effects on Rat Erythrocyte NPSH (Nonprotein Sulfhydryl) Concentration of Certain HMP Stimulating and GSSG-R Inhibiting Compounds.

GSSG-R poison[a]	HMP stim. cpds.[b]	Erythrocyte NPSH concentrations (meq of -SH/liter of cells; means ± SE)				*p* Values for differences:		
		Control flasks (a)	GSSG-R poison only (b)	HMP stim. cpd. only (c)	Stim. cpd. + GSSG-R poison (d)	(b) vs (a)	(c) vs (a)	(d)[c] vs (c)
I. Experiments with intact erythrocytes, and glucose the substrate								
Cr6 (1)	H_2O_2	2.22 ± 0.24	0.29 ± 0.07	2.08 ± 0.14	0.31 ± 0.05	S—	NS	S—
	$H_2O_2 + NaN_3$	2.22 ± 0.24	0.29 ± 0.07	0.56 ± 0.14	0.42 ± 0.16	S—	S—	NS
	NaN_3	2.22 ± 0.24	0.29 ± 0.07	2.09 ± 0.32	0.23 ± 0.06	S—	NS	S—
Cr6 (0.3)	Mb	2.60 ± 0.23	0.92 ± 0.11	1.53 ± 0.14	0.52 ± 0.09	S—	S—	S—
DNBA	Mb	3.32 ± 0.18	2.32 ± 0.24	1.73 ± 0.31	0.61 ± 0.18	S—	S—	S—
II. Experiments with hemolysates, and glucose the substrate								
Cr6 (1)	Mb	2.77 ± 0.18	0.97 ± 0.12	0.55 ± 0.09	0.39 ± 0.06	S—	S—	NS
DNBA	Mb	3.00 ± 0.24	1.74 ± 0.39	0.63 ± 0.15	0.55 ± 0.12	S—	S—	NS
III. Experiments with hemolysates, and glucose-6-phosphate the substrate								
Cr6 (1)	Mb	2.78 ± 0.15	1.38 ± 0.11	0.37 ± 0.08	0.78 ± 0.10	S—	S—	S+
DNBA	Mb	2.94 ± 0.20	1.83 ± 0.19	0.59 ± 0.11	0.60 ± 0.21	S—	S—	NS

[a] Cr6 (1) = 1 m*M* Na_2CrO_4; Cr6 (0.3) = 0.3 m*M* Na_2CrO_4; DNBA = 0.1 m*M* 2,5-dinitrobenzoate.

[b] H_2O_2 = 1 m*M* hydrogen peroxide; NaN_3 = 10 m*M* azide; Mb = 0.01 m*M* methylene blue.

[c] S = significant at 0.01 level; NS = not significant; + after S signifies that mean indicated by upper level letter is significantly greater than mean indicated by lower level letter; — after S signifies the reverse.

HMP rate stimulated by hydrogen peroxide plus azide. However, when the substrate added to the hemolysates was labeled glucose-6- phosphate, the only increases in HMP rate significantly inhibited by chromate was that induced by NADP+ used at the very high concentration of 5 mmoles. DNBA continued to be ineffective in inhibiting HMP stimulations induced by 0.01 m*M* methylene blue, and by NADP+. It was effective in inhibiting stimulations induced by hydrogen peroxide plus azide, and by 1 m*M* GSSG.

Effects of compounds used on rat erythrocyte NPSH concentrations. Data obtained are presented in Table IV. Whether the erythrocytes were intact or hemolyzed, and the substrate glucose or glucose-6-phosphate, both chromate and DNBA induced statistically significant decreases in rat erythrocyte NPSH concentrations; these were more striking for chromate than for DNBA. The NPSH concentrations were also significantly lower for erythrocytes exposed either to methylene blue or to the combination hydrogen peroxide plus azide than they were for erythrocytes incubated in the control flasks. Neither hydrogen peroxide nor azide, used alone, significantly altered the NPSH level.

Effects of compounds used on rat erythrocyte methemoglobin concentrations. Erythrocytes incubated for 1 hr at 37° with 1 m*M* H_2O_2 + 10 m*M* NaN_3 exhibited marked methemoglobinemia (about 50%) whether chromate or DNBA was also present or not; hydrogen peroxide used alone was ineffective (% MethHb did not exceed 6%). No other compound or combination of compounds tested was found to induce methemoglobinemia exceeding 15%.

Experiments to test for mechanism of action of chromate in inhibiting at the glucose phosphorylation step. The ability of 1 m*M* chromate to inhibit 0.01 m*M* methylene blue stimulation of the HMP, rat erythrocyte he-

molysates, was not significantly altered by change in gluose concentration over the range 3–81 m*M*, of Mg^{2+} over the range 0.5–50 m*M*, of added GSSG over the range 0–3 m*M*, and of added ATP over the range 5–10 m*M*, (significant phosphorylation not obtained if ATP <5 m*M*; ATP itself inhibitory if present at concentration >10 mM). Chromate inhibition was reversed on addition of 2.8 units of yeast hexokinase/flask (1 unit = 1 μmole of glucose phosphorylated/hr).

Discussion. For rat erythrocyte hemolysates, when the labeled substrate for glucose oxidation via the hexosemonophosphate pathway (HMP) was glucose itself: (a) NADP+ was at least as potent at stimulating the HMP when used at 1.5 m*M* as when used at 5 m*M* concentration, and (b) 1 m*M* chromate markedly inhibited the NADP+ stimulated HMP, regardless of which NADP+ concentration had been used. In contrast, when the labeled substrate was glucose-6-phosphate: (a) 5 m*M* NADP+ gave a far greater stimulation of the HMP than did 1.5 m*M* NADP+ (or methylene blue or any other compound tested), (b) only that stimulation induced by 5 m*M* NADP+ was significantly inhibited by 1 m*M* chromate. These findings are in accord with the concepts that: (I) when the labeled substrate is glucose, and NADP+ is used in excess, the limiting step is glucose phosphorylation, markedly inhibited by 1 m*M* chromate; (II) when the labeled substrate is glucose-6-phosphate, greater stimulation can be obtained using NADP+ because another reaction beyond glucose phosphorylation becomes the limiting step for the HMP; furthermore, this reaction can also be inhibited by 1 m*M* chromate, but not sufficiently that the overall HMP rate will be measurably affected except when NADP+ itself is used at high concentration to stimulate the HMP.

Koutras *et al.* (3, 4) presented experimental findings which they interpreted to mean that hexavalent chromate compounds are reasonably specific inhibitors of human origin erythrocyte glutathione reductase (GSSG-R). Present data indicate that in rat and other mammalian origin erythrocytes one of these hexavalent chromate compounds, $NaCrO_4$, used at concentrations similar to those used by them, also inhibits at least one and probably two enzymatically catalyzed reactions important in glucose catabolism. While no claim was made by Buzard and Kopko (5) that 2,5-dinitrobenzoic acid is a specific inhibitor of erythrocyte GSSG-R, present data indicate that it comes closer to being so than Na_2CrO_4. The data of Table IV show that when the (labeled) substrate was glucose, 0.3 m*M* chromate was more potent than 0.1 m*M* DNBA in lowering NPSH levels of both intact and hemolyzed rat erythrocytes. This greater potency of the chromate may be due more to its action to inhibit glucose phosphorylation and therefore the HMP, than to its action to inhibit GSSG-R. Similarly, chromate induced damage of erythrocytes, attributed by Koutras *et al.* (3, 4) to chromate inhibition of GSSG-R, may also be due instead to chromate inhibition of the HMP. There is a considerable literature [cf. (14)] which attests to the importance for erythrocyte stability of maintenance of the HMP. In their work on removal of damaged erythrocytes from the circulation, Ultmann and Gordon (15) used ^{51}Cr labeled chromate of specific activity 380 mCi/mg; this permitted them to use chromate as such low concentrations ($1–10 \times 10^{-5}$ m*M*) that it is very unlikely there was significant inhibition of either GSSG-R or the HMP. However, a certain skepticism is justified about conclusions *re* longevities of erythrocytes drawn by earlier workers (16–18) who had used labeled chromate at concentrations which do result in significant inhibition of both GSSG-R and the HMP.

Summary. Increases in glucose oxidation via the hexosemonophosphate pathway (HMP) may be induced in erythrocytes by many and diverse chemical compounds. This research was initiated on the hypothesis that insofar as they are *specific* inhibitors of glutathione reductase, Na_2CrO_4 and 2,-5-dinitrobenzoic acid (DNBA) should have similar discriminatory inhibitory effects against chemically stimulated increases in erythrocyte HMP

rates. These were estimated, for both intact and hemolyzed rat erythrocytes, in terms of $^{14}CO_2$ formed from labeled glucose or glucose-6-phosphate. In the labeled glucose tests, DNBA inhibited only those increases in HMP rate which were induced by hydrogen peroxide plus azide, or by GSSG, while chromate inhibited all chemically induced increases in HMP rate tested. Substitution of labeled glucose-6-phosphate did not result in change in pattern *re* DNBA inhibitions, but did lead to loss of most chromate inhibitions. These various findings are in accord with the concepts that DNBA is a fairly specific erythrocyte GSSG-R inhibitor, while chromate inhibits not only this enzyme but also at least one reaction essential to the HMP, e.g., glucose phosphorylation.

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