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DEPRESSION OF HEME FORMATION AND THE PRODUCTION OF
FREE PORPHYRIN IN DUCK ERYTHROCYTES

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Prothemo formation in the intact erythrocyte is depressed by a variety of substances (1-8). However, the effects of most of the substances on total protoporphyrin formation are not certain; thus, it is not clear whether the depression of heme formation is ascribable to inhibition of some phase of porphyrin synthesis or the conversion of porphyrin into heme. Some of the substances that depress heme production also affect the formation of free, i.e. non-heme protoporphyrin (5,8,9), but in most instances effects on heme and free porphyrin formation have not been established for the same cell system.

For some of the substances known to depress heme formation and others the present work provides a comparison of action on heme and free porphyrin and, thus, total porphyrin formation in the same cell system, namely duck erythrocytes in plasma. The findings suggest that depression of heme production by a divalent lead, as indicated by earlier work (5,8), is accounted for by inhibition of some phase of porphyrin synthesis, while the depressions produced by a number of compounds, e.g. pyridoxine and derivatives, benzimidazole and derivatives, and physostigmine, are attributable to inhibition of some phase of the conversion of protoporphyrin into heme. The compounds also appear to inhibit porphyrin synthesis; but, under certain circumstances, pyridoxamine phosphate and physostigmine increase porphyrin synthesis.

EXPERIMENTAL

Blood taken in heparin from adult Pekin ducks was used. Heme and free protoporphyrin formation were estimated as indicated elsewhere

(10). In brief, blood plus radioactive iron (Fe^{59}) was shaken four hours at 37° under 5% carbon dioxide in oxygen in the presence or absence of added glycine and in the case of each mixture with and without the substances under test. The difference between the level of protoporphyrin in the mixture of blood and other materials before and after incubation was taken as a measure of free porphyrin formation. Heme formation was estimated from the level of labeled and unlabeled iron in plasma before the incubation and the labeled iron recovered as heme after the incubation. The total uptake of iron by the cells was measured similarly. The procedure used for estimation of protoporphyrin (11) appears reasonably specific, at least the final acid extracts obtained from the experimental mixtures all exhibited maximum light absorption at the same wave length (410 m μ) as authentic protoporphyrin and its methyl ester.

Representative results are given in Table I. All effects considered below as positive were greater at twice the concentration indicated in the Table. With all substances the percentage decrease in iron uptake by the cells was practically the same as the decrease in heme formation.

Lead. Incorporation of added glycine into heme in chicken (5) and duck erythrocytes (3), incorporation of acetate into heme in duck erythrocytes (8), and free porphyrin formation in chicken (5) and duck (8) erythrocytes are depressed by divalent lead. At a given level the metal affects free porphyrin more than heme formation (5,8). All stages of heme formation by broken cells are inhibited, but early stages

appear most sensitive (6,8).

In the present tests a level of lead that depressed free porphyrin formation almost completely had essentially no effect on heme production (Test 1a). A higher level depressed heme formation significantly (Test 1b). At comparably concentrations divalent mercury, cobalt, and manganese had no effect on porphyrin or heme formation.

Benzimidazole and derivatives. The 2,5- and 5,6-dimethyl and the 2-ethyl-5-methyl derivatives of benzimidazole inhibit incorporation of added glycine into the heme of chicken erythrocytes. At comparable concentrations benzimidazole and mono-methyl derivatives have a lesser effect (4). Free porphyrin formation in the presence of added glycine is depressed by benzimidazole but not by the 2-ethyl-5-methyl derivative (9).

In the present tests the 5,6-dimethyl compound (Test 4) depressed heme formation but had no effect on free porphyrin formation at the concentration indicated or at twice that concentration. Benzimidazole (Test 2) and the 2,5-dimethyl derivative (Test 3) had lesser effects on heme formation but depressed free porphyrin production appreciably.

Purines and ribosides. Adenosine and inosine depress incorporation of added glycine into the heme of intact duck erythrocytes (3). Inosine, however, and a number of nucleotides increase porphyrin synthesis by broken-cell preparations of chicken erythrocytes (9).

Heme formation in the duck erythrocytes was depressed by adenosine (Test 6) and adenine (Test 5). The purine depressed free

porphyrin formation in the presence and absence of added glycine. The riboside had no obvious effect in the presence of added amino acid, but increased free porphyrin formation slightly in its absence. Xanthine, xanthosine, and adenosine triphosphate at comparable levels ($1 - 4 \times 10^{-3}$ M) produced no effects.

Chloromycetin. The antibiotic has no effect on free porphyrin formation in chicken erythrocytes (9). However, it depressed both heme and free porphyrin production in the duck erythrocytes (Test 7).

Pyridoxine and derivatives. Pyridoxal-5-phosphate and pyridoxamine-5-phosphate, but not pyridoxal, pyridoxamine, or pyridoxine, increase incorporation of added glycine into the heme of intact erythrocytes from pyridoxine-deficient ducklings (1). The effect of the phosphates is considered to derive from increased porphyrin synthesis and to reflect requirement for pyridoxal phosphate in the synthesis of δ -aminolevulinate (1,9). Pyridoxal phosphate, but not pyridoxamine phosphate, depresses incorporation of added glycine into the heme of cells from normal ducklings (1). Deoxypyridoxine, a pyridoxine antagonist, depresses iron uptake by duck erythrocytes; the effect on iron uptake is not reversed by pyridoxine (2). The antagonist also depresses formation of free porphyrin in chicken erythrocytes supplied with added glycine. Pyridoxal phosphate, pyridoxal, and pyridoxine reverse the depression. At certain levels, pyridoxal and pyridoxine, but not pyridoxamine, depress free porphyrin formation (9).

In the present tests pyridoxine and all derivatives examined depressed heme formation in the presence and absence of added glycine

(Tests 8-13). In keeping with earlier findings (1,2,9), deoxypyridoxine (Test 12) had the greatest and pyridoxamine phosphate (Test 13) the least effect. All of the compounds, except pyridoxamine phosphate and deoxypyridoxine, increased free porphyrin formation in the absence of added glycine (cf. Tests 8-11 with 12-13). With addition of the amino acid, free porphyrin production was increased by pyridoxamine phosphate (Test 13), pyridoxal phosphate (Test 11), and pyridoxal in sufficient concentration (Test 10b), but was depressed at lower concentrations of pyridoxal and by pyridoxine and other derivatives (Tests 8,9,12). Deoxypyridoxine depressed free porphyrin formation in the presence and absence of added glycine (Test 12). Pyridoxamine phosphate in the presence of added glycine increased the formation of total, i.e. heme plus free, porphyrin synthesis somewhat (Test 13, glycine added).

Physostigmine. The drug depressed heme and increased free porphyrin production in the presence and absence of added glycine (Test 14) and under certain conditions increased the formation of total porphyrin (Test 14b). The effects of physostigmine probably are not related to its pharmacological activity since neostigmine (prostigmine) at comparable concentration produced no change in heme or porphyrin formation.

DISCUSSION

Interpretation of the effects of the substances tested on the formation of heme and porphyrin in the intact erythrocyte depends upon

the relationship between these processes. The relationship is not completely established; however, simplicity and some evidence (10) suggest as hypothesis that porphyrin and heme synthesis proceed at separately determined rates and, consequently, that free porphyrin formation passively reflects the difference between these rates. The hypothesis interprets the depression of the production of total, i.e. free plus heme, porphyrin by all of the compounds under certain conditions as inhibition of some phase of porphyrin synthesis. Inhibition of porphyrin synthesis alone is sufficient to account for the depression of heme formation by divalent lead. This is indicated by the essentially complete suppression of free porphyrin formation and lack of obvious effect on heme production at one level of lead (Test 1a) and depression of the latter process at a higher level (Test 1b). Results obtained with other cell systems also point to inhibition of porphyrin synthesis as sufficient explanation of the depression of heme formation by the metal (5,8). In contrast to lead, all of the compounds under certain conditions depressed heme and total porphyrin production concurrently which, according to the hypothesis, implies inhibition of some phase of heme formation as well as porphyrin synthesis. Support for the implication that the compounds depress heme formation by relatively direct action rather than through inhibition of porphyrin synthesis is afforded by the experiments with added glycine (Tests 2-14). In these experiments, free porphyrin formation, although depressed by some compounds (Tests 2, 3, 5-10, 12), was in all cases appreciably greater than the decrease in heme formation; i.e. porphyrin was available but was not converted into

heme. The various effects on free porphyrin formation are accounted for by the relative degrees of inhibition of the processes. No change in free porphyrin, as obtained with 5,6-dimethylbenzimidazole (Test 4), signifies equal effects on porphyrin and heme formation. Increase in free porphyrin, as produced by pyridoxal phosphate (Test 11), reflects a greater effect on heme formation than on porphyrin synthesis. Decrease in free porphyrin, as found for deoxypyridoxine (Test 12a), corresponds to a greater effect on porphyrin synthesis. As expected for a substance with more effect on porphyrin than heme synthesis, deoxypyridoxine at a sufficient level stopped free porphyrin formation but still allowed some heme formation (Test 12b). The effect of some compounds on free porphyrin depended upon the presence of added glycine, e.g. pyridoxine increased the formation of free porphyrin in the absence but depressed the formation in the presence of the added amino acid (Test 8). Interpretation of the effect of the added glycine is not obvious.

Total porphyrin formation in the presence of added glycine was increased by pyridoxamine phosphate (Test 13) and by physostigmine when heme formation was relatively low (Test 14b). Consequently, these substances under the conditions indicated stimulate porphyrin synthesis. However, under the other conditions used - pyridoxamine phosphate in the absence^{of} added glycine (Test 13) and physostigmine in the absence of added amino acid and in the presence of the acid at the usual level of heme formation (Test 14a) - the compounds according to the hypothesis depressed porphyrin synthesis.

With a few exceptions the substances tested do not effect heme or free porphyrin formation strikingly. Thus, unless further study indicates otherwise, the main significance of the effects produced may be their bearing on hypothesis relating cellular control of the synthesis of porphyrin to its conversion into heme. The reasonably straight-forward explanations of the effects of most of the substances allowed by the hypothesis considered above provides it appreciable support. The proposal that pyridoxamine phosphate and physostigmine, depending upon experimental conditions, inhibit and stimulate porphyrin synthesis does tend to question the hypothesis. However, other obvious hypotheses propose similar dual effects for more compounds and appear, therefore, less tenable than the first.

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ABSTRACT

The effects of a number of substances that depress protoheme formation and affect free protoporphyrin production in duck erythrocytes in vitro were measured. The findings indicate that inhibition of some phase of porphyrin synthesis accounts for depression of heme formation by divalent lead and that inhibition of some phase of the conversion of porphyrin into heme accounts for depression of heme formation by pyridoxine and certain derivatives, adenosine, adenine, chloromycetin, benzimidazole and certain derivatives, and physostigmine. All of the compounds also inhibit porphyrin synthesis, but under certain conditions pyridoxamine phosphate and physostigmine stimulate porphyrin synthesis.

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TABLE I

Effect of Inhibitors of Heme Formation on Free Porphyrin Production
in Intact Duck Erythrocytes

Heparinized blood plus sufficient Fe^{59} to give about 10^5 counts per minute per ml of mixture was shaken 4 hours at 37° under 5% carbon dioxide in oxygen in the presence or absence of added glycine ($9 \times 10^{-3} \text{M}$ final concentration) and in the case of each mixture with and without the test substance which was added as the solid. Heme formation was estimated from the labeled and total iron in plasma before the incubation and the labeled iron in heme after the incubation. The total uptake of plasma iron by the erythrocytes was also measured. Free porphyrin formation was taken as the difference between the level of protoporphyrin in blood before and after the incubation. The results for the tests with and without added glycine given below derive from different blood samples. The sample of blood used in Test 17b was taken from an animal X-irradiated several days earlier. The results given are observed values adjusted to expectation for the average level of hemoglobin in blood, 7.1 $\mu\text{moles per ml}$, and are expressed as millimicromoles per 100 ml of blood. The percent change in iron uptake by the cells was in all cases practically the same as the change in heme formation.

Test	Substance	M	Heme formation	Free porphyrin formation	Added glycine	
					Heme formation	Free porphyrin formation
1a.	Lead ⁺⁺	0	272	18	494	137
		2.0×10^{-5}	265	1	500	5
1b.		0	506	26	490	152
		4.0×10^{-5}	475	0	432	0
2.	Benzimidazole	0	348	20	625	285
		2.4×10^{-3}	322	14	600	231
3.	2,5-Dimethylbenzimidazole	0	509	25	323	243
		2.8×10^{-3}	482	21	290	222
4.	5,6-Dimethylbenzimidazole	0	385	18	463	227
		2.8×10^{-3}	321	19	415	222
5.	Adenine	0	740	25	158	101
		2.0×10^{-3}	650	13	144	44
6.	Adenosine	0	550	21	435	166
		2.1×10^{-3}	367	29	298	162
7.	Chloromycetin	0	390	13	255	141
		1.7×10^{-3}	325	8	235	88

TABLE I (Cont'd)

Test	Substance	M	Heme formation	Free porphyrin formation	Heme formation	Free porphyrin formation
					Added glycine	
8.	Pyridoxine	0	205	5	285	277
		2.6×10^{-3}	67	12	124	175
9.	Pyridoxamine	0	662	11	350	145
		2.3×10^{-3}	480	55	210	84
10a.	Pyridoxal	0	485	7		
		2.8×10^{-3}	265	41		
10b.		0			355	164
		1.2×10^{-3}			315	97
		2.8×10^{-3}			245	156
		4.9×10^{-3}			148	283
11.	Pyridoxal phosphate	0	405	15	472	53
		2.2×10^{-3}	182	30	285	116
12a.	Deoxypyridoxine	0	310	17	382	201
		3.0×10^{-5}	106	6	173	84
12b.		0	405	16	481	225
		3.0×10^{-4}	30	0	40	0
13.	Pyridoxamine phosphate	0	272	18	250	132
		2.1×10^{-3}	253	14	240	172
14a.	Physostigmine	0	429	8	338	219
		2.0×10^{-3}	219	65	168	292
14b.		0			62	84
		2.1×10^{-3}			21	150