Formation of Porphyrin Isomers from Porphobilinogen by Various Hemolysates of Red Cells from Bovine and Human Subjects with Erythropoietic (Uro-) Porphyrin¹⁾

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(Eingegangen am 6. Januar 1969)

Dedicated to Prof. Dr. Joachim Brugsch on the occasion of his 60th birthday

Hemolysates of erythrocytes from bovine and human subjects having erythropoietic porphyria, and from normal subjects, have been incubated with PBG²⁾. Cells obtained by differential centrifugation or osmotic lysis as well as total cells were hemolyzed. The proportion utilized or unaccounted for after incubation was generally much greater with the porphyric than the normal hemolysates, with the lesser than the greater density fractions, and with the hemolysates of the osmotically more fragile cells. Larger amounts of PBG in relation to cells, less protection against ambient temperature and longer incubation were associated with greater formation of type I porphyrins by normal as well as porphyric hemolysates. With more nearly optimal conditions, only or mainly type III was in evidence in the normal while the porphyric hemolysates formed both I and III. Porphyric hemolysates regularly formed larger amounts of protoporphyrin 9 (III) than the normal. After repeated bleeding of bovine porphyrics, the hemolysates formed larger amounts of porphyrin and significantly greater proportions of type I isomer than before bleeding. This is interpreted as an enhanced deaminase-isomerase imbalance in young cells. Under the same conditions the normal bovine hemolysates formed mainly type III. These observations are considered in respect to the question whether the normoblasts are uni- or bimodal in terms of the genetic abnormality, i. e., the deaminase-isomerase imbalance. The present evidence is in better accord with a unimodal distribution.

Erythrocytenhämolysate gesunder und an Porphyria erythropoetica erkrankter Menschen und Rinder wurden mit PBG²⁾ inkubiert. Zur Hämolysen wurden die Zellen in toto oder nach Differentialzentrifugation bzw. Osmolyse verwandt. Ein im allgemeinen viel stärkerer PBG-Schwund wurde, verglichen mit Normal-Hämolysaten, in Porphyrie-Hämolysaten gefunden, ebenso in denen von leichteren im Vergleich mit schwereren und in denen von osmotisch fragileren Zellen. Wurde im Verhältnis zu den Zellen mehr PBG zugesetzt oder weniger gegen die Umgebungstemperatur geschützt und länger inkubiert, so wurden sowohl von normalen als auch von Porphyrie-Hämolysaten mehr Typ-I Porphyrine gebildet. Wurden dagegen annähernd optimale Bedingungen eingehalten, so wurde von Normal-Hämolysaten nur oder überwiegend Typ III gebildet, während Porphyrie-Hämolysate sowohl Typ I als auch Typ III bildeten. In Porphyrie-Hämolysaten entstanden im allgemeinen größere Mengen Protoporphyrin 9 (III) als in Normal-Hämolysaten. Nach wiederholten Aderlässen bildeten die Hämolysate porphyrikranker Rinder größere Mengen Porphyrin und bedeutend größere Anteile des Typ-I-Isomeren als vor den Blutverlusten. Dies wird durch ein gestörtes Desaminase-Isomerase-Gleichgewicht hauptsächlich jugendlicher Zellen erklärt. Unter gleichen Bedingungen bildeten normale Rinder-Hämolysate hauptsächlich Typ III. Diese Beobachtungen werden in Hinblick auf die Frage diskutiert, ob Normoblasten eine oder zwei genetische Abweichungen in bezug auf das Desaminase-Isomerase-Gleichgewicht aufweisen. Die hier vorgetragenen Ergebnisse stimmen besser damit überein, daß lediglich eine einzige entsprechende Störung vorliegt.

In earlier studies (1, 2, 3) the delineation of erythropoietic porphyria, both human and bovine, depended in part on the presence of large numbers of intensely fluorescing normoblasts in the bone marrow. Since

other normoblasts, morphologically similar but not fluorescing, comprised approximately half of the total, the possibility was considered that two cell types were represented, functionally distinct, one abnormal and fluorescing due to excessive production of type I uro- and coproporphyrins (UP and CP), the other normal, and non-fluorescing as a result of the low concentration of free porphyrin (3).

As suggested elsewhere (4) the fundamental abnormality in this disease may be a regulator gene disturbance with overproduction of porphyrins of both series I and III, the type I porphyrins in relatively great excess as contrasted with the normal state. Recent histologic studies by others (5, 6), both by light and electron microscopy, have favored the existence of two separate varieties of normoblasts in this disease, "porphyroblasts" (6), vs. normal nucleated red cells but the question remains whether the differences observed are acquired, related

¹⁾ Aided by grants from the U. S. Public Health Service, and the Margaret H. and James E. Kelley Foundation.

²⁾ Definitions and Abbreviations: PBG = porphobilinogen; ALA = δ -aminolevulinic acid; UP = uroporphyrin when used in a purely chemical or non-enzymatic context, or uroporphyrinogen in relation to enzymatic formation or conversions. I or III = corresponding isomer types; CP = coproporphyrin, or coproporphyrinogen, as above. I or III = corresponding isomer types; UP $\rightarrow$ CP = decarboxylation of UP to permit determination of isomer ratio; UP I + CP I = sum of UP I and CP I calculated as UP I; Proto = protoporphyrin 9 (series III); Meso = mesoporphyrin 9 (series III); l. d. c. = lesser density cells obtained by fractional centrifugation; g. d. c. = greater density cells obtained by fractional centrifugation; t. c. = total cells C. B. = Cornford-Benson method for chromatographic separation of UP I and III (31); P = porphyric; N = normal; Ht = hematocrit.

to injury, or to temporal functional changes in some of the normoblasts, and whether all are unimodal with respect to the genetic abnormality or whether this involves but one cell type of a bimodal distribution. RIMINGTON (7) observed that on incubation with PBG, a normal bovine erythrocyte hemolysate formed only type III UP, whereas an hemolysate from the red cells of a bull calf homozygous for the trait of erythropoietic porphyria, under the same conditions, formed about equal amounts of types I and III. This finding was compatible with the concept of a bimodal but did not exclude a unimodal distribution.

It was shown in earlier studies (8) that in both the human and bovine disease the osmotically more fragile cells are UP rich, while the more resistant cells contained Proto- but relatively little UP³⁾ or CP³⁾. Reference was made in an earlier paper (4) to the finding that the UP rich cells are lighter and can be concentrated like the reticulocytes, by centrifugation. This method has been used extensively in the experiments to be described.

In a series of unpublished experiments (9) transfusion of whole blood from bovine porphyrics into normal animals was followed by rapid disappearance of the (transfused) erythrocyte UP in the recipient, while the Proto- level³⁾ returned to the pre-transfusion concentration much more slowly (see below). This might also be explained on the basis of bimodal erythrocytes, one from each of the supposed normoblast colonies earlier postulated. In this respect the bimodal concept would embrace the possibilities that the presumably less mature UP rich cells have a short life span or lose their UP to the circulation, without undergoing destruction. It was also conceivable that the normoblasts and erythrocytes are unimodal in respect to the genetic error but that the UP rich cells simply represent "stress" forms, immature and of short life span (10, 11). According to this concept, all of the normoblasts participate in the genetic error but porphyrin production and consequent fluorescence of normoblasts is more dynamic and variable than previously assumed; in other words, at any given time certain of the normoblasts might be in a less, others in a more active state in terms of excessive porphyrin production. If this were true, it would be anticipated that the greater the stimulus to hemoglobin synthesis, the more manifest the enzymatic abnormality and accumulation of UP I and CP I. This, in effect, suggested that an additional stimulus to erythropoiesis might result in a much higher proportion of fluorescing normoblasts than that observed in the animal's basic state. It was postulated in earlier papers (2, 8) that the marked reduction of erythrocyte, urinary and fecal porphyrin concentrations after splenectomy in human erythropoietic porphyria was due to removal of the stimulus to erythropoiesis provided by excessive hemolysis. WASS and SCHWARTZ (12) noted that bleeding

of porphyric animals was shortly followed by marked increases of UP and CP in the circulating red cells. In one animal, for example, the withdrawal of three liters of blood was followed within four days by an increase of the erythrocyte UP from 2.9 $\mu\text{g}/100\text{ ml}$ to 142 $\mu\text{g}/100\text{ ml}$. In a separate communication with W. RUNGE (13) a study is described in which porphyric and normal animals were bled sufficiently to stimulate erythropoiesis, the percentage of fluorescing normoblasts in the bone marrow being determined before and after bleeding. The uniform increase to from 80–90% appears more in accord with the unimodal concept, at least insofar as the bovine disease is concerned.

If the bimodal concept were correct it would be logical to anticipate that the presumably short-lived uroporphyrin rich cells would regularly provide a hemolysate representative of the genetic abnormality, i. e., preponderance of type I porphyrin formation, while hemolysates prepared from the osmotically less fragile cells of greater density might be expected to convert PBG to isomer III. At the same time it is recognized that even with a unimodal distribution some difference in this respect might well be due simply to the relative maturity of the cells represented in these fractions but it is unlikely that the difference would be as great or as consistent as would be anticipated with a bimodal distribution. Experiments designed to study this question have now been carried out and are described in the following.

Material and Methods

Eleven experiments have been completed with hemolysates from bovine (exps. 1–11, incl.) and two from human porphyric blood (exps. 12, 13) with appropriate controls. Tables 1–13 are numbered accordingly.

The porphyric animals studied were homozygous for the bovine erythropoietic porphyria trait, members of a family of purebred Holstein cattle (14). The control animals, with one exception, No. 2121 (see below) had no known relationship to the porphyrics, and their erythrocyte porphyrin values were normal as contrasted with the marked or significant elevations in the porphyric animals, as will be noted in the Tables (see Results).

Hemolysates for incubation with PBG were prepared by lysis with distilled water or hypotonic saline solutions, using total red cells or fractions obtained either by differential centrifugation or differential osmotic lysis. In all experiments the red cells were washed twice with cold physiological saline prior to hemolysis. In expts. 1 and 2, the phosphate buffer — KCl mixture (0.15M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ pH 7.4, containing 0.483M KCl) was prepared according to the earlier method of DRESEL and FALK (15). In all other experiments, bovine and human, the later method of DRESEL and FALK (16) was used, consisting of dilution of the hemolysate to isotonicity by adding 0.604M KCl. The quantitative relationships were in the ratio of 1.0 red cells: 2.0 H_2O : 0.5 of 0.604M KCl and 2.2 of 0.122M KCl. In run 4, exp. 9, the Tris buffer mixture, as employed by VAVRA and co-workers (17) was compared with the KCl diluent. The Tris buffer mixture was composed as follows: 1 ml/ of 2.1% NaCl, 5 ml/ 0.1M Tris buffer, 1.3 ml/ 0.604M KCl, 1.0 ml/ 0.122M KCl and 1.8 ml/ 0.85% NaCl.

In separating erythrocytes on the basis of density, the cells were packed at 2000 RPM for 30 minutes at 4° after which the upper and lower quarters were removed separately, each being resuspended and recentrifuged, the upper and lower quarters again being obtained. The PBG in the amounts noted in the Tables was dissolved in the phosphate buffer — KCl mixture (exps. 1 and 2) or the

³⁾ These abbreviations include both porphyrins and chromogens, it being recognized that biogenesis proceeds only over the porphyrinogens. In the isolations and analyses to be described, however, the latter are included with the porphyrins.

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isotonic KCl solution (exps. 3–13). The PBG used was crystallized from the urine of patients having acute porphyria in relapse or remission, by the method of COOKSON and RIMINGTON (18). The sample of PBG was weighed in exps. 1 and 2, in the others being determined before and after incubation, after precipitation of protein with trichloroacetic acid and application of the Ehrlich reaction, as previously described (19). The solutions, in flasks plugged loosely with cotton, were incubated at 37° for periods ranging from 2–8 hours at a rate of 90–100 shakes/minute. Control runs were made with packed cells from normal bovine or human blood. The control used in exps. 5 and 7 was from animal No. 2121, a heterozygote, on the basis of the breeding record. It has not been possible thus far to detect the heterozygous condition on the basis of porphyrin analyses, whether of blood, bone marrow, or urine. Controls were run without added PBG, or without hemolysate. The bovine reticulocytes were counted by the method of TODD and coworkers (20). As will be noted, the counts in the normal bovines were usually 0, in accord with previous reports (21). At times there was an increase of cells staining diffusely blue, without substantial reticulofilamentosa, but believed to represent equivalent forms (see Table 9, below).

In experiment 1, differential osmotic lysis was carried out at 4° with 120 ml of packed red cells from 580 ml of blood of porphyric cow No. 1589. The total cells contained 3.5% reticulocytes. Three hemolysates, A, B and C, were prepared from the entire sample hemolyzed in sequence for $\frac{3}{4}$ h each at 0.6, 0.52% NaCl and distilled water. Amounts of hemolysate, in terms of equal hemoglobin content (0.29 gms Hb in each) were adjusted with 10% saline to a final concentration of 0.6% after which 10 ml of the phosphate buffer — KCl mixture (see above) containing 1 mg of PBG was added to each and to the control, the latter composed of the saline-buffer-PBG mixture without hemolysate.

Exps. 2–6, incl., were carried out with blood from the bovine porphyric No. 2120. Exps. 2, 3, 6, 8, 9, 10 and 12 included observations on hemolysates obtained after differential centrifugation of red cells, while in exps. 4, 5, 7, 11 and 13, only total erythrocytes were hemolyzed. In exp. 5 the possible effect of GSH and AMP on the amount and type of free porphyrins formed from PBG, was examined. In each of six runs 10 ml of packed cells were used, laked with 20 ml distilled water. GSH was added to give a final concentration of 0.01M in runs 3 and 4, and AMP to a final concentration of 0.003M in runs 5 and 6. In each instance these substances were dissolved in 5 ml of 0.604M KCl. To this was added 22 ml of 0.122M KCl with the dissolved PBG.

Although the amounts of UP, CP and Proto- were determined before and after incubation in all experiments, no data were obtained as to amounts of heme which might have been formed. In exp. 7 (Tab. 7) the effect of variations in K and Na concentration was studied in relation to porphyrin formation and isomer ratio. This was done in view of the variable but relatively high content of NaCl in bovine erythrocytes (22). Ten ml packed cells of porphyric (2955) and heterozygous (2121) bovines were used in each run. One run from each animal was made with the KCl diluent (see above) and one with an isotonic concentration of NaCl and KCl (2:1).

In the earlier experiments, Nos. 1–5, incl., the initial transport and handling of blood and hemolysates was at ambient temperature except that the fractional centrifugations were carried out in a refrigerated centrifuge at 4°. In these the interval was considerably longer than in the later bovine and human experiments as the bleeding of the bovines was done at the Veterinary Farm about 20 miles from our laboratory where the samples were subjected to the experiments to be described. This entailed a variable period of exposure to ambient temperature. In exps. 6–11, incl. the samples were refrigerated in transit except in certain runs to be noted, in which the exposure at ambient temperature was purposely allowed in order to permit comparisons in respect to this factor. In exps. 12 and 13 (human) the exposure to ambient temperature was only brief as the blood was drawn in our laboratory and at once processed.

Exp. 11 was designed to permit comparison of different periods of incubation, in respect to porphyrin formation by normal vs

porphyric bovine hemolysates. The blood and hemolysates were kept cold from collection to incubation. Normal, without added PBG: 45 ml of packed normal red cells (Hamilton No. 1 cow) were hemolyzed and diluted according to the above ratio. The hemolysate was divided in three equal parts, the first being frozen immediately, the second being incubated for four hours and the third for eight hours. Normal, with PBG: 60 ml packed cells were treated similarly. This hemolysate was divided in four parts which were incubated for 2, 4, 6 and 8 hours, respectively. The porphyric red cells from cow No. 2955 were studied in the same way.

Exps. 12 and 13 were carried out with human porphyric blood obtained, respectively, from cases # 71, (D. H., ♀ 24), # 76 (M. H. ♀ age 15) and # 265 (T. H. ♀ age 7). The case numbers relate to our general porphyria roster. Case 76, first described in 1954 (2), has shown the characteristic photosensitivity, hydroa and epidermolysis, hirsutism, reddish brown teeth and red urine. Case 265 is the younger sister of our first case of erythropoietic porphyria, D. H., #71 ♀ who is now 24 and in remarkably good health, having been splenectomized at age 4 (23). T. H. first developed evidence of photosensitivity and red urine at age 2. The father of these girls is dead, the mother living and well. There is no history of consanguinity nor of photosensitivity in the parents or other members of the family. There are three older siblings, all of whom have been examined with respect to physical or biochemical abnormality (urine, fecal and erythrocyte porphyrins). No evidence of porphyria has been found.

Fractional determination of UP, CP and Proto- of red cells or hemolysates from all experiments was carried out by the methods previously used in this laboratory (24, 25). The native free porphyrins and the CP obtained by decarboxylation of UP, were subjected to preliminary purification by Al_2O_3 chromatography, after which the respective methyl esters were chromatographed on either $CaCO_3$ or MgO (26). In certain of the bovine samples the UP fraction was difficult to separate from an unidentified green compound and in these repeated chromatography was necessary. Decarboxylation of UP (27) and quantitative paper chromatography of the CP isomers is indicated in the Tables as UP → CP. The Eriksen method (28) was used and in addition the Turner "door" fluorometer⁴⁾ was employed to determine the proportion of CP isomers (29, 30), native or after decarboxylation of UP. In the later experiments of the present series (Nos. 6, 8, 9, 10, 11, 13) the UP isomer composition was determined by the Cornford-Benson technique (31) not yet described when the earlier experiments were carried out. According to the authors, their method "detects and estimates ratios of the isomers ranging between 17:83 and 100:0 (I:III) with 7% accuracy". In exps. 6 and 8, both this method and decarboxylation were employed. In several experiments (Nos. 5–8, incl., and 10) the Proto- of the hemolysate, after incubation and separation from CP and UP, was converted to Meso- by catalytic hydrogenation with palladium, followed by paper chromatography. The essential features of this method have been described in an earlier report (4).

In general no attempt has been made to render an accurate account on a molar basis of the net differences of both PBG and total porphyrin, before and after incubation. Since the important relationship is that between PBG and UP this calculation first entails conversion of the amounts of CP and Proto- on the basis of their molecular weights to the equivalent amount of UP (Proto- $\times 1.4$ or CP $\times 1.3$ = UP equivalent). This may then be expressed in μM net increase of total porphyrin, as in the sample calculations for the porphyric and normal subjects in Tables 12 and 13. To permit comparison of the net increase of CP I and UP I in relation to isomer III, as in Tables 6, 8, 9 and 10, the amount of each is calculated on the basis of the corresponding % I in the columns on the right; the amount of CP I is then calculated and converted to UP I by the factor 1.3.

Whenever it has appeared helpful in relating procedure to results, additional details of the individual experiments are included in the Tables, under Results.

⁴⁾ G. K. Turner Associates, Palo Alto, California.

Results

In the following description of results, special attention will be given to the utilization or disappearance of PBG, the quantities of porphyrins and proportion of UP and CP isomers I and III, and the amount and identity of the Proto-, formed in the various hemolysates after incubation with PBG, as described in the foregoing.

The essential data, including certain items of protocol related to the individual experiments, are presented in Tables 1—13, incl.

The hemolysates of the more fragile porphyric red cells obtained by fractional osmotic lysis utilized more PBG and formed larger amounts of total and individual porphyrins (Tab. 1).

Tab. 1

Exp. 1 (9-23-59). Four hour incubation of PBG (1 mg in each run) in hemolysates obtained by differential osmotic lysis of red cells from porphyric animal No. 1589 (retic. 3.5 %)

Run	Fractional hemolysis	μg porphyrins (Pre-incubation values in parentheses)			UP % I (UP $\rightarrow$ CP)
		Proto-	CP	UP	
1	Hemolysate A (hemol. at 0.6 % NaCl)	18.0 (6.7)	34.5 (1.5)	92.0 (21.8)	70 %
2	Hemolysate B (remainder at 0.52 % NaCl)	9.6 (6.4)	10.7 (0.6)	54.0 (5.6)	*
3	Hemolysate C (remainder in dist. H ₂ O)	6.2 (5.0)	3.9 (0.4)	19.0 (1.6)	70 %
4	Control (PBG only)	0	0	0.09	

*) Sample destroyed during decarboxylation

Utilization or disappearance of PBG

It is evident in Tables 2—12, incl., that there was greater utilization of PBG and formation of porphyrin, by

porphyric as compared with heterozygous or normal hemolysates. In the case of the hemolysate from the heterozygous bovine # 2121 (Tab. 7) the differences were not great and the amount of UP observed after incubation was actually greater, although interestingly enough, the Proto- was significantly greater in the homozygous hemolysate. In Table 13 no significant difference is evident in the total porphyrins as between porphyric, probable heterozygous and normal hemolysates.

The same type of differences is often observed with the lesser as contrasted with greater density cells (l. d. c. and g. d. c., respectively), (Tab. 2, 3, 8, 10). In exp. 6 (Tab. 6) opposite differences of minor degree are seen but here the separation of reticulocytes was of questionable significance. In exp. 9 (Tab. 9), the normal bovine cell hemolysates in the comparable runs 3 and 6, before and after bleeding, respectively, produced similar amounts of porphyrin despite a significantly greater disappearance of the added PBG in run 6. It is clear that disappearance of PBG is not necessarily related to porphyrin formation although in the porphyric hemolysates generally more than 50% was thus accounted for. The extent to which the fraction unaccounted for relates to heme formation or conversion of PBG to porphobilin has not been determined. (The question of pH effect is discussed below). Studies⁵⁾ with δ -amino-levulinic acid-[4-¹⁴C] (ALA-[4-¹⁴C]) in normal and porphyric hemolysates, carefully protected against heat

⁵⁾ With S. SCHWARTZ and I. BOSSENMAIER, unpublished.

Tab. 2

Exp. 2 (12-2-59). Eight hour incubation of PBG in hemolysates obtained by differential centrifugation of bovine porphyric red cells

Run	Source and type of cells	Reticulo- cytes %	ml packed cells	ml dist. H ₂ O	ml buffer	mg. PBG added	μg porphyrins (pre-incubation values in parentheses)			UP % I (UP $\rightarrow$ CP)	Net increase UP I μg
							Proto-	CP	UP		
1	Porphyric # 2120, l. d. c.	82.4	1.0	15	5	0.4	51 (3)	36 (24)	198 (74)	76	94
2	Porphyric # 2120, g. d. c.	8.2	1.0	15	5	0.4	22 (6)	14 (.4)	138 (3.2)	60	81
3	Normal # 1913, t. c.	< 0.5	5.0	75	25	1.0	9*	8*	39*	—	—
4	Control, no cells	—	0	75	25	1.0	0.2	0.1	18	—	—
5	Control, no cells	—	0	15	5	0.4	0	0.3	21	—	—

* Pre-incubated values not determined

Tab. 3

Exp. 3 (8-24-60). Utilization or disappearance of PBG in hemolysates of greater and lesser density porphyric vs normal erythrocytes. Samples of packed cells from bovine porphyric (P) # 2120, and bovine normal (N) # 1756 hemolyzed and incubated for eight hours

Run	Description	Reticulocytes %	ml packed cells	Added	mg PBG Remaining	μg porphyrins (pre-incubation values in parentheses)		
						Proto-	CP	UP
1	N., t. c.	0	4.0	2.91	2.09	19 (2.0)	38 (0)	114 (0)
2	N., l. d. c.	0	2.0	1.95	1.32	17 (1.0)	56 (0)	100 (0)
3	N., g. d. c.	0	2.0	1.93	1.37	12 (1.5)	18 (0)	88 (0)
4	P., t. c.	3.3	4.0	2.90	0.38	139 (7.0)	419 (2.7)	723 (21)
5	P., l. d. c.	5.5	2.0	1.92	0.30	115 (4.5)	134 (6.0)	640 (28)
6	P., g. d. c.	1.9	2.0	1.97	0.84	65 (6.0)	73 (0.9)	257 (9.0)

Tab. 4

Exp. 4 (10-4-61). Utilization or disappearance of PBG, amounts and types of porphyrin isomers formed with normal vs porphyric bovine hemolysates of total cells, eight hour incubation

Run	Source of cells	ml packed cells used	Added	mg PBG Remaining	μg porphyrins (pre-incubation values in parentheses)			CP % type I	UP (UP $\rightarrow$ CP)
					Proto-	CP	UP		
1	Normal # 1756	9.0	2.77	1.58	21 (6)	26 (0)	162 (0)	45	80
2	Porphyric # 2120	9.4	3.07	0.30	136 (29)	214 (7)	702 (33)	85	100
3	Control — no cells	0	1.88	1.09	0.2	0.2	35	—	65

Tab. 5

Exp. 5 (12-20-62). Utilization or disappearance of PBG on eight hour incubation of hemolysates of red cells from porphyric # 2120 (retic. 12%) vs heterozygous # 2121 (retic. 0); amounts and types of porphyrin isomers formed; effect of GSH and AMP (see Methods); 10 ml packed cells in each hemolysate

Run		mg PBG		μg porphyrins (pre-incubation values in parentheses)			Porphyrin isomers, % I	
		Added	Remaining	Proto-	CP	UP	CP	UP $\rightarrow$ CP
PBG only								
1	# 2120	4.39	0.25	365* (35)	257 (7)	1008 (52)	81	100
2	# 2121	2.5	1.2	42* (6)	19 (0)	280 (0)	95	83
	Control, no cells	1.01	0.79	0	0	3.0	—	—
PBG and .01M GSH								
3	# 2120	4.4	**	97	72	43	97	98
4	# 2121	2.4	**	6	11	14	95	—
PBG and .003M AMP								
5	# 2120	4.5	0.79	238*	338	626	91	95
6	# 2121	2.4	1.58	64*	21	96	95	93

* Proto- to Meso-, all type 9 (III)

** PBG determination prevented due to rapid fading of color caused by GSH

Tab. 6

Exp. 6 (6-25-63). Effect of temperature variations prior to incubation, on utilization or disappearance of PBG and on porphyrin formation by porphyric (# 2120) and normal (# 2126) bovine hemolysates (15 ml packed cells in each run, hemolyzed as described under Methods, and incubated three and one-half hours)

Run	Animal	Type	Cells hemol.	Reticu- lyocytes %	Preincu- bation temp. °C	mg PBG Added to he- moly- sate	mg PBG Remain- ing after incuba- tion	μg porphyrins (preincu- bation values in parentheses)			Net increase μg UP I + CP I	CP % I or III	Isomer data	
								Proto-	CP	UP			UP % I C. B.	UP $\rightarrow$ CP or III
1	2120	P	t. c.	23.5	4	0		42 (50)	31 (28)	104 (129)	-21	100 I	100 I	100 I
2	2126	N	t. c.	0	4	0		6.6 (8.4)	0.3 (0.1)	0 (0)	0	—	—	—
3	2120	P	t. c.	23.5	4	0.77	0.10	175 (50)	91 (28)	172 (129)	125	100 I	100 I	95 I
4	2126	N	t. c.	0	4	0.77	0.25	67 (8.4)	20 (0.1)	53 (0)	0	100 III	100 III	88 III
5	2120	P	t. c.	23.5	A* + 55°, 15'	0.93	0.10	151 (50)	91 (28)	191 (129)	144	100 I	100 I	95 I
6	2126	N	t. c.	0	A* + 55°, 15'	0.93	0.28	14 (8.4)	6.4 (0.1)	90 (0)	98	—	100 I	95 I
7	2120	P	l. d. c.	30.0	4	0.78	0.07	137** (46)	159 (79)	192 (153)	143	100 I	100 I	100 I
8	2120	P	g. d. c.	21.0	4	0.68	0.07	125 (44)	75 (17)	116 (14)	177	100 I	100 I	100 I
9	2120	P	t. c.	23.5	A*	0.79	0.07	82 (50)	60 (28)	156 (129)	69	100 I	100 I	100 I
10	2126	N	t. c.	0	A*	0.80	0.28	36 (8.4)	22 (0.1)	38 (0)	—	—	—	—

* Ambient

** Proto- to Meso-, all type 9 (III)

Tab. 7

Exp. 7 (10-23-63). Effect of Na vs K on utilization or disappearance of PBG and formation of porphyrins by porphyric (# 2955) vs heterozygous (# 2121) bovine hemolysates; incubation three and one-half hours; 10 ml packed cells in each hemolysate

Run	Animal	Conditions	mg PBG Added	mg PBG Remaining	μg porphyrins (pre-incubation values in parentheses)			UP % I (UP $\rightarrow$ CP)
					Proto-	CP	UP	
1	2955	Hemolysate with KCl only	0.42	0.14	131* (85)	27 (.9)	57 (.4)	60
2	2955	Hemolysate with 2:1 NaCl/KCl	0.41	0.14	86 (85)	23 (.9)	52 (.4)	62
3	2121	Hemolysate with KCl only	0.41	0.19	29 (7.5)	15 (.1)	77 (0)	80
4	2121	Hemolysate with 2:1 NaCl/KCl	0.42	0.19	28 (7.5)	25 (.1)	75 (0)	78
5	—	Control, no cells with 2:1 NaCl/KCl	0.51	0.50	0	0	0.8	—

* Proto $\rightarrow$ Meso-, all type 9 (III)

before incubation, indicate that heme formation is small or negligible.

The temperature factor in porphyrin isomer formation

The effect of variation of conditions, especially temperature, in the period between drawing the blood and incubation is highly important as regards isomer distribution. In the present series the effect of exposure to ambient temperatures with or without additional heating at 55°, was first studied in exp. 6, Table 6. Comparison of runs 3 and 4 with 9 and 10 reveal a much smaller total porphyrin formation in the latter in which there was no protection before incubation of the hemolysate. Despite this highly significant difference just as much PBG disappeared in runs 3 and 9 and in runs 4 and 10.

The possibility is not excluded that the difference is accounted for by greater heme formation in runs 9 and 10. In runs 5 and 6 in which there was additional heat exposure at 55° for 15 minutes to destroy isomerase activity (38), the much larger amounts of isomer I formed, together with the entirely comparable disappearance of the added PBG suggest that sufficient isomerase may have been active in runs 9 and 10 to produce some Proto- 9 (III) and heme. This could only be determined with labelled substrate and comparable numbers of reticulocytes among the red cells hemolyzed. The plentiful number in run 9 would undoubtedly have supplied both of the mitochondrial enzymes, coproporphyrinogenase and heme synthetase, but in both runs 4 and 10 the normal bovine red cells had no demonstrable reticulocytes, the only difference being in the

pre-incubation temperature. At the same time it must be noted that in both of these runs a significant amount of new Proto- was formed, at least indicating the presence of some mitochondrial enzyme. Again, this may relate to young cells without the tinctorial properties of reticulocytes (see exp. 9, Tab. 9).

The data in Table 6 reveal an outspoken difference in porphyrin isomer production depending on the pre-incubation temperature. It is seen that only the normal hemolysate kept at 4° before incubation exhibited isomer III formation. In the other runs in which the isomer ratio could be determined, only or mainly type I was observed. The 7-COOH "pseudo-uroporphyrin" was noted only once and then in association with type III UP.

In exps. 6—13, incl. (Tab. 6—13), except exp. 12 (Tab. 12), the pre-incubation temperature of the cells and hemolysate was kept at or near 4°, thus the isomer data are undoubtedly much more reliable than in the earlier experiments 1—5, incl. The data in Table 12 (exp. 12) must be considered individually in this respect as in this instance the (human) blood was drawn in the laboratory and at once processed (see Methods) unlike the bovine bloods in exps. 1—5 which had to be transported a considerable distance during which there was no protection against ambient temperature effect. With the preliminary refrigeration the dominant or at times exclusive formation of isomer III by the normal cell hemolysates is evident and in the same experiments the

porphyrin hemolysates at the end of incubation contained a major proportion at times only CP I and UP I. Nevertheless significant increases of Proto- 9 (III) are also seen, again revealing the presence of isomerase (Tab. 6 as already discussed; Tab. 8, 9, 10).

Electrolyte, pH and other factors

Exp. 7 (Tab. 7) presents comparative data obtained with porphyrin vs heterozygous cell hemolysates with special reference to variation in Na and K concentration. The porphyrin hemolysate in the KCl diluent had a significant increase in Proto- 9 (III) after incubation, otherwise no effect of the variation in diluent was noted. It was unexpected that the porphyrin hemolysate in either diluent contained but 60—62% of UP I while the two heterozygous cell hemolysates contained 78—80% type I. Also, the porphyrin hemolysate in the KCl diluent contained significantly more Proto- (all type 9).

Variations in pH were recorded in exps. 9 and 10 (Tab. 9 and 10). In exp. 9 (normal cell hemolysate) the pH was somewhat lower in the runs after than those before bleeding. The values are reasonably comparable in runs 3 and 6 before and after bleeding, respectively. These are the only runs which may be compared in terms of similar conditions in other respects, as seen in Table 9. The total porphyrin formed was quite similar but the UP before bleeding was considerably greater while in run 6 Proto- was larger in amount. All of the

Tab. 8

Exp. 8 (2-25-64). Utilization or disappearance of PBG and formation of porphyrin isomers after three and one-half hours incubation of hemolysates prepared from bovine porphyrin (No. 2975) and normal (No. 3000) red cells. The porphyrin animal had been bled a total of 4.0 liters in amounts of 500—850 ml on seven occasions between 1-13 and 2-13-64

Run	Animal	Type of cells	ml packed cells	Reticulocytes %	mg PBG		µg porphyrins (pre-incubation values in parentheses)			Total net increase µg UPI + CPI	% Type III		
					Added	Remaining	Proto-	CP	UP		CP	C. B.	UP → CP
1	3000	t. c.	15.0	0	0.96	0.33	67 (7.5)	20 (0.1)	59 (0)	8	91	95	85
2	2975	l. d. c.	12.0	7.7*	0.80	0.077	282** (54)	48 (1.8)	49 (14.6)	75	27	5	10
3	2975	g. d. c.	13.7	0.8*	0.77	0.142	119** (52)	38 (0.8)	44 (2.0)	37	71	50	42
4	Control	no cells	—	—	0.8	0.62	0	0.3	0.5	—	—	—	—

* T. C. 2.5% retic

** Proto- → Meso-, all type 9 (III)

Tab. 9

Exp. 9. Effect of bleeding on utilization of PBG and porphyrin formation by normal bovine hemolysate (# 3247)
1. Before bleeding (6-26-64). Hb 11.4 gm/100 ml, Ht. 35.5%, reticulocytes 0. In the first runs the volumes of packed cells indicated were hemolyzed and diluted as described under Methods. In run 4 the buffer mixture recommended by Vavra and co-workers (17) was used (see Methods). In runs 1, 3 and 4 the blood and hemolysates were refrigerated until incubation. In run 2 no attempt was made to avoid ambient temperature exposure

Run	Hemolysate from	Hours incubation	ml packed cells	pH		PBG added mg	PBG remaining mg	µg porphyrins (pre-incubation values in parentheses)			Total net increase µg UPI + CPI	% III	
				Before incubation	After incubation			Proto-	Net incr.	CP	UP	CP	UP*
1	t. c.	8	10	7.30	7.44	2.31	1.2	60 (4)	56	45 (0)	134 (0)	86	43
2	t. c.	8	10	7.50	7.60	2.27	1.26	40 (4)	36	32 (0)	157 (0)	100	52
3	t. c.	4	15	7.35	7.48	0.63	0.27	68 (6)	62	31 (0)	71 (0)	100	74
4	t. c.	4	2.5	7.40	7.50	0.71	0.57	2 (1)	1	4 (0)	21 (0)	100	63
5	Control, no cells	4	0	7.00	5.20	0.95	0.68	0	0	0	2	—	—
2. After bleeding (7-10-64). (7.7 liters of blood removed between 6-26 and 7-8). Hb 7.2 gm/100 ml, Ht. 22%, reticulocytes 0.5%; 3.8% dark blue supravital staining cells (with brilliant cresyl blue). L. d. c. and g. d. c. fractions had 1.1 and 0.2% reticulocytes, 6.3 and 1.8% dark blue staining cells, respectively (see text); packed cells in each run hemolyzed as in runs 1-3, above													
6	t. c.	4	15	7.30	7.38	0.78	0.08	113 (7)	106	36 (0)	36 (0)	0	100
7	l. d. c.	4	12.3	7.22	7.22	0.8	0.21	13 (1)	12	32 (0)	25 (0)	0	100
8	g. d. c.	4	14.7	7.22	7.27	0.8	0.16	7 (4)	3	35 (0)	41 (0)	0	100

* Differences from 100% represent type I. In addition, small spots were noted in all runs, both before and after bleeding, indicative of minor amounts of "pseudouroporphyrin" (7-COOH)

Tab. 10

Exp. 10. Effect of bleeding on utilization of PBG and porphyrin formation by bovine porphyrin hemolysate (# 3033)

1. Before bleeding (6-29-64). Hb 8.4 gm/100 ml, Ht. 25%. The blood and hemolysates were kept cold throughout. The same proportions of water to the two KCl solutions were used as in exp. 9, with a four hour incubation

Run	Hemo-lysate from	Reticu-lyocytes %	ml packed cells	pH		mg PBG Added	mg PBG Remaining	μg porphyrins (pre-incubation values in parentheses)			Net increase		% III	
				Before incubation	After incubation			Proto-*	Net in-crease	CP	UP	UP I + CP I	CP	UP
1	t. c.	1.5	15	7.32	7.45	0.65	0.08	218 (113)	105	32 (1.8)	91 (0.7)	83	56	27
2	l. d. c.	1.3	10	7.26	7.30	0.74	0.17	191 (90)	101	29 (1.5)	90 (0.4)	84	66	20
3	g. d. c.	0.6	13	7.30	7.33	0.65	0.18	167 (80)	87	30 (1.7)	117 (0.4)	112	52	19

2. After bleeding (7-9-64). 7.9 liters of blood removed since 6-29. Hb 5.7 gm/100 ml, Ht. 18%. Hemolysates prepared as above, with a four hour incubation

4	t. c.	8	15	7.22	7.25	0.71	0.11	249 (93)	156	80 (12)	121 (66)	125	15	10
5	l. d. c.	17	13	7.10	6.98	0.77	0.16	204 (66)	138	227 (54)	151 (93)	266	4	13
6	g. d. c.	4	13.8	7.20	7.20	0.83	0.05	200 (69)	131	62 (5)	94 (21)	127	14	14

* Meso- in all six runs entirely isomer 9 (III)

Tab. 11

Exp. 11 (9-14-65). Porphyrin formation by normal and porphyrin bovine hemolysates in relation to the period of incubation with PBG; 15 ml packed red cells in the hemolysate of each run (see Methods)

	Hb. gm/100 ml	Whole blood Reticulocytes %	Hours	PBG mg	Proto-μg	Porphyrins and isomers			
						CP μg	% I	UP μg	% I
Normal without PBG	13.3	0	0	0.0	10.6	0.45	—	0	—
Incubated (tube 1)			4	0.0	9.7	0.34	—	0	—
Incubated (tube 2)			8	0.0	8.2	0.21	—	0	—
Normal with PBG (each tube)	13.3	0	0	0.80	(see values above)				
Incubated (tube 1)			2	0.47	60.0	27.0	24	48.0	lost
Incubated (tube 2)			4	0.209	84.5	43.6	19	82.3	lost
Incubated (tube 3)			6	0.052	89.0	50.4	17	101.0	42
Incubated (tube 4)			8	0.0	89.0	63.0	26	87.0	54
Porphyrin (# 2955) without PBG	8.9	1.8	0	0.017	119.0	20.6	65	1.7	—
Incubated (tube 1)			4	0.017	117.0	10.0	75	1.6	—
Incubated (tube 2)			8	0.0	120.0	9.0	73	1.7	—
Porphyrin (# 2955) with PBG (each tube)	8.9	1.8	0	0.79	(see values above)				
Incubated (tube 1)			2	0.258	192.0	53.0	19	68.0	32
Incubated (tube 2)			4	0.026	210.0	67.0	27	77.0	55
Incubated (tube 3)			6	0.017	207.0	68.0	44	55.0	55
Incubated (tube 4)			8	0.0	211.0	72.0	52	53.0	57

CP and UP in this run was of isomer series III while in run 3 the value for UP is 74% III. The small porphyrin production in runs 7 and 8 might be related to the relatively low pH. CORNFORD (32) has shown that pH 8.0 is optimal for isomer III production when using a partially purified isomerase. In this connection the lowest pH of hemolysate was noted in run 5, exp. 10, Table 10 (after bleeding of the porphyrin bovine # 3033). Nevertheless the largest net increase of CP + UP I in this exp. is noted in this run. Also a considerable amount of Proto- 9 (III) was formed. We have been unable to correlate the variations in porphyrin formation in Tables 9 and 10 with the pH variations, nor the latter with variations in amounts of red cells hemolyzed, or PBG added. On the basis of CORNFORD's observation (32) the per cent of type III isomer in runs 4, 5 and 6, especially run 5 (Tab. 10), may be unduly low in comparison with the values of runs 1—3 before bleeding. It should be noted again, however, that in run 7 of the preceding exp. 9, with a pH of but 7.22, only type III isomer was observed, though the amount was small. This may nevertheless represent partial suppression of the normally plentiful isomerase, in terms of total isomer III produced. Use of the Tris buffer mixture (17) in run 4, Table 9, was associated with essentially the same net increase of total porphyrin as formed in run 3, taking into account the differing volumes of packed cells used. (These differed by a

factor of 6.0 while the net increase of total porphyrin differed by a factor of 6.3.)

In exp. 5 (Tab. 5) it is evident that GSH or AMP were evidently inhibitory, in that order. Obviously further experiments are needed to confirm and define this more precisely.

The time factor

In three of the earlier experiments (2, 4, 5) in which there was no refrigeration before incubation, the incubation time was 8 hours, thus contributing to a relatively excessive formation of type I isomer since the deaminase would still be available to convert any residual PBG to type I in the absence of isomerase inactivated by heat, both in part before and during the long incubation. As an example, in exp. 5 (Tab. 5) most of the UP and CP at the end of the 8 hour incubation was isomer I, but considerable Proto- was also formed, especially in runs 1 and 2, and this was shown to be entirely type 9 (III). The time factor was more directly examined in exp. 11 (Tab. 11) where it is noted in the lowest set (porphyrin with PBG) that at 2 hours only 32% UP I was recorded but at 4, 6 and 8 hours, 55—57%. The % CPI is seen to have increased in even more convincing fashion with time. The data for the normal with PBG in this experiment are incomplete in respect to UP isomer ratio but there is some indication of the same increase of type I, however, with the progressive

Tab. 12

Exp. 12 (4-3-61). Porphyrin formation by hemolysates of total, lesser and greater density porphyric human red cells, incubated for eight hours with PBG, case 76, M. H. Q 15

Run	Type of cells	Reticulo- cytes %	ml packed cells	mg added	PBG mg left	net diffe- rence μ M	(pre-incubation Proto-	μ g porphyrins values in parentheses CP	UP	Total net increase μ M as UP	% III CP	UP $\rightarrow$ CP
1	Normal t. c.	—	2.0	3.76	1.5	10.0	9.0	189	360	0.74	75	10
2	Porphyric t. c.	3.6	4.0	7.25	1.44	25.7	41.0 (1.0)	545 (1.6)	1321 (5.6)	2.5	54	8
3	Porphyric l. d. c.	5.0	4.0	7.1	1.47	24.9	35.0 (1.6)	262 (5.2)	1657 (14.6)	2.4	55	10
4	Porphyric g. d. c.	2.8	4.0	7.35	1.03	28.0	30.0 (1.2)	383 (0.4)	1973 (2.0)	3.0	57	*
5	Control, no cells	—	0	3.67	2.4	5.6	0.3	0.2	35	0.04	—	—

* Lost during decarboxylation

Tab. 13

Exp. 13. Utilization of PBG and porphyrin formation by hemolysates of human porphyric and normal bloods; 10 ml packed cells in each run (see Methods); four hour incubation of hemolysates

Run*	Subject	Hb	Reticu- cytes %	mg added	PBG mg left	net diffe- rence μ M	(pre-incubation Proto-	μ g porphyrins values in parentheses CP	UP	Total net increase μ M as UP	% theory	% III CP	UP**
1	D. H.	13.3	2.3	0.549	0.053	2.2	43 (7.9)	68 (7.2)	69 (26.4)	.21	38	62	26
2	T. H.	14.1	7.5	0.35	0.026	1.4	31 (7.2)	110 (2.0)	35 (4.8)	.25	71	84	31
3	Mother	11.7	1.6	0.37	0.017	1.6	23 (3.2)	125 (.06)	32 (0)	.27	67	100	68
4	Brother	17.2	2.6	0.37	0.035	1.5	20 (2.3)	124 (.05)	26 (0)	.26	69	100	60
5	Normal control	15.8	2.3	0.36	0.018	1.5	21 (2.4)	149 (.04)	23 (0)	.29	76	100	82
6	Normal control	14.0	1.2	0.513	0.043	2.1	27 (3.3)	120 (.03)	37 (0)	.27	51	100	83

* Runs 2-5 carried out on 11-13-65; runs 1 and 6 on 12-17-68

** Pseudoporphyrin (7-COOH) spots were observed in runs 3-6 but not runs 1 and 2

increase of total CP with time, the variation in proportion of isomers is of doubtful significance.

Coproporphyrin formation

The isomer values for the CP after incubation are of considerable significance, reflecting to an important degree the UP III which was formed in the hemolysate during the earlier period of incubation, as the decarboxylase converting UP to CP⁶) does not share the extreme temperature sensitivity of the isomerase. Examples are seen in Tables 4, 5 and 8-13, incl. Exp. 12 (Tab. 12) is of special interest as it is the first of two experiments with human porphyric hemolysates, and the only one in which preliminary fractionation of the red cells by centrifugation was carried out. The amounts of CP are quite large and a majority in each run was type III in contradistinction to the great preponderance of UP I. The data from the normal control in Table 12 are highly significant because of the relatively large amount of CP III (75% of 189 μ g) as contrasted with the small amount of UP III (10% of 360 μ g). Again, it may be assumed that the UP I is present because of continued deaminase activity after the isomerase has been exhausted, the CP III having been formed early in the course of the 8 hour incubation but not converted to Proto-. The same relatively small conversion of CP III to Proto- is seen in Table 13. This interesting point will be considered in the following.

Reticulocytes and porphyrin formation

It is seen in Table 12 that despite representing 5% reticulocytes the l. d. c. hemolysate contained less UP

⁶) It may be mentioned again that these designations in the present context include the biologically significant porphyrinogens as well as the porphyrins isolated.

and CP than the hemolysate of the greater density cells albeit these had only 2.8% reticulocytes. The porphyrin formation was often greater with the l. d. c. hemolysates, as noted in Tables 2, 3, 8 and 10, runs 5 and 6. In Table 13 correlation of reticulocytes and total porphyrin formation by total cell hemolysates is lacking, although the proportion of type I in the porphyric is greater (see below). In all of the bovine porphyric samples the l. d. c. had significantly greater amounts of native porphyrin. This was likewise true in the human exp. 12 (Tab. 12) but in this instance, unaccountably, the g. d. c. hemolysate formed larger amounts of porphyrin without, however, taking any newly formed heme into account. In run 7 of exp. 6 it is seen that the amounts of native UP and CP were much greater in the l. d. c. hemolysate than in the g. d. c. hemolysate of run 8, yet in the latter there was a greater increase of UP I + CP I than in the former. Again the possibility of a greater heme formation in run 7 cannot be excluded. One must also consider whether the deaminase was limiting because of prior utilization in formation of the large amounts of native porphyrin, although this appears much less likely.

Protoporphyrin 9 (III) formation

Increased formation of Proto- is evident in many of the hemolysates presently studied. The amounts have generally been larger in the porphyric hemolysates and often in those of the l. d. c. as contrasted with g. d. c. or t. c. (Tab. 2, 3, 8). Account must be taken, as in Table 3, of the amounts of packed cells and PBG used in different runs. Thus in run 5 (l. d. c.) only 2.0 ml/cells and 1.92 mg PBG were used; 115 μ g of Proto- were observed as contrasted with 139 μ g in run 4 (t. c.) but in this run 4 ml of cells and 2.9 mg of PBG were used. Another

example is seen in Table 12 in which the much smaller porphyrin formation in the normal control is related to half as many packed cells and about half as much PBG as in runs 2—4 with porphyrin hemolysates. Even taking this into account it is evident that porphyrin formation by the latter was much greater. Collateral data for these experiments including reticulocyte percentages are given in the Tables.

In the single experiment (Tab. 1) in which the hemolysates were obtained by differential osmotic lysis, that of the most fragile formed much more Proto- than that of the most resistant cells.

Discussion

In considering the significance of the present data due attention must be given to more recent knowledge of porphyrin and heme biosynthesis in erythroid cells, a subject which has been dealt with in an excellent review by GRANICK (33). In this particular attention is given to mitochondrial as versus soluble enzymes, the former being present in young red cells, the latter also in older cells not containing mitochondria. It is well known that the synthesis of ALA, Proto- and Heme is related to the number of immature cells and corresponding mitochondrial enzyme content of the blood sample used (33—37). The earlier report (8) that the abnormal content of native UP I and CP I of the erythrocytes in erythropoietic (uro-) porphyria is largely in cells which are of lesser density, reticulocyte rich and osmotically more fragile, receives confirmation in the present observations, and has also been confirmed by others (35, 36, 37).

While in general the l. d. c. hemolysates formed more porphyrin, a correlation of the net increase with percentage of reticulocytes is often not apparent. In Table 2 it is seen that the l. d. c. had ten times as many reticulocytes as those of greater density but the apparent net increases of porphyrins was scarcely greater in the l. d. c. than in the g. d. c. hemolysate. Also, the difference in percentage of UP I in the two hemolysates is barely significant.

It is evident from the present results that the formation of porphyrin from PBG by hemolysates of either bovine or human porphyrin or normal erythrocytes is affected by various factors, of which temperature is one of the most important. Although the heat sensitivity of the isomerase ("UPG cosynthetase") is well recognized in terms of exposure at 55° for 15—30 minutes (38), the necessity of protection against even a mild exposure to ambient temperatures for relatively short periods prior to incubation has not been duly recognized. It is also evident that with longer incubation, such as 8 rather than 2—4 hours, the isomerase is more completely inactivated. If PBG is still available the isolated deaminase activity then gives rise to an increasing proportion of UP I in normal as well as porphyrin hemolysates. When the blood or hemolysate has not been refrigerated prior to incubation, type UP I may be expected to be the main if not sole representative, although even in such

hemolysates significant increase of CP III and Proto- 9 (III) has regularly been noted, indicating that there was still some isomerase activity, at least in the earlier part of the incubation. An excellent example of the progressively greater deaminase and diminished isomerase activity with time is seen in Table 11, the fourth set of data, i. e., porphyrin hemolysates with added PBG. It is seen that as the amount of UP diminishes the proportion of type I stabilizes at about 55% and at the same time CP I is increasing. Most of the increase of Proto- has occurred at the end of 4 hours.

In general the net increases of Proto- were considerably greater in the bovine porphyrin than in the corresponding human hemolysates. This may bear fundamental relationship to the characteristically greater erythrocyte Proto- in the bovine than in the human disease. Whenever the Proto- of the present hemolysates was converted to Meso-, this was shown to be the type 9 series III isomer. With the often considerable net increase of CP III, even though together with Proto-, representing less than that of UP and CP I, it is evident that lack of isomerase in this disease is by no means absolute but only relative to a marked increase of deaminase. This is true even in the earlier experiments in which the red cells or hemolysates were not refrigerated prior to incubation with PBG, with consequent diminution of isomerase in relation to deaminase activity (see Tab. 1, 2, 4, 5).

Uni- vs bimodality of normoblasts?

In accord with a major objective as discussed at the outset, the data may now be considered in respect to the question of uni- vs bimodality of the erythrocytes in terms of the genetic biochemical abnormality, i. e., the imbalance in the deaminase-isomerase function favoring excessive formation of UP I.

The data shown in Tables 1, 2, 8, 10 and 12 are of major interest in this regard. In general they exhibit greater porphyrin formation by the hemolysates representing greater proportions of young cells having larger concentrations of native UP I + CP I. The increase of porphyrin on incubation of these hemolysates with PBG is represented to a larger extent by UP I and CP I but there are also very significant increases of CP III and Proto- 9 (III). Interpretation of the latter increases is highly important for the question of uni- or bimodality of the erythrocytes in respect to the genetic abnormality. Of the five experiments in these tables the last three are more significant and will be considered more fully, as in these, in contrast to exps. 1 and 2, great care was observed in the pre-incubation refrigeration of the blood samples and hemolysates.

In part, at least, the data are compatible with a bimodal distribution. This is especially true of exp. 8 (Tab. 8) in which even with fewer red cells represented in the l. d. c. hemolysate, much larger amounts of both UP I + CP I and Proto- 9 (III) were formed. Here one might justifiably assume that among the 7.7% reti-

culocytes in this run some representing the genetic abnormality accounted for the type I isomer formation while others, entirely normal, were responsible for the increase of Proto- 9. On the other hand, it might equally well be postulated that all of the reticulocytes are characterized in similar fashion by the isomerase-deaminase imbalance and this is favored by the data in Table 10. Before bleeding the red cells contained but minor increases of native porphyrin with insignificant differences in the three runs. The differences in isomer proportions are of questionable significance. The differences between the data before and after bleeding are highly significant. A considerably greater increase in UP I + CP I is noted in the l. d. c. as contrasted with g. d. c. hemolysates but the difference in the increased Proto- 9 (III) formation in the three runs, 4—6, is insignificant despite the considerable fractionation of reticulocytes. While the UP isomer ratio does not vary significantly in these runs the % of CP III is distinctly less in the l. d. c. run 5 and it is seen that CP I comprises the larger proportion of the total type I isomer formed in this run. This marked increase is roughly correlated with reticulocyte percentage. As described elsewhere (13) the bleeding shown in the table resulted in a sharp increase of fluorescing normoblasts in the bone marrow from 46 to 80% (13). This finding, considered together with the porphyrin isomer data in Table 10, is believed to offer strong evidence for a unimodal distribution of normoblasts and erythrocytes in terms of the genetic abnormality. Bovine # 2975, Table 8, had also been bled of 4 liters in the period just prior to the present experiment and although the fluorescing normoblasts were not counted in this instance, it is safe to assume that the same increase to the range of 80—90% had occurred. The data for the human experiment 12 (Tab. 12) are also of interest in this respect as they fail to show significant differences between l. d. c. and g. d. c. hemolysates despite considerable difference in reticulocyte percentages in these runs, also the considerably greater native porphyrin content of the l. d. c. run 3. This is not in accord with a bimodal distribution. Although the UP fraction of run 4 (g. d. c.) was lost before the isomer ratio was determined, it may be assumed from the entirely similar UP isomer ratios of runs 2 and 3 that that of run 4 probably did not differ significantly. Also, the considerable increases of Proto- in the porphyric runs (2—4) did not differ significantly. Exps. 1 and 2 may be mentioned briefly in regard to the question of uni- or bimodality, though because there was no refrigeration prior to incubation, the formation of type I isomer was undoubtedly favored. It is fully evident in Table 1 (exp. 1) that hemolysate A, that of the osmotically least resistant cells contained most of the native UP, hence may be assumed to represent a concentration of the genetic-enzymatic error, especially when the values for UP are compared with those for native Proto-. It may be assumed, on the basis of earlier studies (8), that hemolysate A contained a large proportion of the reticulocytes of the starting sample of

cells. While this hemolysate formed a great deal more porphyrin including UP, CP and Proto-, the isomer ratios of the UP of hemolysates A and C (runs 1 and 3) are identical, i. e., 70% I. As mentioned above, these values are probably too high because of some exposure to ambient temperatures, nevertheless, with a bimodal distribution a distinct difference in the UP isomer ratios for runs 1 and 3 would have been anticipated.

Exp. 2 is of interest because of the marked separation of reticulocytes by fractional centrifugation. The l. d. c. contained most of the native UP and CP, but the net increase of UP I and the UP isomer ratios, differed only slightly between the two hemolysates (runs 1 and 2). The total net increases of porphyrins in these runs were 184 and 165 μ g, respectively. The fact that such small differences were found with hemolysates representing such a marked difference in reticulocytes reveals that the deaminase must have been nearly as adequate with but 10% as many reticulocytes in the g. d. c. hemolysate (run 2). Since the incubation was for 8 hours, it may be assumed that the available isomerase was exhausted during the first 2—4 hours, thus permitting the heat stable deaminase to continue its activity in forming UP I. These results do not appear to support a bimodal concept. The large number of reticulocytes indicates a very active erythropoiesis and, as already noted (13), whenever this has been produced by bleeding, the percentage of fluorescing normoblasts in the bone marrow increases to a degree scarcely compatible with a bimodal distribution.

Additional note on correction of the proof:

1. Note should have been made in the foregoing of the differences in isomer ratios after incubation in Exp. 13. It is seen that UP I was dominant after incubation of the porphyrin hemolysates, while with those of the mother and brother, quite likely heterozygous, the UP was 60—68% III, and in the two normal controls 82—83% III. This suggests that this method may permit detection of heterozygotes. We have not observed differences in native erythrocyte porphyrin concentrations of heterozygote vs normal, such as HEILMEYER and co-workers (40) have described.

2. Since completion of the above studies, LEVIN and COLEMAN (41) have reported observations on the conversion of PBG to porphyrins by a partially purified synthetase (deaminase) from mouse spleen with and without cosynthetase (isomerase) prepared from another fraction of the spleen, or from wheat germ. Using this synthetase with bovine (42) and human (43) porphyric vs normal hemolysates, LEVIN noted a marked deficiency of cosynthetase in the porphyric cells. However, in this study the possibility was not explored that significant fractions of the uroporphyrinogen III as formed might have been converted in considerable part to CP and Proto-, as often observed in the present experiments.

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