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Research Article

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ERYTHROCYTE CARBOHYDRATE METABOLISM IN HEREDITARY SPHEROCYTOSIS *

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Hereditary spherocytosis (HS) has been a subject for studies of erythrocyte carbohydrate metabolism since 1954, when Selwyn and Dacie (1) observed that glucose corrected the increased lysis of HS erythrocytes incubated in autologous serum. Prankerd, Altman, and Young (2) reported abnormalities of incorporation of P^{32} into ATP [1] and 2,3-DPG with correction by adenosine. Tabechian, Altman, and Young (3) found the HS erythrocyte abnormally sensitive to inhibition of P^{32} uptake by sodium fluoride. These findings were interpreted as consistent with a defect in glycolysis, and enolase deficiency was specifically suggested (4). Recent studies of HS, however, have failed to confirm either an abnormality of phosphate partition (5, 6), or any deficiency in glycolytic enzymes (7). Thus, the relationship between erythrocyte carbohydrate metabolism and HS is still undefined.

This paper reports the results of an evaluation of erythrocyte carbohydrate metabolism in HS by a different approach from that of previous studies. The changing concentrations of carbohydrate metabolites have been determined at intervals in incubating plasma-erythrocyte suspensions, and the modifications of carbohydrate metabolism by the additions of methylene blue or iodoacetate have been evaluated.

METHODS

The subjects of this study were 18 patients with HS, ten of whom had been splenectomized. The diagnosis of HS was defined by the usual criteria. The splenectomized patients had normal peripheral blood values for hemoglobin, packed cell volume, and reticulocytes, except for an unexplained elevation of 4.4% reticulocytes in one patient. The hemoglobin values in the non-splenectomized group varied from 8.3 to 12.8 g per 100 ml, and the percentage of reticulocytes, from 5.5 to 20.3. Control samples were obtained from volunteer physicians, students, and laboratory personnel.

Preparation of erythrocyte-plasma suspension. Fifty ml of blood was collected from fasting subjects in 5 mg of dry sodium heparin and centrifuged immediately for 15 minutes at 0 to 4° C and 700 *g*. The plasma, buffy coat, and approximately one-tenth the volume of packed erythrocytes were removed by aspiration. The procedure was repeated after reconstitution with the removed plasma. By this technique, the leukocyte concentration of the erythrocyte-plasma suspensions was reduced to less than 1,500 per mm^3, except in one splenectomized HS patient, where the count was 4,500. The reticulocyte content of the incubation suspensions never exceeded 2% for the splenectomized group.

Composition of incubation suspensions. A 9.6-ml volume of the prepared erythrocyte-plasma suspension was added to each of 3 Erlenmeyer flasks (25 ml). To each flask was added *a*) 1.4 ml of 15 mM phosphate Locke's solution, pH 7.4 (hereafter referred to as the "endogenous" suspension) or *b*) 1.4 ml of 0.02% methylene blue in Locke's solution or *c*) 1.4 ml of 0.02 M neutralized iodoacetic acid [2] in Locke's buffer solution. The suspensions were incubated in a Dubnoff shaker at 37° C adjusted to 90 to 100 oscillations per minute. The flasks were stoppered during the course of the experiment and opened only at 30-minute intervals to permit sampling. The original pH of the mixture was 7.4. Incubation was continued for 150 minutes, while the pH of the suspensions rose from 7.4 to 7.6 ± 0.1.

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[1] The following abbreviations are used: AMP, ADP, and ATP, adenosine mono-, di-, and triphosphate; IMP, inosine monophosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; G-1,6-P, glucose-1,6-diphosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6-diphosphate; G-3-P, glyceraldehyde-3-phosphate; 3-PG, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; 2,3-DPG, 2,3-diphosphoglycerate; DPN, TPN, di- and triphosphopyridine nucleotide; and DPNH and TPNH, reduced DPN and TPN.

[2] Twice recrystallized from ether, Eastman Organic Chemicals, Eastman Kodak Co., Rochester, N. Y.

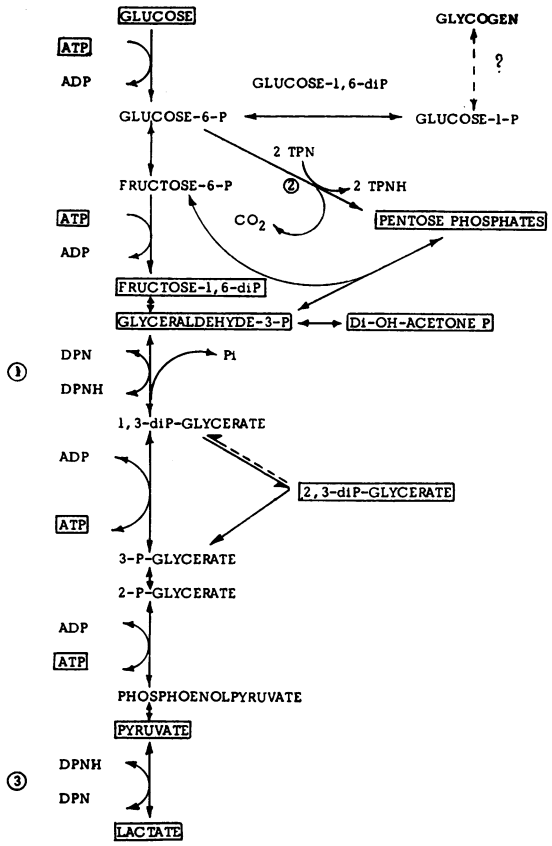

FIG. 1. PATHWAY FOR METABOLISM OF CARBOHYDRATE IN THE MATURE ERYTHROCYTE. Boxed intermediates indicate those measured. In this study, Reaction 1 was inhibited with iodoacetic acid and Reaction 2 was stimulated with methylene blue. Evidence that Reactions 1 and 3 are also influenced with methylene blue is given in the text.

Analytical methods. The carbohydrate intermediates and cofactors selected for analysis represent key reactions of the glycolytic and hexose monophosphate pathways (Figure 1). Glucose was determined in barium hydroxide-zinc sulfate filtrates by the method of Somogyi (8). ATP was determined in neutralized perchloric acid filtrates by the reduction of TPN mediated by glucose-6-phosphate dehydrogenase and hexokinase (9). The following determinations were performed with trichloroacetic acid filtrates: ketose and triose phosphates, by the cysteine-carbazole-sulfuric acid method of Dische and Borenfreund (10); pyruvate, by the total hydrazone procedure of Friedmann and Haugen (11); and total pentose phosphate by the orcinol-ferric chloride reagent of Mejbaum (12) as modified by Ibsen, Coe, and McKee (13). To characterize the pentose components further, free pentose phosphate (not bound to adenine or other bases) was selectively destroyed by boiling in sodium hydroxide (14) before the application of the orcinol reagent. By this procedure, measurements made on unboiled filtrates represented total pentose phosphate, those after boiling represented bound pentose (combined in nucleotide or nucleoside form), and the difference between the two was free pentose phosphate. Lactate was determined in either barium hydroxide-zinc sulfate, or trichloroacetic acid filtrates by the method of Barker and Summerson (15). 2,3-Diphosphoglycerate was determined in trichloroacetic acid filtrates by the resistant-phosphate procedure of Mányai and Várady (16). The procedure depends on the fact that the phosphorus of 2,3-DPG resists 180 minutes of hydrolysis under the conditions employed. Other phosphate esters, however, resist hydrolysis to varying degrees, as shown in Table I, and a correction for their interference must be made should any of these esters vary appreciably during incubation. These corrections were applied as shown in Table II. Inorganic phosphate was determined by the method of Taussky and Shorr (17).

The enzymatic assays of F-1,6-P and DHAP were made with a Beckman DU spectrophotometer and were based on the observed change in absorbance at 340 mμ in cuvettes containing 2 ml neutralized perchloric acid filtrate, 1 ml 0.08 M Tris buffer at pH 7.5, and 0.05 ml 0.003 M DPNH.[3] The assay was initiated by the addition of 5 μl α-glycerophosphate dehydrogenase,[4] 0.5 mg protein per ml, which yielded values for DHAP. After complete depletion of DHAP (approximately 10 minutes), F-1,6-P was determined by the addition of 20 μl aldolase,[4] 1 mg protein per ml.

RESULTS

The results on normal and splenectomized subjects are summarized in Table III, and on nonsple-

[3] California Corporation for Biochemical Research, Los Angeles, Calif.

[4] Sigma Chemical Corp., St. Louis, Mo.

TABLE I

*Resistance of various phosphate esters to acid hydrolysis**

Ester:	G-1-P	G-6-P†	F-1, 6-P	ATP	AMP	IMP	3-PG
% not hydrolyzed:	1.4	86.5	30.1	23.6	71.0	71.0	92.8

* Conditions: 1 N H_2SO_4, 100° C, 180 minutes. Esters listed were commercial preparations. G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-1,6-P, fructose-1,6-diphosphate; ATP and AMP, adenosine tri- and monophosphate; IMP, inosine monophosphate; and 3-PG, 3-phosphoglycerate.

† Repeated assays with G-6-P dehydrogenase showed only trace amounts in erythrocytes throughout this study.

TABLE II

*Estimation of 2,3-diphosphoglycerate (2,3-DPG) from acid-resistant phosphate esters**

		Concentration changes during 150-minute incubation (37° C)		
		Endogenous	Methylene blue	Iodoacetic acid
		μmoles/ml erythrocytes ± SEM		
Total resistant P		+1.06 ± 0.15	+2.00 ± 0.39	−2.40 ± 0.57
Combined pentose P	Total	−0.07 ± 0.04	+0.23 ± 0.07	−0.64 ± 0.08
	Resistant	−0.05 ± 0.03	+0.16 ± 0.05	−0.46 ± 0.06
Ketose P	Total	+3.40 ± 0.40	0.00 ± 0.22	+3.80 ± 0.66
	Resistant	+1.02 ± 0.12	0.00 ± 0.07	+1.14 ± 0.20
Remaining resistant P (by difference)		+0.09 ± 0.19	+1.84 ± 0.40	−3.09 ± 0.61
Estimated 2,3-DPG		+0.05 ± 0.10	+0.92 ± 0.20	−1.55 ± 0.31

* Calculations were made as follows. Total resistant phosphorus equaled the difference between total phosphorus and the phosphorus released as inorganic phosphate by hydrolysis of trichloroacetic acid filtrates in 1 N sulfuric acid at 100° C for 180 minutes. Combined pentose (assumed to be all nucleotide pentose) resistant phosphorus equaled the combined pentose phosphate multiplied by 0.71, the fraction of AMP or inosine monophosphate resistant to hydrolysis (Table I). Ketose phosphate was assumed to be all diphosphate, and therefore its acid-resistant fraction equaled twice the micromolar change recorded in Table IV multiplied by 0.30 (Table I). The sum of both resistant fractions thus calculated was then subtracted from the total resistant phosphorus, and this difference was divided by two to obtain the estimated micromolar changes in 2,3-DPG. No correction was applied for monophosphoglycerates. For composition of the incubation systems and for assay procedures employed, see Methods.

nectomized subjects in Table IV. The values were recorded as the mean change in concentration of metabolite after 150 minutes of incubation. Since the concentration changes, as judged by analyses made at 30-minute intervals, were nearly linear for all of the intermediates except pentose (Figure 2), only the 150-minute values are given. The results on HS subjects were subjected to the *t* test for significant deviation from normal. The algebraic sum of the changes of the measured intermediates represents the balance achieved in the incubation systems. Theoretically, this should be equal to zero under steady state conditions.

Metabolic balance among the intermediates. The total balance figures in Table III indicate that in the normal erythrocyte a surplus of intermediate was found in the endogenous and iodoacetate systems, and a deficit in the methylene blue system. This deficit was expected as a consequence of glucose carbon lost by CO_2 evolution after the activation of the hexose monophosphate pathway. In the HS cell, the degree of metabolic imbalance tended to be less than in the normal cell; in fact, there was no surplus of intermediate found in the HS erythrocyte endogenous suspensions. The positive balance, when it occurred, was largely due to the accumulation of ketose and triose phosphates. In HS erythrocyte suspensions, ketose

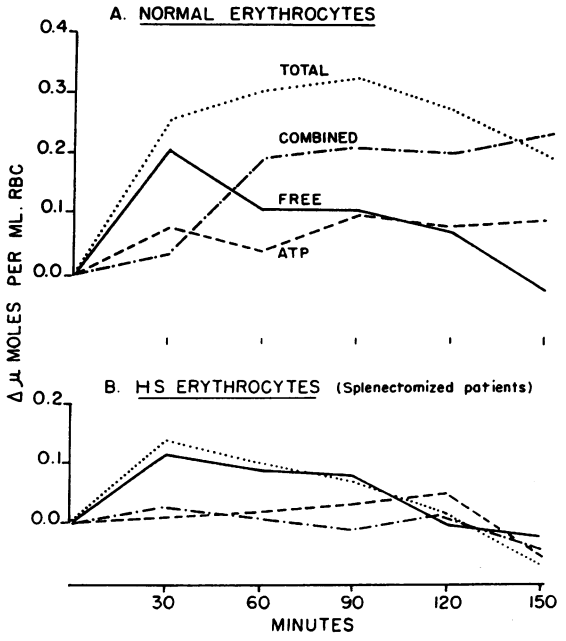

FIG. 2. METABOLISM OF PENTOSE IN INCUBATING (37° C) NORMAL AND HS ERYTHROCYTES IN THE PRESENCE OF METHYLENE BLUE. Total pentose was measured by the orcinol reaction, and further differentiated into fractions of combined pentose (bound as nucleosides and nucleotides) and unassociated free pentose phosphate. ATP, a component of the combined pentose fraction, was measured independently. Procedural details are given in the text.

TABLE III

Changes in concentration of carbohydrate intermediates in erythrocytes from splenectomized patients with hereditary spherocytosis after incubation for 150 minutes at 37° C

Intermediate	Endogenous*					Methylene blue					Iodoacetic acid				
	Patients		Normal			Patients		Normal			Patients		Normal		
	No. of assays	Δ μmoles/ml eryth. ±SEM	No. of assays	Δ μmoles/ml eryth. ±SEM	p	No. of assays	Δ μmoles/ml eryth. ±SEM	No. of assays	Δ μmoles/ml eryth. ±SEM	p	No. of assays	Δ μmoles/ml eryth. ±SEM	No. of assays	Δ μmoles/ml eryth. ±SEM	p
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Glucose	10	−6.80±0.44	12	−6.50±0.52	0.7	10	−9.30±0.63	12	−9.80±0.81	0.7	10	−0.30±0.13	9	−1.00±0.33	<0.01
½ Lactate†	10	+5.32±0.26	11	+5.39±0.21	0.8	10	+4.82±0.38	11	+4.86±0.27	0.9	10	+0.08±0.39	9	+0.87±0.25	0.1
Pentose, total	10	−0.11±0.03	14	−0.05±0.02	0.8	10	−0.06±0.05	14	+0.20±0.06	<0.01	10	−0.40±0.04	14	−0.30±0.05	0.2
½ 2,3-DPG	8	+0.18±0.06	6	+0.02±0.05	0.05	8	+0.40+0.06	6	+0.46±0.10	0.7	8	−0.65±0.10	6	−0.77±0.15	0.6
Ketose	8	+0.40±0.06	10	+1.70±0.20	<0.01	9	−0.23±0.04	7	0.0 ±0.11	0.3	9	+0.98±0.13	10	+1.90±0.33	0.05
½ Triose	5	+1.06±0.06	10	+0.90±0.20	0.5	6	−0.23±0.04	7	−0.08±0.08	0.2	6	+0.94±0.13	10	+0.65±0.17	0.2
½ Pyruvate	8	−0.11±0.06	7	−0.01±0.14	0.3	8	+1.59±0.24	7	+1.50±0.08	0.8	8	+0.49±0.12	7	+0.30±0.10	0.3
Total balance of intermediates		−0.06±0.53		+1.45±0.63			−2.93±0.78		−2.86±0.88			+1.14±0.48		+1.65±0.59	

* The endogenous incubation system is the erythrocyte-plasma-buffer suspension to which neither methylene blue nor iodoacetate was added.
† The micromolar concentrations of all 3-carbon metabolites derived from 6-carbon precursors were taken as half their actual concentrations in order to preserve the mole-to-mole relationship needed to evaluate total balance.

TABLE IV

*Changes in concentration of carbohydrate intermediates in erythrocytes from nonsplenectomized patients with hereditary spherocytosis after incubation for 150 minutes at 37° C**

Intermediate	Endogenous				Methylene blue				Iodoacetic acid			
	No. of assays	Δ μmoles/ml eryth. ±SEM	p_n†	p_s†	No. of assays	Δ μmoles/ml eryth. ±SEM	p_n	p_s	No. of assays	Δ μmoles/ml eryth. ±SEM	p_n	p_s
μmole												
Glucose	7	−8.70±0.49	<0.01	<0.01	7	−10.80±0.49	0.3	0.1	7	−0.30±0.45	0.3	>0.9
½ Lactate	8	+7.55±0.51	<0.01	<0.01	8	+8.21±0.99	<0.01	<0.01	8	+0.33±0.21	0.1	0.5
Pentose, total	8	−0.14±0.05	0.1	0.6	8	−0.08±0.05	<0.01	0.8	8	−0.79±0.10	<0.01	<0.01
½ 2,3-DPG	4	+0.29±0.16	0.2	0.6	2	+0.25±0.05	0.1	0.1	3	−0.70±0.06	0.5	0.6
Ketose	7	+0.65±0.22	0.3	0.3	7	−0.27±0.10	0.1	0.7	7	+1.68±0.15	0.02	0.02
½ Triose	6	+0.73±0.11	0.5	0.05	5	−0.37±0.16	0.2	0.4	5	+0.70±0.36	0.5	0.9
½ Pyruvate	7	−0.10±0.05	0.5	0.9	7	+1.57±0.25	0.8	>0.9	7	+0.50±0.06	0.2	0.2
Total balance of intermediates		+0.28±0.83				−1.49±1.15				+1.42±0.65		

* See footnotes to Table I.
† p_n and p_s are the probability values determined from the means of the normal and nonsplenectomized groups, and from the splenectomized and nonsplenectomized groups, respectively.

rose in the endogenous system to less than one-fourth of the normal level; in the iodoacetate system, approximately one-half of the normal ketose accumulation took place. Specific enzymatic assay for F-1,6-P, G-3-P, and DHAP indicated that an average of 60% of the total ketose measured was F-1,6-P, and 60% of the triose phosphate measured was DHAP. Both the enzymatic and spectrophotometric measurements showed parallel increases in concentration with incubation, indicating that the metabolic changes in ketose and triose concentrations were, in fact, changes in F-1,6-P and DHAP, respectively. Ketose and triose phosphate accumulation, and the metabolic imbalance that results, could be prevented in the normal endogenous system by incubation in an atmosphere of 5% CO_2 instead of air.

Glucose utilization in the presence of iodoacetate. Although the normal and HS erythrocytes had nearly the same rates of glucose utilization (and lactate production) in both the endogenous and methylene blue suspensions, different rates were observed in the iodoacetate suspensions (Table III). Glucose was utilized at only one-third the normal rate by HS erythrocytes. This observation was made in a system where the rate of glycolysis was considerably slowed by iodoacetate. The difference is quantitatively small, but statistically significant ($p < 0.01$).

The metabolism of pentose phosphates. Pentose phosphate changes were not significantly different between HS and normal erythrocytes in the endogenous and iodoacetate suspensions. There was, however, a striking difference observed under the influence of methylene blue (Figure 2). In the normal erythrocytes, total pentose increased during most of the incubation period; in the HS erythrocyte, the increase was considerably less during the first 30 minutes, and then proceeded to decrease. The decreased accumulation of total pentose in the methylene blue suspension was found to be due almost entirely to a decrease in the fraction of bound pentose (combined in nucleoside and nucleotide forms). Free pentose phosphate was observed to rise during the initial 30 minutes of incubation, with a subsequent fall in concentration on further incubation in both HS and normal erythrocytes. Nucleoside and nucleotide pentose did not rise during the incubation of the HS erythrocytes, in contrast to the progressive rise in concentration observed in normal erythrocytes, a rise due to nucleosides or nucleotides other than ATP. Although the changes observed represented less than 10% of the total pool of pentose-containing compounds, the differences were statistically significant ($p < 0.01$).

Metabolism of 2,3-DPG. There was a greater accumulation of 2,3-DPG in the endogenous HS cell suspension than in the normal cell suspension. This increase was statistically significant ($p = 0.05$). In the normal cell, the accumulation of 2,3-DPG in the endogenous system (0.04 μmoles per ml erythrocytes; Table III) represented less than 1% of the total concentration of 2,3-DPG (4.5 μmoles per ml erythrocytes). In the HS cell, where the total concentration of 2,3-DPG was found to be normal, the increased accumulation of 2,3-DPG represented an increase of 7% of the total concentration. In the methylene blue and iodoacetate systems, the metabolism of 2,3-DPG proceeded normally in the HS cell.

Alteration of pyruvate: lactate ratios by methylene blue. In the methylene blue system, lactate production decreased, but pyruvate increased. Lactate was expected to decrease as glucose carbon was lost by CO_2 evolution. The increase in pyruvate, however, indicates that a deficiency in DPNH developed, preventing the conversion of some pyruvate to lactate. Such a deficiency could have occurred if there was an accelerated degradation of some of the lower 3-carbon metabolites leading to pyruvate, such as the degradation of 2,3-DPG in the iodoacetate system, but the degradation of these metabolites was not of sufficient magnitude in the methylene blue system to account for the considerable increase in pyruvate that took place (Table III). Apparently, pyruvate accumulated because methylene blue was instrumental in oxidizing DPNH to DPN, thereby depriving pyruvate of the cofactor necessary for its conversion to lactate. This action of methylene blue as a DPNH oxidant for erythrocytes was recently described by Fornaini, Leoncini, Luzzatto, and Segni (18), and our results tend to confirm their observations.

In the nonsplenectomized group of patients (Table IV), glucose utilization and lactate production were higher than in the normal or splenectomized groups, probably reflecting a younger mean cell age. A specific correlation, however,

could not be made between the degree of increase in glucose utilization and the number of reticulocytes. Despite a wide variation among the individual samples, the results were similar to those observed in studies of erythrocytes from splenectomized patients. There was little ketose accumulation in the endogenous suspension and no accumulation of total pentose in the methylene blue suspension. There was, however, an initial rise in the combined pentose fraction not found in the splenectomized group, followed by a precipitous fall after 60 to 90 minutes to below its preincubation level.

DISCUSSION

The data have shown that both normal and spherocytic erythrocytes have nearly identical glycolytic rates. Therefore, it would seem that the cellular abnormality in HS is not due to an inability to convert glucose to lactate along the classical glycolytic pathway. Nonetheless, the following dissimilarities between normal and HS erythrocytes have been demonstrated: *a*) there is no accumulation of bound (nucleoside and nucleotide) pentose in the spherocyte in the presence of methylene blue, *b*) there is less accumulation of ketose phosphate in the spherocyte, and *c*) glycolysis in the spherocyte appears to be more sensitive to iodoacetate.

At present, the cause of the latter two observations is not clear. The ketose accumulating in the normal endogenous cell system consists principally of F-1,6-P apparently originating from a precursor other than glucose. The precursor's identity is unknown; we were unable to detect any accumulation of G-6-P in any of the incubation systems employed. Other possible precursors include G-1,6-P or glycogen. Bartlett (19, 20) has reported the presence of both of these compounds in normal erythrocytes. As for the increased sensitivity of the spherocyte to iodoacetate, a glycolytic inhibitor, others have shown the spherocyte to be abnormally sensitive to fluoride, also a glycolytic inhibitor (3).

The inability of the spherocyte to accumulate combined pentose could have resulted from a decreased production of free pentose phosphate. The integrity of the hexose monophosphate shunt, however, by which free pentose phosphates can be produced, was unimpaired. The spherocyte oxidized a normal amount of glucose to pyruvate and lactate in the presence of methylene blue (Table III). Also, exploratory experiments in our laboratory showed that the rate of $C^{14}O_2$ production from uniformly C^{14}-labeled glucose in the presence of methylene blue was not significantly altered in HS erythrocytes. The unimpaired ability of the HS erythrocyte to produce free pentose phosphate is further shown by the data illustrated in Figure 2; during the first 30 minutes of incubation, the production of free pentose increased appreciably in the spherocyte, as it did in the normal erythrocyte.

An inability of the HS erythrocyte to utilize effectively the free pentose it produced could also explain its failure to form combined pentose. In that case, free rather than combined pentose might be expected to accumulate. The kinetic data of Figure 2 show no such accumulation. The two utilization curves for free pentose are not markedly dissimilar.

In view of the apparently normal production and utilization of free pentose, the failure of the HS erythrocyte to accumulate combined pentose when methylene blue is available is most likely a failure of some later reaction in the biosynthesis of a bound (nucleotide) pentose. The identity of that compound is unknown. It is unlikely to be ATP, which is effectively maintained at a normal level by the HS erythrocyte. Nor is it likely to be a pyridine nucleotide; Leder and Handler (21) have shown that variations in the concentration of pyridine nucleotides do not occur in the presence of methylene blue, and therefore their synthesis could not account for the increase in combined pentose that is discussed here.

None of the abnormalities reported for hereditary spherocytosis prevent the spherocyte from converting a normal quantity of glucose to lactate, or from maintaining a normal concentration of ATP. Also, the hexose monophosphate pathway is intact, and the significance of the pentose abnormality reported here remains to be established. This raises the question whether the intrinsic defect in the HS erythrocyte is primarily a defect in carbohydrate metabolism, or whether the metabolic abnormalities that we and others have observed are secondary to some other alteration. It is conceivable, for example, that an improperly

constituted membrane could explain many of the abnormalities reported in hereditary spherocytosis; an alteration in permeability or of an active transport mechanism could upset the normal passage of fluoride, iodoacetate, and P^{32} into the cell, and could also produce the structural change in the spherocyte, with its increased osmotic fragility.

SUMMARY

Carbohydrate metabolism has been studied in erythrocytes of hereditary spherocytosis by the measurement of changing concentrations of metabolites in incubating plasma-erythrocyte suspensions, and modifications by the addition of methylene blue or iodoacetate have been evaluated. The most striking difference was the failure of pentose to accumulate in the methylene-blue-treated spherocyte. The possible mechanisms of this finding were discussed. Also, alterations in the metabolism of fructose diphosphate, 2,3-diphosphoglycerate, and dihydroxyacetone phosphate were observed, and there was an increased sensitivity to the inhibitory effects of iodoacetate.

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