Investigation of the Defect in a Variant of Hereditary Methemoglobinemia

By Philip L. Townes and Martin Morrison

A variant of hereditary methemoglobinemia exhibiting dominant inheritance of normal methemoglobin has recently been reported. Although the TPN and DPN methemoglobin reductases were found to be normal, an inability to utilize glucose for the reduction of methemoglobin was demonstrated. The present report describes further attempts to define more definitely the specific metabolic defect responsible for this type of hereditary methemoglobinemia.

METHODS

The hemoglobins were prepared in the manner previously described. A self-recording spectrophotometer (Perkin-Elmer Spectracord) employed for the spectrophotometric analyses was standardized against a hydrogen arc as reported. A Beckman DU spectrophotometer or a Bausch and Lomb Spectronic 20 was used for optical density measurements in the various enzyme assays, which were performed using methods cited below. The terms "subject" and "patient" refer to the same methemoglobinemic individual reported previously.

RESULTS

The methemoglobin present in this variant has been extensively studied and found to be indistinguishable from normal methemoglobin. The following observations were made in order to either confirm that the methemoglobin is normal or demonstrate some subtle abnormality which might have escaped detection. In figure 1 are shown absorption spectra of hemoglobins from the patient and a normal control before and after dithionite reduction. Carbon monoxide was used to stabilize the spectra by preventing the appearance of the 720 mμ peak of reduced hemoglobin which might otherwise obscure the reduction of the 630 mμ maximum of methemoglobin. Dithionite reduction spectra without carbon monoxide, as well as fluoride and azide spectra, were also examined. All spectra were identical with corresponding control oxyhemoglobin-methemoglobin mixtures when examined under the same conditions.

Although the spectral data are characteristic of normal methemoglobin, the possibility remains that the hemoglobin might have a subtle abnormality which facilitates oxidation to methemoglobin. One means of evaluating this possibility is a study of the rates of conversion of hemoglobin to methemoglobin.

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by low concentrations of potassium ferricyanide. To 2.7 ml. of appropriate buffer was added 0.3 ml. (0.4 Gm. per cent) hemoglobin solution and the optical density at 630 mμ was recorded and followed as a function of time after the addition of 0.1 ml. (0.01 M) potassium ferricyanide. It was noted the oxidation proceeded instantaneously under acid conditions (pH 5.0 acetate
0.1 M) while at pH 7.0 and 8.0 (phosphate buffer 0.1 M) the rates were convenient for accurate measurement. There were no differences between the subject’s hemoglobin and normal hemoglobin in rate of methemoglobin formation. The results of this graded oxidation by potassium ferricyanide as a function of pH are depicted in figure 2.

Conceivably, an abnormal tendency to undergo oxidation to methemoglobin may be a property of the patient’s deoxygenated hemoglobin. For this reason, hemoglobins (subject’s and control) were subjected to reduced pressures of oxygen and the rates of methemoglobin formation compared. Partial deoxygenation (50 per cent) was accomplished by placing the solutions in a sealed jar and reducing the partial pressure of oxygen to 30 mm. Hg. After 24 hours the solutions were reoxygenated by shaking with air and the methemoglobin concentrations determined. No demonstrable differences were noted. It seems that the subject’s hemoglobin does not have a greater than normal tendency to undergo oxidation to methemoglobin in the deoxygenated state.

Our observations were limited by the fact that the methemoglobin comprised but a small proportion of the total hemoglobin. To characterize the methemoglobin under more optimal conditions required its separation from oxyhemo-
A VARIANT OF HEREDITARY METHEMOGLOBINEMIA

63

globin. Such separation would then permit "pure" rather than mixed spectra and thereby increase the likelihood of detecting minor spectral aberrations. Employing the fractionation procedure of Morrison and Cook,2 the subject's hemoglobin was placed on an IRC-50 column. The oxyhemoglobin was eluted by phosphate buffer pH 6.3 containing 130 mEq. sodium/liter, while the methemoglobin was isolated with no gross contamination by elution with phosphate buffer containing 425 mEq. sodium/liter (pH 6.3). Absorption spectra of the methemoglobin and cyanmethemoglobin formed by reacting with KCN were identical with normals. The oxyhemoglobin was also spectrally normal. These observations confirm previous results1 from which it was concluded that the methemoglobin under investigation is normal methemoglobin.

Gerald3 and Pisciotta et al.4 have reported that hemoglobin M may be associated with an increase in alkaline-resistant hemoglobin. The subject's hemoglobin was examined in this respect, using the method of Singer et al.,5 and found to contain a normal proportion of alkaline-resistant hemoglobin (0.43 per cent).

Red cell osmotic fragility was determined by the method of Sanford.6 After two hours at room temperature, initial hemolysis was detected in 0.44 per cent sodium chloride and complete hemolysis occurred in all tubes below 0.36 per cent sodium chloride. Identical results were obtained in simultaneously run controls.

While routine examination of the patient's blood smear revealed no abnormality of erythrocyte morphology, it was considered that viewing a fresh suspension of red cells in normal saline through a filter having peak transmittance at 620 m$\mu$ might reveal two distinct populations of cells. Those having a relatively high concentration of methemoglobin would be expected to appear dark while those with low (normal) amounts of methemoglobin would appear brighter. When viewed under these conditions, all erythrocytes were essentially of identical appearance, indicating that the methemoglobin is not limited to certain cells but that it is probably of rather homogenous distribution.

It has been demonstrated1 that red cells of this variant suspended in glucose-containing phosphate-saline do not evidence any increase in rate of methemoglobin reduction, despite the fact that the TPN and DPN methemoglobin reductases are normal. It was proposed,1 on the basis of these observations, that the inability to utilize glucose for the reduction of methemoglobin in a normal manner reflected some specific but undefined metabolic defect of carbohydrate metabolism. The analysis which follows is an attempt to more specifically characterize this defect through an examination of relevant enzyme systems and reducing substances.

Methemoglobin reductase: Hereditary methemoglobinemia has, in some instances at least, been conclusively shown to result from a deficiency of methemoglobin reductase. While this is the only enzyme system so implicated, both TPN and DPN methemoglobin reductase were found to be normal in this subject.1

Catalase: Although acatalasemic individuals are not known to be grossly cyanotic, they have not been extensively examined for possible methemo-
globinemia. It is possible that a deficiency of this enzyme could cause methemoglobinemia by allowing an accumulation of peroxides which might act as oxidants of hemoglobin. Catalase was assayed by the procedure of Beers and Sizer and found to be normal: 68.0 U/mg. Hb, 65.5 U/mg. Hb, for patient and control, respectively.

Glucose 6-phosphate dehydrogenase: This enzyme is an important source of reduced TPN and as such could limit the activity of TPN-methemoglobin reductase. The patient’s blood was found to have high normal glucose 6-phosphate dehydrogenase activity (203 enzyme U/100 ml. RBC) when assayed by the method of Zinkam.

Triosephosphate dehydrogenase: As a major source of reduced DPN, this enzyme, if deficient, could be rate limiting for the methemoglobin reductase requiring DPNH. When assayed by the method of Krebs, only normal levels of triosephosphate dehydrogenase activity were found.

Lactic acid dehydrogenase: Whole blood was assayed for this important source of reduced DPN, using the method of Wroblewski et al., and found to be normal: 15,600 LAD U/ml. RBC and 20,800 LAD U/ml. RBC for patient and control, respectively.

Total pyridine nucleotides: While the various enzymes assayed proved to be normal, all assay methods required addition of the appropriate pyridine nucleotide. As such, a relative deficiency of one of the pyridine nucleotides would not be detected if this were the basis of the defect. The subject’s red cells were therefore examined for total pyridine nucleotide content, employing the acetone condensation fluorometric method of Levitas et al. using N1-methyl-nicotinamide as an internal standard. The fluorescence measurements were made on a Beckman DU spectrophotometer with a fluorometer attachment at 440 µm. The subject’s blood was found to contain 83.3 ¿g. pyridine nucleotide/ml. RBC, while a normal control contained 95.0 ¿g. pyridine nucleotide/ml. RBC. Both values are within the normal range. This determination is a measure of the total pyridine nucleotides and does not reflect the proportions of DPN, TPN and their reduced forms. Employing the method of Bassham et al., the ratio of TPN to DPN was also determined and found to be normal.

Glutathione: The glutathione content of the patient’s erythrocytes was determined, using the method of Beutler, and found to be markedly depressed; namely, 25–28 mg. reduced glutathione/100 ml. RBC. This abnormal value was obtained on repeated examination of different samples of freshly drawn blood and independently confirmed in another laboratory. Despite the markedly depressed glutathione content, the cells did not evidence any glutathione instability when incubated with acetylphenylhydrazine in the standard test method of Beutler. The normal glucose 6-phosphate dehydrogenase activity and normal glutathione stability of these cells clearly indicate that this

*We wish to acknowledge the aid of Dr. J. McWhinney in whose laboratory the determination was confirmed. The value obtained on duplicate analysis (29 mg. per cent) was beyond two standard deviations from normal. Normal range for the laboratory is 54–88 mg. per cent, with a mean of 74 mg. per cent in 13 consecutive determinations of normal individuals.
A variant of hereditary methemoglobinemia is not an example of primaquine sensitivity. A corollary of this inference is that there is no apparent explanation for the low glutathione content in these cells. Furthermore, it raises the question as to what, if any, is the relationship between the low glutathione and the methemoglobinemia. The observations which follow were made in an attempt to better define glutathione metabolism in this variant and therewith provide a basis for determining the cause of the methemoglobinemia.

**Glutathione reductase** is known to be of major importance in maintenance of normal levels of reduced glutathione in the human red cell. This enzyme was assayed using a modification of the methods described by Schrier et al. and Carson et al.

Fresh hemolyzates, both patient's and control, were clarified by high speed centrifugation (25,000 x g) at 5°C and dialyzed against 0.067 M phosphate buffer pH 7.4. To 2.5 ml. 0.2 M tris(hydroxymethyl)aminomethane buffer pH 7.42 were added 1.0 ml. 0.1 M EDTA, 10 μM oxidized glutathione and 1.0 ml. dialysate. TPNH (12.6 μM) was added and final volume made up to 14.0 ml. with resin treated distilled water. Final buffer concentration was 0.036 M. Blanks contained all of the above components except that TPNH was omitted. All assays were made in a 37°C constant temperature bath. Glutathione measurements were made at regular intervals by placing 4.0 ml. aliquots of the incubation mixture in 2.8 ml. 4.5 per cent metaphosphoric acid using the method of Beutler et al. Under these conditions there were no measurable amounts of reduced glutathione in the blank tubes to which TPNH had not been added. The subject's dialysates were found to reduce glutathione at a rate equal to that of normal dialysates, indicating that the glutathione reductase activity was normal.

**Glucose 6-phosphate dehydrogenase—glutathione reductase:** An alternative method of evaluating the glutathione reducing capacity of the patient's cells was accomplished by examining the over-all coupled reaction of glucose 6-phosphate dehydrogenase and glutathione reductase. The conditions of assay were essentially as described above except that TPN (15 μM) and glucose 6-phosphate (10 μM) were substituted for the TPNH. The glucose 6-phosphate dehydrogenase is then the generator of the TPNH required by glutathione reductase. Under these conditions the subject's hemolysates were found to have somewhat higher than normal rates of glutathione reduction. This is due to the fact that the coupled reaction is more directly a reflection of the glucose 6-phosphate dehydrogenase activity, since it is known to be the rate limiting reaction in the coupled system. Since the subject's glucose 6-phosphate dehydrogenase activity has been directly shown to be high normal, it is understandable that the coupled system should be higher than normal. Examination of the coupled system does, however, indicate that the glutathione reductase system is able to utilize the reduced form of TPN as it becomes available from the dehydrogenase activity.

**Total glutathione:** Glutathione determinations as generally performed do not measure the oxidized form and as such are of little value in estimating total glutathione. An approximation of the total red cell glutathione can be made.
by establishing conditions which promote reduction of oxidized glutathione. For this purpose, glutathione determinations were made on fresh hemolyzates and further determinations were made at intervals of time after the addition of TPNH. Controls consisted of equal aliquots of hemolyzates to which no TPNH was added. Since it had been demonstrated that the hemolyzates had normal glutathione reductase activity, the addition of TPNH would be expected to promote reduction of endogenous oxidized glutathione. Comparison of the amounts of glutathione present in the two series indicated that there was an increase of 20-30 per cent in reduced glutathione over that found in the tubes which contained no added TPNH. The glutathione content of the latter remained essentially unchanged throughout the incubation period. Similar results were obtained with hemolyzates of normal red cells.

As a further modification of this procedure, known amounts of oxidized glutathione were added and the initial rates of reduction of the added substrate determined. Again the rates of formation of reduced glutathione in normal hemolyzates and in hemolyzates from the methemoglobinemic patient were essentially the same. From these observations it is concluded that the ratio of oxidized to reduced glutathione is essentially normal; therefore, not only is the reduced form deficient in this variant but the total is proportionally deficient as well.

*Methemoglobin reduction by glutathione:* There are numerous references, primarily indirect, suggesting that glutathione may be directly (non-enzymatically) involved in the normal reduction of methemoglobin. The possibility that the methemoglobin of this variant results directly from a lack of glutathione was investigated through an examination of the efficacy of exogenous glutathione in reducing methemoglobin. Fresh hemolyzates were examined spectrophotometrically, and the spectral changes subsequent to the addition of known amounts of glutathione were followed at regular intervals. The data presented in figure 3 indicate that glutathione is a poor reductant of methemoglobin. This is more clearly seen when glutathione is compared to ascorbic acid (fig. 3). The effect of glutathione on direct methemoglobin reduction was studied both aerobically and anaerobically. In the latter case, a Thunberg-cuvette was employed and glutathione was added from the side arm after evacuation and anaerobic base line spectra had been obtained. The effectiveness of glutathione was the same under aerobic or anaerobic conditions. These findings, while at variance with the brief report of Morrison claiming that methemoglobin was readily reduced by glutathione, are in complete agreement with the findings of Gibson and Jaffe et al., who concluded that it was relatively ineffective. These observations indicate that the methemoglobin does not result from a lack of a glutathione acting as a direct reductant and suggests a more indirect effect.

*Stability of glutathione in hemolyzates:* The possibility that the reduced amounts of red cell glutathione is the result of some ill-defined factor which promotes its degradation was examined and excluded in the following way. To fresh hemolyzates (1 part packed washed cells in 2 volumes resin-treated, distilled water) was added 3 mg. reduced glutathione, to give a final concentra-
A VARIANT OF HEREDITARY METHEMOGLOBINEMIA

Glutathione determinations were then made at regular intervals over a period of three hours. The glutathione content remained essentially unchanged in control and patient's hemolysates. There was no evidence to suggest that there was any abnormal destruction.

Stability of glutathione in washed cell suspensions: To aliquots of washed red cells suspended in phosphate-saline was added reduced glutathione to a final concentration of 100 mg. per cent. Serial glutathione determinations were then made during periods of incubation up to six hours. It was found that the stability of reduced glutathione under these conditions was pH dependent. At pH 6.5, the glutathione underwent very gradual but regular disappearance, while at pH 7.5 the disappearance was much accelerated. From permeability considerations it is assumed that little or no glutathione entered the red cells. The methemoglobin concentration of the red cells remained unchanged.

Effects of reduced pyridine nucleotides on methemoglobin reduction: Both TPNH and DPNH were added to methemoglobin containing hemolysates and found to be ineffective in directly reducing methemoglobin. Ascorbic acid (fig. 3) on the other hand was found to be a good reductant. These observations are in agreement with the results of Jaffe and others.

Effect of lactate on methemoglobin reduction: Washed red cells were nitrated, washed and suspended in saline-phosphate employing methods described. To one series of tubes was added sodium lactate (0.02 M final conc.), to another glucose (0.01 M final conc.) and to the third an equal additional volume of saline-phosphate. The effectiveness of lactate and the ineffectiveness of glucose in promoting reduction of methemoglobin in this variant is shown (fig. 4). The ineffectiveness of glucose under these conditions has also been previously demonstrated. These observations indicate that the metabolic block lies between glucose and lactate.

Serial lactate determinations were made on suspensions of washed cells in saline-phosphate containing 0.01 M added glucose. The results of this experiment are shown in figure 5. The rate of lactate formation is significantly lower that that of normal control cells.

Further evidence of an impairment of lactate production was obtained by incubating washed cells in phosphate-saline containing glucose labeled with C14 at the C1 and C6 position following the procedure of Lerman. Measurements of the labeled carbon dioxide produced indicated that the ratio of C14/C6/C14 is 1:5-3.0 times that observed in normal cells. This observation is further confirmation of a block in the Embden-Meyerhof pathway.

DISCUSSION

The variant of methemoglobinemia described in this paper has been shown to differ from the two generally recognized types of hereditary methemoglobinemia. It differs from the recessive-enzyme deficiency type in that the inheritance is dominant and there is no deficiency of TPN or DPN methemoglobin.

*We wish to acknowledge the kindness of Dr. S. Lerman, University of Rochester School of Medicine, for performing the C14 analysis.
Fig. 3.—Relative effectiveness of glutathione and ascorbic acid in reducing methemoglobin. Plotted curves are changes in optical density (630 mμ) as a function of time after the addition of 0.037 mM reduced glutathione or 0.042 mM ascorbic acid. All final volumes 3.5 ml. in 0.3 M phosphate buffer pH 7.4. Spectra from which plotted points were obtained are shown in inserts. The uppermost curve of each insert was not plotted, for this represents tracing prior to the dilution which occurred when the reductant was added. The lowermost tracing of the GSH insert was also not plotted, for this represents formation of cyanmethemoglobin (zero per cent methemoglobin) resulting from addition of potassium cyanide.

reductase. It differs from methemoglobin M disease in that the methemoglobin is normal.

The findings that the hemoglobin does not have an increased tendency to undergo oxidation in response to low concentrations of ferricyanide, nor auto-oxidation anaerobically, and the fact that the absorption spectrum of the isolated pigment is typical of normal methemoglobin lend support to the previous findings and conclusion\(^1\) that there is no intrinsic hemoglobin abnormality in this variant.

The relative deficiency of glutathione is not due to an inadequacy of factors responsible for maintaining glutathione in the reduced state. This is supported by the fact that glucose 6-phosphate dehydrogenase and glutathione reductase, both individually and as coupled systems, are intact. That abnormal destruction, either oxidation or other degradation, is not involved is shown by the normal glutathione stability, the normal maintenance of reduced glutathione added to hemolysates, and the fact that there is no abnormal increase in the oxidized fraction at the expense of the reduced form. Since there
Fig. 4.—Relative effectiveness of glucose (0.01 M) and lactate (0.02 M) on methemoglobin reduction of patient's nitrited, washed cells suspended in saline-phosphate, pH 7.4.

is no evidence of inadequate reduction nor accelerated degradation, it is most probable that the primary disturbance is one of glutathione synthesis. This would account for the fact that while the ratio of reduced and oxidized glutathione is normal, the total is markedly abnormal.

The possibility that the increased methemoglobin per se is the direct cause of the lowered intracellular content of glutathione is doubtful, but worthy of consideration. Beutler demonstrated that oxyhemoglobin is an essential component of the system which leads to a lowering of red cell glutathione in response to acetylphenylhydrazine and that methemoglobin is not significantly more effective than oxyhemoglobin. Further evidence is provided by Scott and Hoskins who reported normal glutathione values in a patient having severe methemoglobinemia (30 per cent of the total hemoglobin). The methemoglobin in this instance, as in ours, is normal methemoglobin. It is therefore unlikely that a methemoglobin level of 5–6 per cent, as in our patient, could result in a depression of the glutathione to a level of less than 50 per cent of normal. Further confirmation that the glutathione content in this variant is not directly influenced by the methemoglobin level was obtained by discontinuing ascorbic acid therapy for 10 days. During this period the methemoglobin rose from a
Fig. 5.—Comparison of rates of lactate formation in washed red cells suspended in phosphate-saline, pH 7.4 containing 0.01 M added glucose.

level of 6 per cent to 14 per cent while the glutathione content remained unchanged. Conversely, considerable evidence has been presented to indicate that that low glutathione content per se does not result in an accumulation of methemoglobin due to a lack of direct reduction by glutathione. These considerations clearly indicate that the primary disturbance is one of glutathione deficiency which indirectly produces methemoglobinemia in this variant.
A VARIANT OF HEREDITARY METHEMOGLOBINEMIA

The fact that enzymatic reduction of methemoglobin is a property of intact cells and cannot be demonstrated in hemolysates places some limitation on a direct analysis and proof of the nature of the defect. In essence, one cannot simply introduce the required product of the deficient enzyme system and therewith demonstrate with precision the site of the metabolic disturbance. The approach, of necessity, must be less direct and based on deduction and inference.

That methemoglobin reduction is normally dependent on glucose metabolism has been established by many investigators. Gibson clearly demonstrated that such reduction could occur through either the Embden-Myerhof cycle or the hexosemonophosphate shunt. According to Gibson the hexosemonophosphate shunt is normally of minor significance because of the lack of a suitable electron donor. An adequate substitute electron donor is methylene blue. That the patient's hexosemonophosphate shunt is intact has been demonstrated previously by the rapid response to intravenous methylene blue. The Embden-Myerhof cycle then remains as the most probable site of metabolic disturbance producing this methemoglobinemia. Unlike other examples of hereditary methemoglobinemia, this block within the Embden-Myerhof cycle is not at the level of the DPNH-methemoglobin reductase for this has been shown to be normal. Therefore, it must occur somewhere within the cycle at a level of DPNH formation. The two most significant sources of DPNH in the glycolytic scheme are lactic acid dehydrogenase and triosephosphate dehydrogenase. Both enzymes have been assayed and found to be normal. That lactic acid dehydrogenase is also normal in the intact cells is indicated by the fact that red cells incubated in lactate do exhibit accelerated rates of methemoglobin reduction. Presumably this is due to the formation of DPNH which is then utilized by the methemoglobin reductase. In the assay for triose phosphate dehydrogenase, it is necessary to add relatively high concentrations of reduced glutathione. Without it there is no measurable activity. It is concluded that the low glutathione results in impairment of triosephosphate dehydrogenase activity and thereby an impairment of methemoglobin reductase. This interpretation is consistent with and supported by all of the findings in this study. If this is so, one would expect that lactate production would be depressed, and this has proven to be the case.

The proposed mechanism of this variant of hereditary methemoglobinemia is then as follows: the primary defect is one of glutathione synthesis, the deficiency of which then results in an impairment of triosephosphate dehydrogenase activity leading to an inadequate formation of the DPNH required by the methemoglobin reductase. All of our experimental observations are consistent with this hypothesis.

SUMMARY

1. Further evidence has been presented to confirm the fact that the methemoglobin found in a new variant of hereditary methemoglobinemia was normal methemoglobin.

2. The reduced glutathione content of the red cells of this variant was less than 50 per cent of normal.
3. The total glutathione and oxidized glutathione were proportionally deficient.

4. The low glutathione content did not result from abnormal degradation nor lack of adequate reducing mechanisms. The primary defect was considered to be one of inadequate glutathione synthesis.

5. Various enzymes were assayed, including the following: glucose 6-phosphate dehydrogenase, lactic acid dehydrogenase, triosephosphate dehydrogenase, glutathione reductase, glucose 6-phosphate dehydrogenase-glutathione reductase (coupled system) and catalase.

6. This variant of methemoglobinemia was considered to result from inadequate synthesis of glutathione. The deficiency of this essential co-factor apparently results in an impairment of triosephosphate dehydrogenase activity and consequently insufficient reduction of DPN, an essential component of the DPNH-dependent methemoglobin reductase.

**SUMMARIO IN INTERLINGUA**

1. Es presentate nove datos a corroborar le theses que le methemoglobina trovate in un nove variante de methemoglobinemia hereditari es methemoglobina normal.

2. Le contento de reducite glutathion in le erythrocytos de iste variante esseva minus que 50 pro cento del contento normal.

3. Le glutathion total e le glutathion oxydate esseva proportionalmente deficiente.

4. Le basse contento de glutathion non resultava ab un degradation anormal e non ab un manco de mechanismos reductori. Esseva opinate que le defecto primari consisteva in un inadequate synthese de glutathion.

5. Varie enzimas esseva essayate, incluse dishydrogenase de glucosa-6-phosphato, dishydrogenase de acido lactic, dishydrogenase de triosephosphato, reductase de glutathion, sistema accopulato de dishydrogenase de glucosa-6-phosphato e reductase de glutathion, e catalase.

6. Esseva conclutite que iste variante de methemoglobinemia resulta de un inadequate synthese de glutathion. Le deficientia de iste indispensabile co-factor resulta apparentemente in un inadequate activitate de dishydrogenase de triosephosphato e consequentemente in un insufficiente reduction de DPN, que es un component essential del reductase de methemoglobina con su dependencia ab DPNH.

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