Heinz Body Anemia—An Ultrastructural Study.
I. Heinz Body Formation

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Despite the technical refinements of contemporary morphology the structural, and especially ultrastructural aspects of both normal and pathologic red blood cell destruction remain incompletely understood. To gain further insight into the biologic events which occur during hemolysis requires specific criteria for the identification of injured red cells which permit recognition of the terminal events in their destruction. The hemolytic system induced in rabbits by the administration of phenylhydrazine is particularly suitable for this purpose. Like a variety of drug-induced hemolytic anemias, it is characterized by the oxidative denaturation of red cell components, most prominently hemoglobin.1 This results in the precipitation, within the red cell, of relatively insoluble products of hemoglobin denaturation, termed Heinz bodies, which can serve as markers for the drug-injured cell. The observations of Jandl et al.7 suggest, moreover, that oxidative denaturation of red cell components, including hemoglobin, may be part of the process of red cell senescence leading to normal red cell destruction. The present study was, therefore, undertaken to investigate the ultrastructural aspects of Heinz body formation as a model hemolytic system. The process of red cell sequestration and destruction in this hemolytic anemia will be described in a subsequent communication.8

Materials and Methods

Rabbits were injected with phenylhydrazine (25 mg./day, I.M.) on 4–5 successive days and blood samples were taken for morphologic study by ear vein or cardiac puncture at various intervals. Heinz body development was also followed in red cells isolated in vivo within millipore-type diffusion chambers, made according to the method of Algire8 and implanted within the peritoneal cavity of rabbits receiving parenteral phenylhydrazine. Heinz body-containing red cells were produced in vitro by a modification of the method of Beutler et al., substituting phenylhydrazine for acetylphenylhydrazine. Samples were taken from the incubation flask at intervals between 1 minute and 3 hours. Blood was examined by phase microscopy and prepared for electron microscopy by fixation in 1 per cent glutaraldehyde in .067 M phosphate buffer, then washed with buffer, postfixed with osmium tetroxide, dehydrated, and embedded in EPON 812 resin. Sections were then stained with uranyl acetate (1 per cent in 50 per cent ethanol), lead citrate, or both and examined in an RCA EMU-2E electron microscope.

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885

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RESULTS

Heinz Body Formation in Vitro

During exposure of rabbit blood to phenylhydrazine in vitro, Heinz bodies appear rapidly. By phase contrast microscopy they may be observed as small, dense bodies, as large as 0.7 μ in diameter, within 5 to 8 minutes (fig. 1). The first changes seen in electron micrographs of thin-sectioned red cells appear within 3 minutes of the start of incubation with the drug. These earliest alterations consist of collections of small flocculent granular densities, approximately 30 μ in diameter (fig. 2). They are free in the cytoplasm and do not contact the plasma membrane at this stage. Undoubtedly each aggregate of granules is equivalent to one of the dense bodies seen by phase microscopy (fig. 1). The small granular bodies show considerable variation in their electron density (especially notable after staining with lead salts) and ultrastructural organization. The more peripheral, smallest, and youngest floccules in each aggregate are often heavily stained and display a highly ordered arrangement of densities, 50–55 Å in diameter, with a center-to-center spacing of 70 Å suggesting a crystalline structure (fig. 3). At their central and older portions the aggregates are less heavily stained, amorphous, and more confluent. Occasionally, the successive development of fresh areas of hemoglobin denaturation is suggested by such differences in the ultrastructure of adjacent Heinz bodies (fig. 4).

Within 5 to 10 minutes of incubation in phenylhydrazine the small flocculent, crystalline densities which constitute the initial morphologic lesion, become confluent and migrate toward the plasma membrane (fig. 5). This will be termed the intermediate-stage Heinz body. The large, definitive Heinz bodies, which lie directly under and in intimate contact with the plasma membrane, thus appear to result from condensation, coalescence and migration of the initial small bodies (fig. 6). These end-stage Heinz bodies may reach large size, and severely distort the cell surface as a consequence of their rigid structure. Despite such deformation of the plasma membrane, continued observation of these cells by phase contrast microscopy for several hours suggested that little if any direct and spontaneous hemolysis occurred under these conditions.

Heinz Body Formation in Vivo

In the circulating blood of rabbits given 1 or more injections of phenylhydrazine the most prominent lesion is the large Heinz body, adherent to and distorting the plasma membrane, similar to the final stage of Heinz body development observed in vitro (fig. 6). Early forms and intermediate stages are also observed, especially shortly after the initial injection. The sequential stages in development of the Heinz body lesion observed in the in vitro system were confirmed by observing the changes in red cells isolated for up to 24 hours within millipore diffusion chambers implanted in the peritoneal cavities of phenylhydrazine treated rabbits. Under these conditions the coalescence and centrifugal migration of early small granular bodies was apparent. Despite 24 hours of exposure to phenylhydrazine in the diffusion chamber, and progression of the Heinz body lesions from early to late stages, no evi-
Fig. 1.—Phase contrast micrograph of 3 red blood cells, exposed for 5 minutes to phenylhydrazine in vitro, which contain several dense bodies, 0.3–0.7 μ in diameter, comprising the early Heinz body lesions. Mag. x 2400.

Fig. 2.—Portion of a thin-sectioned erythrocyte observed by electron microscopy after 5 minutes incubation with phenylhydrazine. A collection of small (30 mμ in diameter) flocculent densities is observed which constitutes one of the dense bodies seen in figure 1. Mag. x 30,000.

dence for any significant amount of direct hemolysis was observed, as evidenced by the virtual absence of hemolyzed red cell ghosts in this isolated population of red blood cells.

Effect of Phenylhydrazine on Erythrocyte Precursors

Study of the effects of phenylhydrazine upon erythrocyte precursors was facilitated by the brisk hemolytic anemia, reticulocytosis and erythroid hyperplasia induced by the drug. Even after prolonged exposure to phenyl-
Fig. 3.—Higher magnification electron micrograph of the early Heinz body lesion. The precipitated material contains relatively amorphous regions (a), and areas displaying regular arrays of small, 50–55 Å densities (c), with a periodicity of about 70 Å (center-to-center). The very smallest precipitates (arrows) are also composed of small, regularly spaced subunits of the same size and periodicity. Mag. x 165,000.

Phenylhydrazine in vivo, nucleated erythroid cells failed to develop the Heinz body lesion. Likewise, young reticulocytes, identified by their content of clustered ribosomes (polysomes,\textsuperscript{11}) appeared resistant to the denaturative effects of the drug (fig. 7, cell at right). Relatively mature reticulocytes, containing predominantly unclustered or monoribosomes, are sensitive to the drug and develop early (fig. 4), and intermediate stages (fig. 4 and 7, cell at left) of the Heinz body lesion. These small Heinz bodies were very commonly observed in the vicinity of concentrations of mitochondria (fig. 8). The large, condensed and marginated variety of Heinz body was not observed in any but mature red cells.

Alterations in the Plasma Membrane

It has been suggested that in addition to oxidative denaturation of hemoglobin, phenylhydrazine and related drugs produce injury to the erythrocyte
Fig. 4.—Portion of a mature reticulocyte with a high proportion of monoribosomes (arrows). Heinz body material of the early (lower left) and intermediate (upper right) stages of development are observed. Mag. x 45,000.

plasma membrane.\textsuperscript{5,12-14} This damage may, in actuality, be more detrimental to cell survival than the hemoglobin lesion. Evidence for morphologic manifestations of membrane damage was, consequently sought in Heinz body-containing erythrocytes. Figure 9 illustrates an abnormality commonly observed in the region where a large Heinz body contacts the cell surface. In this area the normal bilamellar (or unit) membrane structure appears to be lost and the cell surface is puckered and distorted.

**DISCUSSION**

Previous biochemical studies\textsuperscript{1,3,5,7,15} have suggested that the Heinz body derives from the denaturation of hemoglobin through a sequence of oxidative steps which may involve, as intermediates, methemoglobin and several soluble to insoluble sulfhemoglobin-like denaturation products. The present studies indicated that there are, likewise a characteristic series of ultrastructural alterations in sensitive erythroid cells exposed to a drug capable of inducing the process of oxidative denaturation. The class of compounds capable of inducing Heinz body formation is characterized by a high redox potential. These drugs appear to act as intermediates allowing the oxidative potential of molecular oxygen to act upon hemoglobin.\textsuperscript{1,3,7} It might be anticipated, therefore, that the initial lesions of phenylhydrazine-treated red cells
would be found nearest the cell surface and the source of molecular oxygen. Indeed, observations in a number of laboratories (reviewed in ref. 16) have been adduced as evidence that the Heinz body lesion involves primarily the cell's plasma membrane. To the contrary, however, the earliest morphologic changes, consisting of small, highly ordered, dense precipitates, are observed in the central cytoplasm of both erythrocytes and sensitive reticulocytes (fig. 2). These subsequently enlarge, coalesce, and migrate to lie just beneath and adherent to the plasma membrane (figs. 5 and 6). It is possible that in the intact red blood cell, as opposed to hemoglobin solutions in vitro, some redox intermediate rather than molecular oxygen is the immediate source of oxidative potential. In this regard, it is of interest that in reticulocytes the early Heinz body lesions which develop are found not only in the central cytoplasm, but frequently in close association with clusters of mitochondria (fig. 8). In these cells, which are relatively resistant to the denaturative effects of phenylhydrazine (see below), mitochondria may provide a readily available source of oxidative potential. Alternatively, the significance of the particular locus of early Heinz bodies may lie in a relative deficiency of reduced pyridine nucleotides or reduced glutathione in this region, with a consequentially inadequate repair of oxidative damage.13,17

The chemical nature of the precipitated Heinz body material cannot, of course, be ascertained from its morphologic appearance alone. Nevertheless, the earliest visible lesion has a distinctive, highly ordered and possibly crystal-
HEINZ BODY ANEMIA—AN ULTRASTRUCTURAL STUDY

Fig. 6.—End-stage Heinz bodies lying under and distorting the plasma membrane of a mature erythrocyte. Mag. x 26,000.

line, structure. The repeating electron dense units are 50–55 Å in diameter, a size consistent with that of hemoglobin. This may be adduced as presumptive evidence that an early phase of hemoglobin oxidation results in a uniform molecular population capable of crystallization, with molecular dimensions not too dissimilar to native hemoglobin. It is likely, therefore, that the early crystalline precipitate results from a relatively discrete molecular lesion which does not result in major changes in secondary structure. Oxidative attack at one or more of the porphyrin side chains of the heme moiety is plausible and consistent with the hypothesis that oxidation of heme iron and the local production of H₂O₂ constitute the primary mechanism of drug effect.

Considerable evidence indicates that there is a gradient of increasing susceptibility of erythrocytes to the denaturative effects of phenylhydrazine and related compounds, directly proportional to cell age, and consequent to the progressive loss with age of enzyme systems involved in the protection from and repair of oxidative injury. The present data suggested that this gradient applies, as well, to erythrocyte precursors. Early and intermediate-stage Heinz body lesions are observed in mature reticulocytes (fig. 1), identi-
Fig. 7.—Portions of two adjacent reticulocytes. The cell at upper right is an immature reticulocyte containing numerous polyribosomes. At lower left is a mature reticulocyte containing monoribosomes and a cluster of intermediate-stage Heinz body precipitates. Several small vacuoles and vesicles, which are normally observed in this stage of reticulocyte development, are seen in this cell. Mag. x 32,000.

...fied by their content of monoribosomes. Immature (polyribosome-containing) reticulocytes and nucleated forms appear resistant to the drug. Failure to develop the large, marginated, definitive Heinz bodies, even in mature reticulocytes, probably reflects the partial resistance of these cells to oxidative attack, recognized biochemically by their enhanced capacity to reduce methemoglobin to hemoglobin.

The mechanism whereby the injury induced by phenylhydrazine results in accelerated red cell destruction and anemia is not clearly defined. Suitably large doses of the drug produce a direct lytic effect and massive intravascular hemolysis can be demonstrated. With smaller doses, however, anemia does not appear to occur by this mechanism. It has been suggested that in addition to denaturation of hemoglobin these drugs produce deforma-
Fig. 8.—Numerous early and intermediate-stage Heinz bodies in close association in a relatively mature reticulocyte with 6 mitochondria (m), two large vacuoles (v), and what appear to be small fragments of endoplasmic reticulum (er). Mag. x 35,000.

tions in the cell membrane which accelerate their sequestration and destruction by the spleen and other reticuloendothelial organs. Under the conditions employed in the present studies, on the one hand, no evidence was found for significant spontaneous lysis of drug-treated red cells either in vitro or in vivo. On the other hand, large Heinz bodies in their typical situation just beneath the plasma membrane, produced severe distortion of cell shape and were associated, as well, with an apparent localized loss of the bilamellar unit membrane structure of the cell surface. If the latter entails a significant loss of membrane liproprotein, it may account for the modest decrease in red cell lipid which occurs during drug-induced hemolysis. It is probable that severe distortion of cell shape by the relatively rigid Heinz bodies retards passage of red cells through the splenic cords while injury to the cell membrane proper predisposes to hemolysis by accelerating erythrophagocytosis.

**SUMMARY**

The ultrastructural changes in red blood cells exposed to phenylhydrazine, either in vivo or in vitro, are described. There is an age-dependent gradient of red cell sensitivity to this drug which includes the more mature reticulocytes as well as the population of circulating erythrocytes. Oxidative denaturat-
The surface of a mature erythrocyte. A large Heinz body (hb) distorts the plasma membrane and its bilamellar unit membrane (um) configuration. Mag. x 175,000.

Formation of hemoglobin and the formation of Heinz bodies, which constitute the major drug-induced lesion, are accompanied by a regular sequence of structural changes commencing in the central cytoplasm of erythrocytes and the drug-sensitive reticulocytes. These early changes often appear in close association with clusters of mitochondria. The initial morphologic lesion has an apparently crystalline structure and the significance of this stage is discussed. Heinz bodies grow by coalescence and condensation and finally come to lie just beneath the cell surface. Here they result in considerable distortion of cell shape and deformation of the plasma membrane. Thus, phenylhydrazine administration produces in red blood cells extensive ultrastructural alterations both in hemoglobin and in the cell membrane which may have considerable bearing on the fate of these cells in the circulation.

Summario in Interlingua

Es describite le alterationes ultrastructural occurrente in erythrocytos exponite, in vivo o in vitro, al influentia de phenylhydrazina. Le gradiente notate in le sensibilitate del erythrocytos pro le pharmaco mentionate es correlationate con le etate del cellulas. Le plus matur reticulocytos es afficite sed etiam le population de erythrocytos in le circulation. Le denaturation oxydative de hemoglobina e le formation de corpores de Heinz, le quales constitue le major lesiones inducite per le pharmaco, es accompaniate de un
sequentia regular de alterationes structural comenciante in le cytoplasma central de erythrocytos e reticulocyctos sensibile pro le effecto del pharmaco. Iste precoce alterationes superveni frequentemente in stricte association con cumulos de mitochondrios. Le lesion morphologic initial ha, apparentemente, structura crystallin. Le signification de iste stadio es discutite. Corpores de Heinz cresce per coalescentia e condensation. Finalmente illos jace justo infra le superficie cellular. Hic illos resulta in un distortion considerabile del conformation cellular e in un deformation del membrana plasmatic. Assi l'administration de phenylhydrazina produce in erythrocytos extense alterationes ultrastructural, tanto in le hemoglobina como etiam in le membrana cellular. Il es probable que il se tracta de alterationes de un considerabile importantia in le determination del destino de iste cellulas in le circulation.

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