significantly different in the two groups, that of the tallow group being much shorter than the safflower oil group. The mechanism by which dietary fats influence clotting times including stypven and recalcification times is not known. Studies on the influence of dietary fats on various clotting factors and on rate of thromboplastin generation in miniature pigs are in progress.

Summary. Miniature pigs maintained for one year on diets containing 15% of either beef tallow or safflower oil did not exhibit lipemia in the fasting state, yet the clotting time was much shorter in the tallow group than in the safflower group. The clotting time was shortened 3 hours after ingestion of either fat, but the effect produced by the saturated fat was much more pronounced than that produced by the unsaturated fat. The recalcified plasma clotting time was also a satisfactory measure of the increase in coagulability brought about by dietary fats, and served to detect differences in coagulability brought about by saturated and unsaturated fats.

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Partial Explosion of Erythrocytes, Induced by Hyaluronic Acid.* (28654)

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Inorganic acids and alkalis hemolyse different erythrocytes. Acids may elicit at certain pH a transitory "fixation-effect" without causing any unusual morphological changes; alkalis do not induce any fixation. We assumed a transitory "fixation-effect" with some of the inorganic acids, because the hemolysis observed under phase-contrast was not instantaneous as with alkalis. In studying the action of inorganic and organic acids, we observed a surprising but consistent phenomenon on various erythrocytes, when hyaluronic acid was added. Instead of hemolysis, which might be expected at an acid reaction, the hemoglobin exploded in microscopically well visible granules. In a few seconds several hundred granules were extruded through one sector of membrane. The liberated granules showed a vigorous brownian movement. The membranes retained some of the hemoglobin. The membranes were sharply visible in phase-contrast revealing single or multiple empty splits, or, occasionally, buds filled with hemoglobin.

Materials and methods. Sheep, beef and horse erythrocytes were obtained from defibrinated blood and washed 3 times with 0.85% NaCl solution. One per cent suspension of washed erythrocytes was used for phase-contrast examinations. Human erythrocytes were also examined unwashed in a similar concentration.

Hyaluronic acid (highly polymerized from human umbilical cord) and lyophilized hya-

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luronidase (150,000 U.S.P. units/mg) were purchased from the Mann Research Laboratories, New York.

The microscopical studies were carried out with a Wild phase-contrast condenser (Heerbrugg, Switzerland) by using objective 85 and ocular 10. One drop of the erythrocyte suspension was deposited on a slide and, without applying any pressure, was covered with a cover glass. The washed cells were, under these circumstances, invariably spherical. The objective was centered approximately at the middle of the cover glass. Hyaluronic acid in various concentrations (1 to 0.1% w/v), was dissolved in McIlvain buffer sol. (M/15) at various pH or in isotonic NaCl. One person touched the right side of the cover glass with a capillary pipette and added one small drop; in this way the other person could observe any instantaneous changes which may have occurred in the erythrocytes after addition of hyaluronic acid. The method, in applying the hyaluronic acid solution through capillary action, directly under the microscope, is not strictly quantitative, but has the advantage of ensuring continuously decreasing concentration gradients useful for evaluating the action of hyaluronic acid solutions in different concentrations and at different pH values.

Results. Eight microphotographs were selected for Plate I to illustrate the successive stages in the action of hyaluronic acid on erythrocytes. The phenomenon could be elicited in full sequence in non-neutralized, highly polymerized hyaluronic acid solution diluted up to 0.5% (w/v) in 0.85% NaCl, or in hyaluronic acid diluted in the same concentration in McIlvain citric acid phosphate buffer at a pH-range of 2 to 3.6. The visible reaction was partial at about pH 4, or by applying less than 0.5% hyaluronic acid.

The erythrocytes of sheep, beef, horse and man studied were invariably spherical (Fig. 1). The spherical erythrocytes showed the smallest diameter when isolated from sheep, the largest from human blood. The hyaluronic acid added by capillary pipette caused a current, but the microscopic field usually did not have to be changed, since some of the erythrocytes adhered to the cover glass. These showed, in contact with hyaluronic acid, an instantaneous jerking enlargement (Fig. 2, 3, 4). Depending on the pH and the concentration of hyaluronic acid, 1 to 10% revealed a clear-cut concentric arrangement of the hemoglobin (Fig. 2). In the other erythrocytes the hemoglobin appeared to be homogeneous or apparently retracted (Fig. 3). Various proportions of the enlarged cells were distinctly lemon-shaped (Fig. 4). The enlarged cells may remain unchanged for a long time (several hours or days) at a pH 4 or higher, or in hyaluronic acid diluted 1:1000 or more.

In the full sequence of changes induced by hyaluronic acid all the enlarged cells "exploded" in the next stage. We termed this stage as "explosion" because the enlarged, resting cells instantly evacuated their contents, producing a distinctly visible funnel-shaped foam which consisted of hundreds and hundreds of hemoglobin granules. By applying 0.5% hyaluronic acid at pH 3.2, the explosion of sheep erythrocytes occurs within 10 to 30 seconds. The other erythrocytes (beef, horse, man) explode somewhat later. Since the explosion is finished in a few seconds, we were unable to obtain a distinct microphotograph of this rapid phenomenon in phase-contrast with our equipment (Fig. 5). This phenomenon is one of the most striking which we have ever observed under a microscope.

The granules set free show no tendency of dissolution in the suspending fluid; they are in vigorous brownian movement within the currents of the preparation. The granules are probably expelled through very narrow splits in the erythrocyte membranes, which do not show any visible gap in phase-contrast after termination of explosion. We did not study the partially empty membranes with the electron microscope. If membrane-splits were demonstrable, the partial retention of hemoglobin could be explained: in the case of the larger particles through their size, and the smaller particles through the discontinuation of an increased pressure in the inside of the cell.

The partially empty membranes do not show any change for many hours. Very characteristic secondary changes appear in the

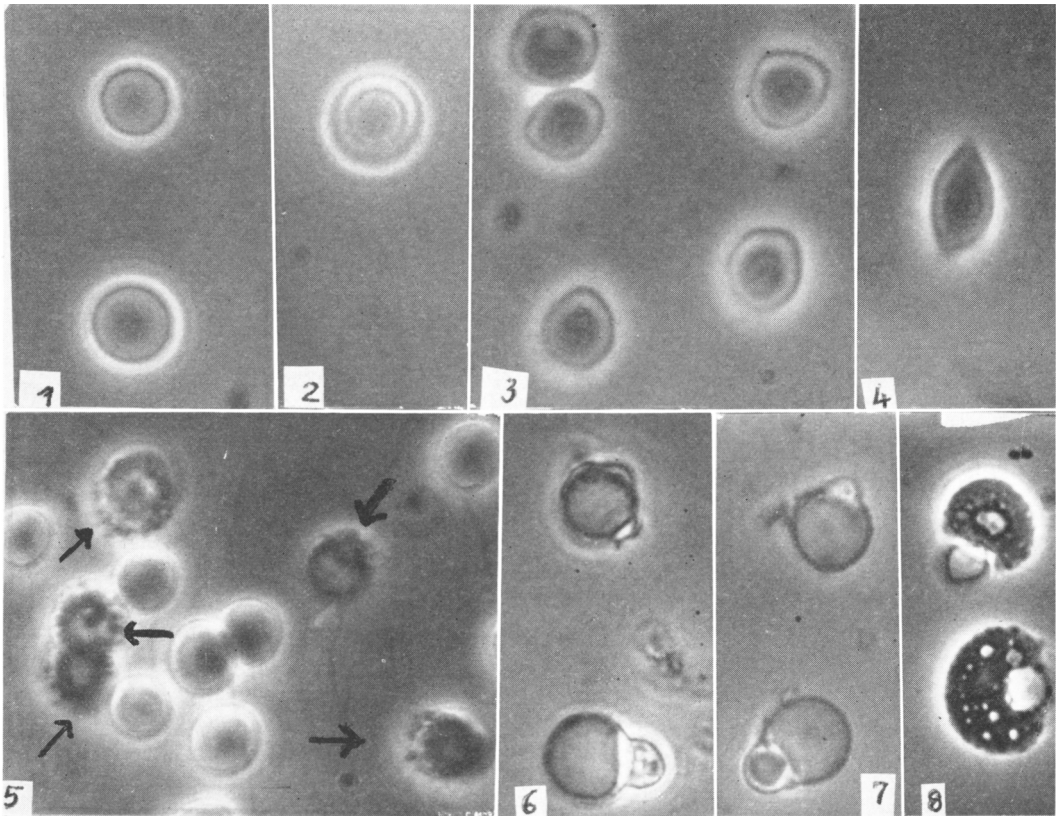

PLATE I. Fig. 1-8. Microphotographs of erythrocytes in phase-contrast. × 1500. Fig. 2-8. After addition of 0.5% hyaluronic acid in 0.85% NaCl solution.

FIG. 1. Washed cells before addition of hyaluronic acid.

FIG. 2. Concentric rings.

FIG. 3. Enlarged cells immediately after contact with hyaluronic acid.

FIG. 4. Lemon-shaped enlarged cell.

FIG. 5. Explosion of hemoglobin.

FIG. 6. Above: Three narrow refringent empty splits in membrane. Below: A large spherical bud, partially filled with hemoglobin and superimposed on one large empty refringent, crescent-formed split.

FIG. 7. Above: One spherical empty bud. Below: One large spherical bud with a hemoglobin-ball.

FIG. 8. Dark-grey masses extruded in a later phase at a less acid pH.

membrane soon after the termination of explosion. The membrane is apparently split through the expelled coagulated hemoglobin granules. The splits can be multiple and 2-3 μ long (Fig. 6, above) or single and 4-10 μ long, in the form of a crescent. These membrane-splits do not contain hemoglobin and are highly refractile in phase-contrast. Depending mostly from the pH, secondary spherical membrane buds (2-5 μ in diameter) appear frequently. Some contain a hemoglobin globule (Fig. 7, below) and some do not (Fig. 7, above). Less frequently 2 large buds were observed on a membrane. The large buds filled with a pink-colored spherical mass (Fig. 7, below) or containing some pink floccules (Fig. 6, below), were often superimposed on long crescent-shaped, narrow membrane-splits. In such cases the bulb or the narrow stalk of the bud was always separated by a fine layer from the narrow refringent membrane-split.

If the pH was 3.2 or higher, sharply limited light dark or grayish masses appeared in the latest stage of hyaluronic acid action outside of the membrane which frequently showed apparent vacuoles (Fig. 8). These masses may correspond to a partially solubilized mix-

ture containing also dissolved membrane substances.

The same weight of lyophilized hyaluronidase was added to highly polymerized hyaluronic acid and the mixture was dissolved in 2% (w/v) concentration in McIlvain buffer at pH 6 or 7. Neither hyaluronic acid nor the mixture exerted any characteristic action on erythrocytes at these pH values. After 5 or 6 hours' incubation at 21°C, the 2% solution of the mixture was brought to 0.5% concentration (computed only for the hyaluronic acid), whereby the pH was adjusted with citric acid or with McIlvain buffer to 3.2. No specific changes (explosion, membrane splits) could be elicited on erythrocytes with hyaluronic acid after treatment with hyaluronidase. A control solution of hyaluronic acid, treated in exactly the same way, gave the characteristic reactions with erythrocytes if it was not digested with hyaluronidase.

Discussion. We cannot explain why highly polymerized hyaluronic acid elicits, in a certain concentration, an instantaneous, jerking enlargement of several erythrocytes, followed by partial explosion of the cells liberating hundreds of hemoglobin granules. The enlarged cells reveal not infrequently a very clear-cut circular structure of the hemoglobin usually after employing an acid fixation as described occasionally in earlier literature. The hyaluronic acid explosion requires an acid reaction at pH values 2-3.6. It may be that in the genesis of concentrically arranged coagulation of hemoglobin certain pH values play the main role. It is, however, certain that the most characteristic event, described here as the explosion of erythrocytes, requires another factor apart from a low pH. We are searching for other substances which may replace the highly polymerized hyaluronic acid. The enzymatic split-products of hyaluronic acid, obtained after digestion of this substance with hyaluronidase, are ineffective.

It is likely that the layers of the erythrocyte membrane, which are separated during the hemoglobin disruption, preexist in the intact cell. If this assumption could be verified with other experiments, the erythrocyte-membrane may be composed of 2 or 3 different layers. We do not think that the loose protein-like envelope thought to be present in beef, but not in sheep erythrocytes,(1,6), plays any role in the hyaluronic acid action on erythrocytes, since neither the explosion nor the consecutive changes of the membrane differ significantly in sheep and beef erythrocytes.

Summary. 0.5% highly polymerized hyaluronic acid added at a pH-range of 2 to 3.6 to 1% suspension of washed sheep, beef, horse and human erythrocytes induces a sequence of regularly demonstrable events: instantaneous jerking enlargement of the cells, explosion of the hemoglobin in hundreds of granules, consecutive changes in the erythrocyte-membranes. The hemolysis is complete at the same pH value using only acids; no hemolysis occurs in the presence of hyaluronic acid. Hyaluronidase digestion of hyaluronic acid abolishes this phenomenon. The explosion of erythrocytes elicited by hyaluronic acid may give further information about the structure of the erythrocyte membrane.

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