is biologically inactive because of some small difference in chemical structure. A final possibility is that the initial content of squalene in these tissues acts as a trap for radioactive squalene formed during the course of sterol synthesis. If this is so, the original source of their squalene is unexplained.

Summary. Preputial glands from rats, when incubated with C^{14} acetate show a marked incorporation of C^{14} into squalene, a sterol precursor, and little into sterols. In this regard, the preputial glands differ from many other mammalian tissues which show incorporation of C^{14} mainly into sterols and little into squalene. Human dermis, which contains sebaceous glands, is another mammalian tissue which shows preferential accumulation of the C^{14} into squalene. The mechanism of the difference is unknown, though it seems likely that factors necessary for conversion of squalene to sterols are missing in these tissues, or that an inhibitor of this reaction is present.

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Studies of Bovine Erythrocytes in Anaplasmosis. I. Flocculating Properties of Stromata in Water.* (26142)

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The erythrocytic membrane has been shown to be intimately connected with phospholipids as structural components(1). Chemical analyses of stromata in various anemias have shown that there are alterations in content of phospholipids(2,3). New evidence of surface structural changes obtained by visual methods and involving the phospholipids of the erythrocyte in bovine anaplasmosis was collected while studying the mechanism of the anemia produced. Observations showed that the flocculating characteristics of erythrocytic stromata prepared from blood of uninfected calves and calves infected with *Anaplasma marginale* were different from each other when suspended in water.

Materials and methods. Stromata were prepared from erythrocytes of 14 normal intact, 6 splenectomized uninfected, and 18 splenectomized infected calves. The latter group of animals was inoculated by the subcutaneous route with 10 ml of infective blood having approximately 30% of the erythrocytes showing anaplasma bodies. Jugular vein blood samples were collected periodically in 50-ml amounts in 0.3 ml heparin sodium solution (1,000 U.S.P. units/ml). The erythrocytes were thoroughly washed and packed in 0.85% sodium chloride solution by centrifugation at 700 × g for 30-minute intervals at 4°C. They were then mixed and lysed in 20 volumes of distilled water at 4°C which was saturated with carbon dioxide. The carbon dioxide saturated distilled water possessed a pH of 3.8 at this temperature. The material was allowed to settle in tall cylinders overnight at 4°C and the clear red supernatant fluid was aspirated off. The fluffy appearing stromata were poured into 250-ml centrifuge bottles and washed in unbuffered distilled water by centrifugation at 700 × g for 10-minute intervals at 4°C. This unbuffered distilled water had a pH of 5.4. Stromata have been prepared by lysing cells and

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flocculating and washing the material in a sodium citrate buffer at pH 5.5(4). Washing in cold unbuffered distilled water at pH 5.4 was continued until the supernatant fluid appeared devoid of hemoglobin. After the last washing approximately 10 ml of unbuffered distilled water at pH 5.4 were then added to initially suspend the sediment by shaking and the centrifuge bottle was completely filled with distilled water and allowed to stand at room temperature. Photographs were taken 10 minutes after the stromata were suspended.

In this laboratory the use of buffered and unbuffered salt solutions produced decreased yields of bovine erythrocytic stromata. This was due to increased solubility of stromata, *per se*(5) and also specifically to increased solubility of lipoidal materials from the stromata(6,7). The supernatant fluids in preparations made with the aid of salt solutions remained cloudy in all instances. The precipitation method of preparing bovine stromata using cold unbuffered carbon dioxide saturated distilled water at pH 3.8 and subsequent washing with cold unbuffered distilled water at pH 5.4 produced adequate yields of material by which the flocculation characteristics could be observed.

Subsequent extraction of total phospholipids from desiccated stromata were made with 3:1 ethanol:ether mixtures(8). The phospholipids included cephalin, lecithin, and sphyngomyelin(3). Estimations of total phospholipids were made on these extracts by using the chemical method of Fiske and Subbarow for phosphorus(9). The factor, total phospholipid phosphorus $\times$ 25, was used to convert the values to total phospholipids(10).

Results. Representative examples of the altered flocculating properties are shown (Fig. 1). In preparations of stromata from normal cells the material agglutinated rapidly and settled in a period of 5 minutes (Fig. 1, a). Stromata prepared from erythrocytes showing anaplasma bodies exhibited varying amounts of suspended and finely divided material which remained in suspension for relatively long periods of time (3-18 hr, Fig. 1, b-f).

The modified flocculating properties began to manifest themselves shortly after the anaplasma body count had reached approximately one-half of its peak value or when the erythrocytic count was just beginning to decrease (Fig. 1, c). An increase in the osmotic fragility index was also observed at this time.

Concentrations of total phospholipids of stromata exhibiting large amounts of suspended matter were as low as 60% of preinfection values (14% down to 6%). It has been reported that normal bovine erythrocytic stromata contain 16% total phospholipids, whereas embryonic bovine stromata contain 11.9% total phospholipids(11). Erythrocytes showed high osmotic fragility indices and in many instances lysed spontaneously in isotonic 0.85% sodium chloride solution. Erythrocytic counts were extremely low (1.5-3.0 million/mm^3) during this time. Anaplasma body counts had already reached peak values and were decreasing and approaching a low level of less than 2%.

Recovery patterns in the flocculating characteristics of stromata were also observed. When erythropoiesis and recovery from the disease were rapid as evidenced by an increasing number of erythrocytes and a low or negative anaplasma body count, the stromata showed progressive increases in ability to aggregate and settle rapidly. This return to normal flocculating properties was also accompanied by an increase in level of total phospholipids of the stromata.

In studying animals undergoing prolonged periods of convalescence fluctuations in anaplasma body counts occurred. Erythrocytic counts showed some variations also, but a trend in an increase in numbers was observed. Although suspensions of stromata appeared cloudy during this phase of the disease in these animals the fragility index was normal. In each of these instances the animals died. Total phospholipid content of the stromata was below normal in all cases in which this phenomenon occurred.

Discussion. A possible explanation for this phenomenon seems to be that a change in isoelectric point of the stromata has taken place which in turn has affected their hydration affinity. The change in chemical com-

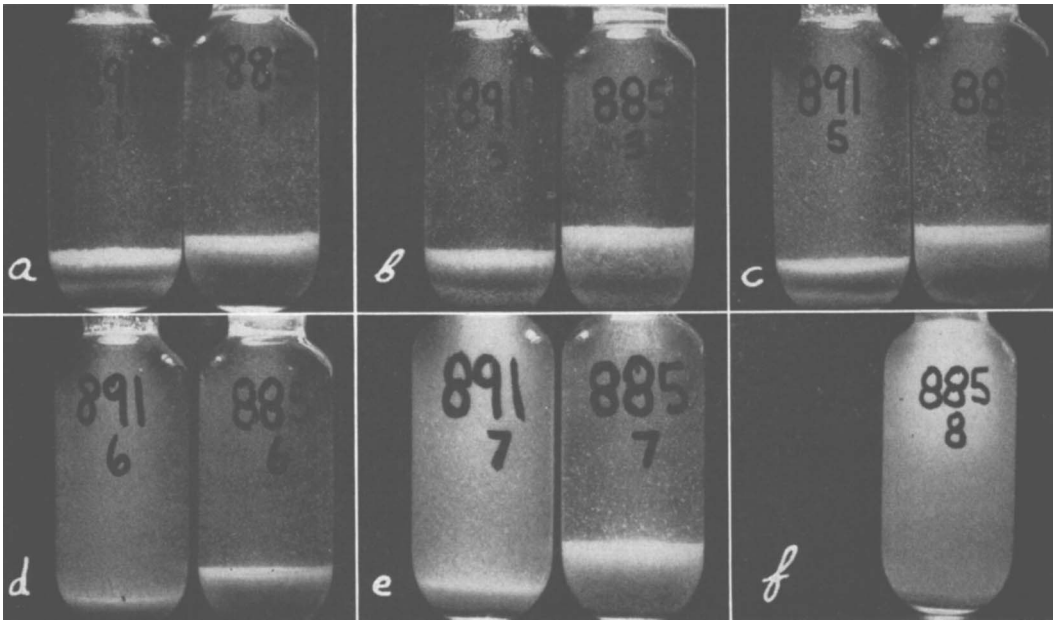

Fig. 1 letter	Designation on centrifuge bottle: Animal No.	Sample No.	ABC	ER	FI	Days after inoculation
a	891	1	negative	9000	.67	5
b	891	3	1.0	7300	.70	9
c	891	5	36.0	6050	.73	14
d	891	6	49.0	3350	.76	16
e	891	7	60.0	2425	.76	17 (died)
a	885	1	negative	7150	.67	5
b	885	3	1.0	6425	.70	9
c	885	5	25.0	6125	.73	14
d	885	6	42.0	4850	.76	16
e	885	7	50.0	3600	.76	17
f	885	8	69.0	1600	.85	19 (died)

FIG. 1. Anaplasma body count in % erythrocytes showing bodies (ABC), erythrocytic count/mm³ in thousands (ER), fragility index in highest % sodium chloride solution showing trace of hemolysis (FI).

position, in this case decreased content of acidic lipids, namely the phospholipids, has effected a change in isoelectric point toward a higher pH value. The isoelectric point of stromata with lipids experimentally extracted has been shown to be at least 3 pH units higher than intact erythrocytes(12). It has also been shown that cells having large amounts of lipoidal material in the surface have low isoelectric points and are capable of becoming the least hydrated while their ability to become aggregated is great(13). Normally the isoelectric point of bovine erythrocytic stromata is approximately pH 3.4(13). The stromata preparations would then tend to remain in suspension for relatively long periods under the conditions cited herein.

The altered flocculating properties, specifically the tendency of stromata showing a decreased total phospholipid content to remain in suspension, might be assumed to be due to presence of reticulum from reticulocytes and immature cells. Ponder(14) has reported that reticulocytes contain more lipid than normal erythrocytes but that total lipid and phospholipid does not differ appreciably from normal when calculated on the basis of the red cell surface which is increased in the reticulocyte. Density of the immature cell is

also less so it presumably contains more water, although it is stated to be stickier than the orthochromatic cell(14). The possibility that reticulocytes and other immature cells may be responsible for the different flocculating characteristics can be discounted by the fact that these immature cells appear shortly after the peak in anaplasma body count has been reached(15). In this case the percentage rise is from a normal of approximately 0.5% to approximately 5% after the peak. Our unpublished observations have indicated that a small number of immature cells also appear at approximately the same time. The change in flocculating properties described herein first appeared prior to the peak in anaplasma body count and continued thereafter only at a time when the content of total phospholipids of the erythrocytic stromata was lower than normal.

As further evidence against immature cells being responsible for this phenomenon it has been found that reticulocytes and immature erythrocytes are less fragile than mature cells (16). In the studies reported here fragility indices were usually high during the appearance of immature cells after the anaplasma body count decreased to a low level, indicating that the cell population as a whole was more susceptible to osmotic lysis.

The differences observed in stromata of animals undergoing long periods of convalescence indicated that imperfect or malformed cells were being produced. Possibly there was a partial disorientation of cell membrane ultrastructure. Hansard and Foote(17) have found that there are malformed cells produced in anaplasmosis during a study of the anemia using Fe^{59}. Although these new cells which possessed a lowered content of total phospholipid had an abnormal structure, they were still physically sound as to osmotic fragility. These imperfect cells may be responsible for an impaired erythrocytic function which indirectly causes anoxia and death even though anaplasma body counts are decreasing and erythrocytic counts are increasing.

It would appear reasonable that this phenomenon may manifest itself in other anemias especially those in which phospholipids are affected. It may have application, also in determination of the type of anemia when chemical composition of the stromata is changed.

These studies are being continued with particular emphasis on partition of phospholipids and other chemical and physical properties of diseased erythrocytes.

Summary. Differences in flocculating characteristics of erythrocytic stromata prepared from blood of uninfected calves and calves infected with *Anaplasma marginale* are described. The differences in flocculating properties are due to a change in isoelectric point brought about by the altered total phospholipid content.

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