TABLE II. Effect of Increasing Amounts of 3-(o-tolyloxy)lactic Acid on Metabolism of Mephenesin.

3-(o-tolyloxy)-lactic acid, μmoles added	Mephenesin metabolized* (by difference) μmoles	μmoles/g	3-(o-tolyloxy)-lactic acid formed, μmoles
0	5.88	16.59	3.40
1.61	5.76	16.07	3.23
5.29	6.15	16.14	2.47
10.47	5.65	15.21	1.58
20.93	5.80	14.79	.13
41.88	5.36	14.29	.0

* K.R. bicarbonate buffer, 60 min. incubation.

tolyloxy)lactic acid formed, these data suggested that an intermediate metabolite was formed and that 3-(o-tolyloxy)lactic acid was not the immediate but the final product of mephenesin metabolism.

Since an aldehyde seemed to be the most likely intermediate, mouse liver slices were incubated with aldehyde trapping agents. Addition of either sodium bisulfite, hydroxylamine or semicarbazide at 10^{-4} and 10^{-3} M concentrations, which had no effect on oxygen consumption of the slices, had no effect on rate or extent of mephenesin oxidation, nor did they significantly alter amount of 3-(o-tolyloxy)lactic acid formed.

Alcohol and aldehyde dehydrogenases have been shown to require intact sulfhydryl groups for activity. Addition of iodoacetic acid to the medium indicated that mephenesin metabolism was sensitive to sulfhydryl inhibitors, for the oxidation was completely inhibited by concentrations as low as 3 mM. Preliminary studies indicated that mephenesin was not metabolized by mouse diaphragm, heart, spleen, or brain slices. Kidney slices oxidized mephenesin slowly.

Berger(9) reported that the LD_{50} for mephenesin, administered intravenously, increased from 195 mg/kg for the rat, to 220 mg/kg for the rabbit, to 322 mg/kg for the mouse. These data suggest that the mouse metabolizes the drug more rapidly than the rat. Our observations on the rapidity of mephenesin oxidation by the rat and the mouse support this conclusion.

Summary. 1. Mephenesin is oxidatively metabolized *in vitro* by rat and mouse liver slices to 3-(o-tolyloxy)lactic acid. 2. Mephenesin metabolism was markedly inhibited by increasing substrate concentration, but was not altered by presence of increasing concentrations of the product, 3-(o-tolyloxy)lactic acid, in the medium. 3. Metabolism of mephenesin was readily inhibited by iodoacetic acid, but was unaltered by aldehyde trapping agents at 10^{-3} M.

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Preparation of Formalinized Erythrocytes. (25444)

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Following discovery by Flick(1) that red blood cells treated with formaldehyde could be preserved for long periods of time, several investigators(2-4) devised methods of preparing formalinized cell reagents in which special properties of red cells were utilized(5). We prepared formalinized red cells by published methods which were not satisfactory for use in agglutination tests by the pattern method (6), because of crenation, inseparable clumps, and non-specific aggregation. Then we devised a method that permitted us to prepare con-

sistently, formalinized red blood cell suspensions of the desired quality. Cells thus prepared have been distributed and used successfully for various purposes(7). Although some improvements in technic have been described(8-11), a general analysis of the factors affecting formalinization is lacking. This paper describes our method and discusses the principles upon which it was designed.

Materials and methods. Bloods collected through a system thoroughly wetted by and into modified Alsever's solution(12) were stored at 4°C up to 4 weeks before use. Processing the cells within a few days after collection was advantageous because fresh cells were less fragile. On day of formalinization the cells were separated (for example from 100 ml of blood) by centrifugation at 600 to 1000 g and washed 5 to 7 times in 10 volumes (500 ml) of cold 0.85% NaCl solution. During washing and at subsequent stages of procedures, care was taken to avoid foaming of the liquid. The washed packed cells (50 ml) were resuspended in not less than 8 volumes (400 ml) of phosphate-buffered saline solution (0.009 M Na_2HPO_4, 0.006 M KH_2PO_4, 0.135 M NaCl) at pH 6.8 to 6.9. Formaldehyde solution, 40%, U.S.P., pH 5.5 to 6.0, equal to ¼ (100 ml) of volume of cell suspension, was poured into a cellophane dialysis sac (flat width 2.5 cm), twisted and knotted at one end. The tubing was cut off so that about one-third of sac remained empty. The air was expelled and the open end tied off. This sac was placed on bottom of beaker, and the cell suspension poured over it. The beaker was agitated at room temperature on mechanically operated orbital shaker. Speed of shaker was adjusted to provide most vigorous mixing action attainable without causing more than a small amount of foam. After 2 hours, the cellophane sac was removed and its contents poured into the beaker. Shaking was continued for additional 12 to 18 hours. Then a mass of clotted cell debris was usually found floating on surface of liquid or adhering to walls of beaker. With care in decanting the suspension into a clean container or filtering it through surgical gauze, it was not difficult to exclude all debris. To the homogenous cell suspension was added ½ volume (250 ml) of saline, then cells were washed 6 times in about 750 ml. More meticulous washing seemed unnecessary, as our observation in agreement with Cox(3) indicated that some formalin may be added without causing further change in the cells; a repeated formalinization had no detectable effect on the cells. Finally, the packed formalinized cells (about 60 ml) were suspended in an equal volume of saline, with or without 0.02% thimerosal, and stored as 50% stock suspension at 4°C.

Results. After many trials with changes and variations in technics, the above method was selected. It has been used satisfactorily in preparation of red cells from 7 species of animals—human, bovine, rabbit, guinea pig, chicken, alligator and sheep. With the latter, however, it was desirable to use 0.85% NaCl solution instead of phosphate-buffered saline solution. With this minor modification, 24 successful preparations have been obtained.

The final cell suspensions typically were brown, changing to reddish-brown upon dilution. For a few days after dispersion, the cells were readily redispersed by gentle shaking. After a longer period, more thorough shaking was necessary to obtain a uniform suspension, but the cells showed comparatively little tendency to stick to walls of vessel or to aggregate nonspecifically. When examined microscopically (Fig. 1a), suspensions contained individual cells (not more than 2% of cells were aggregated), which were slightly larger and more nearly spherical in shape than untreated cells of the same species. The smooth convex periphery of treated cells is illustrated in Fig. 2.

After formalinization, these cells were not subject to hemolysis in hypotonic salt solutions or in presence of complement and specific antibody. Their resistance to mechanical injury was excellent, and they sustained no discernible change in properties after storage for one year or longer at 4°C.

The sharp endpoints and distinct negative patterns of which these cells are capable, are illustrated in the hemagglutination test shown in Fig. 3. Sheep erythrocytes sensitized with

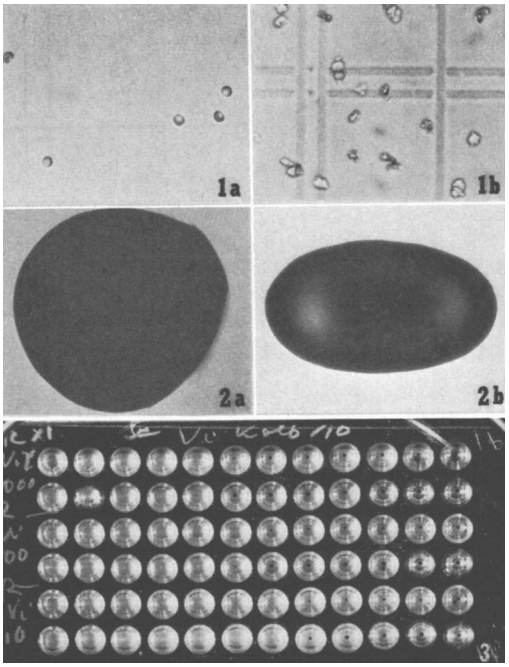

FIG. 1. Formalinized red blood cells prepared by present method (a); and according to published methods(1-4), pool of 4 preparations (b).

FIG. 2. Electron micrographs of a formalinized sheep (a) and chick (b) red blood cell.

FIG. 3. Hemagglutination on Takatsy plate using anti-Vi serum and Vi sensitized formalinized sheep cells.

Vi antigen were used in this experiment in a modification of the Takatsy plate method (13). Two-fold serial dilutions of Vi antibody were made in saline solution (0.025 ml) and the sensitized formalin-treated erythrocytes (0.025 ml of a 0.33% suspension) were added. Homogeneity of treated cells is indicated by sharpness of endpoint. In the second line a double amount of cells (0.05 ml) was used with no change in endpoint. With higher concentrations of Vi antigen, shown in top lines, the titer decreased and negative patterns were more distinct because of the agglutinative protective property of the Vi antigen. This conforms with previous findings(14).

Formalinized cells prepared from blood from different species of animals have been successfully sensitized with Vi antigen. In addition, tannin treated or diazotized formalinized cells have been sensitized with diphtheria and tetanus toxoids, respectively, and sheep cells so treated are used routinely in a study to be published.

Discussion. To produce distinct negative patterns in hemagglutination tests, suspensions of erythrocytes must be not only relatively free of aggregated cells but the individual cells also must have those properties which favor their collection into a compact button-shaped mass at the bottom. Ideally, the cells should be spherical, with smooth surfaces that are not sticky and do not adhere to wetted glass. To obtain formalinized cells with these qualities the following principles were applied. (1) Thorough washing of cells, prior to addition of formaldehyde, avoids stickiness which can result from gelling of plasma proteins on cell surface(15). Further, an hemolysate, free of cells treated with formalin, would readily gel or form clumps. Hence excessive washing which increases cell fragility and hemolysis is to be avoided. (2) High dilution during formalinization decreases concentration of contaminating plasma or hemolysate, and reduces the chances for cell contacts which influence aggregation and distortion. (3) Shaking distributes cells uniformly and helps to eliminate gel forming contaminants by promoting formation of a discrete pellet. Excessively vigorous agitation is disadvantageous, particularly if considerable foam appears, since deformation or lysis of cells may occur at air-water interface. (4) Slow addition of formaldehyde solution by dialysis permits counteractions to take place. Increasing concentration of formalin tends to cause crenation of cells, while declining pH and osmolarity cause cells to swell and assume smooth rounded shapes favorable to subsequent use in hemagglutination tests. On the other hand these factors increase the probability of hemolysis. The method described represents a balance between contesting requirements.

Summary. An improved method for formalinization of erythrocytes from different species of animals is described. Reproducible preparations, relatively free from crenation and non-specific agglutinability, are satisfactory for various agglutination tests by the pattern method, and may be stored for one year or longer at 4°C without loss of capacity

to react in these tests. The principles applied to attain satisfactory formalinized cells are discussed.

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Instability of Metabolic Quotients Obtained from Tissue Cultures.* (25445)

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To compare the metabolism of one tissue culture with that of another they should be maintained under conditions that yield reproducible results. Serum in tissue culture medium introduces a variable. Since serum is not chemically defined, much work has been done to devise synthetic growth media. Some tissue cultures have been grown in absence of serum(3,4) but these conditions are the exception rather than the rule, because most cell strains must still be maintained in serum. Although use of serum in nutritional studies can be questioned, there has been little objection to its use for growing cells for subsequent metabolic determinations, especially if cells are washed and resuspended in a chemically defined solution before the determination is made. We noted previously(7) that HeLa cells grown in medium containing human serum respired and formed lactate at a greater rate than the same cells grown with horse serum. These observations suggested the possibility that tissue cells grown in serum from different members of the same species may respire and form lactate at different rates.

Materials and methods. Earle's Strain L cells were grown in saline solution containing 0.5% enzymatic lactalbumin hydrolysate plus 20% horse serum. Cultures were gassed with 5% CO_2 in air. Saline solution contained in g liter: NaCl 6.5, KCl 0.29, $MgSO_4 \cdot 7H_2O$ 0.21, $CaCl_2$ 0.14, $NaHCO_3$ 2.06, Na_2HPO_4 0.15, glucose 2, phenol red 0.05, lactalbumin 5. This and other solutions used, with the exception of trypsin, contained inorganic salts in about the same ratio as in interstitial fluid (12) and had a tonicity equivalent to 0.93% sodium chloride solution as determined by freezing point depression. All solutions were buffered at pH 7.2. At the start of experimentation, cells were grown in 1.9, 7.5, 15, 20, and 30% horse serum to determine optimal serum concentration for growth. Media was renewed every third day and after 10 days the live cells in each culture counted(8). These results graphed in Fig. 1 indicate that 15-20% horse serum plus lactalbumin medium was optimal for growth of Strain L cells. To prepare cells for metabolic determination, they were freed from T-60 culture flasks(2) with

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