is shared by desoxycorticosterone, the steroid-17-spirolactones, and progesterone, whereas the spirolactones and progesterone actively antagonize the sodium-retaining action of desoxycorticosterone. These discrepancies may follow naturally from the fact that the biological effects of these steroids are mediated on the one hand through the endometrium and on the other, through the renal tubular apparatus. In any case, the apparent overlap between mineralocorticoid, anti-mineralocorticoid and progestational action of these several compounds should provide further understanding of salt and water metabolism in normal and abnormal pregnancy.

*Summary.* The steroid-17-spirolactones (I and II) have been shown to have progestational activity in the estrogen-primed immature rabbit.

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## The Preparation and Use of Formalinized Erythrocytes with Attached Antigens or Haptens to Titrate Antibodies. (24381)

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Red blood cells bearing soluble antigens or haptens attached to the surface have proven a sensitive reagent for detection of antibodies (1,2,3) or antigens(4). For agglutination titrations there are advantages in the use of red cells which have been stabilized by treatment with formalin. When prepared according to published methods(5,6), the cells become very adherent and require prolonged violent homogenization to obtain suspensions for use. The resulting suspensions are reported to agglutinate spontaneously in presence of traces of formaldehyde(5,6), and prolonged washing followed by treatment with bisulfite and dialysis has been recommended to free the cells from loosely bound formaldehyde(6). Cells treated with formaldehyde by the method described below can be dispersed by manual shaking and are not agglutinated by whatever traces of formaldehyde remain after several washes with saline. Some cells have retained their original serological activity after storage in dilute formalin in refrigerator for 15 months.

*Methods. Preparation of cells.** Human type O red cells were used within 2 to 3 weeks after drawing into acid-citrate-dextrose solution. The cells were washed 4 times with about 10 volumes of 0.85% sodium chloride containing 0.005 molar sodium phosphate pH 7.3 (hereafter referred to as saline) and 0.5% dextrose. A 50% suspension of washed cells was made in saline, chilled to 1-5°C and poured into 4 volumes of cold 10% formalin-saline solution. This solution was prepared by diluting 1 volume of neutralized reagent formaldehyde solution† with 3 volumes of 1⅓ times concentrated saline. A final suspension containing 10% red cells and 8% formaldehyde in saline at pH 7 to 7.3 was thus obtained. The suspension was stored at 1 to 3°C in a stoppered flask and the cells resuspended once each day for 10 days by shaking and swirling by hand. Cells treated

* Human cells of various types were obtained through generous cooperation of Miss Narcissa Hocker of Indiana University Medical Center Blood Bank.

† J. T. Baker Co. reagent 37% HCHO containing 11.9% methanol and neutralized to pH 6 to 7 using 1 molar NaOH.

for 18 hours or more at room temperature (25°C) were at least as stable as those treated for 10 days in the cold and were found to be suitable for titrations in which rate of settling was followed photometrically using a method which will be described in detail elsewhere. However, the cells prepared at room temperature suffered microscopically visible alteration and were more prone to agglutinate non-specifically in the settling pattern method than those cells prepared in the cold. The cells can be stored in formalin-saline in the cold for at least a year prior to coupling with antigens.

*Coupling with antigens.* Cells stored in formalin-saline were washed 4 times with at least 30 volumes of saline and coupled with sulfanilazo groups (SA) or with bovine serum globulin (BGG) or albumin (BSA) (Armour & Co.) using methods which have been described for fresh cells(2,3). These procedures were simplified with formalinized cells since there was no problem of hemolysis. The coupled cells were returned to formalin-saline in the manner described for fresh cells and stored in the cold. Some preparations were made by coupling fresh cells as described previously for sheep cells(2) except that 60 ml of a 10% suspension of cells was used. The final suspension was then washed once with saline containing dextrose and treated with formalin as described above. As an alternative to the benzidine method described by Stavitsky(3) for attaching protein antigens, purified serum proteins could be firmly bound by formaldehyde alone. Cells coupled with (BGG) were prepared by mixing 0.5 ml of a 10% solution of protein in saline with 10 ml of a 10% suspension of previously tanned cells in 8% formaldehyde (pH 6 to 7) prepared with 0.4% unbuffered saline. Two ml of pH 7 phosphate-citrate buffer prepared according to McIlvaine's standards(7), but of 2½ times the concentration, was added, and the cells kept in suspension without foaming by rotating them slowly in polyethylene bottle for 3 to 5 days at room temperature. They were then washed 4 times with saline and returned to formalin-saline for storage in refrigerator. Some preparations were stored in the coupling solution and washed just prior to use. *Titration.* Cells stored in formaldehyde were prepared for use in titrations by washing 4 times with at least 30 volumes of cold saline on day they were used. Final suspension in saline was adjusted to give a density of 0.4 at 620 mμ when diluted with an equal volume of saline and read in 10 x 75 mm cuvettes in Coleman Jr. Spectrophotometer using a holder masked out to leave a 1 $cm^2$ window as described by Leiner(8). This suspension contained 8 x $10^7$ cells/ml and was approximately 0.7% by volume. Settling pattern titrations were performed using 14½ x 75 mm tubes selected to give uniform small rings or buttons with cells in saline containing 1:100 normal rabbit serum (NRS). Tubes were soaked in mild detergent and swabbed out with cotton after each use. For titrations racks having 12 mm holes in the bottom shelf were used and serial two-fold dilutions of serum were set up in 0.5 ml amounts with 1:100 NRS in saline as diluent. One-tenth ml of cell suspension was added to each tube, the racks shaken and left at room temperature. After 3 hours and 18 to 24 hours the patterns were observed with the aid of a stand having a front-silvered mirror at an angle below the tubes and a fluorescent lamp above. Titers are reported as the reciprocal of the highest dilution of serum in 0.5 ml which caused a pattern clearly distinguishable from the controls. Sometimes the patterns were more distinct after 40 hours owing to more complete clearing of the backgrounds in controls and negative dilutions. The NRS for diluent was selected by testing with cells to be used, since it was found that some normal serums agglutinated some cell suspensions. The normal serum was stored at −20°C in small aliquots and thawed as needed since it sometimes developed the property of agglutinating cells non-specifically after standing in dilute solution. Non-specific agglutination by test serums was controlled by running a parallel titration with cells which had not been coupled with antigen or by specific inhibition of agglutination in the presence of soluble antigens or haptens. To find out whether more careful elimination of formaldehyde might lead to improvement of the negative patterns, portions of 5 human cell

TABLE I. Agglutination Titers of Several Serums with Various Cell Preparations.

| Cells | Antiserums: SA-stromata | SA-BGG #52 | SA-BGG pool |
|---|---|---|---|
| | | (× 1000) | |
| SA-H6 | 32,000 | 14 | 2 |
| -H7 | 16,000 | 7 | 1.5 |
| -H14 | | 4 | 1 |
| BGG-H7 (BDB) | < 50 | 32 | 64 |
| -H8A (HCHO) | | 1,500 | 4,500 |
| -H8B (HCHO) | 100 | 1,300 | 5,600 |

All data for SA-stroma serum and for SA-H14 cells are for single titrations. Other figures are averages of 2 or more titrations with a range of ± one double dilution or less. Please refer to *Results* for further description.

preparations were washed and treated as described by McKenna(6) except that the saline taken for each wash was 30 times or more the volume of the packed cells. The density of each suspension was adjusted to 0.40 ± .02 and triplicate portions of the cells were set up with 1:100 NRS in parallel with portions which had simply been washed 4 times with 30 volumes or more of saline.

*Results.* Data for 3 rabbit serums titrated using several cell preparations are given in Table I. The SA-stromata serum was a pool of 15 bleedings from 3 rabbits injected with sulfanilazo-sheep stromata. The SA-BGG #52 was from a single rabbit and the SA-BGG pool was from 9 bleedings of 4 rabbits injected with sulfanilazo-bovine gamma globulin. These antigens were prepared as described previously(9). To test for anti-sulfanilazo activity of the serums, cell preparations sulfanilazo-human #6 (SA-H6) and SA-H7 were made by coupling formalinized human red cells with sulfanilic acid. Preparation SA-H14 was coupled with sulfanilic acid prior to treatment with formalin. The ratios of titers of these 3 serums with each of the cell preparations are within a 2 fold range. To test for anti-globulin activity, cell preparation BGG-H7 was made using the benzidine method (BDB) to attach bovine globulin to formalinized human cells and preparations BGG-H8A and BGG-H8B were made using the formalin coupling technic described in this paper. Cells prepared using formalin to attach the bovine globulin were 50 to 100 times as sensitive as those prepared using benzidine. These cells are apparently also about 100 times as sensitive and also more stable than those which have been prepared using tannic acid to bind proteins to formalinized cells(6).

The titer of 14,000 for serum SA-BGG #52 with SA-H6 is the average of 10 titrations with a range from 8,000 to 24,000. One series of 15 serums from 11 rabbits which had been injected with SA-sheep stromata was retitered with SA-H6 cells after the serums had been stored at –20°C for 18 months following an original titration by another observer using fresh SA-human cells.‡ The original titers ranged from 320 to 64,000, and the retests were within one 2-fold dilution for 13 of the 15 serums while one differed by 2½ and another by five-fold.

Pictures of patterns obtained with cell preparations SA-H6, SA-H7, SA-H10, and uncoupled cells H7 using 4-fold dilutions of serum SA-BGG #52 starting at 1:100 are shown in Fig. 1. Cell preparation SA-H10 was made by coupling with sulfanilic acid before formalin treatment. The patterns shown here are very similar to those pictured for fresh cells in the paper by Stavitsky(3). They appear to be more distinct and easily interpreted than those which have been obtained using cells prepared with prolonged violent

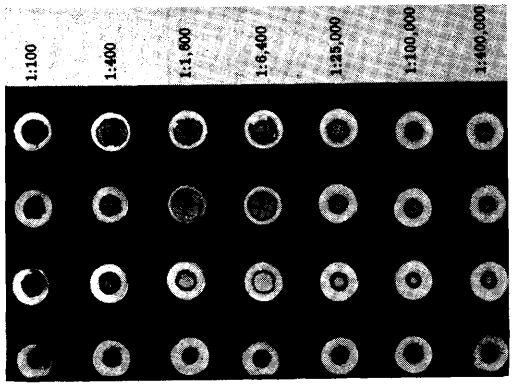


FIG. 1. Agglutination patterns of sulfanilazo-formalinized human cells with anti-sulfanilazo-bovine gamma globulin serum #52 diluted in 4-fold steps. All tubes contain 1:100 NRS. The cells are, from top to bottom, SA-H6, SA-H7, SA-H10 and control H7. This picture is of the reflections of the bottoms of the tubes as described under *Methods*.

‡ The original titrations were done by Dr. H. O. Foley, formerly student at our Center.

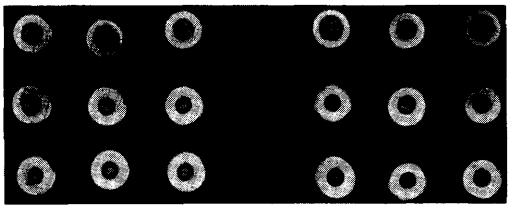

FIG. 2. Effect on spontaneous agglutination of treatment to eliminate formaldehyde. Cells in top row were washed and treated with bisulfite as described by McKenna(6). Those in second row were washed 4 times with saline at room temperature and those in bottom row were washed 4 times in the cold. The group to left are SA-H6 and those to the right are BGG-H8. Tubes are set up in triplicate with 1:100 NRS. Please refer to *Methods* for further details.

homogenization(10). The agglutination behavior of formalin-treated cells differs from that of fresh cells in that even those appearing most strongly agglutinated according to the patterns can be resuspended easily and smoothly with no evidence of clumps as would be observed with fresh cells. This phenomenon is under investigation and a brief report has been given(11).

These cells can be stored at 3-5°C in 8% formalin-saline for extended periods without loss of serological reactivity. Preparation SA-H6 has been stored for 15 months and BGG-H8A and BGG-H8B for more than 2 months with no change in sensitivity detectable by a two-fold dilution agglutination test.

Cells prepared as described here have the further advantage of being insensitive to traces of formaldehyde. Five different preparations of these cells gave just as good or better negative patterns with 1:100 NRS when simply washed four times with 30 volumes or more of saline in the cold as when subjected to the prolonged and relatively involved procedure described by McKenna(6) to eliminate traces of formaldehyde. With preparations SA-H6 and BGG-H8 simple washing led to distinctly better negative patterns as shown in Fig. 2.

The methods described have been applied to rabbit cells with satisfactory results. However, the rabbit cells seemed to be no less prone than human cells to be agglutinated by some normal rabbit serums. Dilute buffered formalin was used for treatment of cells by Moskowitz and Carb(12) but they found the treated cells to be non-agglutinable under their conditions. These methods have been used successfully by J. F. Lawlis working in my laboratory(4), and are now being used in work on the formation in rabbits of antibodies homologous to the sulfanilazo hapten.

The reactions which may take place on treating proteins with formalin under conditions similar to those used here have been investigated in detail by Frankel-Conrat and Olcott(13). On the basis of their results, one might expect proteins to be coupled to red blood cells by methylene bridges between various amino acid side chains. It seems possible that the sensitivity to agglutination by formaldehyde of cells which have been homogenized for an hour in the Waring blender might result from the exposure of surfaces not saturated with formaldehyde which could then couple with portions of other cells having aminomethylol groups on them.

*Summary.* 1) Improved methods for preparation of formalinized red blood cells and a simple method using formalin to bind proteins to these cells yielding a stable, highly sensitive antigen have been described. 2) Cells prepared by these methods give agglutination patterns almost as distinct as those obtained using fresh cells, they are not agglutinated by traces of formaldehyde and are stable on storage at 3-5° C. 3) When cells were coupled with sulfanil-azo-groups a convenient and sensitive antigen for the estimation of antibody against this hapten was obtained.

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## Some Factors Affecting Blood Formation in Turtles. (24382)

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The fundamental factors which influence blood formation in cold-blooded animals are unknown. Hypoxia, generally considered the universal stimulus for erythropoiesis, has no effect in Pisces, Amphibia, and in Reptilia (1,2). Cobalt readily produces polycythemia when administered to birds and mammals, but in frogs an increase in erythrocyte count was produced only by a highly toxic dose(3). Liver extracts, saponin, and even experimental hemorrhage, did not stimulate production of new blood cells in the hypoplastic frog marrow(4,5). It is quite unusual that factors which normally stimulate blood formation in warm-blooded animals fail to do so in cold blooded forms. Further investigation on the physiology of blood formation in cold-blooded animals is needed. Since most of the previous work has been on amphibians, it seemed desirable to study another class such as the reptiles. Experiments have been conducted on the effects of cobalt, cobalt with iron, copper and manganese supplements, crude liver extract, a folic acid antagonist, aminopterin, and of repeated bleeding on blood formation in box turtles.

*Materials and methods.* Adult box turtles (*Terrapene carolina carolina*) of both sexes were housed indoors with room temperature between 20 and 23°C. They were fed horsemeat, greens, bread and tomatoes. Large pans of water were kept in the cages. Experiments were conducted between August and March, a period of sexual rest. Cobalt chloride ($6H_2O$), iron ammonium citrate, copper sulphate, and manganese sulphate were administered in aqueous solution in amounts indicated. The liver extract (crude) was obtained from Lederle Laboratories. Each cc contained Vit. $B_{12}$ activity equivalent to 2 $\mu$g of cyanocobalamin. The folic acid antagonist, aminopterin,* was dissolved by adding 0.4 cc of 1 M KOH/100 mg aminopterin in 100 cc $H_2O$. In the bleeding experiment blood samples were obtained by cardiac puncture using a 2-inch 20 gauge needle inserted near the forelimb. Repeated removal of 1 to 2 cc of blood proved adequate to stimulate blood formation. In other experiments each turtle was used for only one blood sample obtained by decapitation. Erythrocyte and leucocyte counts were made by using a diluting fluid previously described(6), except that 0.1% toluidin blue O replaced Wright's stain. Reticulocyte counts were made from smears stained with new methylene blue N following reticulocyte formula described earlier(2). A Klett-Summerson photoelectric colorimeter was used for hemoglobin determinations. Hemolysis was obtained by use of 0.3 M urea or 0.1% $Na_2CO_3$. Tissues were fixed in 10% buffered solution (pH 7.0) of formalin. Bones were decalcified by 5% formic acid. Paraffin sections were stained routinely with hematoxylin azure eosin.

*Results. Control blood.* Mean hematocrit values, hemoglobin concentration and erythrocyte counts of male turtles are significantly higher than in females (Table I). Leucocyte counts showed no significant sex difference. Thrombocytes were included in the leucocyte counts since they were difficult to distinguish in suspensions from lymphocytes and showed little tendency to clump. Differential analysis of leucocytes in blood smears was made. Mean control values for 13 turtles (both

* Aminopterin kindly supplied by Dr. J. M. Ruegsegger, Lederle Laboratories, Pearl River, N. Y.