satisfactory method for reasons mentioned above. Our method is far more sensitive and can be adapted to measurement of circulating ADH under various physiologic conditions.

Summary. A method of bioassay for small amounts of antidiuretic hormone in plasma of a dog is presented. The method utilizes rats rendered unconscious with ethanol, under constant water load, and with intravenous injections of all standards and unknowns. Change in specific gravity of rat urine expressed as antidiuretic index is used as criterion of potency. This method appears to be the most sensitive bioassay for ADH available in that small dose changes of reference vasopressin, or endogenous circulating ADH, can be differentiated.

1. Ames, R. G., Moore, D. H., Van Dyke, H. B., *Endocrin.*, 1950, v46, 215.

2. Van Dyke, H. B., Adamsons, K., Jr., Engel, S. L., *Recent Prog. in Hormone Research*, 1955, v11, 1.

3. Hare, K., Hickey, R. C., Hare, R. S., *Am. J. Physiol.*, 1941, v134, 240.

4. Ginsburg, M., Heller, H., *J. Endocrin.*, 1953, v9, 274.

5. Jeffers, W. A., Livezey, M. M., Austin, J. H., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 184.

6. Blackmore, W. P., Chester, H. T., *Endocrin.*, 1956, v59, 493.

7. Kleeman, C. R., Rubini, M. E., Lamdin, E., Epstein, F. H., *J. Clin. Invest.*, 1955, v34, 448.

8. Crawford, J. D., Pinkham, B., *Endocrin.*, 1954, v55, 521.

9. Thorn, N. A., *J. Exp. Med.*, 1957, v105, 585.

10. Barbour, H. G., Hamilton, W. F., *J.A.M.A.*, 1927, v88, 91.

11. Verney, E. B., *Proc. Roy. Soc. London*, Series B, 1947-48, v135, 25.

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Hemosiderin. Iron Extraction Studies.*† (24606)

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Preparations of hemosiderin from horse spleens vary considerably in iron concentrations(1). Schwietzer(2) stated that part of hemosiderin iron may be present in crystalline form (limonite) as FeO(OH). After removing iron from hemosiderin with reducing agent, Greenberg(3) noted presence of apoferritin, thus indicating that this protein was originally present as ferritin. No other data are available concerning the form in which iron is present. This investigation is concerned with solubility of iron-containing compounds of hemosiderin in a chelating agent.

Methods. Hemosiderin was isolated from frozen horse spleens by fractional sedimentation as previously described(1). The procedure was modified by washing final product 4 times with distilled water. The washed hemosiderin pellet was changed to pasty consistency by addition of a few drops of water and the material stored in refrigerator in stoppered container. An aliquot of each sample was analyzed for total solids, iron and nitrogen. About 600 mg of "wet" hemosiderin were transferred to polyethylene bottle and 4 ml 9.2% disodium ethylenediamine tetraacetate (Na_2EDTA)/mg of iron were added. This mixture, to which 1 ml toluene was added, was continually agitated by magnetic stirrer. After centrifuging, 0.5 ml aliquots were removed, as a rule, after 1, 5, 15 and 25 days of stirring, for determination of iron. To release apoferritin from hemosiderin, we removed iron according to procedure of Greenberg(3). About 10 g of "wet" hemosiderin, equivalent to about 2 g of dried material, were suspended in dialyzing bag containing 60 ml of 1 M acetate buffer (pH 4.6), the bag then placed in 500 ml stoppered graduate filled with $Na_2S_2O_4$-acetate buffer and the cylinder rotated at slow speed. The reducing solution was changed every 12 hours. At end of 3 days the dialysate was practically free of iron. Subsequently, the material was dialyzed

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against cold tap water for 48 hours. To obtain a clear supernatant solution, the contents of bag were centrifuged at about 17,000 x g for 20 minutes; the residue washed twice with water and the washings combined with supernatant solution. Apoferritin was crystallized from the yellow-brown supernatant solution according to method of Granick and Michaelis (4). After adding cadmium sulfate, a brown amorphous precipitate which formed immediately, was removed by centrifuging at 17,000 x g for about one hour. A similar precipitate was not obtained when using a solution of apoferritin. The first crystals were observed after about 48 hours and continued to form on prolonged standing. Our routine procedure allowed this mixture to stand 6 days, centrifuge off the crystals, and allow the clear solution to stand for 3 more days. Additional crystals which formed, were removed and on further standing, crystals were no longer observed. Nitrogen content of collected crystals was determined. Iron was analyzed by wet ashing about 25 mg of dried hemosiderin in 2 ml concentrated H_2SO_4 and subsequent addition of several drops of 30% H_2O_2; 20 ml water were added and the mixture boiled 10 minutes to dissolve insoluble iron sulfate. This solution was brought to volume and aliquots analyzed for iron by adaptation of Laurell procedure(5). Iron extracted by Na_2EDTA could be determined without prior ashing provided excess phenanthroline was added. Nitrogen determinations were done by micro Kjeldahl procedure.

Results. Fig. 1 shows amount of iron extracted from "wet" and lyophilized dried hemosiderin suspended in 9.2% and 4.6% Na_2EDTA. An appreciable amount of iron, varying 24 to 28%, is extracted during first 24 hours; then iron is removed at a much slower rate. These results show that the most effective extraction of iron is attained with 9.2% Na_2EDTA- "wet" hemosiderin mixture. After 20 days 95% and 88% of total iron of "wet" and dry hemosiderin, respectively, is dissolved. Varying the ratios of volume of chelating agent/mg hemosiderin iron (2:1, 4:1, 8:1) did not affect amount of iron extracted.

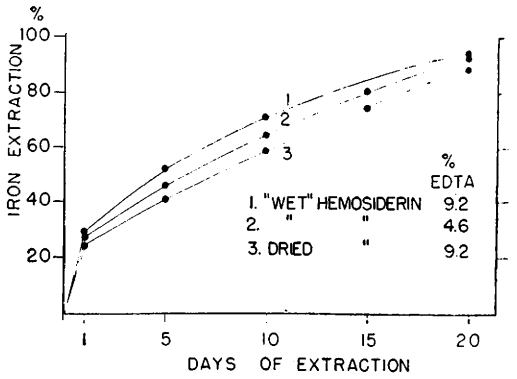

FIG. 1. Iron extraction from hemosiderin by Na_2-EDTA.

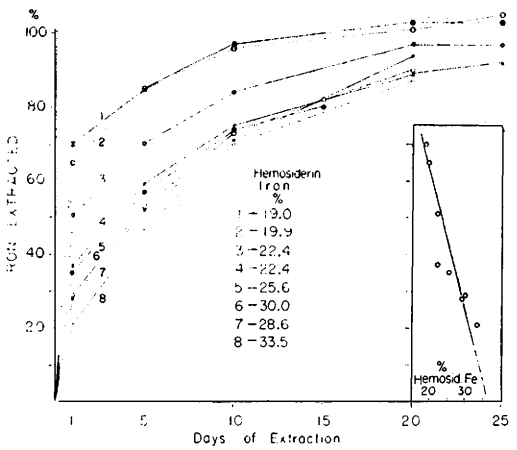

FIG. 2. Extraction of iron from 8 different hemosiderins by 9.2% Na_2EDTA. Data in insert show relationship between iron extracted during first 24 hr and total hemosiderin iron (19-33.5%).

Eight different preparations of "wet" hemosiderin from individual spleens were stirred in 9.2% Na_2EDTA solution for 25 days and per cent of extracted iron plotted against time (Fig. 2). There is an inverse relationship between amount of iron extracted at end of 24 hours and iron concentrations of hemosiderins. For example, 70 and 21% of hemosiderin iron is extracted from preparations containing minimum and maximum values of 19 and 33.5% iron. Parenthetically, it might be mentioned that iron concentrations for 9 different samples of hemosiderin were previously found to vary between 24.5 and 36.5%(1). The rate at which iron is dissolved after first day is related to initial hemosiderin iron concentration. The slow steady extraction continues until 90 to 100% of iron is removed

from hemosiderin in 25 days. The results indicate that iron may be present in more than one form in hemosiderin. Iron that is readily dissolved during the first 24 hours undoubtedly represents a compound(s) which differs in composition from a relatively insoluble iron-containing residual material. To explain the gradual increase in iron after first day of extraction, it may be assumed that iron is firmly bound to an insoluble protein which undergoes structural changes during extraction or that an insoluble iron complex is gradually converted to a hydrate(6). The relatively insoluble iron residue is probably not identical with limonite(2) since this material is insoluble in Na_2EDTA.

In 6 samples of water-soluble fraction of iron-free hemosiderin, the apoferritin nitrogen concentration constituted 1.2 to 3.5% of total hemosiderin nitrogen. These results represent .5 to 1.5% of ferritin in hemosiderin. These results may not be quantitative since the most reliable data would be obtained by immunological technics. If it is assumed that all ferritin iron reacts with Na_2EDTA during the first 24 hours, it represents only a small portion of extractable iron.

Summary. 1. It has been demonstrated that iron is extracted from horse spleen hemosiderin at different rates by disodium ethylenediamine tetraacetate. During the first 24 hours of extraction, between 21 and 70% of total hemosiderin iron was removed by chelating agent from 8 hemosiderin preparations which contained between 33.5 and 19% iron, respectively. The amount of iron removed is inversely proportional to total hemosiderin iron concentration. Subsequently, iron is dissolved at a slow rate until practically all is removed during 25 days extraction. 2. Analytical data for amount of ferritin present in hemosiderin are presented.

1. Ludewig, S., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 514.
2. Schwietzer, C. H., Acta Haemat., 1953, v10, 174.
3. Greenberg, D. M., 19th Ross Pediat. Research Conf., Portland, Ore., Oct. 20-21, 1955.
4. Granick, S., Michaelis, L., *J. Biol. Chem.*, 1943, v147, 91.
5. Laurell, C. B., *Acta Physiol. Scand.*, 1947, v14, Suppl. 46.
6. Schwietzer, C. H., *Deutsche med. Wschr.*, 1952, v77, 17.

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Strain Differences in Mercury Retention by Chicks.* (24607)

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Extensive information on pharmacology and toxicology of mercury is available(1,2). However, in a study of metabolism of organic mercury compounds, a difference in retention of mercury in strains of chicks was noted. This difference in strains of chickens selected for susceptibility and resistance to lymphomatosis is reported here. No reports have been found indicating a relationship between mercury retention and resistance or susceptibility to lymphomatosis. Pending further study, we do not imply such a relationship exists. No information is available on m.l.d. of phenylmercury acetate (PMA) with chicks. Löliger (3) fed a fungicide containing 5.1% phenylmercury pyrocatechol to hens at 1 g (26.5 mg mercury)/kg body weight without deaths. Hagen(4) reported the dose of same phenylmercury compound injected intravenously causing death to mice was 50-100 mg (25-50 mg mercury)/kg body weight. Basic phenylmercury nitrate injected intravenously at 8.6 mg mercury/kg body weight was fatal to rabbits(5). At Western Washington Exp. Station (WWES), intramuscular injections of 9 mg mercury at PMA/kg body weight caused

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