in distilled water. Rinse 3 to 4 times in 0.5 per cent. aqueous solution of hydrochloric acid. Rinse thoroughly in water. Leave for 1 minute in a weak solution of lithium carbonate (3 drops of a saturated aqueous solution per 100 cc of water). Rinse thoroughly in water.

3. Rinse in distilled water, then in 50 per cent., 70 per cent., 80 per cent. and 95 per cent. alcohols.

4. Stain for 1 minute in the following solution (OG 6):[8]

5. Rinse 5 to 10 times in each of two jars containing 95 per cent. alcohol, to remove excess stain.

6. Stain in EA 36 or EA 25 for 2 minutes.

7. Rinse 5 to 10 times in each of three jars containing 95 per cent. alcohol. (Do not use the same alcohol which was used after Orange G.) Rinse in absolute alcohol and xylol. Mount in Clarite, Canada Balsam or Gum Damar.

The advantages offered by this staining method are the following: (1) The epithelial cells and the erythrocytes are more transparent. Overlapping cells can be more easily differentiated. (2) The color of the acidophilic cells varies from red to orange. This helps in the identification of certain smear types. Basophilic cells stain green or blue-green. (3) Cells or fragments of tissue penetrated by blood take a characteristic orange or orange-green color which permits an easier recognition of small amounts of blood, even when erythrocytes are not distinctly seen. (4) Smears which were subjected to partial or even complete drying can be stained fairly satisfactorily. The differential coloring is not entirely lost.

Stains EA 36 or EA 25 can be used for short staining by those who want to make an immediate examination of a slide. No fixative needs to be used. The slides are dipped directly in the staining solution or covered by stain contained in a dropping bottle. The smears are thus fixed and stained simultaneously within a few minutes, although they may be kept in the stain for a longer time without being overstained. The excess stain is washed off in 95 per cent. alcohol and then the slides are carried through absolute alcohol and xylol and mounted in Clarite. The nuclei are stained faintly, but the cells show good differential staining and retain their transparency. Smears stained by this simple method can be restained by a repetition of the procedure described in this paper, including hematoxylin. This will improve the nuclear staining as well as the cellular differentiation and will permit the use of the same smears for a more detailed cytological study.

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time. The loss from filtering and evaporation is gradually replaced by the addition of fresh stain.

[8] The addition of phosphotungstic acid to the Orange G solution intensifies the orange color. For normal slides a slight acidification of 0.010 gm per 100 cc (OG 8) or 0.015 gm per 100 cc (OG 6) is suggested. For cancer diagnosis a higher acidification of 0.025 gm per 100 cc (OG 5) is often preferable, as it gives a sharper contrast of the abnormal cell types.

Orange G	National Aniline and Chemical Co.	0.5 per cent. solution in 95 per cent alcohol	100 cc
Acid phosphotungstic	Merck		0.015 gm

FERRITIN AND APOFERRITIN

LAUFBERGER[1] discovered in 1937 an iron-containing protein extracted from horse spleen, containing about 20 per cent. iron. The compound was studied in more detail by Kuhn, Sorensen and Berkofen.[2] Our own investigation was started primarily with respect to the magnetic properties of this compound. In this preliminary note, however, we restrict ourselves to the statement of the result that the iron atom, which is in the ferric state, has a dipole moment of 3.8 Bohr magnetons per g-atom of Fe and thus is probably another representative of those rare ferric compounds with three unpaired electrons of which Pauling and Coryell[3] discovered one in the form of ferrihemoglobin hydroxide (alkaline) methemoglobin and we[4] another in the form of catalase. This note is more concerned with another very remarkable property of ferritin found on the occasion of these studies.

The crystals of ferritin obtained by adding $CdSO_4$ to its solution are, although rather insoluble in pure water, yet easily soluble in 0.5 to 1.0 per cent. ammonium sulfate solution from which they can be recrystallized by addition of $CdSO_4$. The iron in the ferric state is rather stable but can be gradually reduced to the ferrous state by sodium hydrosulfite ($Na_2S_2O_4$, sodium dithionite). When a solution of ferritin is mixed with $Na_2S_2O_4$, a sufficient amount of α, α'-dipyridyl and some acetate buffer of pH 4.6 is added to avoid alkaline reaction, the iron is gradually removed from the protein in the form of the soluble red ferrous dipyridyl complex which can be separated from the protein by dialysis. According to the period of time used for this process one can obtain protein solutions which after addition of $CdSO_4$ within a minute or so yield crystals of the same shape as originally, but more or less decolorized, and finally even quite colorless solutions and crystals. This iron-free protein will be referred to as *apoferritin*. The possibility of obtaining crystals of variable iron con-

[1] Laufberger, *Bull. Soc. Chim. Biol.*, 19: 1575, 1937.
[2] Kuhn, Sorensen and Berkofen, *Ber.*, 73: 823, 1940.
[3] Pauling and Coryell, *Proc. Nat. Acad. Sci.*, 22: 159, 210, 1936.
[4] Michaelis and Granick, *Jour. Gen. Physiol.*, 25: 325, 1941.

tent will explain why the iron analyses of various preparations may differ somewhat from each other. Taking 23 per cent. Fe as the maximum value, one may say that 23 per cent. dry weight of the crystals is iron. On removing Fe^{+++}, one may either imagine their replacement by protons, or their removal in the form of a hydroxide such as FeO(OH). Accordingly, either 23 per cent. or 36 per cent. of the mass of the crystal can be removed without essentially altering the very characteristic crystal shape. One may imagine how large is the volume occupied by non-protein interstices within the crystal lattice made up by the protein part. These interstices may be filled either by water or by ferric hydroxide without any obvious influence on the crystal lattice. One may imagine what amount of "impurities" or non-protein constituents can adapt themselves into the interstices of a protein crystal, and how critical one should be with respect to the homogeneity and "purity" of a crystallized protein. This critical aspect, of course, is not all novel. So, pepsin may be more or less acetylated[5] without influence on the shape of the crystals. Yet the present case shows this phenomenon to an extent unappreciated heretofore.

On the other hand, it would be quite absurd to consider the iron of ferritin simply as a contamination of the protein. Rather may one say that the specific structure of apoferritin is adapted to the task of fixing iron atoms in the ratio of about one half to one Fe to each peptide link, without losing the specific structure. It is easy to understand that the space between the four N atoms of a porphyrin allows one Fe atom to be enclosed without loss of the porphyrin structure, but it is hard to imagine a structure of a protein allowing the building in of iron to that enormous extent as found in ferritin. One can not exclude the possibility that a cluster of many molecules of ferric hydroxide such as are known to represent a micelle of a colloidal ferric hydroxide solution, form a center surrounded by apoferritin molecules, reminding one of the very open structures of the zeolites. Such an assumption would dispense with the necessity of linking almost each peptide group with an Fe atom. Study of the crystal structure may be able to decide this point.

The iron content of ferritin is rather constant, *i.e.*, within 2–3 per cent. Ferritin from a normal horse was compared with that from a horse which had been subjected to many bleedings. Although the yields of ferritin obtainable from the two horses differed by 10 : 1, the Fe content of the ferritin was the same. At present we have no evidence for the existence of apoferritin as such in the spleen. That ferritin is not an artefact due to the method of preparation can be seen from the fact that the juice of spleen mixed with $CdSO_4$ without any other treatment yields crystals of ferritin.

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[5] Northrup, "Crystalline Enzymes," Columbia University Press, 1939.

THE PRODUCTION OF ANTIBODIES IN VITRO

BY following the general procedure proposed in connection with a theory of serological phenomena,[1] we have succeeded in making antibodies in the laboratory.

The procedure consists in subjecting normal globulin or other protein to the action of denaturing reagents or conditions in the presence of an antigen. The protein molecule unfolds, and then refolds in such a way as to assume a configuration complementary to that of the antigen, thus acquiring the properties of a specific homologous antibody.

Bovine γ-globulin was the protein used in most of our experiments. Some success was obtained also with other serum globulin fractions and with serum albumin. As antigens there have been used the triphenylmethane dye methyl blue (a mixture of the *p*-trisulfonated and disulfonated triphenylpararosanilines), the azo dye 1,3-dihydroxy-2,4,6-tri(*p*-azophenyl-arsonic acid) benzene, and pneumococcus polysaccharide type III. Successful experiments were made by addition of alkali (to pH 11) and slow return to neutrality, by addition and slow removal of urea, and by heating to about 65° and slowly cooling; some success was also obtained by surface denaturation. The most satisfactory of the treatments tried was that of holding a solution of protein and antigen for several days at about 57°; this temperature seems to be high enough to cause the protein chains to unfold and to refold under the influence of the antigen into specific complementary configurations.

In one experiment a solution containing 0.01 per cent. of the azo dye mentioned above and 1 per cent. of bovine γ-globulin was held at 57° for about 14 days, then removed from the bath, and dialyzed through Cellophane against 1 per cent. salt solution. Some precipitate formed during the heating and more during the dialysis. The mixture was then dialyzed against a 1 per cent. solution of the haptene arsanilic acid, which was changed several times. Most of the precipitate dissolved during this dialysis; that which remained was discarded. The solution, which was free of dye, was then dialyzed against 1 per cent. salt solution to remove the haptene.

[1] L. Pauling, *Jour. Am. Chem. Soc.*, 62: 2643, 1940.