centage of nucleated red cells in the marrow smears was not increased. This was also true of normal rats injected with cobalt(7). This does not mean, however, that there was not an absolute increase in the number of red cell precursors in the marrow since sections of the marrow were not studied.

Conclusions. The anemia of hypophysectomy was repaired by the administration of cobalt. The increase in red cell volume was of the same magnitude as that reported in normal rats. Apparently the pituitary is necessary for a normal erythropoietic response to hypoxia but is not necessary to obtain a normal erythropoietic response from cobalt. In this way the mechanisms responsible in each case appear to differ.

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A Simple and Sensitive Histochemical Method for Calcium. (19661)

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A histochemical method for staining calcium should be both specific and sensitive. The commonly used Von Kossa silver nitrate stain(1) has great sensitivity but lacks specificity for calcium: its use depends upon the fact that it stains the anions usually in association with calcium, namely phosphate and carbonate. Some of the alizarin dyes were shown by Cameron(2) to have considerable specificity but lacked sensitivity, a defect that has not been remedied by a number of modifications(3-6). Because of low sensitivity this test was found to be inapplicable to the study of early pathological calcification during the period when only minute foci were present.

In conjunction with chemical and anatomical studies(7,8), investigations were undertaken aiming at a stain that was specific, sensitive, and simple. These studies have led to the development of an alizarin staining technic which is almost as sensitive(8) as the Von Kossa and yet which is specific for calcium under the usual conditions of normal and pathological calcification. With it, studies have shown very early intracellular calcification at a time when the Von Kossa test was completely negative, thereby revealing the inadequacy of the latter as an index of the calcium present. Calcium deposits have been demonstrable in tissues from 12-48 hours before microanalytical procedures revealed the presence of excess calcium. Its use with more heavily calcified tissues has shown that the technic gives a satisfactory relative estimate of the amount of calcium present, correlating well with quantitative estimates.

Reagents. 1. Fixative—95% ethyl alcohol. 2. 1% alizarin red S† (sodium alizarin sulfonate) in 0.10% NH_4OH, final pH 6.3-6.5. Stir 0.5 g of the dye into 45 ml of distilled H_2O so that only a few small grains of dye remain undissolved. Add 5 ml of a 1:100 dilution of C.P. 28% NH_4OH slowly with constant stirring. The pH which results is

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† Obtained from National Aniline Division, New York City.

TABLE I. Calcium Preservation by Different Fixatives.

Fixative	pH	Ca stain
10% formalin, aqueous	3.3	0 to +
saturated with marble chips	7.2	+
" " $Pb(OH)_2$	7.20	+
" " $MgCO_3$	8.9	2+
" " Na_2CO_3	10.83	2+
in .05 M veronal buffer	8.7	+ to 2+
in .2 M borate buffer	8.35	+ to 2+
95% alcohol		3+ to 4+
95% alcohol saturated with Na_2CO_3		3+ to 4+

These results were obtained in 3 series of rats inj. with 2 or 10 mg/kg of $UO_2(NO_3)_2 \cdot 6H_2O$ and killed after 12-48 hr at which times the accumulation of calcium is limited, and differences therefore are apparent more easily. Samples from the same kidney were placed in each of the fixatives and handled in the usual fashion thereafter. The relative ratings represent avg of the 3 series.

6.36-6.40 as measured by a Beckman glass electrode pH meter. This solution is stable for at least one month if kept covered when not in use. After several hundred slides have been stained, the need to renew the solution will be shown by the formation of a precipitate, which is caused by the introduction of alcohol into the solution. 3. Xylol. 4. Absolute and 95% ethyl alcohols. 5. Distilled H_2O—pH *ca* 5.8.

Procedure. Specimens not exceeding 2-3 mm in thickness are fixed and dehydrated by exposure to 4 changes of 95% ethyl alcohol over a 36-hour period, followed by overnight immersion in absolute ethyl alcohol. Embedding is done directly into paraffin from which sections are cut at the desired thickness and placed without adhesive on unscratched, clean slides. Elimination of adhesive from the usual technic of mounting sections was found to be important because both gelatin and egg albumen caused fixation of the stain in a variable manner. The staining procedure is carried out at room temperature. After removal of paraffin with 2 rinses of xylol, a section is washed in 2 changes each of absolute ethyl alcohol and 95% ethyl alcohol, drained for a few seconds on a paper towel and placed in the stain for 2 minutes. Surplus stain is removed by irrigation with a gentle stream of distilled water (pH 5.8), and subsequent washing of the section in a jar of distilled water for 5-10 seconds, by which time maximum decolorization should be obtained. The stained section is dehydrated again by rinsing in 95% alcohol then absolute alcohol (2 changes of each), clarified by immersion in xylol (2 changes), and mounted in cedar wood oil. With this technic any deposits of calcium salts are revealed by an intense reddish-orange coloration, set against an almost imperceptible pink background. The specific stain should be so sharply defined that granules as small as a few micra in diameter can be seen as loci of calcification. Slight diffusion of the color occurs during immersion in the stain and subsequent wash with distilled water. This diffusion can be minimized by reduction of the staining period to one minute and of the wash to the least interval required to make the background a faint pink. Further increase of definition may be obtained by rinse with 10^{-3} M HCl in 95% ethyl alcohol (concentrated HCl 1 part, 95% alcohol 10,000 parts) for 15 seconds after the wash with water; although not generally required, this additional step may be of advantage in localizing minute foci of calcification.

Observations. Fixation of tissue. The results of fixation with a variety of solutions are shown in Table I. It had been appreciated early that acid fixatives would lead to loss of calcium salts, but not that there would be a significant difference between the neutral or alkaline formalins, as compared with 95% ethyl alcohol. As the table indicates, 95% alcohol was far superior to the other fixatives listed, and because the alkaline alcohol caused cellular swelling without enhancing the stain, neutral 95% alcohol was chosen as the fixative of choice. The considerable increase in

TABLE II. Solubility of Various Alizarate Complexes.

Salt	pH at which ppt. first appeared	pH range of max ppt.	pH range at which ppt. present	Color of alizarate
$CaCl_2$	3.90	4.28–6.75	3.90–8.05	Reddish-orange
$MgNH_4PO_4$	0	—	0	—
$MgCl_2$	0	—	0	—
$MgSO_4$	0	—	0	—
$FeCl_2 \cdot 4H_2O$	5.62	5.62–8.05	5.62–8.05	Deep purple
$Fe(NO_3)_3 \cdot 9H_2O$	5.62	5.73–6.26	5.62–8.05	Purple-black
$Ba(C_2H_3O_2)_2$	4.08	4.08–4.50	4.08–8.05	Reddish-orange to magenta
$SrCO_3$	4.50	4.50–7.38	4.50–8.05	Reddish-orange
$BeSO_4$	5.73	6.26–8.05	5.73–8.05	Magenta
$CdCl_2$	5.11	5.11–5.73	5.11–8.05	Reddish-orange to magenta
$La(C_2H_3O_2)_2$	4.50	5.73–8.05	4.50–8.05	Cherry to light purple
$Pb(C_2H_3O_2)_2$	3.50	4.08–4.50	3.50–8.05	Reddish-orange to purple
$HgCl_2$	0	—	0	—
$UO_2(NO_3)_2 \cdot 6H_2O$	4.08	4.50–5.62	4.08–8.05	Purple
$ZnCl_2$	0	—	0	—

To 2 ml of buffer (.2 M acetate buffer pH 3.50–5.87, or .1 M histidine buffer pH 5.73–8.05) in a small test tube, .5 ml of a 1% aqueous solution of the salt was added and shaken, followed by 1 uniform drop of the ammonium-alizarin staining solution, then mixed thoroughly. The tube was then centrifuged for 2 min, and precipitate and supernatant compared with a control tube containing the same buffer and alizarin, but .5 ml of H_2O in place of salt solution.

the number of stained foci with this fixative suggested the possibility that the enhanced binding of the dye might be due to a change in the tissue proteins, rather than to an improved preservation of calcium salts. This problem was first studied by noting the effect of a 1 N HCl wash, applied to kidney sections from a series of uranium-poisoned rats(7), specimens from which were preserved in both alkaline formalin and alcohol. In all instances the stained foci were shown to be present in sections which had not been exposed to the acid, whereas they were absent from the alternate sections that had received the acid wash. Further investigation of this problem was made by exposing sections to the chelating agent di-sodium-ethylene-diamine tetra-acetate which forms soluble non-ionic complexes with most di- and tri-valent metallic cations in such a manner that they no longer behave as metallic ions(9). If there were no focal areas of stain after subjecting sections to the effects of this agent, it would suggest that the ion originally responsible for combining with the stain had formed an undissociated complex with the chelating agent. Control serial sections were exposed to 0.17 M ethanolamine buffer(10) and test sections to equal parts of buffer and chelating agent (0.45 g/100 ml H_2O), brought to pH 10.4 with HCl, for 30 minutes with gentle continuous agitation. After being stained, the control slides were completely comparable with untreated stained sections whereas the test sections were either absolutely negative or at most contained a rare stained area. Finally, the observation that tissues from control (normal) rats had the same staining characteristics whether fixed in the formalins or 95% alcohol demonstrated that fixation with alcohol did not cause a general increase in the non-specific fixation of dye.

Specificity. Since spot tests with a number of magnesium salts had shown magnesium alizarate to have colors similar to calcium, it was of significance to find that calcium alizarate was almost completely insoluble in buffers over the pH range 4.0-6.8, whereas the magnesium alizarate was freely soluble (Table II). As a result the washing with distilled water, which contains traces of CO_2, eliminates interference due to the presence of magnesium. This specificity is fortunate because magnesium may accumulate in significant amounts in tissues undergoing pathological calcification(11).

Strontium, barium, beryllium, lead, and cadmium are not distinguishable from calcium (Table II); in the special problems that require consideration of their presence in the

tissues, supplementary differential procedures must be employed. Iron, although it yields a purple-black precipitate when present as an ion, produces no color when present in hemosiderin, a non-ionic compound. This was verified by the absence of stain in sections of a liver‡ from a patient with hemochromatosis, despite the fact that massive deposits of hemosiderin could be demonstrated by the Prussian blue reaction.

The results with the present method have been compared with those obtained using the Von Kossa silver nitrate stain. Although long used to identify calcium, the Von Kossa procedure is not specific for that ion, but instead indicates a number of anions of which that most commonly in association with calcium, namely phosphate, is one(2). In early calcification in which analytical data(7) clearly indicated that at least part of the calcium was associated with an anion other than phosphate, the present alizarin technic revealed calcium deposits in the absence of a stain with the Von Kossa technic. In contrast, areas found positive to the Von Kossa stain were invariably positive to alizarin. Thus in each case in which an accumulation of calcium has been indicated by an independent method, it has been demonstrable with the alizarin stain.

Sensitivity of the method. When employed as a spot test on filter paper, the present method is positive for calcium above a limiting surface density of 0.02-0.002 $\mu g/mm^2$. A study with the usual 1% aqueous alizarin stain(6) revealed the present technic to be some 50 times more sensitive. This high sensitivity is dependent on proper adjustment of acidity: without neutralization a 1% solution of the dye has a pH of about 3.6 and a light brown color whereas with adjustment of the pH to 6.3 the medium is brought into the range of insolubility of the calcium alizarate and of maximum color intensity of the indicator dye. For neutralization of the stain NH_4OH was found to be superior to KOH and NaOH, since the non-specific background stain in tissue is much less when NH_4OH is employed. In use as a histochemcial procedure the stain has a greater sensitivity than manifest in spot tests. Although magnification of a field reduces the depth of color, the focal distribution of calcium deposits allows the recognition of minute areas of intense stain. From studies in which early pathological calcification of the kidneys was quantitated by direct analysis(7), it was found that the histochemical test became positive at a time when no more than 0.05 mg Ca/g tissue had accumulated; calculated for a section of 7 μ thickness this is equivalent to a surface density of 0.0004 $\mu g/mm^2$. Mordants, which have been recommended for use with alizarin dyes(5), were found to give no increase of sensitivity under the conditions of the present test. The following salts in concentrations of 0.01-10.0% were without effect: $AlCl_3$, $AlK(SO_4)_2$, $Al(OH)_3$, KCl, $K_2Cr_2O_7$, KI, $KHCO_3$, Na_2CO_3.

Among a variety of attempts to remove the pink background stain while preserving the calcium alizarate, none proved as successful as a distilled H_2O wash. Included as soaks or rinses in different concentrations before or after staining were the following: $(NH_4)_2SO_4$, urea, KOH, NH_4OH, the chlorides of Na, Hg, Mg, Zn, and Al, $La(C_2H_3O_2)_2$, 10 and 20% formalin in 95% alcohol. The relative permanence of the present stain is indicated by the oldest slides stained with the present technic: after 5 months there has been no loss in intensity of the color, and diffusion of the dye has been, at most, very slight.

Results with the stain. This stain has been used to localize pathological accumulations of calcium in the kidneys of rats poisoned with uranium, and physiological deposits in cartilage from rat tibiae;§ it has shown excellent definition of calcified areas in human kidney, aortic valve with attached myocardium, and abdominal aorta,‖ all of which showed gross calcification (Fig.1-6). In the case of the

‡ Obtained from Dr. William Eisenmenger of the Hospital of The Rockefeller Institute for Medical Research.

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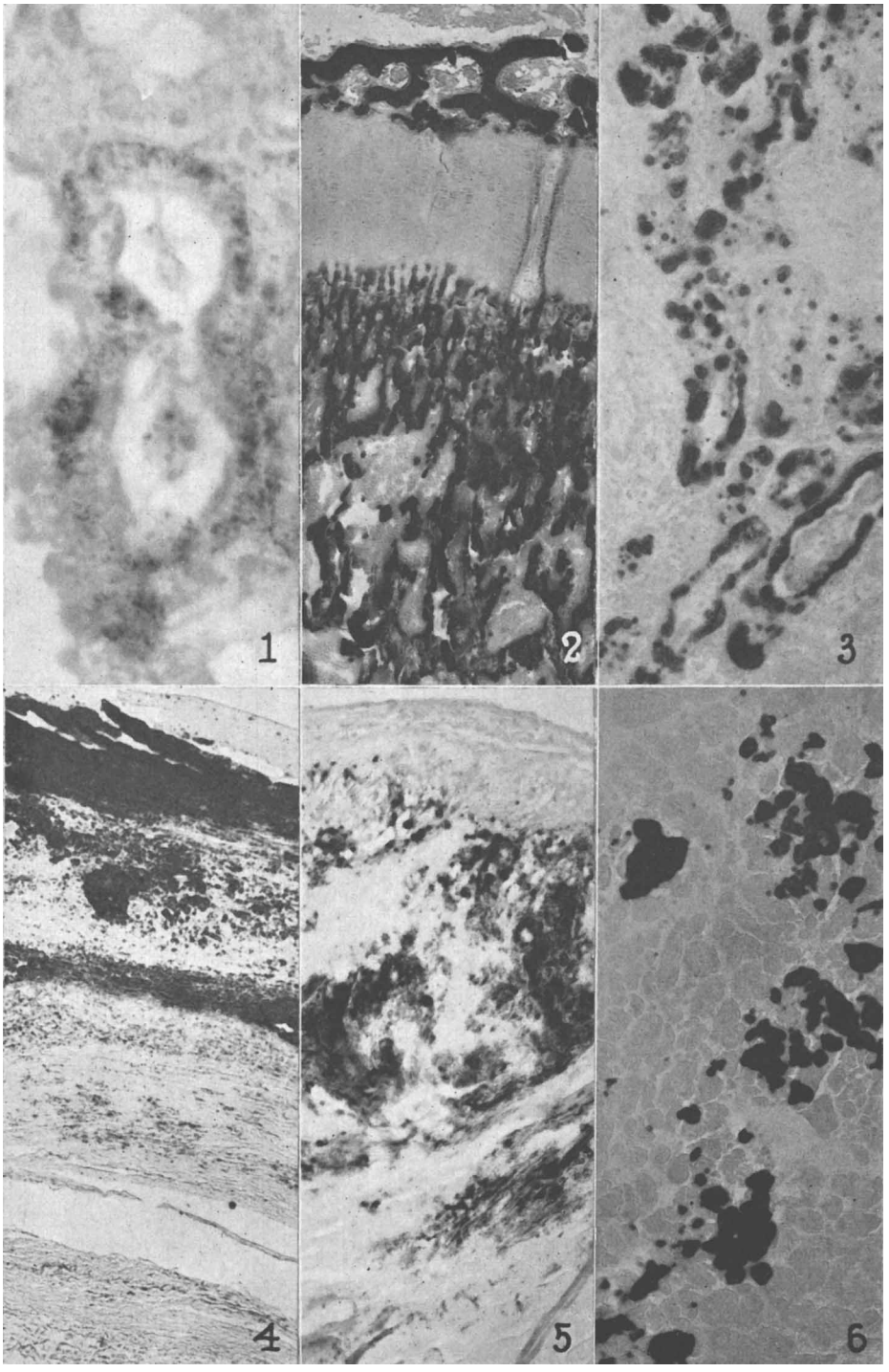

FIG. 1. Rat kidney, showing early calcium deposits in a single proximal convoluted tubule. Alizarin. × 710.

FIG. 2. Normal 51-day rat tibia. Alizarin. × 56.

FIG. 3. Calcification in human kidney. Bellevue Hospital Autopsy #39383. Alizarin. × 128.

FIG. 4. Atherosclerotic plaque—human abdominal aorta. Bellevue Hospital Autopsy #39551. Alizarin. × 56.
FIG. 5. Calcification of aortic valve. Same case as in Fig. 4. Alizarin. × 128.
FIG. 6. Calcification of myocardial fibres. Same case as in Fig. 4. Alizarin. × 128.

uranium damaged kidneys, the method revealed minute foci of accumulation from 12-48 hours before an increase could be unequivocally demonstrated by quantitative microanalysis. The correlation of the histochemical test with quantitative analysis of the tissue was investigated in a series of sections that had been taken from the kidneys of rats after injury with uranium. On the basis of the alizarin stain 8 kidneys were ranked from greatest to least calcification. The sequence thus obtained correlated closely with the sequence based upon quantitative analysis (Table III).

TABLE III. Comparison of Tissues According to Amounts of Calcium Present.

Quantitative analysis mg Ca / g tissue	Rank	Alizarin stain Rank
8.82	1	1
6.48	2	3
5.06	3	2
2.87	4	4
2.60	5	6
1.78	6	5
1.27	7	8
1.17	8	7

The kidneys from 8 uranium-damaged rats(7) were subjected to microanalysis for calcium. Amounts of calcium found are shown at left. Sections of kidneys from each of these animals were stained with alizarin red S, and the amounts of calcium shown by this stain were ranked from greatest to least. The column on the right indicates this ranking, which is to be compared with the absolute rank, shown in the middle column.

Summary. A technic is described for histochemical definition of calcium. It is specific for calcium in the presence of magnesium, approximately 50 times as sensitive as previous methods based upon the use of alizarin, and capable of localization to within a few micra.

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Linkage Studies of Genes for Sickling and MNS Blood Groups: Negative Findings. (19662)

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Snyder, Russell and Graham(1) presented evidence of linkage between the genes for MN blood types and the gene for sickling of erythrocytes. Subsequent studies by Snyder, Clark and Moore(2), and Snyder(3) gave additional supporting evidence for linkage between these two genes. The above studies were based on a total of 85 families, 12 of which were usable for linkage studies. Race *et al.*(4), in studies on the MN and S groups in 123 English families observed close linkage between the genes for these two blood groups, no recombination being observed in 82 children capable of disclosing it. The

‡ Deceased July 14, 1952.