which may result in an inordinate loss of this cation.

It is well known that chronic alcoholism is associated with liver damage. Alcoholic addiction combined with the poor nutritional status of the alcoholic engenders fatty infiltration of the liver with subsequent irreversible hepatic fibrosis. Since the liver is commonly thought to be the site of plasma protein production, and in view of the great probability of serious liver damage in alcoholics, it is not surprising that markedly reduced plasma protein concentrations were encountered in these patients.

The signicant increment in erythrocyte K in the chronic alcoholic agrees with Nicholson and Taylor's conclusion(1) " . . . potassium must be retained in the cells." Their studies, however, were of short duration and involved a relatively limited alcohol consumption, whereas the current experiments involved the long-term, intemperate use of alcohol. Nevertheless, calculations assuming an extracellular fluid volume of 20% of body weight and using average weight of the alcoholic patients (71.6 kg) indicate that an average of 3 meq of extracellular K is lost. Erythrocyte K gain, based on an assumed red blood cell volume of 4% of the body weight, equals 8 meq. From such approximations it is clear that K is either being retained in the chronic alcoholic, or that some marked redistribution of K, associated with altered cell membrane permeability, has occurred.

Bianco and Jolliffe(8) have pointed out that quantitative anemia occurred in 61% of 184 cases of alcoholic addiction, which is not surprising in view of possible complications (stomatitis, pellagra, cirrhosis etc.). In this study average reduction in hematocrit from the control equaled 5% in the alcoholics. While this value was not seriously low (42%), it constitutes a statistically significant departure from the control.

Summary. Plasma and erythrocyte Na, K and Cl have been determined in 64 long-term chronic alcoholics before administration of therapy. Plasma Na, K, Cl and red blood cell Cl were significantly reduced below control values. Erythrocyte K was significantly elevated, while Na remained unchanged. Plasma protein concentration and hematocrit were significantly reduced from control values.

1. Nicholson, W. M., Taylor, H. M., *J. Clin. Invest.,* 1938, v17, 279.

2. Strauss, M. B., Rosenbaum, J. D., Nelson, W. P., III., *ibid.,* 1950, v29, 1053.

3. Rubini, M. E., Kleeman, C. R., Lamdin, E., *ibid.,* 1955, v34, 439.

4. Kleeman, C. R., Rubini, M. E., Lamdin, E., Epstein, F. H., *ibid.,* 1955, v34, 448.

5. Huang, K. C., Knoefel, P. K., *Arch. Int. Pharmacodyn.,* 1957, v109, 90.

6. Klingman, G. I., Haag, H. B., *Quart. J. Stud. Alcohol.,* 1958, v19, 203.

7. Peters, J. B., van Slyke, D. D., *Quantitative Clinical Chemistry: Methods,* Baltimore, Williams and Wilkins, 1932, v2, 829.

8. Bianco, A., Jolliffe, N., *Am. J. Med. Sc.,* 1938, v196, 414.

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Observations on Vitamin C Activity of D-Ascorbic Acid.* (24827)

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Previous studies have shown marked differences in retention of D-ascorbic acid-1-C^{14} and L-ascorbic acid-1-C^{14} when administered in small intraperitoneal doses to normal and Vit. C-deficient guinea pigs (1). D-Ascorbic acid is rapidly metabolized and excreted; most of the compound disappears from the body within 12 hours after the dose. In contrast, L-ascorbic acid is

* The authors are grateful to Carole Evans for synthesis of D-ascorbic acid and to Constance Glasgow for assistance in bioassay experiments.

present in the guinea pig for a considerable period of time, disappearing with an average half-life of 3 to 4 days(2). The observation that D-ascorbic acid is retained in guinea pigs to a considerably lesser extent than L-ascorbic acid suggests a possible explanation for the lack of Vit. C activity reported for the D-isomer(3,4). To test this hypothesis, bioassay experiments were carried out in guinea pigs under conditions in which essentially comparable tissue concentrations of D-ascorbic acid and L-ascorbic acid were maintained.

Methods and materials. A modified curative bioassay for Vit. C activity(5) was employed in 2 experiments. *Bioassay 1.* Fifty-four male guinea pigs weighing from 150 to 210 g were given a scorbutogenic diet. On the fifteenth day of the experiment the animals were treated daily for 10 days as follows: One-third received 12 mg of D-ascorbic acid, one-third received 2 mg of L-ascorbic acid and the remaining served as negative controls. *Bioassay 2.* Twenty-three guinea pigs, weighing from 250 to 350 g were placed on the scorbutogenic diet and after 18 days they were treated daily for 8 days as follows: Eight received 4 mg of D-ascorbic acid, 8 received 2 mg of L-ascorbic acid and 7 served as negative controls. In the case of both bioassay experiments, the daily dose of each compound was divided into 2 equal doses and administered by intraperitoneal injection at 12-hour intervals. Negative control animals received injections of saline at the same time intervals. The guinea pigs were weighed daily. At time of sacrifice they were examined for hemorrhages, particularly in the region of the knee joints. The lower jaws were immediately fixed in Lillie's aqueous neutral calcium acetate formalin solution(6) for 24 hrs, decalcified in 5% formic acid, dehydrated, embedded in paraffin and sectioned mesio-distally at 6 μ. Sections were stained with the Rinehart method for acid mucopolysaccharides(7), the alloxan-Schiff method for proteins(8), and Lillie's periodic acid-Schiff method for carbohydrates(6). The Vit. C free diet† was prepared as described by Woodruff, *et al.*(9). D-Ascorbic acid was synthesized by addition of cyanide to D-xylosone(10).

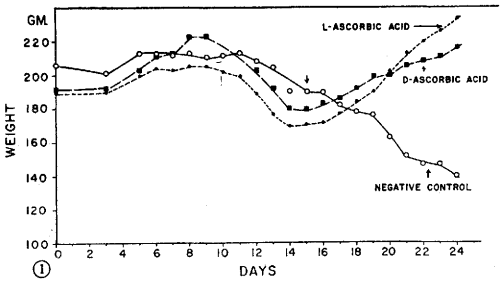


FIG. 1. Wt changes in guinea pig from each of the 3 groups in Bioassay I.

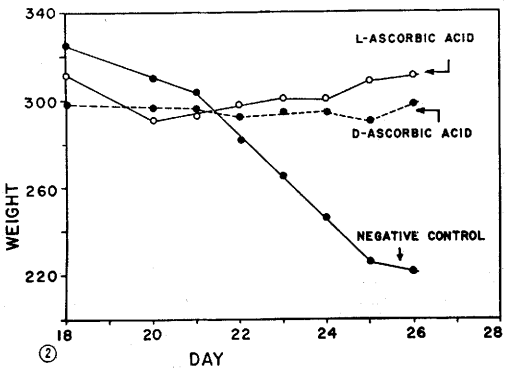


FIG. 2. Wt changes in guinea pig from each of the 3 groups in Bioassay II.

Results. The pattern of weight change in a representative animal from each group in Bioassay I is given in Fig. 1. The animals initially gained weight until about the eleventh day on diet when their weight declined. On the fifteenth day the animals were treated as described in Methods Section. The animals receiving D- and L-ascorbic acid showed comparable weight gains from the fifteenth to twenty-fifth day, averaging 30 g for each group. The guinea pigs treated with D-ascorbic acid had normal appetites and could not be distinguished in gross appearance from those receiving L-ascorbic acid. However, negative control animals all showed a marked loss in weight and had typical scorbutic appearance‡ (5). Out of 18 guinea pigs in the negative control group, only 4 survived at end of experiment, compared to 17 and 16 animals in

† Obtained from Nutritional Biochemicals Co., Cleveland, O.

‡ Sections of liver stained by periodic acid-Schiff method showed that animals in D- and L-ascorbic acid group had normal glycogen content whereas no glycogen was detected in the negative control group.

PLATE I. Photomicrographs of teeth.

A. Apical portion of a molar root from a normal guinea pig. D, dentin; P, pulp; E, enamel; O, odontoblasts. Rinehart stain. × 73.

B. Apical portion of a molar root from a normal guinea pig. Alloxan-Schiff stain. × 73.

C. Apical portion of a molar root from a guinea pig in negative control group. Rinehart stain. Note strong reactivity of stain for acid mucopolysaccharides in the abnormal osteodentin and connective tissues generally. × 73.

D. Apical portion of a molar root from a guinea pig in negative control group. Alloxan-Schiff stain. Note lack of reactivity of stain for proteins in dentin. × 73.

D-ascorbic acid and L-ascorbic acid groups, respectively. Despite ability of D-ascorbic acid to produce a comparable weight gain and survival rate as L-ascorbic acid, upon autopsy the animals receiving D-ascorbic acid showed hemorrhages about the joints similar to those observed in the negative control group. No hemorrhages were detected in the animals receiving L-ascorbic acid.

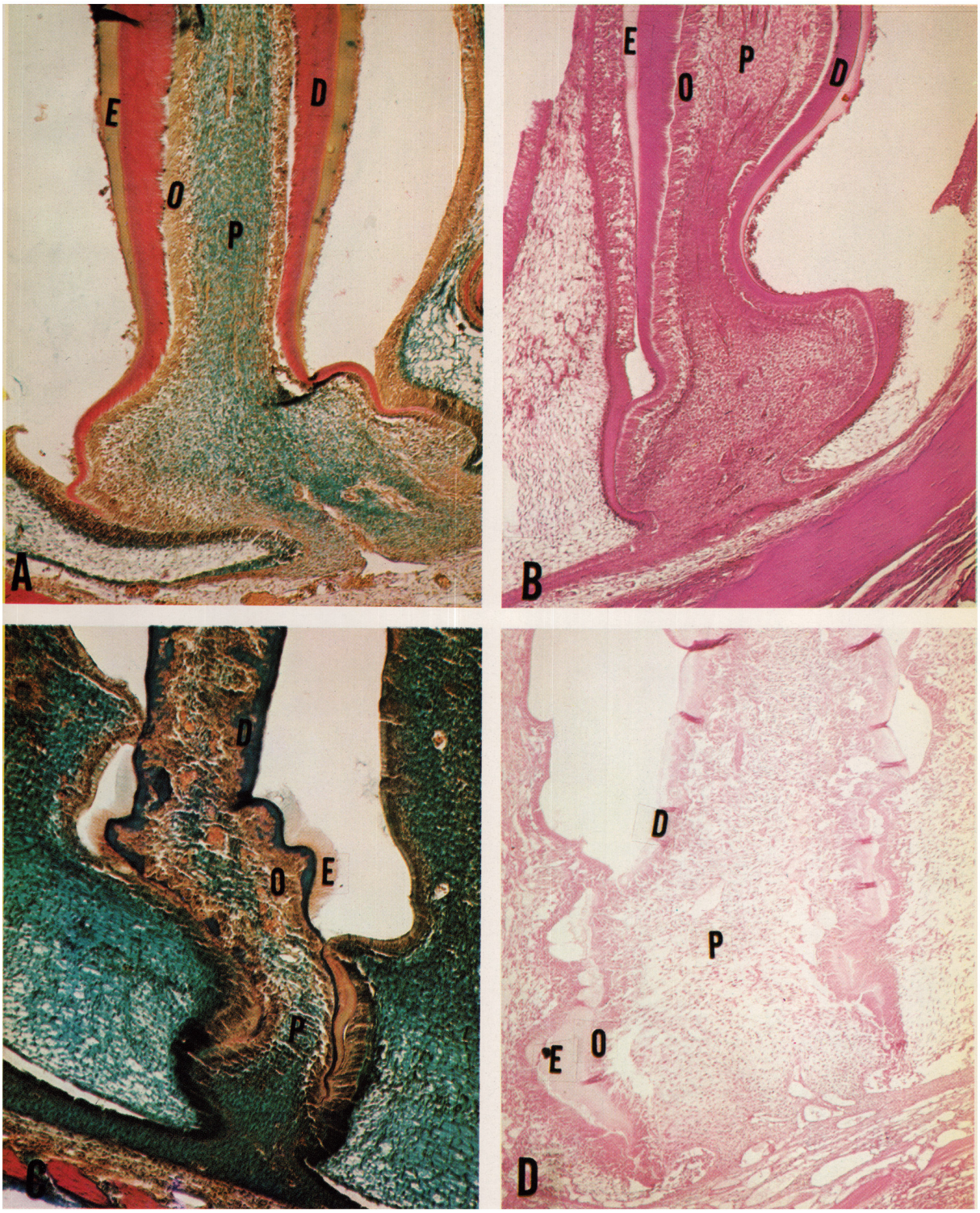
The pattern of weight change in an animal from each group in Bioassay II is given in Fig. 2. The results indicate that even the relatively small dose of D-ascorbic acid employed in this experiment was sufficient to maintain the weight of guinea pigs. In contrast, negative control animals all showed marked loss of weight. Seven out of 8 guinea pigs in each of the D- and L-ascorbic acid groups survived at end of the experiment, compared to 2 out of 7 in the negative control group. Upon autopsy, animals receiving D-ascorbic acid had hemorrhages about the joints to about the same extent as observed in Bioassay I.

Since changes in dental structures are a sensitive index of Vit. C deficiency(5), histochemical tests were made of the teeth of guinea pigs in Bioassay I. Dentin obtained from normal guinea pigs§ is tubular and stains red with both the Rinehart (Plate 1A) and alloxan-Schiff methods (Plate 1B). Dentin produced in the negative control group was atubular osteodentin, characteristic of scurvy. This abnormal dentin stained brilliant blue with Rinehart method (Plate 1C), indicating strong reactivity for acid mucopolysaccharides, and remained unstained or lightly stained with alloxan-Schiff method indicating lack of reactive proteins (Plate 1D). The small amount of bone produced during the scorbutic period was also strongly reactive with Rinehart stain for acid mucopolysaccharides and unreactive with alloxan-Schiff method for proteins. Thus the Rinehart stain proved to be a valuable method to detect the scorbutic state of guinea pigs.

Dentin produced in animals treated with L-ascorbic acid was tubular and stained the normal red with Rinehart (Plate 1E) and alloxan-Schiff methods (Plate 1F). Dentin produced in animals receiving D-ascorbic acid was also normal in that it had tubular structure and stained red with Rinehart (Plate 1G) and alloxan-Schiff methods (Plate 1H). However, predentin in this group usually reacted abnormally since it still retained the blue stain with Rinehart method (Plate 1G), and was unreactive with alloxan-Schiff method (Plate 1H).

Conclusion and summary. 1) Some activities of L-ascorbic acid can be replaced by D-ascorbic acid when administered in relatively small doses under conditions of these experiments. For instance, weight and survival of scorbutic guinea pigs can be maintained with D-ascorbic acid, but hemorrhages about the joints are not prevented. The most striking effect of D-ascorbic acid was revealed with histochemical studies of teeth. The dentin produced in animals receiving D-ascorbic acid was normal in morphology and staining properties. However, the Vit. C effect of D-ascorbic acid was not complete in that predentin formed in these animals was still abnormal in its reactivity to acid mucopolysaccharide stain though it was eventually converted to normal staining dentin. 2) These results furnish further evidence for multiple actions of Vit. C; those that are specific, for which only L-ascorbic acid will serve, and those that are non-specific in which D-ascorbic acid and presumably other compounds with the same

§ Guinea pigs which had been maintained on lettuce and Rockland Farm guinea pig diet, *ad lib.*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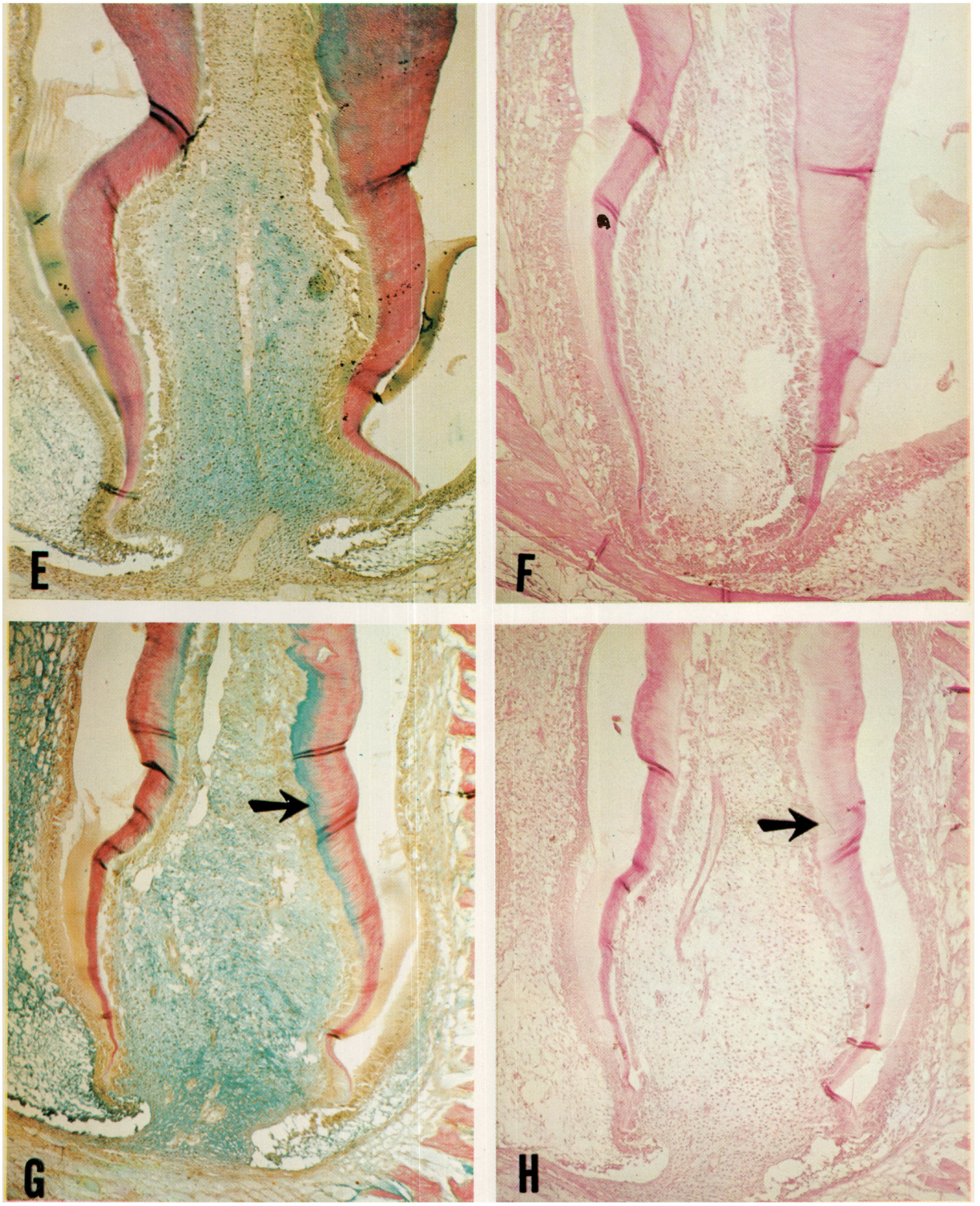
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E. Apical portion of a molar root from a guinea pig treated with L-ascorbic acid. Rinehart stain. Note normal morphology and staining characteristics of dentin formed during restoration period. × 73.

F. Apical portion of a molar root from a guinea pig treated with L-ascorbic acid. Alloxan-Schiff stain. Note normal morphology and staining characteristics of dentin formed during restoration period. × 73.

G. Apical portion of a molar tooth from a guinea pig treated with D-ascorbic acid. Rinehart stain. Note normal morphology of dentin and predentin. Note also that predentin (arrow) tends to retain the stain for acid mucopolysaccharides, whereas predentin in Plate IE does not. Dentin stains normally. × 73.

H. Apical portion of a molar tooth from a guinea pig treated with D-ascorbic acid. Alloxan-Schiff stain. Note normal morphology of dentin and predentin. Note also that predentin (arrow) tends to remain unstained, indicating lack of reactive proteins, while dentin stains normally. × 73.

redox properties can substitute. Separation of biological activities of Vit. C has been indicated by studies with D-araboascorbic acid in guinea pigs(4) and with D-ascorbic acid in monkeys(11). One of the best examples of non-specific role for the vitamin has come from studies of tyrosine metabolism in which L-ascorbic acid can be replaced by various other structurally unrelated compounds that are susceptible to oxidation and reduction(12, 13). Further studies with D-ascorbic acid in guinea pigs may throw light on the nature of non-specific functions of the vitamin.

1. Dayton, P. G., Burns, J. J., *J. Biol. Chem.,* 1958, v231, 85.

2. Burns, J. J., Dayton, P. G., Schulenberg, S., *ibid.,* 1956, v218, 15.

3. Demole, V., *Biochem. J.,* 1934, v28, 770.

4. Reichstein, T., Demole, V., *Festschrift,* Emil C. Barell, Basel, 1936, v107.

5. György, P., *Vitamin Methods,* Vol. I, Academic Press, N. Y., 1951, 244.

6. Lillie, R. D., *Histopathologic Technic and Practical Histochemistry,* 2nd ed. Blakiston Co., N. Y., 1954.

7. Rinehart, J. F., Abul-Haj, S. K., *Arch. Path.,* 1951, v52, 189.

8. Yasuma, A., Ichikawa, T., *J. Lab. and Clin. Med.,* 1953, v41, 296.

9. Woodruff, C. W., Cherrington, M. E., Stockell, A. E., Darby, W. J., *J. Biol. Chem.,* 1949, v178, 861.

10. Solomon, L. L., Burns, J. J., King, C. G., *J. Am. Chem. Soc.,* 1952, v74, 5161.

11. May, C. D., Hamilton, A., Stewart, C. T., *Blood,* 1952, v7, 978.

12. LaDu, B. N., Jr., Greenberg, D. M., *Science,* 1953, v117, 111.

13. Knox, W. E., *Amino Acid Symposium,* 1955, W. D. McElroy and H. B. Glass, Baltimore, Md., 850.

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Use of Bromelin to Demonstrate Erythrocyte Antibodies.* (24828)

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Technics to demonstrate circulating incomplete erythrocyte antibodies fall into 3 categories: (A) coating cells and suspending them in media containing anisometric molecules (albumin, etc.)(1), (B) an indirect Coombs' test(2), (C) pretreating erythrocytes with proteolytic enzymes (trypsin, papain, ficin)(3). The first technic is generally inadequate in range of activity and sensitivity. The Coombs' procedure is sensitive and possesses widest range of activity. However, a prozone effect, high expense, and the tedious nature of the test make it undesirable. Pretreating with proteolytic enzymes eliminated the prozone effect, but the tedious procedure persisted. By prior activation of papain with L-

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