enough to bring both the benzene and the toluene to constant specific activity. Further passages through either carbowax or silicone lead to no diminution in the specific activity, and the front and back parts of a given peak, or fraction, of benzene have the same specific activities. Within a factor of two (the insecurity in our knowledge of the C^{14} ion beam intensity) we have found that approximately 2% of the C^{14} ions striking the benzene replace a C^{12} atom and form a C^{14}-labeled benzene molecule. About half this figure, or 1%, of the ions are found as C^{14}-labeled toluene. At the present time no other products of irradiation have been identified.

Our experiments indicate that organic compounds may be labeled to a comparatively high specific activity by this technique. A methyl homolog, such as toluene, may be 100% labeled. In the case of the benzene, we find that a 10^{-9} amp. beam running for 10 hours gives about 1,000 dis./min. in the chromatographically purified product. If one were to use a microamp. beam one should be able to get approximately one microcurie of C^{14} into a few milligrams of benzene in one day's irradiation.

[8] U. S. Foreign Operations Administration Fellow, 1954–56.

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DEMONSTRATION OF INTERMEDIATE FORMS OF CARBONMONOXY- AND FERRIHEMOGLOBIN BY MOVING BOUNDARY ELECTROPHORESIS

Sir:

Oxidation of carbonmonoxyhemoglobin to ferrihemoglobin by ferricyanide results in the acquisition of a positive charge at acid *p*H and the release of carbon monoxide by each heme (Hb) oxidized. Since each hemoglobin molecule contains four hemes, the reaction occurs in four successive steps: $(HbCO)_4 \rightarrow (HbCO)_3Hb^{+1} \rightarrow (HbCO)_2Hb_2^{+2} \rightarrow (HbCO)Hb_3^{+3} \rightarrow Hb_4^{+4}$.

Although attempts have been made in the past to obtain physical evidence for intermediates in hemoglobin reactions,[1] the present experiments, in which $(HbCO)_3Hb^{+1}$, $(HbCO)_2Hb_2^{+2}$, and $(HbCO)Hb_3^{+3}$ were separated by moving boundary electrophoresis, provide the first conclusive demonstration of their existence.

Aliquots of normal human adult carbonmonoxyhemoglobin solution were diluted to about 1 g./100 ml. with potassium phosphate buffer of ionic strength 0.01 and *p*H 6.85 (measured at 25°), and 0.01 *M* $K_3Fe(CN)_6$ was added in volumes equivalent to about 25% to a three-fold excess of hemes present. The reaction mixtures were further diluted with buffer to a hemoglobin concentration of 0.5 g./100 ml. and allowed to stand overnight under carbon monoxide at 4° after which they were dialyzed for at least 40 hours with two or three changes of buffer to remove ferricyanide and ferrocyanide ions. The dialyzed solutions were analyzed in the same buffer in a Spinco Model H electrophoresis instrument at 0.5°. The percentage of each sample oxidized to ferrihemoglobin was determined in a Cary model 14 recording spectrophotometer. Spectrophotometric measurements in a series of phosphate buffers of ionic strength 0.01 indicated that ferrihemoglobin is almost entirely in the acidic form at *p*H 6.85.[2] When mixtures prepared from carbonmonoxyhemoglobin ($(HbCO)_4$) and ferrihemoglobin (Hb_4^{+4}) were analyzed electrophoretically, neither the alteration of proportions nor the appearance of components of intermediate mobility was discernible; thus, no detectable intermolecular reaction occurs during electrophoresis. On the other hand, each of the samples prepared by partial oxidation of $(HbCO)_4$ had components with intermediate mobilities. Since boundary anomalies may interfere with the interpretation of electrophoretic patterns obtained in dilute buffers,[3] control experiments with known mixtures were performed. It was found that proportions obtained from ascending limb patterns agreed well with the true proportions present. Therefore, it was possible to identify the components in each sample by direct comparison of electrophoretic and spectrophotometric analyses. For example, Fig. 1 shows the pattern obtained from a preparation that was 22% oxidized according to its absorption spectrum. The only interpretation consistent with both analyses is that, of the three major components, the slow, intermediate, and fast components are $(HbCO)_4$, $(HbCO)_3Hb^{+1}$, and $(HbCO)_2Hb_2^{+2}$, respectively. The small component migrating ahead of the others is probably $(HbCO)Hb_3^{+3}$. As the percentage oxidized was increased, components identified as $(HbCO)Hb_3^{+3}$ and Hb_4^{+4} appeared in increasingly higher proportions. More detailed descriptions and analyses of these experiments will be published.

Fig. 1.—Ascending limb pattern of 22% oxidized carbonmonoxyhemoglobin after electrophoresis for 22,440 sec. at 9.08 volts/cm. Total hemoglobin concentration 0.5 g./100 ml. in potassium phosphate buffer of *p*H 6.85, ionic strength 0.01; δ-boundary at right.

[1] For discussions, see J. B. Conant, *Harvey Lectures*, Ser. 28, 159 (1932–1933); R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949, p. 271.

[2] For *pK* determinations of ferrihemoglobin at low ionic strength, see P. George and G. Hanania, *Biochem. J.* (*London*), **55**, 236 (1953).

[3] L. G. Longsworth, *J. Phys. Colloid Chem.*, **51**, 171 (1947).

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A CONVENIENT METHOD OF LOCATING SUBSTITUENTS ON THE HYDROCARBON CHAIN OF MOLECULES ADDUCTING WITH UREA

Sir:

Attention is called to the series of continuous layer lines which form when a Laue X-ray diffraction pattern is taken of a urea (or thiourea) adduct single crystal. Those lines may be interpreted as