too anticomplementary for satisfactory analysis.

9. Five grams each of *E. coli* K 12 with and without chloramphenicol (500 mg/g) were broken in a French press, centrifuged (Spinco Model L) consecutively at 15,000, 30,000, and 105,000*g* for 10, 30, and 90 minutes. The final pellets (approximately 125 mg each) were standardized by dilution in buffer and analysis at 260 mμ in the Zeiss spectrophotometer.
10. Dr. J. Kumate, Hospital Infantil, and Dr. C. Biro, National Institute of Cardiology, Mexico, D.F., provided 84 serums currently under study from patients with hematologic disorders who had been exposed to chloramphenicol.
11. With the technical assistance of J. H. Douglass and editorial advice from Professor S. E. Mills. Supported by PHS grant AM 09207.

24 January 1966

Iodoinsulin Used To Determine Specific Activity of Iodine-131

Abstract. *Anodal mobility of iodoinsulin on starch-gel electrophoresis increases progressively as the number of iodine atoms substituted in the molecule increases. The iodine content of iodine-131 solutions is determined by comparison of autoradiographic patterns of insulin heavily labeled with iodine-131 and of insulins lightly labeled with iodine-131 and known quantities of iodine-127.*

A simple experimental determination of the specific activity of nearly carrier-free I^{131} has heretofore been lacking. Since I^{131} itself contains only about 8 μg of iodine per curie, direct chemical measurement of the iodine content of I^{131} solutions requires a prolonged wait for physical decay to lessen the radiation hazard involved in handling measurable amounts of iodine. The amounts of the isotopes I^{127} (stable), I^{129} (half-life, 1.6×10^7 years), and I^{131} (half-life, 8.05 days) produced by neutron irradiation of tellurium targets can be calculated theoretically from the isotopic abundance of tellurium isotopes and their cross sections for neutron capture. However, considerable uncertainty exists in the cross-section data, and small but significant and unknown amounts of I^{127} may be present originally in the tellurium targets or picked up as contaminant during processing. Even greater difficulties may confront the estimation, from fission yields, of the specific activity of I^{131} obtained as a fission product.

We now present a relatively simple method for determining the specific activities of I^{131} preparations. We have aimed at accuracy only within 30 percent, but greater accuracy should be possible with little additional effort. Our method is based on the fact that insulins labeled with small amounts of I^{131} in the presence of varying quantities of I^{127} exhibit series of discrete spots on starch-gel electrophoresis; the series appear to correspond to iodinated insulins having different ratios of iodine to protein (*1*). The chemical iodine content of a batch of I^{131} can thus be estimated by comparing the electrophoretic pattern of highly labeled I^{131}-insulin with patterns obtained with known amounts of I^{127}.

Freshly dissolved bovine crystalline insulin (Lilly lot No. 719106) was iodinated by the method of Hunter and Greenwood (*2*), with minor modifications (*3*). The contents of the reaction vessels were immediately transferred to tubes containing 40 to 300 μl of plasma so that the final I^{131} concentrations were approximately the same for all preparations. Forty microliters of each mixture were immediately placed in slits of a starch gel for electrophoresis (*4*), and a small sample was further diluted in plasma and applied to paper strips for analysis by hydrodynamic-flow chromatoelectrophoresis (*5*). In the latter procedure, undamaged I^{131}-insulin remains absorbed to the paper at the origin, damaged labeled fractions migrate with the plasma proteins, and iodide migrates further anodally.

After approximately 6 to 7 hours at 8 v/cm or 12 to 14 hours at 4 v/cm, starch gels were removed from the mold, wrapped in Parafilm, and placed in contact with x-ray film for autoradiography. Multiple films were obtained with exposures varying from 3 to 30 minutes; in this way the relative densities of the major spots could be evaluated from the short exposures, and the presence of less-active spots could be brought out in the longer exposures. Although different observers using naked-eye comparison were in good agreement on the iodination levels of highly labeled I^{131}-insulin, the intensities of spots on the autoradiographs were more objectively evaluated by transmission densitometry (*6*).

When starting ratios of I^{131} to insulin were about 1000 to 1800 mc of I^{131} per milligram of insulin, the efficiency of iodination was usually about 70 to 90 percent as determined by chromatoelectrophoresis on paper. Most of the radioactivity was distributed between the first and second "pre-albumin" spots

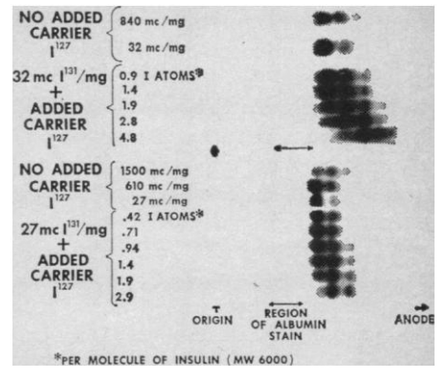

Fig. 1. Autoradiograph of starch-gel electrophoresis of insulin-I^{131} preparations. The numbers given for iodine atoms per molecule of insulin indicate the average value for the preparation; they are calculated as the product of the starting ratios and the percentage iodination. The molecular weight of the beef insulin monomer was taken as 6000 (approximation). Since the molecular weight is in fact 5734, the iodine numbers in this and succeeding figures are more precisely 4.5 percent higher than shown.

on starch-gel electrophoretograms, but a more-anodal third spot was always evident (Fig. 1); in more prolonged exposures a weak fourth spot was frequently brought out and a still weaker fifth spot was occasionally detectable (Fig. 2). When the same I^{131} was used at 15 to 35 mc of I^{131} per milligram of insulin, most of the insulin-bound radioactivity migrated in the first pre-albumin spot and only minute amounts were ever present beyond the second spot; occasionally, the efficiency of iodination was less than 70 percent in these preparations, possibly as a consequence of the very low iodine concentrations. When the same small quantity of I^{131} was supplemented with increasing quantities of I^{127} the iodination yields again increased (*7*) and an electrophoretic pattern showed an increasing number of spots, with a progressively more-anodal localization of radioactivity (Fig. 1, *8*).

The average ratio I^{127} : insulin was calculated as the product of the starting ratios (usually an integral or half-integral number of I^{127} atoms per molecule of insulin monomer—molecular weight, 6000) and the percentage of I^{131} bound to the insulin, as determined from the chromatoelectrophoretic analysis.

At 1 atom or less of I^{127} per molecule the intensity was always greatest in the first spot. At 1.5 atoms per molecule the second spot was slightly more

intense than the first spot, and with greater iodine substitution the site of most intense radioactivity moved further anodally (Figs. 1 and 2).

In the experiment shown at the top of Fig. 1, the highly labeled preparation (840 mc of I^{131} per milligram) produced a pattern almost identical with that obtained with insulin labeled with 32 mc of I^{131} per milligram of insulin and 0.9 atom of I^{127} per molecule of insulin. Since the latter contained 19 μg of iodine per milligram of insulin (*9*), if one neglects the small amount of I^{131}, the specific activity of the I^{131} must have been 0.8 c per 19 μg, or 23 μg of iodine per curie I^{131}. Thus I^{131} comprised 8/23 or 35 percent of the total iodine present at the time of iodination (approximately 4 days after removal of the tellurium target from the reactor, after an irradiation cycle of 102 hours). The extents of iodination of the two highly labeled I^{131}-insulins shown at the bottom of Fig. 1 were estimated by comparison with the relative-density histogram of I^{127}-insulins (Fig. 2) obtained in the same experiment. The pattern observed with I^{131}-insulin at 1500 mc per milligram yielded a value of 38 percent for I^{131} abundance; with I^{131}-insulin at 610 mc per milligram, 44 percent. A third shipment of I^{131} proved to have an I^{131} abundance of 35 percent. These values are about twice as high on the average as those calculated theoretically at the reactor (*10*). Occasionally shipments of I^{131} have been unsatisfactory for labeling hormones at high specific activity; in one such lot I^{131} abundance proved to be less than 5 percent.

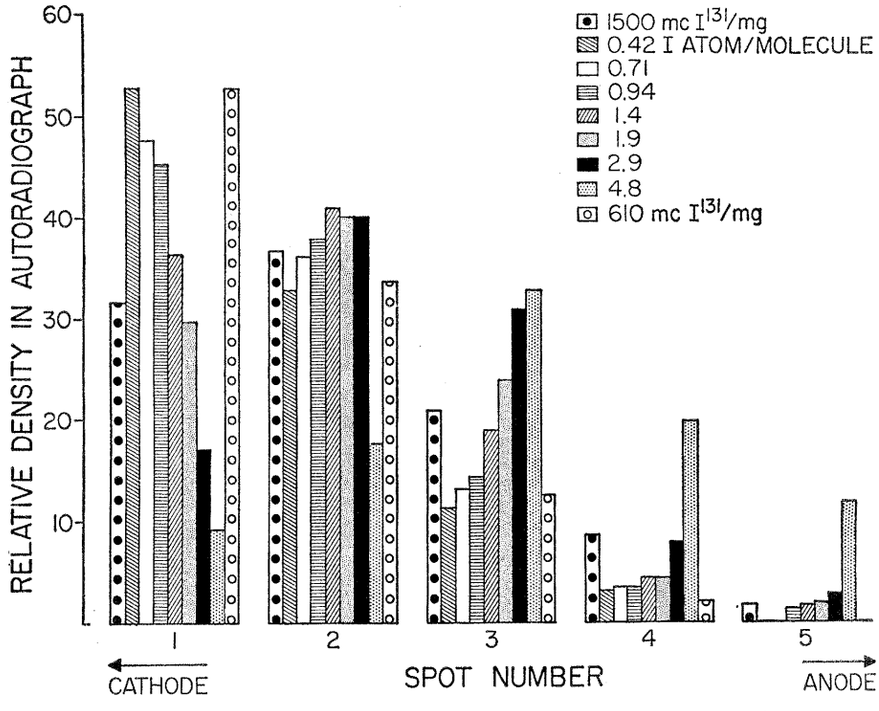

Fig. 2. Relative intensities of radioactive spots obtained from densitometric measurements in autoradiographs shown in Fig. 1; they were obtained by summation of the density readings for all the spots in the particular preparation, and division of the total density by the density reading for the individual spot.

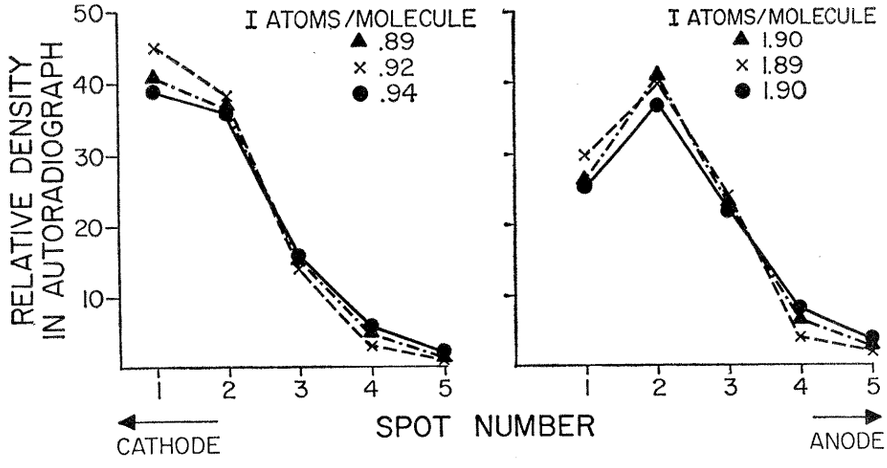

Fig. 3. Relative intensities of radioactivity in spots 1 to 5 of insulins labeled with about 1 or 2 atoms of iodine per molecule in separate experiments.

Since the relative intensities among the various spots (especially for the most critical spots 1 and 3) in separate experiments were satisfactorily constant at the same degree of iodination (Fig. 3), the results shown in Fig. 2 are considered suitable as a set of permanent reference standards for the insulin preparation and conditions employed.

It cannot be asserted unequivocally that the spots 1, 2, 3, . . . represent insulin molecules labeled with 1, 2, 3, . . . atoms of iodine, respectively. Indeed, one or more of the spots may possibly contain insulins of more than one iodine number. However, we have measured by radioimmunoassay (*11*), the insulin in spots 1 to 5 of one highly labeled preparation using unlabeled crystalline bovine insulin as a standard, and have found that the insulins eluted from spots 1 and 2 retain unimpaired immunoreactivity and contain 1 and 2 atoms of iodine per molecule of insulin, respectively: insulin in spot 3 appears to contain 3 atoms of iodine per molecule but shows slight impairment of immunochemical integrity; greater decreases in immunoreactivity of insulins eluted from spots 4 and 5 did not permit accurate estimation of insulin content, but results obtained in both suggested iodine numbers as great as or greater than three (*12*).

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References and Notes

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2. W. M. Hunter and F. C. Greenwood, *Nature* **194**, 495 (1962).
3. Iodinations were carried out in 2-ml conical flasks; the amounts, volumes, and order of addition of reagents employed were as follows: 10 μl phosphate buffer, 0.25*M*, *p*H 7.5, containing 0 to 27μg KI^{127}; 5 to 8 μl of I^{131} solution containing 0.2 to 2.5 mc; 2 to 20 μg insulin (1 mg/ml) in *p*H 2.3 water; 35 μg

chloramine-T (oxidant) in 10 μl phosphate buffer; 48 μg sodium metabisulfite (reductant) in 10 μl phosphate buffer. All reagents were added with micropipettes, with thorough mixing by bubbling through the pipettes. Generally less than 15 to 20 seconds elapsed between addition of insulin and completion of the procedure. The I^{131} employed in all our experiments was produced at the Sterling Forest Research Center and supplied through Iso/Serve, Cambridge, Mass.

4. O. Smithies, *Advan. Protein Chem.* **14**; 65 (1959).
5. S. A. Berson, R. S. Yalow, A. Bauman, M. A. Rothschild, K. Newerly, *J. Clin. Invest.* **35**, 170 (1956); S. A. Berson and R. S. Yalow, *Ann. N.Y. Acad. Sci.* **70**, 56 (1957). In this study, 100 μl of plasma containing portions of the iodination mixtures was placed on horizontal strips of Whatman-3MC paper, 3.7 cm wide, for chromatoelectrophoresis in veronal buffer 0.1*M*, *p*H 8.6, 16 to 18 v/cm. Strips were left exposed to the atmosphere at 4°C.
6. Densitometric readings were taken only in the region of strict linearity with radiation dose, as determined from measurements made on a test spot of radioactivity exposed for increasing periods. Agreement was excellent regarding the relative intensities of spots obtained from films after different exposures to the same gel. Since the various spots differ in width and trailing of radioactivity produces spurious enrichment of radioactivity in the more cathodal spots, the relative *intensities* are not measures of the relative *amounts* of I^{131} in the spots. However, the same spot usually shows about the same width in all samples and the amount of trailing does not vary appreciably.
7. At 10 atoms of iodine per molecule and higher ratios, iodination efficiency decreases and substitution asymptotically approaches 8 atoms of iodine per molecule. Each insulin monomer contains 4 tyrosine residues, each with two available sites for iodine substitution.
8. Increase in anodal mobility with increase in iodination of tyrosyl residues would be expected in view of the lower *p*K of the phenoxy group of diiodotyrosine than of tyrosine [*Handbook of Chemistry and Physics* (Chemical Rubber Publishing, ed. 38, 1956–7), p. 1649].
9. [(127 mg of iodine-127) × 0.9]/(6000 mg of insulin).
10. H. Arino and W. H. Wahl, Sterling Forest Research Center, personal communication.
11. R. S. Yalow and S. A. Berson, *J. Clin. Invest.* **39**, 1157 (1960).
12. After prolonged storage at −15°C various mammalian insulins dissolved in 0.005*M* HCl develop anodally migrating components in the unlabeled state, probably as a consequence of slow deamidation. Therefore only freshly prepared insulin solutions should be used for these studies.

5 January 1966

Hemoglobins M: Identification of Iwate, Boston, and Saskatoon Variants

Abstract. *Hemoglobin M variants, M Iwate, M Boston, M Saskatoon are easily and accurately identified by electron spin resonance with small amounts of patients' blood. In hemoglobin M Iwate and M Boston the electron spin resonance of both fresh blood (unprocessed) and isolated pure ferrihemoglobin M revealed similar signal shapes; whereas that of hemoglobin M Saskatoon was doublet in fresh blood and triplet in pure ferrihemoglobin M.*

A methemoglobin with a unique absorption spectrum was found by Hörlein and Weber (*1*) in a family whose members exhibited cyanosis. This abnormal hemoglobin, designated by Singer (*2*) as hemoglobin M (Hb M), is a genetically dominant character. Several investigators have shown that Hb M is heterogeneous with respect to certain physicochemical properties (*3*), and characteristic abnormalities of its amino acid sequence occur in some variants (*4*). The primary structures of the variants are represented as $\alpha_2{}^{58Tyr}\beta_2{}^{A}$ for Hb M Boston; $\alpha_2{}^{A}\beta_2{}^{63Tyr}$ for Hb M Saskatoon, $\alpha_2{}^{A}\beta_2{}^{67Glu}$ for Hb M Milwaukee-I, and $\alpha_2{}^{87Tyr}\beta_2{}^{A}$ for Hb M Iwate (*5*), respectively. Some variants of Hb M have been identified as belonging to one of above types: for example, Hb M Osaka to Hb M Boston (*6*), Hb M Kurume to Hb M Saskatoon (*7*) and Hb M Kankakee to Hb M Iwate (*8*). The structures of other variants, such as Hb M Chicago (*9*), Hb M Oldenburg (*10*), Hb M Leipzig-I (*11*), and so on, remain unknown.

Usually the classification of these variants is based on the difference of absorption spectra in the visible region, on their electrophoretic properties, and sometimes on their reaction rate with cyanide (*3*). These procedures demand pure material and very skillful technique, yet often fail to give accurate information. The primary structures of the variants have been determined by amino acid analysis after two-dimensional or column chromatographic separation had been made.

We now report the use of electron spin resonance (ESR) as a simple and accurate means for identification of Hb M Iwate, M Boston (Osaka), and M Saskatoon (Kurume).

Heparinized blood from the patients was used without further treatment. The blood (0.2 ml) was placed in a 3-mm quartz tube and inserted into the resonant cavity of Varian ESR spectrometer V-4500 (100 kcy/sec magnetic field modulation). The ESR was measured at liquid-nitrogen temperature, and the signal was recorded on the *Y*-axis of an *X*-*Y* recorder. The output voltage of a Hall-effect element, which was proportional to magnetic field intensity, was measured on the *X*-axis. The microwave frequency was measured by cavity wave meter for the determination of *g*-value.

The ESR signals in the region $g \approx 6.0$ are important because the ESR of Hb M variants revealed remarkable abnormality in this region; in the amorphous state the detection of the ESR in the region $g \approx 2.0$ was difficult owing to the low intensity of the signal. Hemoglobin of normal human blood is in ferrous state, so its ESR was not observed. In Hb M of patients' blood the ESR signal was strong at about $g \approx 6.0$ (Fig. 1), an indication of ferric hemoglobin (ferri-Hb). There are distinct differences among the Hb M variants, and the shapes of the curves appear to be characteristic of each variant. The shapes of Hb M Boston (Osaka) and M (Saskatoon Kurume) signals are doublet. The shape of Hb M Iwate signal, though it closely resembles that of ferri-Hb A, is also

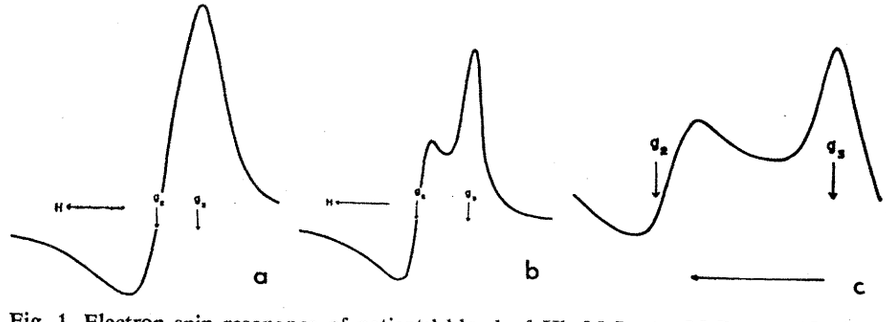

Fig. 1. Electron spin resonance of patients' blood of Hb M Iwate, M Boston (Osaka), and M Saskatoon (Kurume). Magnetic field modulation is 12 gauss and microwave power attenuation is 10 db. Horizontal arrow represents direction of magnetic field (110 gauss). Vertical arrows: left, g_2; right g_3. (*a*) Hb M Iwate, 9.1 × $10^{-3}M$, 160 times the sensitivity of the output of the ESR spectrometer. (*b*) Hb M Boston (Osaka), 9.0 × $10^{-3}M$; sensitivity 80 times. (*c*) Hb M Saskatoon (Kurume), 8.5 × $10^{-3}M$, sensitivity 400 times.