Determination of Protein-Bound Iodine (PBI) In Human Plasma by Thermal Neutron Activation Analysis[2]

Edward M. Smith, James M. Mozley and Henry N. Wagner, Jr.[1]

Baltimore

In this communication, we shall attempt to demonstrate that thermal neutron activation analysis is a precise and rapid method for measuring the protein-bound iodine (PBI) concentration in human plasma. In its present form, the procedure is not economically competitive with the chemical methods, but further development, including automation, would make it so. Furthermore, the finding of a systematically higher value by the neutron activation analysis method when compared to the Barker chemical method is consistent with the viewpoint that other PBI compounds, in addition to iodothyronines, may be present normally in plasma.

Chemical procedures currently used for determining PBI are technically difficult and subject to the problems of contamination from beginning to end. Acland (1) has reviewed the various chemical methods of determining the PBI and has discussed the difficulties associated with each.

Kellershohn *et al* (6) utilized thermal neutron activation analysis to eliminate the colorimetric determination of iodine as a final step in the chemical procedure, but the method remained technically complex.

In the present study, a sample of human blood plasma is passed through an ion-exchange column to remove greater than 99 per cent of the cations and anions normally present. The column effluent containing the PBI is then irradiated together with iodide standards in a nuclear reactor. After the irradiation is complete, major radiochemical contamination (Na^{24}, Cu^{64}, N^{13}, A^{41} and Cl^{38}) in the activated plasma is removed by stable isotope dilution and differential precipitation. The induced I^{128} activity in the sample and iodide standards is measured The residual radioactive contamination (Cu^{64}, N^{13}, and Cl^{38}) is eliminated by gamma-ray spectrometry. The iodide concentration in the final samples is determined by comparing the I^{128} radioactivity to the activity in the iodide standards.

EXPERIMENTAL

Pre-irradiation Treatment: Dowex 1 × 8 (50-100 mesh) resin in the chloride form is converted to the acetate form; Dowex 50W × 8, 50-100 mesh resin in the hydrogen form is converted to the ammonium form. The resin column is made of glass tubing 1 cm, i.d., and the lower orifice of the column is 0.5 to 1.0 mm^2 in area. The size of the orifice is not critical since the flow rate through the column is determined primarily by the resistance of the resin bed itself. Glass wool is placed in the bottom of the column to support the resin bed. In the final step in preparation of the column one hundred ml of 10 per cent ammonium acetate solution is passed through the column followed by 50 ml of demineralized distilled water.

A 10 ml heparinized plasma sample is obtained from each subject. Duplicate 5 ml aliquots are pipetted onto two resin columns. The optimal flow rate is 2 to 3 ml per minute. The initial effluent is not collected; immediately after the water displaced by the plasma has drained off, 3¾ ml of demineralized distilled water is pipetted onto the resin bed. The resultant 2¾ ml of effluent is collected in a 3 ml disposable Lusteroid tube. The Lusteroid tube is sealed with a polyethylene cap, and made water proof by dipping the top of the tube in molten paraffin.

The 5 ml plasma sample saturates the resin column. The demineralized distilled water is used to wash the plasma off the column. The PBI concentration in the first 3 ml of the column effluent is constant; 2¾ ml of demineralized distilled water is used to accommodate the size of the irradiation container.

At this stage, the concentration of the PBI in the eluate is 85.8 per cent (± 2.9%, relative standard deviation, 25 determinations) of that in the original plasma. This reduction in PBI concentration is due to the adsorption of it on the resin. These values were obtained using plasma to which radioactive I^{131}-thyroxine (Abbott Laboratories) was added prior to passing through the resin column. Similar values were obtained when the PBI determination was performed by the chemical method (Bio-Science Laboratories) on identical samples before and after passage through the resin column.

Reagent grade NH_4I was dissolved in demineralized distilled water is preparing iodide standards. Cupric nitrate and NH_4Cl solutions were also irradiated for use as standards in the gamma-ray stripping procedure (see below).

Samples and standards were placed in 100 ml Lusteroid tubes and arranged in two tiers each containing four samples and an iodide standard (Fig. 1).

Irradiation: The samples were irradiated for approximately 50 min at a thermal neutron flux density between 1 and 2 × 10^{11} neutrons/cm^2-sec. There was no apparent neutron flux density variation within a tier of samples; however, there was a significant variation between tiers. This necessitated an iodide standard in each tier to evaluate the samples. The problem of self-shielding of the samples was found to be insignificant.

In Fig. 2 serial gamma-ray spectra are shown for a 1 ml plasma sample that has been passed through the resin column, irradiated, and then counted
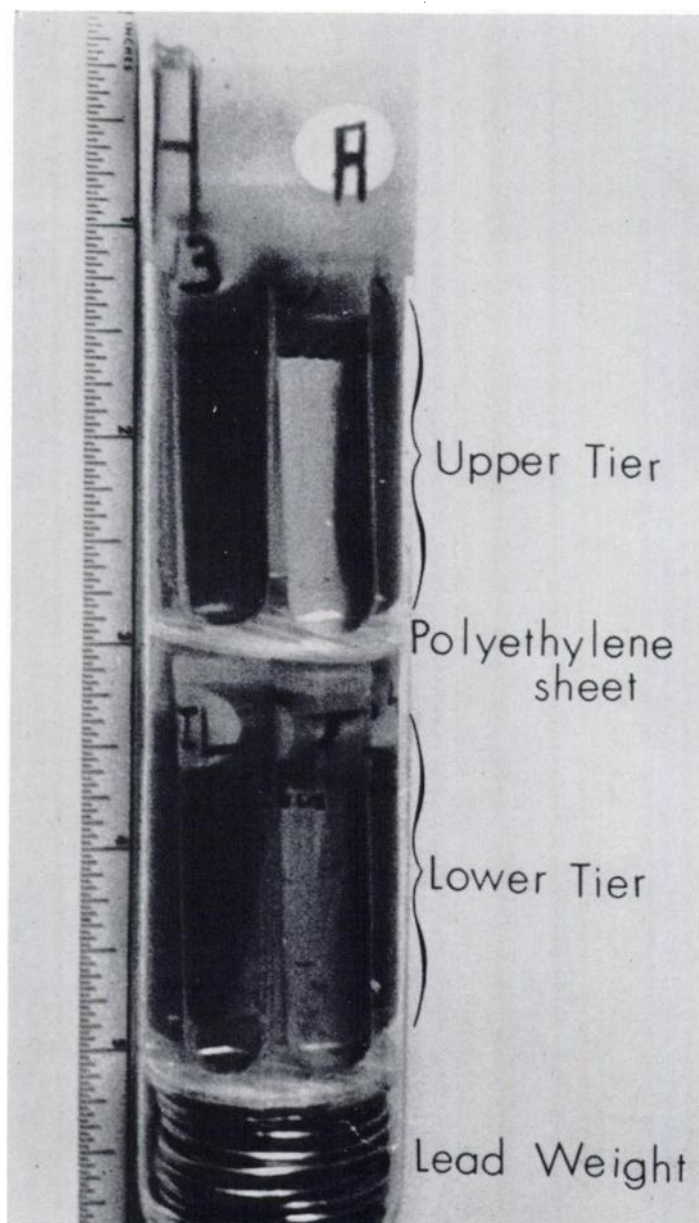
Fig. 1. Samples prepared for activation.

with no post irradiation chemistry. Argon[41] is present due to the dissolved air in plasma. Nitrogen[13] results from the n,2n reaction on N^{14}.

Post Irradiation Chemistry: Post irradiation chemistry was completed as rapidly as possible since the half-life of the product radionuclide, I^{128}, is 25 minutes. A 2 ml aliquot of each sample is pipetted into a Lusteroid counting tube. This step is the only quantitative transfer in the procedure. The use of a blowout pipette is necessary since the viscosity and surface tension of the plasma changes significantly as a result of radiation damage.
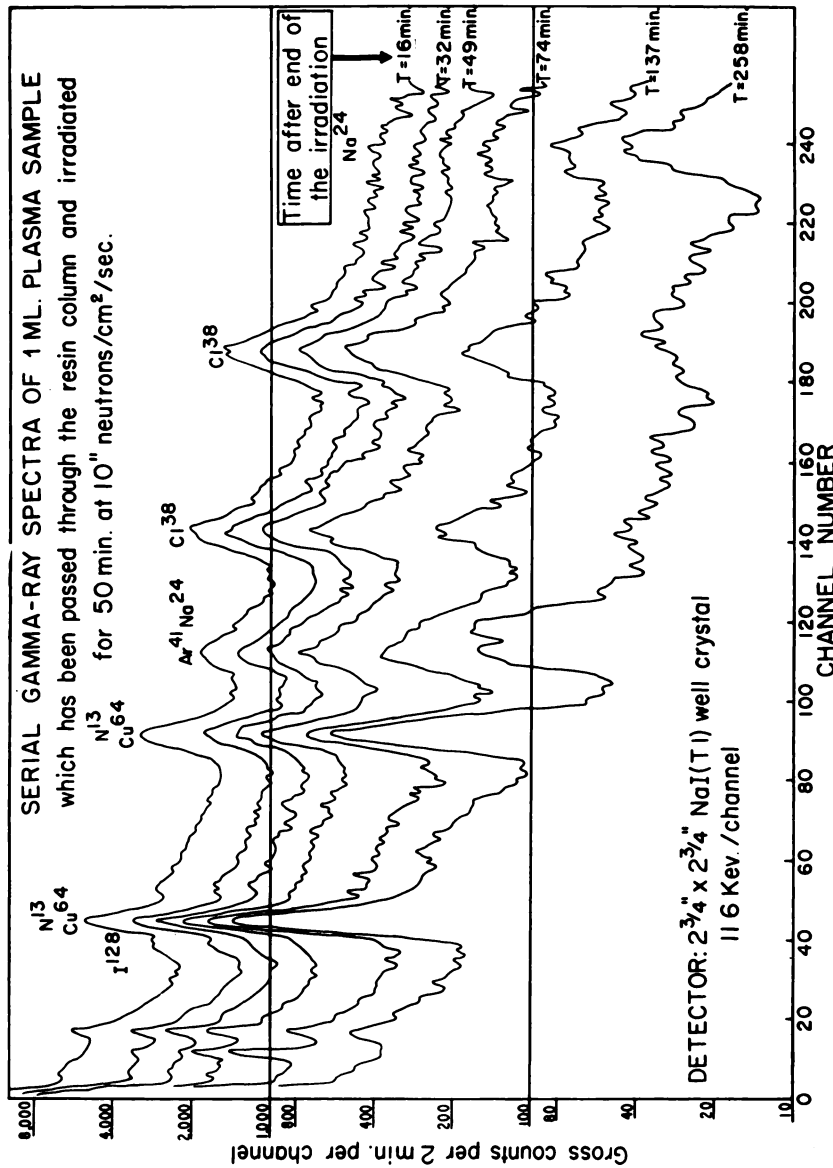

Fig. 2. Serial Gamma-ray spectra of 1 ml plasma sample.

The following reagents are added serially: 0.1 ml-1N NaHSO$_3$, 1 ml-0.04 meq I as NaI/ml in 1M NaNO$_3$, 1 ml-12N HCl, 2 ml-5% trichloroacetic acid (TCA) and 2 ml-0.1N.AgNO$_3$. The rods remain in each tube throughout the rest of the procedure to insure that no precipitate is lost. Wooden stirring rods are used so that they may remain in the Lusteroid tube during the centrifugation steps.

The samples are heated in boiling water for one minute, and then cooled to prevent deformation of the tubes during centrifugation at 2,000-3,000 rpm for 5 minutes. The precipitates are washed twice with 3-5 ml of 5% TCA in 1N NaNO$_3$. After the supernates from the last washing are decanted, the outsides of the counting tubes are wiped with absorbent tissue prior to counting. Removal of the samples from the reactor and postirridation chemistry, requires slightly less than 30 minutes.

The hydrochloric acid hydrolyzes the plasma proteins liberating any protein-bound cations, such as copper, and also provides 12 meq of chloride carrier. The TCA denatured proteins act as a mat for the small mass of silver halide precipitate. Since AgI is more insoluble than AgCl, the AgI will be precipitated first. Silver iodide and AgCl are precipitated by the addition of 0.2 meq of AgNO$_3$. To decrease the amount of Cl^{38} precipitated, 12 meq of chloride are added. Thus by isotopic dilution the amount of Cl^{38} precipitated is decreased by approximately a factor of 60.

The silver iodide and silver chloride precipitate is heated in boiling water to promote coagulation and facilitate rapid settling of the precipitate during centrifugation. The supernatant liquid contains a major fraction of the radioactive contaminants, A^{41}, Na^{24}, Cl^{38}, Cu^{64} and N^{13}.

Measurement of Induced Radioactivity: The gamma activities of the samples and standards are measured in a shielded 2¾ in × 2¾ NaI(Tl) well crystal-photomultiplier tube assembly connected to a Nuclear Data 512 multichannel pulse height analyzer having IBM computer typewriter digital readout and an analog type oscilloscope display. The use of a well crystal is essential if one is to obtain a significant 1.02 mev annihilation photopeak which is needed for the spectrum stripping procedure.

Spectrum Stripping: Post irradiation chemistry has eliminated the major portion of the radioactive contaminants except Cl^{38} and two positron emitters, Cu^{64} and N^{13}. Before the I^{128} can be determined, the Compton contribution from the gamma-rays of residual Cl^{38} and annihilation radiation must be subtracted from the 0.45 mev photopeak of I^{128}. This is done by "spectrum stripping". Figure 3 is the gamma-ray spectrum of a plasma sample at various stages in the post irradiation treatment.

Calculation of PBI: The net counts in the 0.45 mev photopeak of the stripped gamma-ray spectrum of the plasma sample, are compared to the net counts in the 0.45 mev photopeak of the iodide standard, and the PBI calculated. A correction is made for the loss of PBI (approximately 15%) during the first step, passage through the resin column.
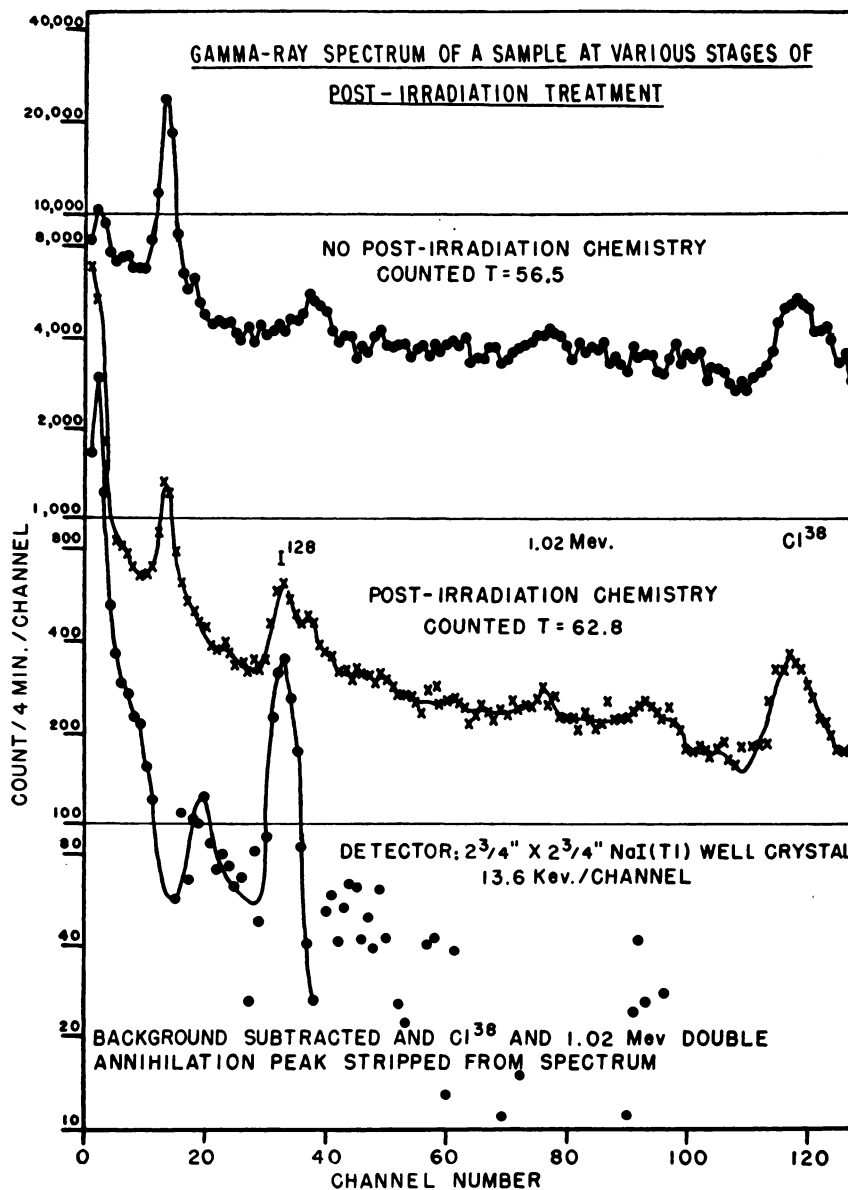
Fig. 3. Gamma-ray spectrum of a sample at various stages of post irradiation treatment.

RESULTS AND DISCUSSION

Precision: The PBI was determined on 9 aliquots of the same plasma sample. The mean of these determinations was 6.84 μg/100 ml with a standard deviation of 0.45 μg/100 ml, and a relative standard deviation of 6.8 per cent.

The mean difference between duplicate determinations on 43 plasma samples was 0.31 μg/100 ml with a standard deviation of 0.24 μg/100 ml. The data are presented graphically in Fig. 5.

To determine whether the random error between the activation analysis technique and the chemical procedure is of the same order of magnitude as the random error between two laboratories using the same chemical procedure, the following study was performed. The PBI was determined on 48 human plasma samples by the Bio-Science Laboratory and our laboratory using the activiation analysis procedure. The PBI was also determined on 29 human serum samples by the Bio-Science and Bio-Analytical Laboratories using the modified Barker chemical method (8). The ratio of the standard deviation of the mean difference for the value of the PBI between two laboratories over the mean value of the
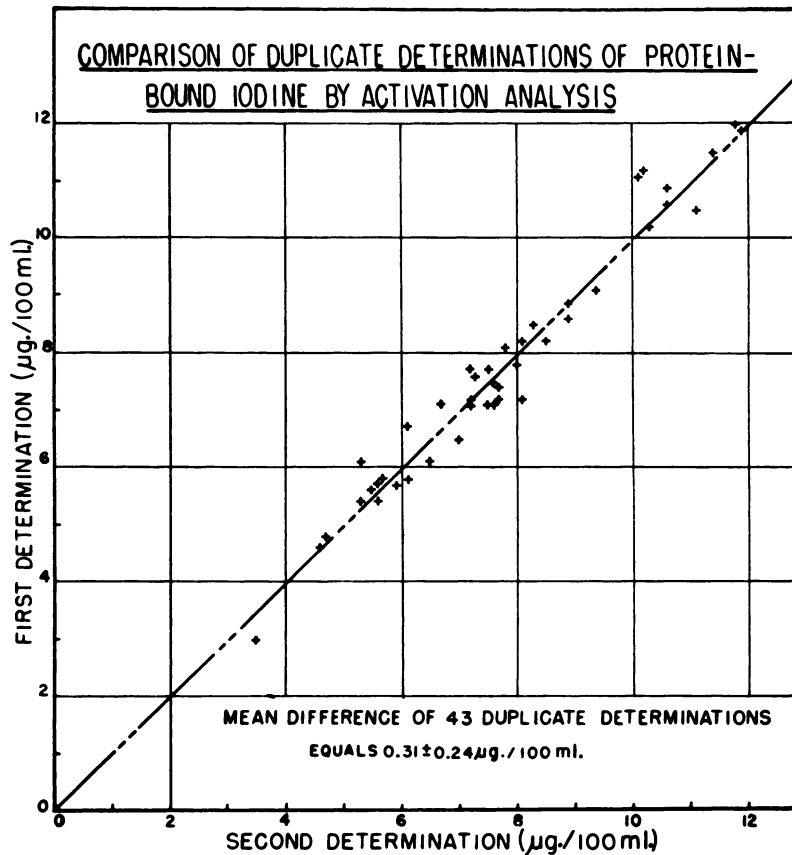

Fig. 4. Comparison of duplicate determinations of PBI by activation analysis.

PBI should be indicative of the random error between the two laboratories. The mean difference between the activation analysis method and the modified Barker chemical method as performed by the Bio-Science Laboratory on 48 plasma samples was 1.80 μg/100 ml. The standard deviation of this difference is 0.79 μg/100 ml, and the mean value of the results of the two laboratories is 7.07 μg/100 ml. For the 29 determinations of the PBI performed by the two commercial laboratories using the chemical method, the mean difference was 0.05 μg/100 ml. The standard deviation of this difference is 0.57 μg/100 ml, and the mean value of the results of the two laboratories is 5.65 μg/100 ml. The ratio for the Bio-Science Laboratory and our laboratory is 0.111 and the ratio for the two commercial laboratories is 0.101.

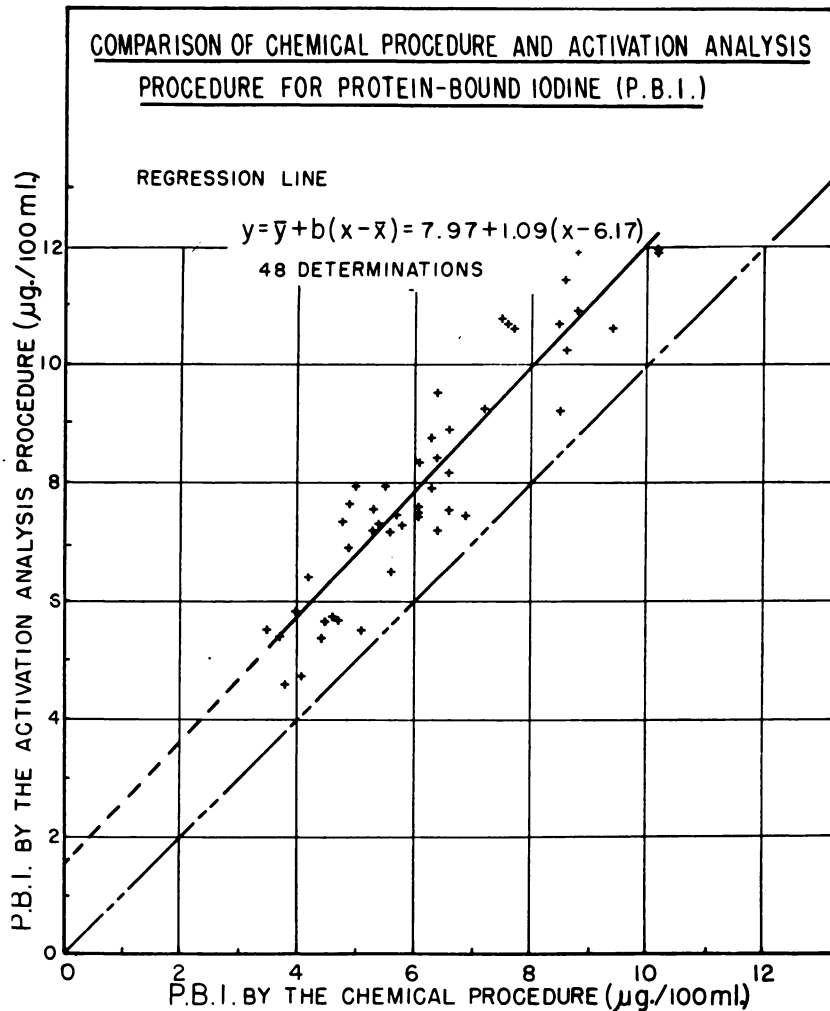The relative standard deviation of the net I^{128} counts was between 3 to 6

Fig. 5. Comparison of chemical procedure and activation analysis procedure for PBI.

per cent, which includes the statistical errors introduced in the gamma-ray stripping procedure, for a four-minute counting period when the sample was counted within the first 80 minutes after the end of the irradiation.

Recovery Study: A 500 ml blood sample was obtained from a single donor. Four 35 ml fractions of the plasma were transferred to separate containers. Two ml of thyroxine in 1% albumin with different thyroxine concentrations lying between 0.68 and 5.39 μg/100 ml were added to each container. Each of the fractions were analyzed in quintuplicate by the activation analysis method and in duplicate by the Bio-Science Laboratory (modified Barker method). Nine aliquots of the original plasma sample, without added thyroxine, were analyzed by the activation analysis method and 3 aliquots by the chemical method to serve as controls. The quantity of iodine recovered was calculated by subtracting the average value of the PBI of the control from the average value of the PBI for fractions A, B, C and D for both the activation analysis and chemical methods. The results are, presented in the Table.

The fraction of the added thyroxine recovered was acceptable in all cases. The value of the PBI by the activation analysis method for each fraction of the plasma, including the control, was consistently about 2 μg/100 ml higher than the corresponding value obtained by the chemical method.

Comparison of the Activation Analysis Procedure to the Chemical Procedure: Forty-eight blood samples were collected from patients who were to have the PBI determination performed. An aliquot of the plasma sample from each was sent to the Bio-Science Laboratory to have the PBI determined by the modified

TABLE—RECOVERY STUDY

Activation Analysis Method

Fraction	Average PBI (μg I/100 ml)	μg I/100 ml Added as Thyroxine	μg I/100 ml Recovered
Control	6.84	——	——
A	7.57	0.68	0.73
B	8.09	1.35	1.25
C	9.85	2.70	3.01
D	11.88	5.39	5.04

Chemical Method

Fraction	Average PBI (μg I/100 ml)	μg I/100 ml Added as Thyroxine	μg I/100 ml Recovered
Control	4.67	——	——
A	5.15	0.68	0.48
B	6.2	1.35	1.53
C	7.4	2.70	2.73
D	10.2	5.39	5.53

Barker method (3) and an aliquot of each plasma sample was used to determine the PBI by activation analysis. The results of these determinations are shown in Fig. 5.

The activation analysis method gave a consistently higher value than the chemical procedure. The 95 per cent confidence interval for the slope, b, is: 0.946 to 1.232. A slope of unity is included in this interval. There was a systematic difference between the two procedures which appeared to be constant, i.e. the difference was independent of the value of the PBI, within the range of PBI values studied. The range of the activation analysis determinations was 4.6 to 12.6 μg/100 ml, and the range of the chemical determinations was 3.5 to 10.2 μg/100 ml. Several experiments were performed to determine whether their systematic difference could be attributed to errors in the activation analysis procedure.

To determine whether the inorganic iodide was getting through the resin column, a plasma sample was tagged with I^{131} as NaI, and various concentrations of iodide were added. The fraction of the added iodide remaining in the plasma after passage through the resin column was 2×10^{-4} and was independent of the concentration of iodide added to the plasma over a wide range of iodide concentrations (0.012 μg/ml to 1400 μg/ml).

After post irradiation chemistry and gamma-ray stripping are completed, the net counts in the I^{128} region of the gamma-ray spectrum of a sample should arise from only disintegrations associated with I^{128}. If this is not the case then the post irradiation treatment has not produced radiochemical purity in the I^{128} region, and would give rise to erroneously high values of the PBI. To determine whether radiochemical purity was achieved by the post irradiation treatment, samples which had undergone post irradiation chemistry were counted several times, and the net counts in the I^{128} region were calculated. The net I^{128} counts were plotted on semi-log paper versus time after irradiation plus one-half the counting time. The net counts in the I^{128} region of the samples decayed at a rate following the 25 min half-life of I^{128} for a period of time (approximately 80 min) during which the samples were normally counted, indicating that adequate radiochemical purity was achieved.

The possibility of iodide contamination prior to the activation step was evaluated. A demineralized distilled water sample was used in place of the plasma sample, and it was treated as such in all of the steps of the procedure. The magnitude of the reagent blank was always less than 0.2 μg I/100 ml with a relative standard deviation of the order of 50 per cent.

Bowen (4) and other investigators have reported that halides made radioactive may become adsorbed on the walls of the container in which they are irradiated. This is due to the highly reactive state of the activated halide, and the large kinetic energy they have acquired as a result of the neutron capture reaction. An experiment was carried out to determine if the phenomenon were of importance in explaining the high results by adsorption in the standards under the irradiation conditions used in determining the PBI. The surface area of the material of the irradiation container was deliberately increased since the phenomenon would be more pronounced, by addition of strips of Lusteroid to the

irradiation container to increase the surface area presented to the iodide solution during the irradiation period. The samples were then processed in the regular manner. These results indicate that adsorption of I^{128} to the walls of the irradiation container does not occur under the irradiation conditions used in determining the PBI.

There are no interfering nuclear reactions producing I^{128} in plasma.

The radiochemical recovery was evaluated by tagging plasma with I^{131}-thyroxine, and determining the ratio of I^{131} activity in an irradiated plasma sample before and after post irradiation chemistry. The results indicate the radiochemical recovery is better than 98 per cent.

In view of our failure to find a technical error as a possible reason for the higher values of PBI that we obtained, our findings suggest that there may be another iodine-containing constituent in plasma that is not measured by the Barker alkaline ash method.

Pileggi, Segal and Golub have recently reviewed the controversy concerning the final elucidation of the chemical nature of blood iodine in normal persons (11). These authors failed to find detectable amounts of thyroxine precursors, monoiodotyrosine and diiodotyrosine, in normal human serum. However, they did find that between 6 and 11 per cent of the PBI in normal subjects could be eluted from a chromatographic column in a region distinct from thyroxine.

Further study in this laboratory is being directed to elucidating the possible source of the additional PBI detectable by activation analysis.

SUMMARY

Neutron activation analysis was found to be a precise method for measuring PBI in plasma. Passage of human plasma through ion exchange resins in the ammonium acetate form prior to neutron activation removed greater than 99.9 per cent of the inorganic iodide as well as most other inorganic ions. Neutron bombardment of the treated plasma in a nuclear reactor with a flux density of approximately 10^{11} thermal neutrons/cm^2 sec activated I^{127} to the radioactive isotope I^{28}. After irradiation, major radiochemical impurities were eliminated by simple radiochemical techniques (stable isotope dilution and differential precipitation). The induced I^{128} radioactivity was measured and compared to activated iodide standards by gamma-ray spectrometry using spectrum stripping to eliminate interference from remaining radioactive contaminants. The activation analysis technique yielded results which were systematically 1.8 $\mu g/100$ ml higher than the Barker chemical method although thyroxine recovery studies run on both procedures were identical. This systematic difference could not be attributed to any systematic error in the activation analysis procedure indicating this technique may be measuring an additional iodine component of plasma such as iodotyrosines. Advantages over chemical methods of measuring PBI are elimination of the protein digesting step, elimination of the reagent blank and a reduction in the problem of iodine contamination.

REFERENCES

1. ACLAND, J. D.: The Nature, Determination and Clinical Importance of Blood Iodine: A Review, *J. Clin. Path.*, 11: 1958, p. 195.
2. ATCHISON, F. J. AND BLAMER, W. H.: Neutron Activation Analysis with the van de Graaf Accelerator—Application to the Halogens, *Anal. Chem.*, 28, No. 2, Feb., 1956, p. 237.
3. BARKER, S. B.; HUMPHREY, M. J. AND SOLEY, M. H.: Clinical Determination of Protein-Bound Iodine, *J. Clin. Invest.;* 30, 1951, p. 55.
4. BOWEN, H. J. M.: The Determination of Chlorine, Bromine, and Iodine in Biological Materials by Activation Analysis, *Biochemical J.*, 73, No. 2, 1959, p. 381.
5. COSGROVE, J. F.; BASTIAN, R. P. AND MORRISON, G. H.: Determination of Traces Mixed Halides by Activation Analysis, *Anal. Chem.*, 30, No. 11, Nov., 1958, p. 1872.
6. KELLERSHOHN, C.; COMAR, D. AND LE POEC, C.: Determination de l'Iode Sanguin par d' Activation; *Inter. J. of Applied Rad. and Isotopes*, 12, 1961, p. 87.
7. KLEINBERG, J. AND COWAN, G. A.: The Radiochemistry of Fluorine, Chlorine, Bromine and Iodine, NAS-NS-3005, Jan., 1960.
8. WAGNER, H. N., JR.; NELP, W. B.; AND DOWLING, J. H.: Use of Neutron Activation Analysis for Studying Stable Uptake by the Thyroid, *J. of Clin. Invest.*, 40, No. 11, Nov, 1961, p. 1984.
9. WAHL, A. C. AND BONNER, N. A.: Radioactivity Applied to Chemistry, John Wiley and Sons, Inc., New York, 1951, p. 244.
10. ZIEVE, L.; DAHLE, M. AND SCHULTZ, A. L.: Comparison of Incineration and Chloric Acid Methods for Determination of Chemical Protein-Bound Iodine, *J. Lab. and Clin. Med.*, 44, No. 3, Sept. 1954, p. 374.
11. PILEGGI, V. J.; SEGAL, H. A. AND GOLUB, O. J.: Determination of Organic Iodine Compounds in Serum. III. Iodotyrosines in Normal Human Serum. *J. Clin. End. and Metab.* 24:273, 1964.