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A Micro Modification of the Automatic Determination of Serum Iron

DONALD D. CLARKE and RUTH NICKLAS

In last year's symposium we described the automation of the assay for transglutaminase in connection with our studies of the elevation in the level of this enzyme caused by injection of bacterial endotoxins into mice (1,2). At the same time Barry reported on the determination of serum bound iron as a measure of the dose of bacterial endotoxin injected into rats and mice (3). We decided to attempt to correlate these measurements; i.e., decrease in the level of serum bound iron and the increase in the liver transglutaminase activity on mice. The use of automated serum iron determination of Young and Hicks (4) which was used by Barry for his study of rats would have required pooling sera from many mice. We preferred to attempt to get data for individual mouse sera, and therefore investigated the scaling down of the procedure of Young and Hicks. We wish to report the results of this effort here.

In our experience double dialysis usually adds more than proportional increase in the noise of the baseline of a procedure than is the corresponding increase in efficiency of dialysis, and we decided to eliminate it. Furthermore, the flow rate through the system in the scaled down procedure is about 1/6 that of the manifold of Young and Hicks; hence the residence time in the single dialyzer is correspondingly increased and double dialysis is of reduced utility. To take care of any needed increase in sensitivity which is due to the lower ratio of sample to diluent we used a range expander to effect electrical amplification of the recorder response. In addition the chart speed of the recorder was slowed to 6 in./hour so that a more compact series of peaks were obtained on each chart than was obtained with the more usual chart speed of 20 in./hr we used in our other procedures in this laboratory. The manifold which was finally adopted is shown in Fig. 1.

The reagents used are essentially those of Young.
and Hicks (4) with some minor modifications of concentrations. Thus the ammonium acetate solution was reduced in concentration from 10% to 3.5% and the flow rate of this sample was scaled down by a factor of 2 as compared to Young and Hicks’ procedure. This avoided problems with back pressure encountered when smaller pump tubing was used for this line. The segmenting air bubbles were also scaled down to 1/5 the values used by Young and Hicks (4).

In order to decrease the hold-up time in reagent lines from reagent bottles to the pump tubing, small-diameter polyethylene tubing (0.034 in. I.D.) was used in place of standard transmission tubing which is generally used for this purpose. Single glass mixing coils were found to be satisfactory in place of double or triple mixing coils used by Young and Hicks (4).

Another observation that helped to improve the performance of this manifold was to keep a separate 15 mm tubular flowcell for use with this manifold. Our Technicon AutoAnalyzer is used with the manifold described in last year’s symposium for transglutaminase assays (1) in which the light absorption of a ferric hydroxamate colored complex is employed. It is also used for ninhydrin determinations of amino acids. The traces of iron left in the system have to be washed out thoroughly, and it takes a considerable time of pumping of reagents for the baseline to reach a steady level when using the same flowcell employed in the above procedures. For those who do not use the AutoAnalyzer for other purposes, this problem will not come into consideration.

The interaction between samples was sufficiently large that it was found necessary to put sample cups with distilled water between each sample. The sampler was run at 40/hr with a 2:1 sample-to-wash ratio cam; thus samples are analyzed at a net rate of 20/hr. At this flow rate 0.1 ml of sample is consumed per assay. Another slight modification is the use of a 590 m\textmu filter instead of a 600 m\textmu filter to correspond more nearly with the absorption maximum of the Fe-TPTZ colored complex (7). The ammonium acetate and TPTZ reagent streams could be interchanged with a slight increase in sensitivity of the method. We did not consider the change sufficient to adopt this modification in the manifold finally accepted for our studies.

The data in Fig. 2, a comparison of steady state levels with those obtained with the normal sample schedule, indicate that with the manifold described the measured peaks are approximately 60% of the height of the steady state values shown here for an iron concentration of 150 \mu g/100 ml. To the right of this figure is a series of standards covering the useful working range of 50 to 500 \mu g/100 ml.

If the percentage transmission data from such a run is converted to optical densities and plotted versus the iron concentrations an excellent straight line is obtained as shown in Fig. 3. The solid line is drawn using the slope and intercept calculated by the method of least squares.

In Fig. 4 we see a series of standards followed by values for a serum pool which contained 300 \mu g of Fe/100 ml and mixtures of such a serum....
pool with a standard containing the same level of iron. It is evident that the recoveries of Fe from the serum are close to 100% of the value obtained with standards. As a more direct check of absolute efficiencies of the recovery of iron, standards were run with the dialyzer bypassed. When corrected for the increased volume due to mixing of the 2 streams it could be calculated that the absolute efficiency of dialysis was approximately 31%. Some approximate studies with a manifold made up to the specifications of Young and Hicks but using single dialysis indicated that efficiency of dialysis under those conditions was approximately 20%.

In Table I is shown some of the data obtained after varying levels of a *Salmonella typhosa* endotoxin were injected into mice. Sixteen hours after the injection the mice were decapitated and the serum collected. The decrease in serum iron level is proportional to the dose of endotoxin when the probit transformation is used. Our results agree reasonably well with those reported by Barry (3) in last year's symposium.

**Materials and Methods**

Lipopolysaccharide from *Salmonella typhosa* was obtained from Difco Laboratories. A weighed quantity was dissolved in physiological saline, suitable dilutions made, and 0.1 ml of the appropriate dilution was injected intraperitoneally into each mouse (usually between 4 to 5 P. M.). The following morning the mice were stunned by a blow to the head and decapitated with a guillotine (Harvard Apparatus Co.). The blood was allowed to drip onto a watch glass, immediately pipetted into a 2-ml centrifuge tube, capped with parafilm, and left to clot in the refrigerator. After the clot had retracted, the serum was centrifuged and transferred to a 2-ml sample cup using a Pasteur pipette with a finely drawn out tip (0.5-ml sample cups would have been more satisfactory). The samples of serum were then placed on the Auto-Analyzer. The livers were also taken and homogenized as described previously (1).

For the preparation of a serum pool the sera from 24 mice were pooled and kept refrigerated in small batches. This pool assayed for 300 ± 8 μg % Fe. In any one run the standard deviation was less than ± 6 μg % Fe. The larger value given represents the average over many runs on different days.

**Animals**

Female Swiss albino mice of approximately 20-g body weight were used in most of these experiments. The results given in Table I were carried out using a special strain of inbred mice (SSRB3) obtained from Dr. Joanna Lee of the Rockefeller University.

**Reagents**

*Sample Diluent:* 1% Ascorbic Acid; 1 g Ascorbic acid is dissolved in 100 ml of 0.1 N HCl with 1 drop of Brij-35 (1/2 cc/liter).

*Recipient Solution:* 10 mg% 2,4,6-Tripyridyl-s-triazine; 10 mg of TPTZ is dissolved in 100 ml of 0.1 N HCl with 1 drop of Brij-35.

*Alkaline Solution:* 3.5% ammonium acetate; 3.5 g ammonium acetate is dissolved in 100 ml water.

*Iron Standards, Preliminary solution:* 4.32 g of Ferric ammonium sulfate is dissolved in water, 10 ml of conc. HCl is added, and the solution brought to 100 ml with water: Final conc. = 5 mg. Fe/ml.
Iron Standards, Stock solution: 1 ml of above solution, 10 ml of conc. HCl added, and brought to 1000 ml with water. Final conc. = 5 µg Fe/ml.

Iron Standards, Working solutions: 50, 75, 100, 150, 200, 250, 300, 400, and 500 µg Fe/100 ml. One drop of conc. HCl is added to each level.

Water—Distilled water is used at all times.

Discussion

Independently of the work of Young and Hicks other workers have automated assays for iron in serum and in urine (5,6). These procedures have a number of similarities. In all of them iron is reduced from the ferric to the ferrous state in an acidic solution and bathophenanthroline or its sulfonate or tripyridyl-s-triazine used as the chromogenic reagent (7). TPTZ has its absorption maximum at a longer wavelength than the phenanthroline derivatives and in addition is the less expensive reagent. Hence it would seem to be the reagent of choice for these determinations as suggested by Young and Hicks (4).

Zak and Epstein (5) have reported that 1 N HCl in the reducing solution gave better results than the 0.1 N HCl used by others. We did not find this acid concentration to make a difference in sensitivity or reproducibility with the manifold described here. The accuracy of the method was not exhaustively investigated in this work as the prior work of Young and Hicks appeared to adequately establish this point. This method will evidently appeal to those examining small samples of serum, as for example infants, and the preceding papers of Garry and Owen bear this out.

References