Experience of more than one year with a commercially available insulin kit is reviewed. While the method is adequate as delivered, it suffers from higher than desired nonspecific binding (NSB) values and greater than desired intra-assay and interassay variations. Modifications can be made, however, which will reduce the NSB % and enhance the precision—40% reduction in standard deviation (s.d.) at 70 μU—and accuracy of the assay. Modifications involve incubation times, precooling tubes before addition of reagents, and changes in technique, making them particularly well suited to small departments without facilities and personnel to do more extensive assay optimization.

Insulin, which is secreted by the beta cells of the pancreas, produces a variety of metabolic effects, particularly in the regulation of carbohydrate metabolism. In that regulation, insulin exerts its main effect by governing the entry of glucose into the cell. Insulin was the first protein hormone to be characterized structurally, synthesized chemically, and measured by radioimmunoassay (1-5). In recent years, further development and refinement of technique has permitted routine serum insulin levels to be available for correlation with glucose levels during tolerance testing. Insulin testing is now used to determine the following: "juvenile diabetic" response; "maturity-onset" response; insulin excess; insulinoma; and occult diabetes. A review of insulin patterns has been presented by Kraft (6).

Materials and Methods

As in all radioimmunoassays, the Amersham insulin RIA test is based on an antigen-antibody reaction. The kit's components are described in the protocol supplied by the manufacturer as follows: buffer component—a freeze-dried stabilized phosphate buffer; insulin binding reagent—a freeze-dried preparation of anti-insulin serum (guinea pig) precipitated by anti-guinea pig serum (rabbit), which also contains phosphate buffer, preservative, EDTA, and bovine serum albumin (the pre-precipitate is a modification of the double antibody method of Hales and Randle [7,8]); iodinated I-125 insulin—prepared from purified bovine insulin dissolved in 2 ml of phosphate buffer; and human insulin standard—a freeze-dried preparation of human insulin.

The incubation schedule is 45 min for the first incubation, and 2 hr and 15 min for the second, both at 2-4° C.

If the protocol is followed precisely, the assay yields useful information. However, about one year ago, a series of trials led to a new test protocol. The first trials involved comparing test tubes to determine if tubes of different compositions would yield different results. Duplicate experimental assays were performed using the same reagents, pipets, and personnel; only the tubes, polypropylene and polystyrene, were changed.

In the second series of trials, bovine serum albumin (BSA) was added to the tubes to coat the inner surface and prevent adsorption of insulin to the tube wall, in an attempt to lower the NSB. A bovine serum albumin specially prepared for radioimmunoassay was used. Various concentrations of BSA (2%, 5%, and 10% by volume) and volumes (20 μl, 50 μl, and 100 μl) were used with experimental standard curves and reference serums.

The next areas scrutinized were pipetting systems, delivery, and incubation times. While these three areas may seem diverse, in fact they are not because the amount of time spent pipetting is critical when assaying some 100 to 150 tubes per test. The primary consideration was to maintain the most accurate and reproducible pipetting possible while delivering the volume rapidly, so as to reduce first-to-last tube variation to a minimum.

A repeating dispenser (Hamilton Co., Reno, NV), dispensing at 50 tubes per min, and the SMI pipet (Scientific Manufacturing Industries, Emeryville, CA), a

<table>
<thead>
<tr>
<th>Standard</th>
<th>Mean % B/T</th>
<th>2 s.d.</th>
</tr>
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<tbody>
<tr>
<td>Bo</td>
<td>25.0</td>
<td>5.6</td>
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<td>3.4</td>
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<td>40</td>
<td>11.7</td>
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<td>80</td>
<td>6.1</td>
<td>2.2</td>
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<tr>
<td>160</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>NSB</td>
<td>5.5</td>
<td>2.2 (N=10)</td>
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Ortho Sera Mean Concentrations—s.d. and c.v.

<table>
<thead>
<tr>
<th>Mean Concentration Ortho I (N=42)</th>
<th>s.d.</th>
<th>c.v.</th>
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<td>34.4</td>
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<td>14.6</td>
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<th>Mean Concentration Ortho II (N=42)</th>
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<td>73.4</td>
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<td>12.5</td>
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<table>
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<th>Standard</th>
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<tr>
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<tr>
<td>160</td>
<td>4.5</td>
<td>1.2</td>
</tr>
<tr>
<td>NSB</td>
<td>2.9</td>
<td>0.5 (N=10)</td>
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</tbody>
</table>

Ortho Sera Mean Concentrations—s.d. and c.v.

<table>
<thead>
<tr>
<th>Mean Concentration Ortho I (N=84)</th>
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<th>c.v.</th>
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<table>
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<tr>
<th>Mean Concentration Ortho II (N=84)</th>
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<tbody>
<tr>
<td>72.9</td>
<td>5.2</td>
<td>7.2</td>
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</table>

Results and Discussion

The trials performed throughout the year built a successful assay "ladder style." Each succeeding trial incorporated the best of previous trials. The first trials involving test tubes resulted in a consistently lower nonspecific binding for the polystyrene tube than for the polypropylene tube (9). The NSB % was still felt to be too high (typically 3–7%); this led to the second series of trials involving the addition of BSA. The volume and concentration which resulted in the greatest decrease in NSB was a 50-μl volume of a 5% solution. When added to all the tubes prior to assay, it effectively prevented adsorption of insulin to the tube wall without significantly contributing to the overall volume of the assay.

We next experimented with pipetting systems, delivery, and incubation times. The repeating dispenser is a rapid system but because of the force of the dispensed stream, some of the reagent is splashed onto the sidewall of the tube. This is a portion not previously coated with BSA.

The SMI system is much slower than the Hamilton system yet it allows the technologist to add the reagent precisely to the BSA in the very bottom of the tube. To compensate for the long pipetting time (typically 120 tubes in 12 min), an additional 15 min was added to the first incubation (now 1 hr) and an additional 45 min to the second (now 3 hr) maintaining the 1:3 ratio.

The revised protocol requires addition of 50 μl of 5% BSA solution, using a repeating dispenser, into empty polystyrene tubes. The tubes are vortex mixed and the standard or specimen using an SMI pipet is added directly into the bottom of the tube. The binding reagent is added with an SMI pipet directly into the bottom of each tube. These samples are mixed and incubated for 1 hr at 2-4°C in a refrigerator. The labeled antigen is pipeted directly into the bottom of the tube, vortex mixed, and incubated for 3
hr at 2-4° C. Then 700 μl of cold buffer (as supplied by
the manufacturer) is added to each tube. The tubes are
centrifuged for 40 min at 2500 × g at 2-4° C. The
supernatant is decanted as directed by manufacturer.
This revised protocol takes approximately 1 hr and 30
min longer than the manufacturer suggests, but can still
be completed in an 8-hr day.

Statistically, the maximum binding (B₀) showed a
slight increase from a range of 22-28% to 24-29%, the
percentages calculated as [(B/T)-NSB] × 100. The NSB
showed a somewhat greater change by decreasing from
4.5-6.5% prior to modification to 2.7-3.2% following
modification. Table 1 illustrates the mean% B/T (after
subtracting NSB) and two standard deviations for the six
standards done in triplicate from ten assays performed
prior to modification. Additionally, the Ortho reference
sera (Ortho Diagnostics, Raritan, NJ) lots 10N403 for
Ortho I and 10N503 for Ortho II were assayed and the
mean concentration, s.d., and c.v. derived from the same
ten assays are listed. Table 2 illustrates the same para-
metrics for ten assays following modification. The same
lots of Ortho sera were used.

As a part of routine quality control for this assay, the
New York State Department of Health and CAP
unknowns were assayed. One such unknown from New
York State was assayed prior to final assay modification
and a result of 19 μU was obtained. This did not compare
favorably to the target value of 35 μU. New York State
graciously supplied us with an additional vial of the
specimen and the value obtained using the fully modified
protocol was 37 μU. Recently, additional unknowns
from New York State were assayed (Table 3).

**Conclusion**

It is worth noting that the mean concentration of the
Ortho II reference sera did not change significantly
because of modification, but that the s.d. is much improved. This is due in part to the much improved s.d.
of the standards themselves following modification.

While the ultimate accuracy of insulin may or may
not be more important than the shape of the insulin curve
or its comparison with an accompanying glucose
tolerance curve, I believe that the 1 hr greater incubation
time and slightly increased technologist involvement is
worth the 40% decrease in s.d. at the 70 μU level and
greater accuracy at all levels.

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