then some of the peroxide produced in the enzyme reactor would be lost, and the amount of color formed in the indicator reaction would decrease. Longer enzyme coil loops contain proportionally more catalase, which could account for the plateau in the plot of assay sensitivity vs. IME reactor length.

To test this hypothesis, we added sodium azide, a known inhibitor of catalase (6), to the buffer, and repeated the study of assay sensitivity vs reactor length. In addition, we did several other experiments designed to determine the effect of azide on the system. The results are given below.

Glucose oxidase (GO) was immobilized on nylon tubing by a method adapted from procedures described by Morris et al. (7) and Sundaram et al. (8). Four 25-cm enzyme coils with almost identical activities were prepared and used, alone or in tandem, for this study. Sodium azide solutions were prepared in a pH 6.85 phosphate buffer and were stable for several weeks at room temperature. A miniature continuous-flow system, developed in this laboratory, was used in all the experiments.

Addition of azide improved the linearity of the curves dramatically (Figure 1, curves B and C). To prove that the effect of azide was to inhibit catalase, we did additional experiments.

First, the immobilized GO coil was removed from the continuous-flow manifold, and standard peroxide solutions were assayed. The instrumental response was reduced by only 3% at an azide concentration of 100 mmol/L. Thus, the indicator reaction was not significantly inhibited by azide. Next, a 25-cm GO-IME reactor (assumed to contain co-immobilized catalase) was added to the manifold. The peroxide standards now gave a much smaller instrumental response than before, presumably owing to reaction with co-immobilized catalase. As azide was added in increasing amounts to the buffer, the loss of peroxide decreased as shown in Figure 2, curve A. At a concentration of 100 mmol/L azide, the instrumental response was 98% of that with no catalase present (the GO coil removed). Thus, the presence of co-immobilized catalase in the IME reactor was confirmed.

Curve B of Figure 2 shows the combined effect of azide on glucose oxidase and catalase. Glucose replaced peroxide as the sample in these experiments. The decrease in response when the azide concentration exceeded ~5 mmol/L might be due to inhibition of glucose oxidase, but we did not investigate the nature of this inhibition.

Three other facts deserve mention. First, the effect of azide on glucose oxidase and catalase was completely reversible. Enhanced sensitivity was observed in less than 5 min after azide was added, while the sensitivity returned to "normal" after the azide was removed. Second, we assayed control sera, using a dialyzer and a 25-cm GO-IME reactor. The results showed that there were no significant differences between the values obtained with and without azide, except that the assay sensitivity was increased by a factor of 1.7 with added azide. Third, the effect of azide would be less apparent if the analytical and indicator reactions were not separated, because peroxidase could compete favorably with catalase for the peroxide produced in the GO-catalyzed reaction.

Thus we recommended the addition of azide to the buffer reagent in continuous-flow determinations of glucose by use of immobilized glucose oxidase-colorimetric methods. The optimum azide concentration actually in contact with the immobilized glucose oxidase (considering dilution in the manifold) is 1.67 mmol/L. With the addition of azide and the use of a 75-cm GO coil, the assay sensitivity was increased by a factor of 4.8 over that observed with no azide and a 25-cm coil.

Fig. 1. Assay sensitivity vs enzyme coil length curves at various azide concentrations

Curve A, 0.0 mmol/L azide; curve B, 1.0 mmol/L azide; curve C, 10.0 mmol/L azide

Fig. 2. Relative sensitivity vs log azide concentration with hydrogen peroxide (curve A) and with glucose (curve B) as the sample

Reference
1. Chirillo, R., Caenaro, G., Pavan, B., and Pin, A. The use of immobilized enzyme re-
Table 1. Graded Hemagglutination Inhibition (GHI) and Radial Immunodiffusion (RID) Compared for Quantitation of Human IgM in Sera from 15 Patients

<table>
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<th>Dilution of serum (fold)</th>
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References


Contact

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Creatine Kinase BB in Human Amniotic Fluid

To the Editor:

Of the three common isoenzymes of creatine kinase (CK; EC 2.7.3.2), CK-BB is reportedly the major isoenzyme in fetal skeletal muscle; it decreases steadily throughout embryonic life with a concomitant increase in CK-MM, the predominant isoenzyme in adult skeletal muscle (1). CK-MM, CK-MB, and CK-BB are known to be increased in the serum of patients and some carriers of Duchenne's muscular dystrophy (2–5); moreover, CK-BB is significantly increased in biopsy samples taken from patients with this disease (2).

Investigating the possibility of early detection of fetal abnormalities, we determined the CK-BB concentration in amniotic fluid of 103 women, selected without conscious bias from patients being evaluated in the Prenatal Genetics Clinic at North Carolina Memorial Hospital. The gestational age of the fetus was measured from the mother's last menstrual period or by ultrasound real time measurement of biparietal diameter and crown rump length. Where menstrual dates and ultrasound scan values differed by more than 10 days, the scan was taken to be correct; otherwise, the date of the last menstrual period was used. Amniotic fluid was obtained by intra-uterine puncture. The first 10 mL of amniotic fluid withdrawn was used for cytogenetic studies. The next 10 to 20 mL was centrifuged (1500 \( \times \) g) to remove any cells.

Lyophilized, stabilized human serum containing, per liter, 1 g of sodium azide and 0, 5, 10, 20, or 100 \( \mu \)g of human CK-BB was obtained commercially (Mallinkrodt, Inc., St. Louis, MO 63134). We used a RIA-Quant CK-BB test kit (Mallinkrodt, Inc.) to determine the CK-BB content of amniotic fluid. Kit directions were followed except that the specimens were first diluted fourfold in stabilized human serum standard ("0" std.).

We quantitated alpha-fetoprotein (AFP) in amniotic fluid by rocket immunoelectrophoresis (7–8 V/cm, overnight), using Seakem agarose gel (Bio Products, Rockland, ME 04841), 12 g/L, containing a titered, AFP-specific antibody obtained from rabbits immunized against AFP purified from cord serum. The slab gels were stained with Coomassie Brilliant Blue G (Sigma Chemical Co., St. Louis, MO 63178), 50 g/L of a water/methanol/acetic acid (45/45/10, by vol) mixture. Concentrations of the AFP standards ranged from 8.4 to 67.2 mg/L.

Regression analysis showed little correlation between gestational age (between 14 and 26 weeks) and CK-BB concentration in human amniotic fluid (Figure 1): \( r = 0.03, p \geq 0.05 \), \( n = 103 \). Assuming gaussian distribution of the data, we established a reference interval of 0–50 \( \mu \)g/L (mean ± 2 SD) for CK-BB in amniotic fluid over this gestational period.

There was also little correlation between concentrations of AFP and CK-BB in human amniotic fluid (Figure 2): \( r = -0.09, p \geq 0.20, n = 103 \). As previously observed, however, gestational age correlated well with concentration of AFP (\( r = 0.70, p < 0.0001 \)).

CK-BB and AFP concentrations in-

from the same mold by a special order, from a local manufacturer. The test was performed as previously described (2). We found that, to prevent some spontaneous nonspecific agglutination of the erythrocytes, sera to be tested should be filtered through a fiber-glass filter (Whatman Ltd., Springfield Mill, Maidstone, Kent, England). This filtration does not alter the protein concentration of the serum. In plotting inner ring diameter (Figure 1) vs the log of IgM concentration, a linear standard curve results. This linear relation is well reproducible. In our trial 15 sera were tested, each in four different dilutions. The same sera were also tested with use of commercially available radial immunodiffusion (RID) plates. Table 1 summarizes the results. The mean within-run CV is 6.8%, quite comparable with more conventional methods of Ig determination. By increasing the dilution of the serum, there is no outstanding tendency towards any unexpected increase or decrease in the reading of results. The agreement between results by our method and by RID was tested by statistical correlation analysis. The correlation coefficient \( r = 0.795 \) \( (p < 0.01) \), the slope was 0.923, and the intercept −12.9. These values reflect good agreement between the two methods.

Fig. 1. Graded hemagglutination inhibition of known concentrations of IgM inner ring diameter is inversely related to IgM concentration. Numbers are IgM in mg/L.

References

