The main point of discussion concerns the relatively large number of cases where, according to our criterion, myocardial infarction is present (see Methods section), and where the criteria of the B.M.C. kit indicate that there is no suspicion of myocardial infarction (43 vs 24 cases). This difference is caused mainly by the fact that the kit criterion, stating that the percentage CK-MB of total CK must be >6, is not fulfilled. This criterion implies total CK activity in the enzymatic diagnosis of myocardial infarction and thereby re-introduces the disadvantage of its nonspecificity. Thus part of the benefit gained by the high cardiospecificity of CK-MB is lost. If CK-MB determinations are carried out serially, together with electrophoresis if required, routine determination of total CK does not contribute to the diagnosis of myocardial infarction.

We gratefully acknowledge the critical assistance of Sara Tas on preparing the manuscript.

References

Reference Intervals for Amylase Isoenzymes in Serum and Plasma of Infants and Children

Balba Kurins Gillard, Janet A. Simbais, and Lee Goodglick

Measurement of pancreatic (P) and salivary-like (S) amylase isoenzyme activity in serum of adults is useful as an indirect indicator of pancreatic and salivary gland exocrine dysfunction. To extend the use of this assay to the pediatric population, we measured amylase isoenzymes in 546 serum and plasma samples and defined normal reference intervals for the P and S isoenzymes as a function of age in newborns, infants, and children. The mean activity of P isoenzyme in newborns is 3% of that of adults, begins to increase at seven to eight months, and reaches adult values by five years. The mean activity of S isoenzyme in serum is 32% of the adult mean at birth, begins to increase by three to four months, and reaches adult values by 19 months; children five to 12 years old have slightly higher values than adults. These changes with age underscore the importance of the use of age-matched reference intervals when serum amylase isoenzyme activities are measured as diagnostic indicators.

Additional Keyphrases: changes with age from birth to adulthood · plasma/serum comparison · saccharogenic (soluble starch) assay · changes in relative proportions of isoenzymes · pedi atric chemistry · newborns · exocrine function index

Total serum amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) consists of pancreatic (P) and salivary-like (S) isoenzymes. The activities of the P and S isoenzymes in serum are indirect indicators of pancreatic and salivary gland exocrine function (1–3), and are used clinically as diagnostic indicators for acute pancreatitis, pancreatic insufficiency, mumps, and other disease states (reviewed in 4 and 5). Several studies (6–9) have shown that infants and children have lower values for both P and S isoenzymes in serum than do adults. To allow better use of assays of circulating amylase isoenzymes in the diagnosis of exocrine gland dysfunction in the pediatric population, we have determined the normal reference intervals of these activities in infants and children.

Materials and Methods

Sample collection. Blood was sampled by venipuncture or skin puncture from nonfasting, ambulatory children and
adults seen in the UCLA Pediatric Outpatient and Acute Care Clinics, as well as from nonpatient adult volunteers, according to a protocol approved by the UCLA Human Protection Committee. After centrifugation, serum or plasma was removed and stored at −20 °C until assayed. Fifty-one percent of the donors were male. Patients known to have diseases of the pancreas, parotid gland, or kidney were excluded from this study, because altered function of these organs affects serum amylase isoenzymes (4, 5).

Comparison of serum and plasma determinations. Circulating amylase and amylase isoenzyme activities are usually determined in serum. However, most blood samples taken in the UCLA Outpatient Clinics are drawn into tubes containing anticoagulant (EDTA or heparin). To determine whether this larger sample population could be used for our study, we measured the effect of these anticoagulants on amylase and amylase isoenzyme activities. Blood was sampled from 18 adult volunteers and each specimen was divided into three aliquots, in red-top (serum), green-top (heparin), and purple-top (EDTA) Vacutainer Tubes (Becton Dickinson, Rutherford, NJ 07070). The serum and plasma samples were assayed for total amylase and amylase isoenzyme activities as below, and the differences between plasma and serum pairs were analyzed by Student's paired t-test.

Total serum amylase was determined by a modified Nelson-Somogyi saccharogenic method (10) with soluble starch substrate (Sigma Chemical Co., St Louis, MO 63178). This method was used because of its high sensitivity and small (140 μL) sample requirement. CaCl₂ (0.5 mmol/L final concentration) was added to the assay mixture to ensure sufficient calcium for maximum amylase activity in EDTA-treated plasma (see Results). For comparison of methods, some samples were also assayed by the Phadebas Amylase Test (Pharmacia, Piscataway, NJ 08854) and the Du Pont aco (Du Pont Instruments, Wilmington, DE 19898) assays.

Serum amylase isoenzymes. We used electrophoresis on polyacrylamide gel to separate the isoenzymes in 10 to 20 μL of serum, followed by specific staining of the gels for amylase activity and densitometry, both as described previously (10). The relative amount (%P and %S) of the isoenzymes is calculated as %P = 100 − %S = P band area/(P + S band areas), and the absolute activity is calculated as %P (or %S) times the value for total serum amylase activity. This method can detect activity as low as 0.2 starch units/L per band, with a 10-μL sample size. We used 20-μL samples from infants (< one year old) to increase the detection limit to 0.1 starch units/L per band. The method has a reproducibility (CV) of 4% and a precision (CV) of 3% for samples from normal adults (10).

Statistical analysis of the data was done with the aid of the BMDP statistical software package (11).

Results

Anticoagulant Effect on Amylase Activity

As reported previously (9), total amylase activity in heparin-treated plasma samples was the same as in serum samples (Table 1). In contrast, EDTA-treated plasma samples had values an average of 20% lower than the corresponding serum samples (p < 0.001), due to binding of calcium by EDTA. Free calcium is required for maximum amylase activity (12).

Addition of 0.5 mmol/L CaCl₂ to the assay mixture used for measurement of total amylase activity overcame the EDTA effect (Table 1). This concentration of CaCl₂ saturates the EDTA introduced into the assay mixture by plasma samples from at least half-filled blood-collection tubes. As much as twice this concentration of CaCl₂ (1.0 mmol/L) in the assay mixture did not alter serum amylase activity (p > 0.5). Therefore, addition of CaCl₂ allows interchange of the three sample collection conditions for determination of total serum amylase activity.

Anticoagulants did not affect the electrophoretic measurement of the relative amounts of P and S isoenzymes (calculated as %P, Table 1). Therefore, modification of the method to quantify the isoenzyme distribution was not necessary.

| Table 1. Comparison of Total Amylase and Isoenzyme Activities of Paired Serum and Plasma Samples |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Samples | CaCl₂ | Total amylase assay | Isoenzyme assay (%) |
| (n = 18) | mmol/L | t | p | t | p |
| Serum (control) | 0.0 | | | | |
| Heparinized plasma | 0.0 | 0.76 | >0.4 | -0.05 | >0.9 |
| EDTA plasma | 0.5 | 0.67 | >0.5 | | |
| EDTA plasma | 0.5 | 0.59 | >0.5 | | |

* Each sample type was compared with the serum control by Student's t-test for paired samples.

** Final concentration of CaCl₂ added to amylase assay mixture.
Comparison of Assays

Total serum amylase was measured by the Nelson–Somogyi assay because this assay has a low detection limit and therefore can measure the low activity in infant samples. To facilitate the use of our reference ranges in other laboratories, we compared values obtained by this assay with those obtained by the commonly used Phadebas Amylase Test and Du Pont aca methods. Linear correlation lines for paired samples were as follows: Phadebas Amylase Test vs Nelson–Somogyi, \( y = -23.4 + 15.3 \times x, r = 0.98 \), \( n = 17 \), with amylase range of 12.9 to 48.3 starch units/L; Du Pont aca vs Nelson–Somogyi, \( y = 9.51 + 3.25 \times x, r = 0.92 \), \( n = 11 \), with amylase range of 10.8 to 40.1 starch units/L.

Isoenzyme Banding Pattern

One P and one S band were detected in most serum samples after electrophoresis and staining. However, a variant pattern with two strong P bands occurred in 10.8% (56/520) of the samples in which P isoenzyme activity was detectable (see below). This incidence is similar to the 13% we reported previously for a smaller number of adult samples (10). Age of the donor did not have a significant effect on the incidence of the doublet P pattern: \( \chi^2 (6 df) = 4.921, p > 0.1 \).

Only a single electrophoretic band was detected after staining samples from some infants less than nine months of age. This single band co-migrated with added internal S isoenzyme standard (from saliva), confirming the band to be S isoenzyme. This indicates that some normal newborns have P isoenzyme activity below the detection limit of 0.1 starch unit/L.

Effect of Age on Isoenzyme Activities

The mean and SEM values for %P and total, S, and P activities from 546 serum and plasma samples as a function of age are shown in Figure 1. For the age groups indicated, male and female values were not significantly different (Wilcoxon Rank Sum test).

Analysis of variance over groups was performed to determine when adult values are reached. Total amylase activity reaches adult values by age three to four years, absolute activity of P isoenzyme reaches adult values by five years, and S isoenzyme activity reaches adult values by two years. The relative distribution of pancreatic and salivary isoenzymes in serum (%P and %S) continues to change significantly until age eight to nine years. Mean %P and %S values have a smaller variance than absolute S and P values, perhaps because P and S isoenzyme activities tend to be correlated for a given individual (3). This smaller variance contributes to the significance of the age effect on %P and %S values in older children, and is consistent with the upward trend reported in the adult population (13) in which %P values increased to a plateau at age 25 to 45, then decreased in yet older donors.

Adjacent age group values that were not significantly different were pooled to define reference intervals for total amylase and isoenzyme activities in the pediatric population (Table 2). Mean values for each age group were calculated from either a normal or log normal fit to the data. The range of values observed for each age group is indicated by the 5th, 50th, and 95th percentiles (14).

Discussion

The definition of reference intervals for serum amylase isoenzymes in newborns, infants, and children provides the

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Table 2. Reference Intervals for Serum Amylase Isoenzymes

<table>
<thead>
<tr>
<th>Age</th>
<th>Mean (starch units/L)</th>
<th>95th (starch units/L)</th>
<th>P isoenzyme, starch units/L</th>
<th>S isoenzyme, starch units/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5th</td>
<td>50th</td>
</tr>
<tr>
<td>0.1 mos</td>
<td>2.3</td>
<td>0.5</td>
<td>2.7</td>
<td>6.3</td>
</tr>
<tr>
<td>4.3 mos</td>
<td>4.6</td>
<td>1.8</td>
<td>4.5</td>
<td>9.1</td>
</tr>
<tr>
<td>7.3 mos</td>
<td>5.6</td>
<td>2.3</td>
<td>5.6</td>
<td>12.6</td>
</tr>
<tr>
<td>10.7 mos</td>
<td>7.3</td>
<td>2.9</td>
<td>6.9</td>
<td>15.5</td>
</tr>
<tr>
<td>1.3 yr</td>
<td>7.3</td>
<td>3.5</td>
<td>8.0</td>
<td>17.9</td>
</tr>
<tr>
<td>2.2 yr</td>
<td>10.7</td>
<td>5.0</td>
<td>11.0</td>
<td>21.3</td>
</tr>
<tr>
<td>3.9 yr</td>
<td>13.7</td>
<td>7.2</td>
<td>14.2</td>
<td>24.2</td>
</tr>
<tr>
<td>7.9 yr</td>
<td>15.1</td>
<td>8.5</td>
<td>15.1</td>
<td>26.0</td>
</tr>
<tr>
<td>15.2 yr</td>
<td>14.4</td>
<td>8.0</td>
<td>14.6</td>
<td>26.4</td>
</tr>
<tr>
<td>33.6 yr</td>
<td>14.1</td>
<td>7.8</td>
<td>13.8</td>
<td>22.7</td>
</tr>
</tbody>
</table>

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Table 2 provides reference intervals for serum amylase isoenzymes in newborns, infants, and children. Mean values were calculated from a normal or log normal distribution. Percentiles were obtained from a smoothed curve fit to the data. Number of samples with P activity below the detection limit of 0.1 starch units/L are *21/53; †1/20; ‡4/23. Statistical analysis of each age group compared to the adult (20–72 yr) group indicates significant differences of *p < 0.001; †p < 0.005; ‡p < 0.01. Bottom half of Table arranged according to age groups in top half.

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basis for clinical application of these isoenzymes for assessment of exocrine function in the pediatric population. We used the Nelson-Somogyi saccharogenic assay (modified for the presence of EDTA) in this study to measure total serum amylase because of its high sensitivity. Correlation between activity measured with this assay and the commonly used Phadebas Amylase Test and Du Pont aca assays were determined to facilitate use of the reference intervals in other laboratories.

However, problems with amylase assay interconvertibility should be kept in mind. For example, we use the same soluble starch substrate for both total amylase and amylase isoenzyme determinations. If instead a soluble substrate were used in one assay and an insoluble one in the other, a bias might be introduced, especially at low total amylase activity (15, 16).

The results of this study are consistent with most previous reports of serum amylase activity in children (6, 9, 13). Total serum amylase is low at birth (18% of adult values), increases by age two to three months, and reaches adult levels by three years, as reported by Otsuki et al. (7). This study is also consistent with a study of older children and adults (13), which reports no significant age effect.

S isoenzyme development parallels total serum amylase: it is one-third of adult values at birth, increases at three months of age, and reaches adult values by three years of age. This trend with age is similar to those reported by Skude (6) and by O'Donnell and Miller (9). At five to 12 years the slight increase of S isoenzyme activities over adult values supports a previous report of increased serum S isoenzyme in this age range (9). Amylase activity in saliva has also been reported (17) to be higher in older children than in adults. These studies indicate salivary gland amylase secretion reaches a maximum during late childhood, and subsequently decreases.

Serum P enzyme development is similar to, but lags behind, S isoenzyme development. It is lower than S isoenzyme at birth, increases by seven to nine months, and reaches adult values by five to nine years, consistent with Skude's report (6). Although O'Donnell and Miller (9) also found low P isoenzyme activity in infants, they report mean P isoenzyme values for the 0 to 10-month age group to be greater than the mean S isoenzyme values.

The inhibitor method used by O'Donnell and Miller for measurement of isoenzyme activity may account for their difference in the ratio of P to S activity in infants. Rammeloo et al. (18) have reported that the inhibitor method gives higher P isoenzyme values than the electrophoretic method. Berk et al. (19) have noted that the inhibitor method yields imprecise quantitative results when the percent P isoenzyme is greater than 80% or less than 10%, as in newborn sera.

The low serum amylase isoenzyme activity in infants is consistent with previous studies of exocrine function development. Both amniotic fluid (20, 21) and urine samples from newborns (22) have lower P than S isoenzyme activity. Direct assessments of pancreatic function also indicate low output of amylase in infants. For example, Lebenthal and Lee (23) reported that duodenal fluid in infants up to four months of age contains very little, if any, amylace. Hadorn et al. (24) found that the pancreas in babies up to three months old, when stimulated with pancreozymin and secretin, secretes only 6% as much amylase as the pancreas of the five- to 13-year-old.

In this study we have defined reference intervals for serum amylase isoenzymes for the pediatric population. The parallel development of serum isoenzyme activities and exocrine function suggests serum isoenzyme values will prove useful as a simple indirect measure of pancreatic and salivary exocrine function in infants and children (25), as is the case for adults (1-5, 26).

Supported by National Institutes of Health Grant AM25107, Cystic Fibrosis Foundation Grant G721B, and the Ramer Cystic Fibrosis Research Fund. Phadebas Blue Starch tablets for micro assay of serum amylase were provided by Pharmacia.

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References
This simple, rapid, sensitive kinetic bioluminescent method for the assay of bile acids in serum involves use of 3α-
hydroxysteroid dehydrogenase (EC 1.1.1.50) in combination
with a new, commercially available NADH Monitoring Rea-
gent (LKB-Wallac) containing a low activity of NADH:FMN-
oxidoreductase and a high activity of bacterial luciferase. Interfering dehydrogenases in serum are inactivated in the
test tube with trichloroacetic acid before the assay. The
standard curve is linear for concentrations of bile acids up to
about 300 μmol/L. With a sample volume of 20 μL, the
detection limit is about 0.2 μmol/L. The within-run precision
(CV) is about 10%, both at high and low concentrations of
bile acids in serum. Correlation is good (r = 0.996) between
results by this method and an enzymatic method based on
spectrophotometry. However, the latter method is considera-

Additional Keyphrases: liver disease · enzymic methods ·
kinetic methods · cholate

Increased concentrations of bile acids in serum, the result
of shunting of systemic portal blood or a decreased hepatic
uptake, are an early and sensitive sign of liver disease (1, 2).

In spite of the documented diagnostic sensitivity and
specificity, determination of bile acids has not yet been
included in the battery of tests in general use for diagnosis
of liver diseases (1), in part because the commonly used
methods for bile acids in serum are fairly complicated. Gas-
liquid chromatography is highly specific and can be used to
determine individual bile acids (3, 4), but is rather insensitive
and very time-consuming. Radioimmunoassay (1, 5) is
relatively specific and sensitive, but is expensive and labori-

HSD, EC 1.1.1.50) (6–9) compete with the classical liver
tests with respect to simplicity and practicability (1).2 These
methods are, however, insensitive and require relatively
large volumes of serum. Attempts have been made to
increase the sensitivity by using fluorescence (10, 11) or
enzyme recycling (12). Such modifications increase the
sensitivity but often decrease the practicability.

We report here our efforts to develop a sensitive but
simple and practical assay for bile acids in serum by use of
the 3α-hydroxysteroid dehydrogenase reaction, coupled to a
bacterial bioluminescence system. The potential for high
sensitivity in the latter step is well established (13).

Assay of NADH by bacterial bioluminescence involves
two enzymes: NADH:FMN-oxidoreductase (OR) reduces fla-
in mononucleotide (FMN) to FMNH₂, which is oxidized in
the light-producing reaction catalyzed by bacterial lucifer-
ase (BL). In the second reaction, oxygen and an aliphatic
straight-chain aldehyde with eight to 14 carbon atoms are
required as illustrated in the reaction schemes:

NADH + H⁺ + FMN OR NAD⁺ + FMNH₂

FMNH₂ + O₂ + RCHOBL FMN + RCOOH + H₂O + Light

Bacterial bioluminescence has been used to analyze nu-
merous substrates and dehydrogenase activities involved in
NADH-producing reactions, e.g., malate (14, 15), malate
dehydrogenase (14, 16), lactate dehydrogenase (16), alcohol
dehydrogenase (13, 16, 17), ethanol (13, 16, 18) and oxaloac-
etate (14). Steroids have been assayed by use of dehydrogen-
ases, BL, and OR co-immobilized on Sepharose 4B (19).

In most of the above studies, the composition of the
reagents has not been defined in terms of activities of BL
and OR, and the detailed kinetic properties of the prepara-
tions have not been known.

If a purified reagent with a low OR activity and a high BL
activity is used, the light emission stays almost constant for
several minutes in response to 10⁻¹⁰ to 10⁻⁶ mol of NADH
per liter, because the low OR content results in negligible
consumption of NADH during the reaction. Substrates and
enzymes in NADH-producing reactions can thus be kine-
tically determined by continuously monitoring the changes in
light intensity (13).

In the present work we have used 3α-HSD and a com-
mercially available purified BL/OR reagent that seems to fulfill
the above requirements for assay of bile acids in serum.

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2 Nonstandard abbreviations: OR, NADH:FMN-oxidoreductase; BL, bacterial luciferase; FMN, flavin mononucleotide; 3α-HSD, 3α-
hydroxysteroid dehydrogenase; BSA, bovine serum albumin; and TCA, trichloroacetic acid.

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Bioluminescent Assay for Total Bile Acids in Serum with Use of Bacterial
Luciferase

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