the large variation in Schale's results (94.5%); however, the results do show that workers should be cognizant of the fact that large spectral bandwidths can cause significant errors in their absorbance measurements. At this time we are not prepared to speculate on other sources of errors in colorimetric measurements of NADH. We simply suggest that anyone working with medium- and wide-band instruments be aware of the inherent error in absorbance values obtained when the spectral bandwidth becomes much greater than 10% of the natural bandwidth of the compound being analyzed.

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Differences between Manufacturer-Assigned Values and Observed Values of Alkaline Phosphatase Activity in a Commercial SMA Calibration Serum

To the Editor:

The purpose of this communication is to report our findings of unreliability in manufacturer-assigned values of a commercially available serum "standard" used in the calibration of the SMA 12/60 (Technicon) method for measurement of serum alkaline phosphatase activity. The SMA 12/60 method is based on the AutoAnalyzer method of Morgenstern et al. (*1*), a modification of the manual procedure of Bessey et al. (*2*), and is similar in principle to the kinetic procedure of Bowers and McComb (*3*). In the SMA method, *p*-nitrophenyl phosphate is hydrolyzed as a result of phosphatase activity at pH 10.25 in 625 mmol/liter 2-amino-2-methyl-1-propanol buffer, and the rate of appearance of the colored end product, *p*-nitrophenol, is determined at 410 nm after a short incubation period (about 3–5 min) at 37.5 °C.

Primary standardization of the SMA method is best achieved by use of a standard solution of the end product in the buffer. An equivalent "enzyme activity" value is calculated by dividing the original concentration of the standard solution by the "incubation time" interval in the continuous-flow system (*4*). This calculated value is used to calibrate a scaling device on the SMA analyzer while the absorbance of the standard solution is being measured. Calibrated in such a manner, the SMA analyzer is reported (*5*) to produce analytical results that agree reasonably with results derived from the kinetic procedure of Bowers and McComb.

Although the use of pure end-product standards represents the best available analytical approach in standardizing enzyme methods on multichannel analyzers, routine standardization of SMA methods, to be practical, requires the use of a multicomponent serum "standard." Such serum "standards," available commercially from several manufacturers, attempt to provide adequate standardization of each test method on SMA analyzers by analysis of a single sample. A calibration value is usually assigned for each chemistry test by the manufacturer and, at least in the case of SMA Reference Serum manufactured by Technicon Instruments Corp., such values are said to be confirmable by various methods. With regard to alkaline phosphatase activity, assigned values in Technicon SMA Reference Serum may be confirmed by reference to the method described above, with use of a standard solution of *p*-nitrophenol (*5*).

Our initial experience with the SMA 12/60 analyzer involved the use of Technicon SMA Reference Serum as a calibrating material. When the SMA 12/60 analyzer was calibrated with SMA Reference Serum with use of manufacturer-assigned values, we observed that the histograms of patient data were shifted upward. We also observed that when alkaline phosphatase activity in SMA Reference Serum was determined by the established AutoAnalyzer method standardized with *p*-nitrophenol standard, results from analysis were substantially lower than calibrating values assigned by the manufacturer. These preliminary observations prompted a more rigorous study.

Materials representing several available lots of Technicon SMA Reference Serum (carefully reconstituted and refrigerated according to manufacturer's instructions) were analyzed for phosphatase activity by the SMA 12/60 method calibrated with the standard as well as by the kinetic procedure of Bowers and McComb conducted at 37 °C. Both reference methods were performed in accordance with analytical details specified by Technicon (*5*). Table 1 compares manufacturer-assigned values with values we obtained experimentally with the SMA method and kinetic procedure. Our values, obtained by the SMA method and by the kinetic method, were fairly consistent, in agreement with statements by Technicon (*5*), but were about 15% lower than values assigned by Technicon.

In arriving at our enzyme activity values for the Technicon Reference Serum, we were aware of the rapid decline in activity (almost 1% per minute) of alkaline phosphatase at 37 °C in pH 10.2 buffer solution (*3*). Since the incubation time in our SMA 12/60 procedure is 4.7 min, our results could be low by a few percent, and the same would be true for the kinetic Bowers-McComb procedure run at 37 °C. If Technicon had calibrated their SMA 12/60 with *p*-nitrophenol and if their and our calibration procedures were the same (including extinction coefficient), then both of us should have obtained the same activity on the Reference Serum (although both results could have been a few percent low). That our results were substantially lower than those of Technicon suggests that: (*a*) our calibration procedure differs significantly from that of Technicon; (*b*) there was loss of enzyme activity between the time Technicon assayed the material and the

Table 1. Alkaline Phosphatase Activity of Technicon SMA Reference Serum

	Alkaline phosphatase activity (μmol/min/liter)		
		Observed	
Lot no. of reference serum	Assigned by manufacturer	SMA 12/60[a]	Kinetic[b]
B3D-265	88	76, 76, 74	78, 73, 77
B3D-266	88	75, 75, 72	74, 72, 72
B3F-287	87	75, 75, 72	74, 72, 71

[a] The SMA 12/60 method was calibrated with *p*-nitrophenyl phosphate, 386 μmol/liter in buffer (625 mmol/liter, pH 10.25) [estimation of concentration was based on the molar extinction coefficient (*3*) of 18750 at 405 nm and 30 °C]. "Incubation time" in the continuous flow system was found to be 4.7 min. Buffer pH's were measured at 30 °C.

[b] The kinetic method studied was the manual procedure of Bowers and McComb (*3*) at 37 °C.

time we repeated the assay; or (c) Technicon assigned the values by a method other than the primary calibration of the SMA 12/60 with *p*-nitrophenol or the Bowers–McComb kinetic procedure.

These observations strengthen the point that, for reliable analysis, manufacturer-assigned values in multicomponent-serum "standards" be adequately confirmed by suitable reference methods before their routine use in standardizing clinical chemistry procedures. This caveat is necessary both to keep results from different laboratories somewhat comparable and also to guard against a manufacturer's complacency.

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Fluorometric Detection of Methadone Metabolite on TLC Plates

To the Editor:

When testing for the presence of drugs of abuse in urine, questions sometimes arise with regard to the possibility of interferences from other drugs and their metabolites. The detection of methadone and (or) its metabolite(s) in urine is of interest in monitoring patients on methadone maintenance (treatment) programs or to detect illicit use of methadone. A method for detecting a methadone metabolite on thin-layer chromatographic plates is the pink color produced with a ninhydrin spray reagent and the reddish-purple color produced by overspraying with an acid iodoplatinate reagent (*1*). Interfering substances in some urine samples may limit the use of one or both of these detection methods. We report here another way to detect a methadone metabolite on thin-layer chromatographic plates, which depends on the reaction between a methadone metabolite and ninhydrin in the presence of triethylamine in acetone to form a red product that fluoresces under a long-wavelength (~366 nm) ultraviolet lamp, which is intensified in the presence of an acid iodoplatinate reagent.

Urine samples are taken through a direct extraction concentration and thin-layer chromatographic separation procedure (*1*). After the plates (LQDF TLC plates; Quantum Industries, Fairfield, N.J. 07006) are dried at 100–110 °C for 5 min, they are sprayed with a freshly prepared solution of ninhydrin in *n*-butanol (2 g/liter), dried for 5 min at 100–110 °C, and then placed under a short-wavelength (~256 nm) ultraviolet lamp for 5 min. A methadone metabolite will appear as a pink spot with an R_F of about 0.89 after development in the solvent system ethyl acetate:absolute ethanol:*n*-butanol:concentrated ammonium hydroxide (28:14:2:0.4, by vol). The plate is then oversprayed with an acid iodoplatinate reagent (*2*), which gives a reddish-purple spot both for methadone and a methadone metabolite (methadone added to a drug-free urine gives this later reaction). If the same plate is now heavily oversprayed with a solution of triethylamine in acetone (20 g/liter), the metabolite of methadone will give a red fluorescence when exposed to a long-wavelength ultraviolet lamp. Methadone added to a drug-free urine does not give this red fluorescence.

Urine samples from patients taking methadone orally were taken through the above procedure and 20-, 10-, 5- and 2-μl aliquots of the reconstituted residues were spotted on plates, to compare the relative sensitivity of ninhydrin and the fluorometric reaction (20 μl is spotted in the routine method). With ninhydrin, a pink color appeared in each case. After overspraying with iodoplatinate a reddish-purple spot appeared in each case. When the plate was oversprayed with the triethylamine reagent, this second spot showed a red fluorescence that could be seen for the 20-, 10-, and 5-μl aliquots. This indicates that the sensitivity of the fluorescence produced with the triethylamine spray is less than that of the color produced with the other two reagents, but still may be useful for confirming the use and abuse of methadone. We have not determined which metabolite(s) reacts to form the pink color with ninhydrin and (or) the red fluorescent product with the triethylamine reagent.

If the plate is sequentially sprayed with the ninhydrin spray and the triethylamine reagent, a red fluorescence appears under a long-wavelength ultraviolet lamp at the position of the metabolite but is much less intense. We have found no false positives with the method.

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Zero-Order Kinetics?

To the Editor:

One criterion applied to the design of reference methods for the estimation of clinical enzyme activity is that the substrate concentrations used should give rise to zero-order kinetics. While it is true that under this condition—that is, where $[S] \gg K_m$, in the usual Michaelis–Menten equation (1)

$$v = \frac{V_{max}}{1 + K_m/[S]}$$

(where the symbols have the generally accepted meaning), the initial rate of change of substrate concentration is constant, giving rise to linear outputs of substrate concentration against time. The opposite deduction—that linear outputs indicate zero-order kinetics—does not necessarily hold.

When $[S] < K_m$ the reaction will follow pseudo-first-order kinetics and be described by the usual exponential equation (2). By applying a linear least-squares analysis to the output from this equation, it can be shown that the initial 20% of reaction can be represented by a straight line of coefficient of correlation 0.9956 and a standard error in the slope of less than 3%. This calculated error decreases as smaller extents of reaction are taken or when fractional orders of reaction are approached as [S] is increased relative to K_m.

This small error is much less than that inherent in many commercial enzyme analyses currently in use employing recommended methods. It is important therefore that a distinction be made, by committees and other bodies making recommendations on enzyme assay procedures and by firms marketing assay kits, between those methods that are de-