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Standardization of clinical enzyme assays: a reference method for aspartate and alanine transaminases

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This is the second report produced by the Working Party set up under the auspices of the Standards Subgroup of the Laboratory Development Advisory Group of the Department of Health and Social Security. The Working Party includes representatives of the Association of Clinical Biochemists, the Association of Clinical Pathologists, and the Royal College of Pathologists. Its first report, devoted to the standardization of the assay of alkaline phosphatase, has recently been published (Moss, Baron, Walker, and Wilkinson, 1971).

Aspartate and alanine transaminases have long been known in clinical biochemistry as glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase respectively, but are officially recognized by the International Union of Biochemistry as L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1.) and L-alanine: 2-oxoglutarate aminotransferase (EC 2.6.1.2.). Aspartate transaminase and alanine transaminase are approved trivial names and in this report these are abbreviated to AST and ALT respectively.

Several colorimetric, fluorimetric, and spectrophotometric techniques have been devised for their determination but for the purposes of standardization a spectrophotometric procedure is recommended as a reference method.

The measurement of aspartate and alanine transaminase activities, described in this report, are examples of reaction-rate techniques in which the progress of the enzyme reaction is followed throughout the period of observation. Absorbence readings are taken at intervals timed with a stopwatch or, preferably, with the aid of a recording spectrophotometer. By such means it can be verified that the reaction rate is constant. Such verification is clearly impossible with a two-point assay system. Nevertheless, within certain limits methods of the latter type can be used satisfactorily, provided they are calibrated with the aid of a suitable reference method, such as those proposed in this report. The principles of calibration were discussed in the first report of the Working Party.

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Principle

The reactions involved in the determination of aspartate transaminase (AST) activity are as follows:

$$\text{Aspartate} + \text{2-oxoglutarate} \rightleftharpoons \text{glutamate} + \text{oxaloacetate} \quad (1)$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{dehydrogenase}]{\text{malate}} \text{malate} + \text{NAD}^+ \quad (2)$$

The oxaloacetate produced by the transaminase serves as substrate for malate dehydrogenase by which it is reduced to malate in the presence of dihydronicotinamide-adenine dinucleotide (NADH), which is simultaneously oxidized. NADH has an absorbence peak at 340 nm which is not shown by the oxidized form, and the decrease in absorbence at this wavelength provides a means for the measurement of the transaminase activity. The methods suggested are based upon those of Henry, Chiamori, Golub, and Berkman (1960) who re-investigated and improved the technique originally introduced by Karmen (1955).

The spectrophotometric procedure has an important advantage over colorimetric procedures in which the oxaloacetate produced in reaction 1 is allowed to react with a chromogenic reagent such as 2,4-dinitrophenyl-hydrazine (Reitman and Frankel, 1957) or a diazonium reagent (Babson, Shapiro, Williams, and Phillips, 1962). Oxaloacetate is a potent inhibitor of AST (Boyd, 1961) and in the spectrophotometric procedure is removed as fast as it is formed, whereas in the colorimetric techniques it is allowed to accumulate.

The spectrophotometric procedure is standardized by reference to the molar absorbance of NADH ($6{\cdot}22 \times 10^3$) at 340 nm. The decrease in unit time

(1 min) during the rectilinear (zero order) phase of the reaction provides a means for the calculation of the enzyme activity in international units (micromoles NADH oxidized per minute) per litre[1] under the reaction conditions defined.

Alternatively the NADH may be measured fluorimetrically (Laursen and Hansen, 1958), and this procedure has been adapted to an end-point system for use in the AutoAnalyzer (Levine and Hill, 1965) but it is unsuitable for use as a reference method.

Similar principles are employed in the measurement of alanine transaminase (ALT) activity. In the spectrophotometric procedure (Henley and Pollard, 1955), the pyruvate produced in the transaminase reaction (3) is reduced to lactate by the NAD^+-dependent lactate dehydrogenase (4):

$$\text{Alanine} + \text{2-oxoglutarate} \rightleftharpoons \text{glutamate} + \text{pyruvate} \quad (3)$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons[\text{dehydrogenase}]{\text{lactate}} \text{lactate} + \text{NAD}^+ \quad (4)$$

Substrate Concentrations

Henry *et al* (1960) modified the AST technique by adjusting the substrate concentrations to give considerably higher enzyme activities. The L-aspartate concentration (0·033 moles/litre) employed in Karmen's original spectrophotometric procedure for the determination of AST (Karmen, 1955) was found to be suboptimal, and Henry *et al* (1960) recommended increasing this to 0·125 moles/litre. DL-aspartate cannot be used as the D- form inhibits this transaminase.

Although Henry *et al* (1960) increased the final concentration of L-alanine to 0·167 moles/litre in the measurement of ALT activity, experience has since shown that this concentration is suboptimal (Arvan and Coyle, 1969; Laudahn, Hartmann, Rosenfeld, Weyer, and Muth, 1970). Arvan and Coyle (1969) recommended the use of 0·25 moles/litre L-alanine, since higher concentrations (such as the 0·81 moles/litre L-alanine, recommended[2] by Laudahn *et al* (1970)) caused little further enhancement of activity and led to difficulty in preparing stock solutions, owing to the limited solubility of this amino acid. Since D-alanine does not act as a substrate for ALT and in our experience does not appear to inhibit the enzyme, DL-alanine may be used in place of L-alanine. The necessary two-fold increase in concentration, however, complicates the use of the DL-form, which is liable to crystallize from stock solutions during storage in a refrigerator.

The reference methods recommended in this report are those of Henry *et al* (1960), slightly modified in that measurements are made at 25°C, and a final concentration of 0·25 moles/litre L-alanine is used for the ALT reaction.

Temperature

Karmen (1955) performed his reaction at 'room temperature' (23°C) and Henry *et al* (1960) specified 32°C, the temperature reached in the cell compartment of the Beckman DU spectrophotometer. The International Union of Biochemistry originally recommended 25°C, but later 30°C, for enzyme determination, but few clinical laboratories in Britain carry out enzyme measurements at 30°C, since in this country it is quite practicable to maintain a temperature of 25°C in a thermostatically controlled cuvette compartment of a spectrophotometer for most of the year without the aid of an external cooling unit other than tap water.

The development of semi-automated enzyme rate analyzers, such as the LKB, or spectrophotometers specially adapted for microanalysis, such as the Eskalab, which operate at 35°C or 37°C, has necessitated further review of operating temperatures. There is much to be gained by performing all NAD- or NADP-dependent enzyme measurements at a single temperature, but the Working Party has not been able to recommend a universally satisfactory temperature. The choice appears to lie between 25°C, 30°C, 35°C, and 37°C. We at first considered 37°C as the most suitable, since the phosphatases and other enzymes are regularly determined at this temperature and every clinical laboratory is equipped with a 37°C water bath. However, doubts were felt about the stability of certain enzymes at 37°C, in particular liver lactate dehydrogenase (LD-5) and glucose 6-phosphate dehydrogenase (GPD). While the Working Party has been unable to obtain evidence of loss of activity of the former at 37°C, the latter loses about 15% of its activity during preincubation for 20 minutes at 37°C.

The Working Party has some reservations about proposing a temperature other than that recommended by the International Union of Biochemistry and endorsed by the International Federation of Clinical Chemistry, namely 30°C, but the fact has to be faced that this figure has not proved acceptable in most British clinical laboratories.

[1]The international unit of enzyme activity (U or iu) has not been adapted for use with the SI system (Système Internationale d'Unités). The IUPAC/IUB Commission on Biochemical Nomenclature have instead recommended the 'katal' as the unit of 'catalytic amount', defined as the amount of a catalyst (including an enzyme) that brings about the transformation of one mole of substrate per second under defined conditions. Thus 1 katal per litre (kat/l) = 60×10^6 international units per litre (U/l) or 1 U/l ≙ 16·67 nkat/l.

[2]The Boehringer optimized test combination.

The original IUB recommendation of 25°C has been criticized mainly because of the technical difficulty of maintaining it in hot countries and especially in the USA, but during the past decade, several moderately priced refrigerated water baths or portable refrigerator units have been marketed which overcome this problem. We therefore consider that it is practicable to standardize the determination of NAD- and NADP-dependent enzymes at 25°C. The procedures described are designed for use at 25°C, but the conditions recommended may not be optimal at higher temperatures. The following conversion factors have been experimentally determined with the suggested method at 37°C on six sera with AST and ALT activities ranging from 47 to 184 U/litre and 46 to 139 U/litre respectively.[3] While these enable results obtained at 37°C to be compared with those measured at 25°C, such factors should be used with caution.

Cuvette Temperature	*Factors to Convert to*	*AST*	*ALT*
25°C	37°C	2·18	1·96
37°C	25°C	0·46	0·51

Factors for the interconversion of AST and ALT activities determined at 25°C or 37°C

The Working Party considers that individual laboratories should use whatever temperature is appropriate to their equipment, but should calibrate their procedures with the aid of a suitable reference method. Whatever temperature of estimation may be chosen in a particular laboratory, it is essential to use a spectrophotometer equipped with effective means of controlling the temperature of the sample accurately and precisely.

Recommended Method

REAGENTS

Distilled or demineralized water should be used for the preparation of the following reagents:

1 *Phosphate buffer (1·0 moles/litre), pH 7·4*
One hundred and thirty-six g KH_2PO_4 and 33 g NaOH dissolved in water and the volume adjusted to 1 litre.

2 *Phosphate buffer (0·1 moles/litre), pH 7·4*
One hundred ml of 1M-phosphate buffer made up to 1 litre and adjusted to *p*H 7·4 at 25°C.

[3]A working party of the Association of Clinical Biochemists is currently investigating the reproducibility of temperature coefficients for AST and ALT determined by the methods described.

3 *NADH solution*
2·5 mg per ml reagent 2. Best prepared fresh each day. The development of inhibitors of some dehydrogenases in certain NADH solutions is not a critical factor when used for transaminase determination as the dehydrogenase used as indicator enzyme is present in considerable excess. Slight variations between batches of good quality NADH have negligible effects on the results of transaminase assays.

4 *2-Oxoglutarate (0·1 moles/litre)*
0·73 g 2-oxoglutaric acid is dissolved in 35 ml water. Five ml reagent 1 is added and the *p*H adjusted to 7·4 at 25°C by the addition of NaOH (1 mole/litre), after which the volume is made up to 50 ml with water. May be stored up to one month at 4°C.

5 L-*Aspartate (0·375 moles/litre)*
5·0 g L-aspartic acid is dissolved with the aid of heat in a mixture of 50 ml water and 35 ml NaOH (1 mole/litre). After cooling to room temperature, 10 ml reagent 1 is added and the *p*H adjusted to 7·4 at 25°C with NaOH (1 mole/litre). The volume is then made up to 100 ml. May be stored up to one month at 4°C.

6 L-*Alanine (0·75 moles/litre)*
6·67 g L-alanine is dissolved in 75 ml water and 10 ml reagent 1, and the *p*H is adjusted to 7·4 at 25°C with NaOH (1 mole/litre). The volume is then made up to 100 ml. May be stored for one month at 4°C.

7 *Malate dehydrogenase 5 U per ml*
A commercial preparation should be diluted with water to provide a day's supply. Each batch of malate dehydrogenase should be checked for the presence of AST activity by performing a blank reaction, ie, omitting serum, and also for apotransaminase by carrying out a blank reaction in which serum is replaced by 0·2 ml solution containing 0·2 μg phosphopyridoxal (Rosalki and Wilkinson, 1959). The presence of the apotransaminase must be excluded since it would be activated by the phosphopyridoxal of serum to give a spuriously high transaminase activity.

8 *Lactate dehydrogenase 5 U per ml*
The rabbit-muscle enzyme should be diluted with water to provide a day's supply.

PROCEDURE

The following mixtures are prepared and incubated for 15 min. at 25°C ± 0·2°C.

	AST (ml)	*ALT (ml)*
Phosphate buffer (0·1 moles/litre)	1·3	1·3
Serum	0·2	0·2
NADH solution	0·2	0·2
L-aspartate	1·0	—
L-alanine	—	1·0
Malate dehydrogenase	0·1	—
Lactate dehydrogenase	—	0·1
Total volume	2·8	2·8

An ultra-violet spectrophotometer, preferably a recording instrument, is switched on and set for recording changes in absorbance at 340 nm. The cuvette compartment should be thermostatically controlled so that the cuvette temperature is 25°C ± 0·2°C. When the instrument is ready the incubated solutions should be transferred to cuvettes. In each case, the reaction is started by the addition of 0·2 ml 2-oxoglutarate solution followed by careful mixing. Recordings for the purpose of calculation should not be made until the reaction obeys zero-order kinetics, ie, the decrease in absorbance becomes steady. (There is often a lag phase of 2 to 5 min.) The rate of change in absorbance per minute is measured (Δ A/min), and the transaminase activity calculated in U per litre:

$$\text{Transaminase activity} = \frac{\Delta \text{ A/min}}{6{\cdot}22 \times 10^3} \times \frac{1000}{0{\cdot}2} \times 3000$$
$$= \Delta \text{ A/min} \times 2400 \text{ U/litre}$$

The spectrophotometric unit introduced by Karmen (1955) is the enzyme activity in 3 ml reaction mixture producing a change in absorbence at 340 nm of 0·001 per minute per ml of serum. Thus Karmen units/ml can be converted into International units per litre by multiplying by 3/6·22 or 0·48.

It is important to check the absorbence scale of the spectrophotometer, since the value of the method depends upon accurate measurement. Methods for checking accuracy with spectrophotometric standards have recently been discussed by Rand (1969).

Normal Ranges for Serum Transaminase Activities

The normal ranges were obtained at 25°C on healthy blood donors and laboratory personnel by the methods described:

Aspartate transaminase	males	5-15 U/litre
	females	5-12
Alanine transaminase	males	5-19
	females	5-12

Calibration of Automated and Manual Procedures

The problems involved in the calibration of automated transaminase procedures are similar to those encountered in the case of alkaline phosphatase discussed in the first report of the Working Party. The most commonly used automated systems include the AutoAnalyzer methods with 2,4-dinitrophenylhydrazine (Fingerhut, Ferzola, Marsh, and Levine, 1962) or a diazonium reagent (Schaffert, Kingsley, and Getchell, 1964), but the fluorimetric AutoAnalyzer method (Levine and Hill, 1965) also requires calibration.

For this purpose it is suggested that the transaminase activities of a series of about five sera[4] be determined by the reference methods described in this report. The activities should span the effective range of the automated technique, ie, from about 10-300 U/litre. The reference sera may be stored frozen in small containers for periods of up to eight months, or at 4°C for up to one week without significant loss of activity. Nevertheless we recommend that the activities of such sera be checked at least twice a week.

Non-automated manual versions of these colorimetric or fluorimetric techniques can be calibrated in a similar manner using sera whose transaminase activities have been measured by the reference method.

Comments and Recommendations

1 Spectrophotometric methods are considered to be the most suitable reference procedures for transaminase determination. The rate of oxidation of NADH, when measured by the change in the absorbance at 340 nm is a much more dependable standard than any of the alternatives which have been proposed, eg, pyruvate, 'standard' sera, etc. Since the indicator enzymes are in considerable excess the presence of possible dehydrogenase inhibitors in the NADH (Fawcett, Ciotti, and Kaplan, 1961; Dalziel, 1961) has little or no effect on the transaminase reaction which remains the rate-determining step.

2 Transaminase activities determined by a colorimetric technique should not be reported in spectrophotometric units, eg, Karmen units, unless the method has been calibrated by reference to the recommended or other appropriate spectrophotometric procedure.

3 The techniques recommended have been demonstrated to give maximal activities and to be repro-

[4]If pools of human sera are used, care should be taken in case some specimens contain hepatitis virus. Horse serum with added liver extracts free from Australia antigen may be used. See First Report of Working Party (Moss *et al*, 1971). If liver extracts are employed they should be centrifuged to remove mitochondria since mitochondrial glutamate dehydrogenase might catalyse the reductive amination of 2-oxoglutarate by ammonium salts present in the malate dehydrogenase preparation.

ducible, a day-to-day coefficient of variation of ±5% being obtained on sera with borderline and moderately elevated transaminase activities.

4 The methods of standardization described in this report are applicable to other enzyme procedures dependent upon the oxidation of NADH or NADPH, eg, lactate dehydrogenase, or the reduction of NAD^+ or $NADP^+$, eg, glucose 6-phosphate dehydrogenase.

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