REVIEWS

SOME REFINEMENTS IN DIAGNOSTIC ENZYMOLOGY*

By I. K. Tan

SYNOPSIS

This paper discusses the recent developments and various factors which have contributed to the advancement in the field of Diagnostic Enzymology. It briefly reviews the application of several enzymes/isoenzymes studies in clinical diagnosis and in the detection of inherited metabolic disorders. It also draws attention to recent work on quality control and standardisation of enzyme assays.

Within a period of 10 years, great strides have been made in the field of diagnostic enzymology. I remember in the early 1960s, relatively few requests for enzyme determinations were received from the clinicians. They were confined to the limited range of enzyme tests available at that time, namely, amylase, acid and alkaline phosphatase, the aminotransferases (transaminases)—SGOT and SGPT, and an occasional aldolase or caeruloplasmin. By the end of the decade, the repertoire of routine enzyme tests has widened considerably to include some 20 different types of enzyme investigations. Fig. 1 shows the total number of the more commonly requested enzyme determinations performed in the Clinical Biochemistry Laboratories of the Department of Pathology in Singapore over the period 1963 through 1973. It is noted that the total number of enzyme assays performed in 1973 was approximately 9 times greater than that carried out 10 years ago.

Over the decade, the increasing refinements in equipment and methods of enzyme determinations have resulted in improvements both in the reproducibility and the accuracy of enzyme measurements. This has in turn made possible more reliable detection of small but significant alterations in enzyme levels in sequential enzyme studies.

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*Presented at The Royal Australian College of Physicians Annual Scientific Meeting held in Singapore on March 6, 1974.

FIG. 1. NUMBER OF ENZYME ASSAYS PERFORMED 1963 - 1973
(Clinical Biochemistry Laboratories, Government Department of Pathology, Singapore)

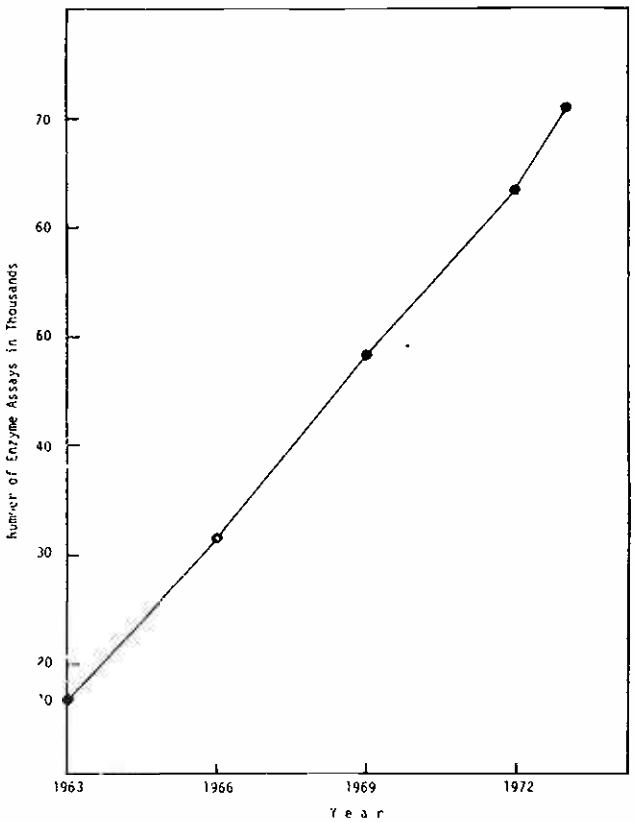

Fig. 1.

In this comparatively brief presentation, it is proposed to consider the various factors and developments which have contributed to the advancement in the field and to review the present state of knowledge.

EFFECT OF BETTER UNDERSTANDING OF THE DISTRIBUTION OF ENZYMES IN BODY TISSUES AND THE PROPERTIES OF THESE ENZYMES

For a considerable time, it has been assumed that human serum alkaline phosphatase (SAP), lactate dehydrogenase (SLDH), creatine phos-

phokinase (SCPK), SGOT and a host of other clinically important enzymes were single entities. When the application of a variety of electrophoretic and chromatographic techniques made it possible to separate what appeared to be single entities into multiple components, it became clear that these enzymes exist in multiple molecular forms; in fact experimental evidence have indicated that it is the exception rather than the rule for an enzyme to exist in only one form (Kaplan, 1968). The term 'isoenzyme' has been recommended for each of these forms. At the present state of knowledge, it has been decided most appropriate to retain a broad definition of isoenzyme as "one of a series of different proteins with similar enzymatic activity". The discovery of isoenzymes and the availability of simple means of their separation stimulated numerous detailed studies on their physical/chemical properties and their distribution in various human tissues. Distribution patterns of various isoenzymes in individual human tissues and organs have now been firmly established and the results of comparative studies on serum isoenzyme patterns in various diseased states have greatly enhanced the value of diagnostic enzymology.

It is now well recognised that in some instances, the assay of total serum enzyme activity is not sufficiently specific or sensitive for the differential diagnosis of diseases or identification of specific organ damage. One example of such lack of specificity is the observation of elevations in total serum lactate dehydrogenase levels in a wide variety of pathological states, including cardiac, hepatic, malignant, haematological, muscular and renal diseases. Methods for detecting alteration in the isoenzyme pattern in serum have been found more useful as the serum isoenzyme pattern closely mimic that of the diseased tissue, even when the total serum enzyme activity is within the 'normal' limits.

According to the specific properties of the isoenzyme employed, such methods may be separated into the following groups:—

(1) Electrophoretic methods
   (a) Acrylamide
   (b) Agar
   (c) Agarose
   (d) Cellogel
   (e) Cellulose acetate
   (f) Starch

(2) Physical method
   Heat stability

(3) Chemical methods
   (a) specific inhibitors
   (b) substrate analogues
   (c) coenzyme analogues
   (d) pH variation

(4) Immunological method

The choice of any particular method depends on a number of factors: availability of appropriate instrumentation, number of specimens to be assayed, availability of reagents as in the case of immunological assays, and the relative cost of reagents.

EFFECT OF IMPROVED METHODS FOR ENZYME DETERMINATION

In most clinical enzyme procedures, the enzyme solution (usually serum) is mixed with coenzymes and activators in the appropriate buffer at the optimum pH. The reaction is started by the addition of excess substrate.

$$\text{Substrate} + \text{oxidised/reduced coenzyme} \xrightarrow[\text{Optimum pH}]{\text{Enzyme, Activator}} \text{product} + \text{reduced/oxidised coenzyme}$$

Measurement of enzyme activities may be made by estimating the rate of substrate utilisation or product formation, or the rate of coenzyme conversion. The rate of enzyme reaction may be followed continuously (kinetic method) or calculated from measurements made before and after a fixed period of enzyme reaction (two-point assay).

There are various factors which control enzyme reaction rates: (1) the substrate concentration; (2) the substrate and product inhibition; (3) pH; (4) the temperature; (5) the presence of coenzymes and activators; and (6) the absence of inhibitors. Due to limitation of instrumentation and the lack of detailed investigations into the effect of one or more of the above factors when devising conditions for enzyme procedures, many earlier methods of enzyme assay have been found to give inaccurate and poorly reproducible results. For example, an early method for the determination of serum

lactate dehydrogenase with lactate as substrate and NAD^+ as coenzyme (Wacker *et al*, 1956) gave a reaction curve which was non-linear at any stage of the reaction.

$$\text{Lactate} + NAD^+ \xrightarrow[\text{Lactate Dehydrogenase}]{} \text{Pyruvate} + NADH + H^+$$

This procedure was subsequently modified by greatly increasing the NAD^+ concentration (Amador *et al*, 1963).

Another example of unsatisfactory methodology is the determination of aspartate aminotransferase (SGOT).

$$\alpha\text{-Ketoglutarate} + \text{L-Aspartate} \xrightarrow[\text{SGOT}]{} \text{L-Glutamate} + \text{Oxaloacetate}$$

The product oxaloacetate is a potent inhibitor of the amino-transferases (Boyd, 1961). Techniques which allow the accumulation of oxaloacetate (Babson *et al*, 1962; Reitman and Frankel, 1957; King, 1960) tend to give lower results, particularly at higher enzyme activities. Because of high background and low sensitivity, suboptimal substrate concentration and prolonged incubation time have to be used in methods which depend on hydrazone formation (Reitman and Frankel, 1962; King, 1960). These conditions further add to the undesirability of the methods. Fortunately, with the development of sophisticated instruments, the unsatisfactory colorimetric methods have been replaced by kinetic methods employing optimum conditions and an indicator enzyme which removes the oxaloacetate as soon as it is formed. Using the superior method, it is possible to interpret borderline enzyme values with greater confidence.

THE EFFECT OF RECENT DEVELOPMENT OF SOPHISTICATED INSTRUMENTS

In recent years, a number of sophisticated spectrophotometers or fluorimeters specifically designed for rapid and accurate quantitation of enzyme activities have appeared. Many of these, now in routine use in Clinical Chemistry Laboratories, incorporate facilities or devices for thermostatic control, automatic dispensing and diluting, and recording/printing out of calculated results. Some recorders are fitted with scale expansion to increase sensitivity. In one popularly used system—the LKB Reaction Rate Analyser, one needs only to load the samples into disposable cells which are also used as cuvettes for spectrophotometry, and allows the system to automatically add reagents, monitor initial rate reaction and print out enzyme activities. Besides improving accuracy and reproducibility these instruments enable a larger number of enzyme determinations to be performed. As simultaneous determination of several enzymes can be carried out on some automated instruments, laboratories with the appropriate equipment are able to offer enzyme profiles such as the simultaneous determination of SCPK, SLDH, and SGOT for all patients suspected of myocardial infarction. It is also practical to screen for hereditary enzyme deficiencies which are prevalent among certain populations.

THE INFLUENCE OF ENDOGENOUS AND EXOGENOUS FACTORS ON ENZYME LEVELS

A number of endogenous and exogenous factors are now known to affect enzyme activities. These include: (a) age (McQueen *et al*, 1973), (b) sex (McQueen *et al*, 1973; Hughes, 1962), (c) various physiological conditions: pregnancy (Curzen, 1970), muscular exercises (Griffiths, 1966), (d) diagnostic procedures: prostate palpation (Ishibe, 1971), intramuscular injections (Hess *et al*, 1964; Schwartz, 1971), vascular catheterisation (Guder *et al*, 1972), surgical operations (Guder *et al*, 1972), (e) treatment regime such as administration of albumin (Hansten, 1971). Caution is therefore required in the interpretation of enzyme levels. Two examples will be given.

Increased levels of alkaline phosphatase are found in children and in pregnant women. The former is due to increase in the bone isoenzyme while the latter is due to production of isoenzyme by the placenta. Intravenous administration of albumin (Hansten, 1971) has been reported to give rise to transient elevation in serum alkaline phosphatase due to the presence of placental isoenzyme in the albumin preparation.

INFLUENCE OF DRUGS

A number of drugs used for patient treatment may themselves cause transient elevation of certain enzyme levels. This can occur either by (1) direct interference with the method of assay, (2) causing damage to organ(s) or (3) induction of enzyme synthesis. Tables I and II

TABLE I

DRUG-INDUCED ELEVATION IN SERUM ALKALINE PHOSPHATASE LEVELS

Drug	Effect/Comments	Reference
Acetylsalicylic acid	Prolonged use may cause hepatic toxicity	42
Anabolic steroids	cholestatic syndrome	17
Anticonvulsants	occurs in 24% children (90% bony origin)	25
Antifungal agents	hepatotoxicity may occur	16
Gentamicin	hepatotoxic effect	17
Isoniazid	probable cholestatic effect	32
Penicillamine	possible hepatotoxicity	10
Pyrazinamide	hepatotoxic effect	33
Sulfanilamide	may cause reversible cholestasis	32

TABLE II

DRUG-INDUCED ELEVATION IN SERUM SGOT LEVELS

Drug	Effect/Comments	Reference
Acetylaminophenol	At therapeutic conc may affect SMA 12/60 method	44
Acetylsalicylic acid	prolonged admin. may cause hepatic toxicity	42
Aminophenol	at 1 mmol/l affects SMA 12/60 method	44
Aminosalicylic acid	at 1 mmol/l affects SMA 12/60 method	44
Amphotericin B	hepatotoxicity reported	33
Anabolic steroids	cholestatic syndrome (intrahepatic cholestasis)	32
Antifungal agents	hepatotoxicity may occur	16
Carbenicillin	effect of drug? hepatotoxic	16
Chloramphenicol	hepatotoxic-cholestatic effect	32
Chlordiazepoxide	may produce hepatotoxic effect	1
Chloroquine	reported effect no mechanism cited	33
Chlorthiazide	may cause cholestatic jaundice	33
Clofibrate	transiently elevated during early therapy	5
Griseofulvin	may be hepatotoxic	32
Isoniazid	at therapeutic conc may affect SMA 12/60 method	44
Methotrexate	hepatotoxicity (drug induced cirrhosis)	33
Phenothiazides	may cause cholestatic hepatitis (in up to 4% pts)	33
Pyridoxine	significant increase in elderly after admin.	35
Tolbutamide	at 1 mmol/l affects SMA 12/60 method	32

list a number of drugs which cause elevation of serum alkaline phosphatase and SGOT levels. For a comprehensive list, you are referred to the excellent compilation of references by Young *et al* (1972).

Although interference with serum enzyme levels cannot be said to occur in every instance of drug administration, nevertheless, these examples serve to emphasize the importance of exercising caution in the interpretation of enzyme values on patients known to receive drug treatment of one form or another.

LACTATE DEHYDROGENASE (LDH) ISOENZYMES IN DIAGNOSIS

Electrophoretic separations of tissue homogenate and blood serum have shown up to 5 bands of LDH. Each tissue shows a different distribution of activity in the individual enzyme fractions. These fractions have been named LDH_1, LDH_2, LDH_3, LDH_4 and LDH_5 in the order of highest negative charge and migrating farthest towards the anode during electrophoresis. The percentage distribution of these isoenzymes in tissues are shown in Table III.

It is noted that in heart, brain, kidney and erythrocyte, LDH_1 and LDH_2 predominate; in adrenal, lymph node, pancreas, LDH_3 predominates; while in liver and skeletal muscles, LDH_5 exists as the major isoenzyme. When LDH isoenzyme electrophoresis is carried out on patients' sera, characteristic isoenzyme patterns are obtained which provide useful information on the tissue origin of the increased isoenzyme(s). Table IV summarises LDH isoenzyme alteration in various diseased states.

The presence of additional LDH isoenzyme bands in the serum of a patient who had an inoperable carcinoma of the esophagus with multiple secondary tumors in the liver was reported by Wilkinson *et al* (1968) and Wilkinson (1970). Using acrylamide gel electrophoresis, they found well-marked extra bands of LDH activity between LDH_1 and LDH_2 and between LDH_2 and LDH_3. The explanation offered for these extra bands was the possible synthesis by the tumor tissue of LDH abnormal subunits which, in combination with the normal subunits gave rise to bands of unusual mobility.

TABLE III

DISTRIBUTION OF LDH ISOENZYMES IN VARIOUS TISSUES

Tissue	Percentage distribution of LDH isoenzymes					References
	LDH_1	LDH_2	LDH_3	LDH_4	LDH_5	
Heart	35	36	12	16	11	54, 55
	67	29	4	<1	<1	38
	71	27	2	—	—	51
	49	45	6	—	—	58
Kidney	12	14	24	25	23	54, 55
	42	48	9	1	—	51
	30	50	15	5	—	58
Erythrocytes	39	56	—	5	—	54, 55
	39	46	15	—	—	38
	46	39	11	4	—	51
Brain	21	26	26	20	8	55
Adrenal	3	20	75	—	2	58
Lung	10	20	30	25	15	58
Lymph node	10	25	60	—	5	58
Pancreas	30	15	50	—	5	58
Skeletal muscle	4	7	21	27	41	54, 55
	—	—	2	1	97	38
	—	—	17	10	73	51
Liver	2	4	11	27	56	58

TABLE IV

LDH ISOENZYME ALTERATION IN DISEASES

Disease	Increased Isoenzyme Fraction (s)
Myocardial infarction	LDH_1
Acute rheumatic myocarditis	LDH_1 and LDH_2
Megaloblastic anaemia (B12 or folate deficiency)	LDH_1 and LDH_2
Haemolytic anaemia	LDH_1 and LDH_2
Muscular dystrophy (Duchenne type)	LDH_1
Hepatocellular diseases	LDH_5
	LDH_4 and LDH_5
Malignant disease (especially widely disseminated forms)	LDH_3 (minor change)
	LDH_3 and LDH_4 (minor change)
Malignant disease with liver metastases	LDH_5

Measurement of LDH isoenzyme activity has been claimed to be of value in the diagnosis of cardiac transplant rejection, particularly in the first month when ECG changes are unreliable. Rejection may be signified by LDH_1 activity exceeding that of LDH_2 (Nora *et al*, 1969).

Quantitative electrophoretic separation of isoenzymes is a tedious operation and not so easily adaptable for automation. For routine purposes, a number of simpler techniques have been devised which permit the assessment of the relative proportions of the fast and slow isoenzymes in the serum. Among these are the serum 'α-hydroxybutyrate dehydrogenase' (SHBD) test, the relative heat-stability test and oxalate or urea inhibition test. In our laboratories we have found routine SHBD assays particularly useful in the diagnosis and follow-up studies of megaloblastic anaemia and myocardial infarction. Both LDH and SHBD levels are much higher in megaloblastic anaemia than in myocardial infarction and unless given folic acid, the levels do not decrease spontaneously as in the latter condition.

ALKALINE PHOSPHATASE (AP) IN DIAGNOSIS

It is now known that human serum contains at least 5 isoenzymes of AP. They are the bone, liver, intestine, bile and placental isoenzymes. It is extremely rare to find all 5 fractions together in the same sample of serum. Placental AP is present only in pregnant women, while the intestinal AP is found in individuals of blood groups O and B (Beckman *et al*, 1966; Stolbach *et al*, 1967).

As the electrophoretic techniques are too cumbersome and relatively difficult for routine use, a number of simple techniques have been developed, which are based on the differences in isoenzyme response to heat treatment and to a number of inhibitors. These methods include the L-phenylalanine sensitivity of intestinal (Fishman *et al*, 1963) and placental AP (Fishman *et al*, 1968a), the sensitivity to heat of liver and bone AP (Posen *et al*, 1965; Small, 1969), the heat stability of placental AP (Neale *et al*, 1965) and the use of urea for differentiating liver and bone enzyme Horne *et al*, 1968).

Since Coryn (1934) first reported that maternal serum AP was raised in late pregnancy, there have been numerous confirmatory reports. Many workers have used the serial measurement of serum placental AP to determine the state of placental function and fetal prognosis. However, results obtained so far have not been encouraging (Curzen, 1970; Tan *et al*, 1971).

Although measurement of intestinal AP using the L-phenylalanine technique has been reported to be useful in the diagnosis of cirrhosis (Kriesher *et al*, 1965; Fishman *et al*, 1965), we have not been able to confirm this observation (Tan and Moss, 1969).

The most useful study of AP isoenzyme appears to be in the differential diagnosis of bone and hepatic diseases and we have recently reported the use of a simple heat stability test in the differential diagnosis of these conditions (Tan *et al*, 1972).

Mention must be made of the presence of unusual AP isoenzyme in sera of certain cancer patients. This unusual AP isoenzyme which

cannot be distinguished from placental AP was first demonstrated in a patient with a bronchial carcinoma. The isoenzyme, called Regan isoenzyme after the patient, was found both in the serum and in the tumor (Fishman *et al*, 1968b). Subsequently, it was found in the serum of 27 out of 500 patients with various malignant diseases (Stolbach *et al*, 1969) and was attributed to the ectopic synthesis by tumour cells of an enzyme protein not normally produced in the tissue of origin, due to repression of specific genomes in the malignant cell. Other reports of abnormal AP isoenzyme have been made by Warnock and Reisman (1969) and more recently by Higashino and his co-workers (1972) in hepatocellular carcinoma.

ENZYME ASSAY IN THE IDENTIFICATION OF INHERITED METABOLIC DISEASES

As a result of our interest in identifying the contributory causes of neonatal hyperbilirubinemia in the local population in Singapore, we studied the possible inherited deficiency in the following enzymes in erythrocytes of infants:

Glucose-6-phosphate-dehydrogenase
6-Phosphogluconate dehydrogenase
NAD-linked glutathione reductase
NADP-linked glutathione reductase
Glutathione synthetase (Reduced glutathione assay only)
Phosphoglucomutase
Triose-phosphate isomerase
Glyceraldehyde-3-phosphate dehydrogenase
Pyruvate kinase
Lipoyl dehydrogenase

The most significant finding has been the high incidence of glucose-6-phosphate dehydrogenase deficiency amounting to 2-3% of the population. It is now an established routine practice to assay this enzyme in all infants born in the hospitals. Once enzyme deficiency is detected, the affected infant is retained in the hospital to avoid its possible contact with risk factors that may precipitate the development of severe jaundice.

Contrary to a report on pyruvate kinase deficiency in Chinese neonates in Hong Kong (Fung *et al*, 1969), the enzyme deficiency was not detected in 250 cases of infants admitted to hospital with neonatal jaundice in Singapore. A possible explanation for the difference in observation could be the use of a screening method rather than a quantitative method by Fung *et al* (1969).

STANDARDISATION OF UNITS OF ENZYME ACTIVITY AND ENZYME ASSAYS

The measure of the activity of enzymes is the enzyme unit. The definitions of many units have their origin in the method of measurement and are accordingly varied. This variation has been the cause of a great deal of confusion in clinical enzymology. In order to minimise this variation, the Enzyme Commission of the International Union of Biochemistry (IUB) and the Clinical Chemistry Commission of the International Union of Pure and Applied Chemistry (IUPAC) recommended the use of the 'International Unit' which has been defined as the amount of enzyme which converts 1 micromole of substrate per minute (King and Cambell, 1961; Report of the Commission of Enzymes, 1961; IUB, 1964). The other conditions are 25°C, optimal substrate concentration, optimum ionic strength of the buffer and optimum pH. Unfortunately, for a long time, due to the limitations of equipment and the older enzyme methods, it has not been possible to apply this definition universally. However, more recently, with improvements in instrumentation and refinements in enzyme assay techniques, the use of a uniform unit is becoming increasingly feasible. In late 1973, The Royal College of Pathologists of Australia and The Australian Association of Clinical Biochemists have jointly recommended the adoption of the International System of Units (S.I.) for all clinical chemistry measurements in Australian hospital laboratories (Edwards *et al*, 1973). As the term International Unit has been misconstrued as conveying a warranty of uniform methodology, the S.I. unit for enzyme measurement has been recommended as the amount of enzyme which converts 1 micromole of substrate per minute per liter under defined assay conditions.

Even with the use of a single enzyme unit, there can be variation in measured enzyme levels because of differences in methodology. In view of the growing mobility of populations and the likelihood of a patient attending more than one hospital, there is a need for different laboratories to report comparable results. It is therefore desirable to standardise enzyme methods.

As enzyme activity is dependent on the conditions under which it is measured, including

the concentration of the reactants, temperature, and pH, the universal adoption of an agreed standard method would necessarily mean the precise definition of all the conditions. This may not be practical for some laboratories as the particular instrumentation or reagent(s) necessary for the method may not be available due to various reasons. The alternative is the provision of stable reference preparations of enzymes of known activity which may be used as calibration standards in secondary methods of enzyme estimation, or as quality control preparations to assess analytical performance. The existence of such reference enzyme preparations presupposes that these have been assigned values on the basis of activity determination using a standard reference method. At present, agreement on standard methods of analysis appears to be more easily attainable and is the objective actively pursued by the Expert Panel on Enzymes created by the International Federation of Clinical Chemists Committee on Standards.

QUALITY CONTROL OF ENZYME MEASUREMENTS

Increasing consciousness in the calibration of spectrophotometric instruments, better thermostatic control for enzyme incubation, use of single cuvette for continuous recording of enzyme reaction, and increasing sensitivity of newer methods, have contributed to a general improvement in the standard of enzyme assay.

For the routine quality control program, most laboratories rely on the repeated analysis of 'carry-over' specimens of patient's serum or frozen serum pools prepared in the laboratory for monitoring within-batch and between-batch variations. Unfortunately, unlike the quality control preparations for other non-enzymatic constituents, commercial freeze-dried preparations of enzymes to which values of activity of various enzymes have been assigned have not been accepted with the same degree of confidence. This is because it has been often difficult to obtain results which agree with the assigned values for the control preparations, even though the laboratory's own quality control program suggests that analytical methods are operating satisfactorily. Furthermore, different batches of material from a single source have been shown to give different values. Consequently, there have been doubts as to the stability of the reference preparations, and the accuracy of their original calibration. It would appear that a comprehensive quality control program can only be implemented when standard methods of enzyme assay and stabilised enzyme preparations of defined characteristics are available.

ACKNOWLEDGEMENT

The author wishes to thank Dr. Edward Jacob for his helpful criticism and Mrs. T. H. Koh for typing the manuscript.

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