CLINICAL BIOCHEMISTRY REPORTS

of the

Department of Biochemistry,

Makerere University, Kampala.

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DEPT: OF BIOCHEMISTRY
MEDICAL SCHOOL

9th July 1971.

To

Heads of Depts, Medical School
Dean of Medicine.
Medical Superintendent Mulago Hospital.
Clinical Pathologist.

Clinical Biochemistry Service

In view of the changed relationship between this department and the "Biochemistry" laboratory of Mulago Hospital we have examined the possibilities of providing an alternative service from this building. It now seems practicable and will be available from Monday July 12th. The service will be continued and expanded in range, level, and accessibility if there is demand and if the physical facilities permit. Please note:

1) This experimental and provisional: there is yet no guarantee of continuance.

2) Only serum Na,K and GOT will be available until further notice. Sample containers will be provided from this building when practicable, but any suitable container will be accepted for the time being.
N.B. Serum preferred.

3) Samples and request forms must be brought to the University laboratory. Other arrangements may be possible at later date.

4) Request forms from the University departments must be used. Copies are enclosed and will be replaced on delivery of samples.

Intitially at least the service will be free. It will be necessary later to explore how to provide financial support.

We intend to keep users informed of our procedures, quality control results, etc., through the medium of an occasional (perhaps monthly) journal. Copies will be deposited in the Albert Cook Library and we may be able to supply "back issues"

Comment and criticism will always be welcome.

MCBJ/l

T.R.C. Boyde.

Editorial

The service was inaugurated 2 months ago, because of our conviction that Mulago Hospital deserves, and must have, a better Clinical Biochemistry service than it has ever seen. We began, and intend to continue, modestly, undertaking only what we believe we can do well and maintain indefinitely. Even so we offer no guarantees, only facts. This first issue, of what we hope will become a regular publication, exemplifies the policy of total exposure of the practice of the laboratory.

We aim to explain our methods, our choices, our results and our changes of mind openly for everyone to see. Clinicians are commonly not competent to judge the quality of the output of a laboratory. Yet they do make such judgements and right or wrong their opinions are therefore ill-based. "Clinical Biochemistry Reports" is intended to provide a better basis.

We invite comment, criticism and suggestions - for publication in the Reports if you wish. In particular, the laboratory needs clinical opinion to assist in deciding what to do next. The present plan is to introduce assays of serum Ca, PO_4, protein, bilirubin and alkaline phosphatase over the course of the next 6 months. Possibly 'Astrup' equipment will be available, allowing determinations of whole blood pH and PCO_2 and plasma HCO_3. Urinary steroids will follow and also serum Mg, Cu, Fe, and Zn. In addition to 'routine' the laboratory may be well placed to do special assays in cooperative research programmes. Ask.

Very few samples have yet been submitted to this laboratory for analysis. Perhaps this is due partly to insufficient publicity and accordingly we are making arrangements for wide distribution of this first issue of the Reports. Subsequent issues will be sent to departments and to individuals who wish it. A number of copies will be lodged in the Albert Cook Library and back issues will be available from the department, but may be charged for if reprinting is found necessary.

Whatever arrangements are made in the future, it will always remain the responsibility of the clinician to see that his sample reaches the laboratory. If you want us to do the assay <u>make sure we get the sample</u>.

The Editor.

BIOCHEMISTRY DEPT: MAKERERE UNIVERSITY (Medical School)

Clinical Biochemistry Service.August 1971.

Serum GOT (Glutamic-oxaloacetic transaminase, aspartate transaminase)

1) SUMMARY OF PROCEDURE

i) Switch on spectrophotometer or 'techometer' to allow warm-up
Switch on water-bath and circulating pump;
Check that temperature setting is correct, (30°C or as instructed).
Warm up L-aspartate soln.to assay temperature

ii) Prepare common soln. as follows (for up to 9 tests-scale up appropriately if more are to be done);
Weigh out 3 mg NADH
Add 10 ml phosphate buffer (bulb pipette)
Add 2 ml 2-oxoglutarate (bulb pippette)
Add 50 µg MDH as suspension.(Calculate vol, from stated concn. of susp., e.g. 5 ul of 10 mg/ml). (Beckman micropipette)
Add sufficient distilled water to bring volume to 20 ml . (i.e.8 ml , less volume of MDH) (10 ml grad pipette). Mix.

iii) Distribute by bulb pipette 2.0 ml of the common soln. to each cuvette Add 0.5 ml serum sample (Ostwald pipette or Beckman micropipette)
Add distilled water to bring total volume to 2.9 ml - usually 0.4 ml (1 ml graduated pipette.)
Mix by inversion or with paddle.Allow to stand 15 min at assay temperature.

iV) Transfer cuvette to instrument, avoiding temp.loss.Wipe optical faces of cuvette with tissue.
Add 0.1 ml L-aspartate. Mix by inversion or with paddle and immediately commence optical density (absorbance) readings. (If by hand, at intervals of 1 min, measured by stopclock)
Continue until absorbance (A) change of 0.100 has been recorded but in any case not more than 15 min or less than 7 min. Record temperature in the water-bath and in the cuvette (without removing from the instrument).

v) If optical density readings are by hand, plot on plastic-covered graph paper. Determine slope of best straight line and express as ΔA/min. Neglect points corresponding to slower apparent rates at the beginning and end of the readings. Use at least 4 points. Rely essentially on readings between 0.8 and 0.2 A, but first check whether the instruments are accurate even over this range.

vi) If rate exceeds 0.090 /min repeat on a smaller volume of sample (say 0.2 ml)

If curve flattens out before a sufficient ΔA has been recorded, repeat on a smaller volume of sample (say 0.2 ml). If absorbance is too high for accuracy, repeat on a smaller volume of sample (say 0.2 ml), or offset zero by using a blank of up to 0.5 A (spectrophotometer).

vii) Calculate result as follows.

$$\frac{\Delta A/min \times 1000 \times 0.483 \times \text{correction factors}}{\text{Volume of sample (ml)}} = IU/l.$$

Notes, difficulties and precautions

1) The test may be run on the SP500, the techometer or the SP 8000 (special permission), Familiarity with operating procedures of the instrument concerned is assumed in the above instructions and the analyst may only use an instrument for which he is cleared.

2) Correction factors.

a) Instrumental. The biochemist-in-charge may from time to time direct that a factor is to be used with readings from a given instrument to correct to true A.

b) Temperature. If cuvette temperature differs from 25°C by 0.5°C or more, a correction factor is to be applied, derived from the table provided.

c) If there is evidence that the laboratory results are incorrect and that this is not attributable to (a) or (b) and the cause cannot readily be eliminated, the biochemist-in-charge may from time to time direct that an empirical correction factor be applied in addition to the above.

All three correction factors may be multiplied together to give a factor applied to all tests to which the same conditions of instrument, termperature,etc., apply:

3) Contamination Make quite certain that substrates are not conveyed from test cuvettes to "waiting" cuvettes onpipettes, cuvette covers, paddles used for mixing, or in any other way. Otherwise all your NADH may be destroyed before readings begin.
Rinse possible offenders with distilled water.

TEMPERATURE CORRECTION FACTORS

Multiply observed activity by factor given below to convert to activity at 25°C. Interpolate for intermediate values. Factors are based on experiments conducted here and correspond to an activation energy of 10.66 Kcal/mole, which is rather low (cf refs. 6 and 8). It seems that activation energy is medium dependent. The present figure was determined with the assay medium in use and may thus be accepted for the present.

°C	FACTOR	°C	FACTOR	°C	FACTOR
15	1.85	25	1.000	35	0.562
16	1.74	26	0.943	36	0.531
17	1.63	27	0.888	37	0.503
18	1.53	28	0.838	38	0.476
19	1.44	29	0.791	39	0.451
20	1.36	30	0.746	40	0.427
21	1.27	31	0.705		
22	1.20	32	0.665		
23	1.13	33	0.628		
24	1.06	34	0.594		

II REAGENTS, PREPARATION, STORAGE

1) Phosphate buffer 0.1 M pH 7.4

Prepare stock solutions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4. Precise weighing directions are not given, since each salt may be found in 2 or 3 different crystalline forms with different water content (Calculate from the M.W. given on the bottle.)

Weigh to within 10 mg using the top loading balance. Usually, make up one litre of each at a time. Discard if bacterial or algal growth occurs. Store in screw-cap glass or plastic bottle.

Mix 81 ml 0.1 M Na_2H-PO_4 with 19 ml 0.1 M NaH_2PO_4

Store in screw-cap glass or plastic bottle in the refrigerator

Discard if bacterial or algal growth occurs. Check pH(meter).

2) 2-oxoglutarate, 0.1M, pH 7.4

Weigh out 1.46 g 2-oxoglutaric acid (Sigma or Boehringer) Use analytical balance. Dissolve in approx 50 ml water. Titrate to pH 7.4 with approx 2.0 M NaOH (2 equivalents, approx. 10 ml) using a pH meter.

Great care is needed in the final stages to avoid overshooting. Transfer quantitatively to a 100 ml flask and make to 100 ml.

Store in glass or plastic bottle in the refrigerater <u>over chloroform</u>(i.e add approx 1 ml chloroform to prepared solution). Store bulk dessicated at room temperature.

2) L-aspartate 1.0M pH 7.4

Weigh out 13.3g L-aspartic acid (Sigma)

Add 45 ml approx.2.0 M NaOH and 30 ml water.

Warm to dissolve. Cool to room termperature and titrate to pH 7.4 with further 2.0 M NaOH.
A total of 1 equivalent (approx 50 ml) NaOH is required, so about 5 ml is needed at this stage. Once again, great care is needed to avoid overshooting in the final stages.
Transfer quantitatively to 100 ml flask and dilute to the mark.
Storage - as for 2- oxoglutarate.
Store bulk L-aspartic acid dessicated at room temperature.

4) Malate dehydrogenase. Use commercial suspension (sigma or Boehringer) as supplied. Store in refrigerator. Make sure only one container is open at a time and avoid contmination.
Mix carefully by inversion before withdrawing a portion for use.
5) NADH. Use commercial powder (Sigma) as supplied.
The bulk supply is to be stored at 0-4°C over silica gel and is only to be opened after thoroughly warming the entire dessicator or other container to room temperature (say 1 hour). The supply in use is to be stored in an open tube within a screw capped vessel containing silica gel. This too must be warmed to room temperature or above (say 10 min in the pocket) before opening.

From time to time check that the silica gel is active in each container.
Transfer approx 50-100 mg to the 'supply in use', when exhausted.
For assay, weigh out to ± 5% using an analytical balance.

III QUALITY CONTROL PROCEDURES

i) At least once a week, as directed, the analyst must secure from the Q.C.O. a test soln. which should be assayed just as any other serum sample and the result reported to the Q.C.O. This soln. will usually be a commercial quality-control preparation made up according to the makers' instructions or more dilute or more concentrated. The 'true value' against which the analyst's result is being compared is calculated from a value allotted to the preparation when made up strictly according to the makers' instructions and which may not be identical with the value stated by the maker. (The allotted value is determined by experiment with full regard to thepossiblitities of systematic error due to instruments, temperature, incorrect preparation of reagents, or differences in assay conditions, overt or covert.)
Note allowance to be made for specific volume of protein in lyophilized sample.

The Q.C.O records 'true' and assay values and may use the difference in a Cusum plot.

ii) Daily mean

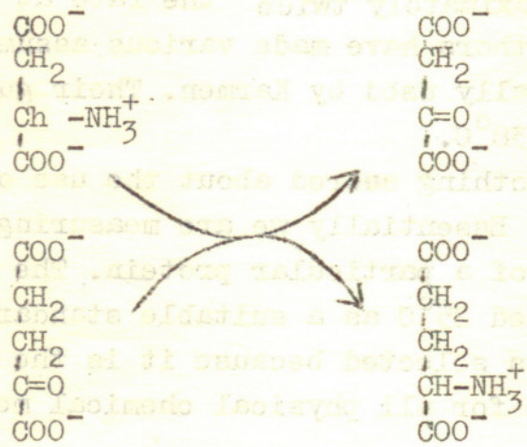
iii) Monthly mean.

The Q.C.O. is to bring to the attention of the biochemist-in-charge any gross discrepancies or apparent systematic shifts in assay results appearing from the above. The limits, or indications for action, will be set when sufficient experience has accumulated.

IV BACKGROUND

Transamination was discovered by the Russian workers Braunstein and Kritsmann (1). The reaction catalysed by GOT is :-

L-aspartate + 2 oxoglutarate ⇔ oxaloacatate + L-glutamate



The estimation of GOT activity as a clinical procedure was introduced in 1954-5 by a U.S.group headed by Wroblewski (2-4). Their choice of the enzyme for study was based on the observation that the heart and liver exhibited especially high activities (5). An enormous literature has since accumulated but as so often in the medical field most papers are repetitive, tendentious, or plain bad. Elevated serum levels have been found in a variety of clinical conditions, but nothing has turned up to shake the original conclusion that really clear-cut rises are due to release of the enzyme form damaged heart, or liver, or both. This review will be confined to technical matters affecting the numbers reported to clinicians and therefore affecting the interpretation to be placed upon those numbers.

First, however, a brief digression. It is perfectly true that glutamic-pyruvic transaminase activity in liver is higher than GOT*, that the reverse is true in heart, and that in most cases of liver disease with elevated enzyme levels serum GPT/serum GOT>1. It does not follow that both transaminases must be assayed by laboratories.

*(Soluble fraction enzyme).

We do not live in an ideal world, especially those of us who work in Clinical Biochemistry laboratories, and we find it hard to believe that the differential diagnosis of myocardial infarct vs. hepatitis is often a serious problem to the clinician.

Accordingly serum glutamic-pyruvic transaminase estimations are not offered as routine.

The first reasonably convenient and accurate assay was given by Karmen (4) Most regrettably it is not possible to accept his paper as setting a strictly standard assay, because:-

1) He did not specify the temperature used or indicate the necessity for temperature control.

We may point out that

i) The rate of transamination varies approximately as 6% per ^{o}C temp.rise (compound), so that, for example, the rate at 37^{o}C is approximately twice the rate at 25^{o}C.

ii) Various authors have made various assumptions about the temperature actually used by Karmen. Their guesses have ranged from 22^{o}C to 38^{o}C.

iii) There is nothing sacred about the use of 37^{o}C for enzyme measurements. Essentially we are measuring a parameter related to quantity of a particular protein. The enzyme Commission recommended 25^{o}C as a suitable standard reference temperature: this was selected because it is the accepted standard temperature for all physical chemical measurements.

The Commission has since adopted 30^{o}C as an alternative standard, especially because of the difficulties of tropical laboratories. Neverthless it has not been possible to enforce this as a standard temperature for world-wide use.

For the time being at least, all enzyme assay results from this laboratory will be in terms of activity at 25^{o}C, whatever the actual temperature used in the analytical procedures.

2) He did not specify whether he used L- or DL-aspartate.

Authors have made one or the other assumption according to taste. Notwithstanding claims to the contrary it would seem that D-aspartate is not significantly inhibitory under the conditions of Karmen's assay. The question, therefore, is only whether 16.7 mM and 33.3 mM L-aspartate will give significantly different rates. From Karmen's own results the answer would appear to be, yes, but to the extent of a few per cent only.

Subsequent work has increased the difficulty of deciding on a proper set of standard assay conditions.

The Enzyme Commission advocates that as far as possible enzymes should be assayed under kinetically optimal conditions.

This recommendation leads to endless difficulties, essentially because whenever some bright spark finds conditions where an enzyme works better he presumably invalidates all previous assays. In the case of GOT it has been found that phosphate (used in all previous assays as the buffer) is strongly inhibitory. Assays in tris-acetate buffer give results 15-25% higher than in phosphate (7), and it cannot be guaranteed that some favourable buffer yet will not be found.

In addition to the many previous interpretations of the temperature used by Karmen, authors have varied widely thephosphate buffer concentration, apparently regarding this as unimportant - a pH stabilizer only.

Chosen Technique - Summary

In summary, it is not possible to set up assay conditions strictly according to Karmen, because we do not know what his conditions were. Commercial quality control preparations traditionally quote assays by the Karmen method and it is usually impossible to discover exactly how the assays were done, hence the attitude taken in Section III towards allotting a value to these preparations.
(The Warner Co. "weighs in " the unknowns added to their 'Versatol' , including enzymes. In the circumstances surrounding GOT assay this makes no difference to the problem).
A choice of assay conditions is forced upon us. The choice in this laboratory is made. i) Because it is reasonably close to Karmen which is universally quoted as standard. ii) Because there is evidence that, by pure chance, it treats the isoenzymes alike. Each operates at about 80% of the highest rate observed under conditions ideal for each individually (8).

The technique is referred to as "Karmen-Boyde variant" and is not identical with Karmen's original procedure nor with that of Boyde (7) . The known significant differences may be listed thus. (Concentrations are final concentrations in the test cuvette):-

	Karmen	Boyde variant
Aspartate	33 mM (?L- or DL)	33 mM L-aspartate
Phosphate	56.5 mM	33 mM
NADH	0.080 mM approx.	0.119 mM approx
	(should make no difference, but the higher concn. is more convenient for rate measurements over a longer period)	
Temperature	unspecified	25°C

ISOENZYMES

Fleisher, Potter and Wakim (9) were the first to recognise two distinct enzymes with GOT specificity but with different kinetic behaviour and different sub-cellular distribution. Karmen's work and much of that based on him was done in ignorance of this fact and many of the assays in use repond effectively only to the cell-sap isoenzyme. True enough that, to date, variations in serum level of this enzyme form have been much the most important, but a practicable technique for at least semiquantitative assay of the mitochondrial form does now exist (10), and it has been shown to vary independently of the other in some disease states (11,12,). This field remains to be fully exploited.

Other possible assay methods

Reitman and Frankel (13)

The most widely used. It is simple but very liable to random and systematic errors:

Random, because i) high blank ii) small range of accessible absorbance values. iii) non-linear calibration curve iv) difficulty in controlling the extent of decarboxylation of oxaloacetate to pyruvate.

Systematic, because i) the absorbance values are extremely sensistive to the precise normality of NaOH used, ii) it is "calibrated" by reference to the Karmen method which we have seen is wide open to misinterpretation, iii) there are many snags in making up one's own calibration solutions, as has been done routinely at Mulago, iv) underestimates the mitochondrial enzyme by approx. three-fold.

Babson (14)

Morgenstern (Autoanalyser) (15) Superior to Reitman and Frankel but subject to many of the same criticisms. Performance with respect to the mitochondrial isoenzyme has not been tested.

Units and Calculations.

The Enzyme Commission of the International Union of Pure and Applied Chemistry has recommended as the International Unit of Enzyme Activity, "That quantity of enzyme which will catalyse the transformation of one micromole of substrate into products per minute under optimum conditions." The question of choice of temperature and the problem of what constitutes optimum conditions have been discussed above. Perhaps the most disturbing feature of all this is that it is perfectly possible for two excellent laboratories to quote different results in IU, and for both to be right.

Nothing can be done about this except to be aware of all the circumstances surrounding the assay and to use the "normal range" quoted by the laboratory concerned.

In the assay in question NADH is present in excess and is used as an indicator of oxaloacetate produced, via the reaction: oxaloacetate $+$ NADH $\xrightarrow{MDH}$ malate $+$ NAD
Thus one micromole of oxoaloacetate produced leads to conversion of one mole of NADH to NAD.

The molar absorbance of NADH has been very exactly determined and is 6.22×10^{3} at 340 nm. Thus a millimolar solution would have an absorbance of 6.22.

In our assay, the reaction is conducted in a volume of 3 ml, so that for an absorbance due to NADH of 6.22 we would require 3 micromoles of NADH.

What is actually observed is a rate of change of absorbance; a rate of 1 absorbance unit per minute would correspond to the disappearance of

$$\frac{1.0 \times 3.0}{6.22} = 0.483 \text{ micromoles/min NADH} = 0.483 \text{ IU}$$

Thus the number of IU of enzyme in our assay system is ΔA/min x 0.483 and the concentration in the original serum sample is

$$\frac{\Delta A/\text{min} \times 0.483}{\text{vol. serum sample in litres}} = \frac{\Delta A/\text{min} \times 0.483 \times 1000}{\text{vol. serum sample in ml}} \text{ IU/l}$$

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10/7/71 QUALITY CONTROL PROCEDURES

The following are to be carried on from the outset. The pattern will be modified in the light of experience. 1,3, and 4 will be reported in the occasional newsletter.

1) Daily mean. If more than one assay is done in a day, record number done and mean. Provide also for calculation and recording of weekly and monthly mean.

2) Cusum (Difficult for enzymes and any other substances which are not stable in solution). For each test, provide a solution giving a result in the optimal range of the assay (not necessarily in normal range). The actual value is unimportant. Have this assayed with each batch until there are sufficient results for the calculation of a valid mean result (or-less satisfactory-rely on the theoretical value). Thereafter, have this solution assayed with each batch and record graphically the deviation from the previously established mean result, the deviation to be cumulative. Usually, the pattern seen will be a sloping line, not horizontal, and changes of slope indicate changing conditions of assay sufficient to affect results.

It is important that the assayist should not be attempting to bring the result to a certain figure and he must be reassured that the actual result he obtains does not matter.

3) Assay of unknown aqueous solutions. (Not possible for enzymes etc.) From an accurate stock solution, the Q.C.O. each day makes up a solution for assay whose value is known only to himself but which lies in the optimal range of the assay. Record theoretical value, assay result, and deviation. At intervals calculate the standard deviation from the formula,

$$S.D. = \sqrt{\frac{\Sigma dev.^2}{n-1}},$$

i.e. Sum the squared deviations, divide by n-1 where n is the number of observations being considered, and obtain the square root.

The results will be used to test the accuracy and precision of the individual assayist and individual batch.

If the deviation is sufficiently bad the biochemist-in-charge may choose to withhold the reports. For the time being, however, no rules will be set to govern this.

The analyst is on his mettle with regard to this particular solution, but care must be taken to keep the emotional temperature to a minimum. If it seems desirable for morale, analysts may be told the magnitude, but not the direction of the deviation they achieve.

It may prove surprisingly difficult to choose proper theoretical values, covering the range but in a random fashion and not giving round number values. The problem must be reassessed at intervals.

4) Commercial quality control preparations. (At least weekly while stocks are available and more often if practicable.) The Q.C.O. need not stick to the dilution proposed by the manufacturer , but should consult the biochemist-in-charge. If a more concentrated soln. is made up, allowance must be made for the specific volume of dried protein (if using lyophilized serum). Treat results as for (3). Note that for enzyme tests the accepted theoretical values may differ from those given by the manufacture.

Possible future variant or additional procedures.

5) Use of commercial preparations as analytical standards (not strictly quality control, and intuitively unappealing - but may become necessary in practice, especially with SMA 12/60.)

6) Completely blind assay of natural or natural-seeming solutions, i.e. the analyst is unaware which specimens are for Q.C. purposes.

7) Blind assay of divided samples. Arrangements are made with a clinician to divide a single sample and submit as two completely separate specimens. He later discloses to the laboratory the results received by him.

These two procedures overlap to some extent: (7) cannot test for accuracy but otherwise is a test of the entire practice of the laboratory. Both have the virtue of testing precision on samples which cannot be treated by the analyst as "special".

The results should therefore give the true precision for a typical assay of an unknown sample. The objection to these procedures is that they are relatively cumbersome,(6) being especially difficult to operate in a small laboratory. If (7) is to be used as the principal Q.C. method, or to give its full possible contribution, a very large number of divided samples would be required.

Perhaps the use of these two procedures will be confined to proving that they are unnecessary.

(8) Separate Cusum samples may be unnecessary. We should test whether deviations obtained from procedures (3) & (4) give adequate Cusum patterns and if so abandon procedure (2).

The objective is to obtain the best possible information in a manner simple enough to be workable in a small laboratory.