

SIMPLE METHODS FOR MEASURING SERUM LEVELS OF THE GLUTAMIC-OXALACETIC AND GLUTAMIC-PYRUVIC TRANSAMINASES IN ROUTINE LABORATORIES

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Measurements of the levels of serum glutamic-oxalacetic transaminase (SGO-T) and serum glutamic-pyruvic transaminase (SGP-T) have become useful procedures in the diagnosis of some cases of myocardial infarction and of liver disorder (for instance, Shabetai, Iglauer, and Anderson, 1957; Wróblewski and La Due, 1956a and b).

The procedures described here are the result of work done with the object of establishing simple and economical techniques which would not require the use of a spectrophotometer. Hitherto, methods applicable to ordinary colorimeters have been developed by Cabaud, Leeper, and Wróblewski (1956) and by Frankel and Reitman (1956), to whom must go the credit for perceiving the possibility of a technique operating without precipitation of protein. The latter method is unpublished,* and is available only in the shape of reagents marketed commercially.

The method of Cabaud *et al.* is more complex, involving protein precipitation and toluene extraction, and has been used as the standard reference procedure in the work reported here. The method used of estimating SGP-T was that of Wróblewski and Cabaud (1957).

The principle adopted in the method of Cabaud *et al.* consists in the toluene extraction of the pyruvic dinitrophenyl-hydrazone derivatives of the products of the enzyme reactions and their measurement in alcoholic alkaline solution by colorimetry. This procedure has been adapted to meet the requirements imposed by the desire to avoid both the precipitation of proteins (with consequent need for centrifugalization) and the need for extraction in toluene not miscible in water, with the consequent need for alcoholic KOH, and more troublesome cleaning procedures.

The optimal absolute and relative concentrations of substrate acids were determined for both SGO-T and SGP-T estimations by exploring the effect of varying the amounts of each acid over a wide range. It will be seen that the figures for SGO-T substrate acids differ greatly from those for SGP-T substrate.

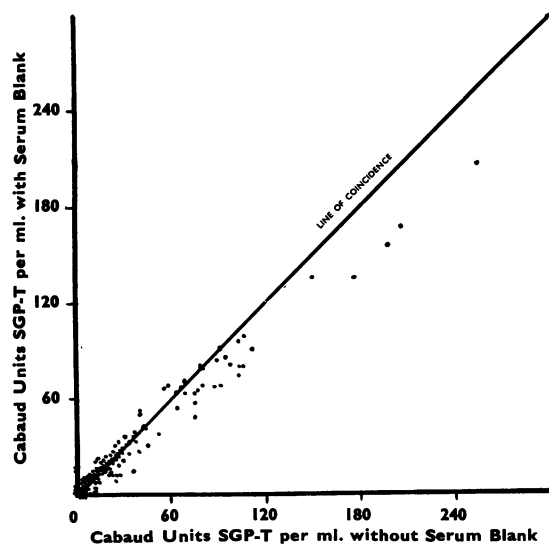
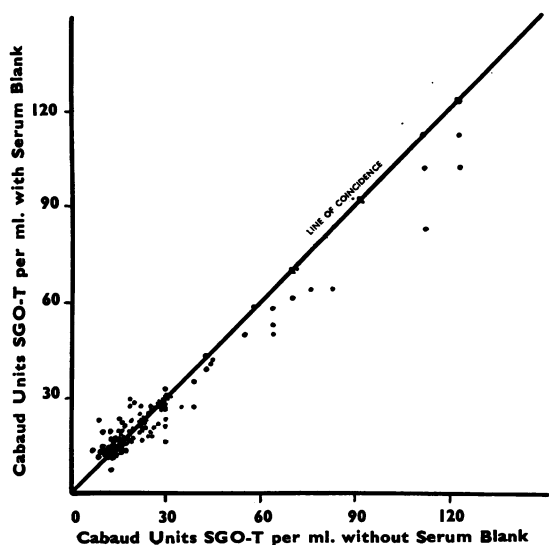
As the work progressed it became clear that there was no need for added co-enzyme (pyridoxal-5-phosphate) in the cases studied: the possibility has not been excluded that serum from a patient severely deficient in the B group of vitamins might require the addition of co-enzyme to produce maximum apparent transaminase content. (This caveat presumably applies to the technique of Cabaud *et al.* also.) In the rare case of this type it might be advisable to run the test with the addition of pyridoxal-5-phosphate to give final concentrations (in the substrate-mixture) of, say, 0, 1, and 10 $\mu\text{g. per ml.}$

The provision of the step involving the addition of 0.07 ml. (two drops) of the aniline-citrate reagent to the reaction mixture enables a true serum blank to be included if required. This reagent inactivates the transaminases instantly; its subsequent function is to convert oxaloacetate into pyruvate. Aniline citrate as an inactivator is retained in the SGP-T technique (where it is not necessary for any other reason) to provide for cases where it is desired to measure a serum blank. The effect of omitting the serum blank is shown in Figs. 1 and 2.

Methods

Serum Glutamic-oxalacetic Transaminase Determination.—One millilitre of SGO-T substrate mixture is placed in a 6 in. by $\frac{3}{8}$ in. test-tube or other convenient tube capable of holding 15 ml. of fluid, and the tube is placed vertically in a rack in a water-bath at

* Written in Feb.-June, 1957.



FIGS. 1 and 2.—Comparison of results of 109 SGO-T and 127 SGP-T measurements with and without inclusion of serum blanks by the technique of the present authors. Perfect agreement would result in all points lying on the line of coincidence. It will be seen that the main effect of omitting the serum blank is to over-estimate those sera with high transaminase content.

37° C. After allowing time for the tube and contents to warm up to 37° C. (10–15 minutes) 0.20 ml. of the serum is pipetted directly into the substrate mixture and a stop-watch or clock started at this moment. If a batch of tests is being done, the sera should be pipetted into their respective tubes of substrate at fixed convenient intervals, say 20 or 30 seconds, so that the first serum is delivered exactly at zero time, the remainder following at the chosen intervals.

Exactly 60 minutes after starting the test, 2 drops (0.07 ml.)* aniline-citrate reagent are added to the tube and mixed. If a batch of tests is being run, the aniline-citrate is added to the first tube at 60 minutes from the zero time, and to the remaining tubes at 20- or 30-second intervals so that each tube has had exactly 60 minutes for the enzyme reaction to proceed. The tubes can be removed from the water-bath at the end of this stage. From now on, the time intervals are not critical; it is only necessary that they be not appreciably less than those given below. Twenty minutes after adding the aniline-citrate reagent, 1 ml. of the 2-4-dinitrophenylhydrazine reagent is added, mixed, and left for 20 minutes, then 10 ml. of 0.4 N.NaOH is added and mixed by inversion, using a clean rubber bung or rubber fingerstall to stopper the tube. The tubes are allowed to stand for 10 minutes.

The colour developed is then compared with that developed in a "reagent blank" consisting of a similar tube (in which water replaces serum) treated in exactly the same way as the test. The galvanometer is set at zero with distilled water.

The volume of 0.4 N.NaOH which is added in the final step of the test can be increased if the intensity of the colour developed is inconveniently great. A similar change must be made in preparing the calibration curve.

Using the 10 ml. volume of 0.4 N.NaOH described above, the intensity of colour developed by normal sera gives convenient galvanometer deflections (around 30–40% of the maximum) when the fluid is placed in an EEL "portable" type photo-electric colorimeter, using a blue filter (No. 303) and tubes of diameter 10 mm. In these conditions the reagent blank gives a galvanometer deflection of 26 to 30% of the maximum, the zero setting being made with distilled water.†

The difference between the colorimeter reading of the test and that of the reagent blank is related to the amount of enzyme originally present by reference to the calibration curve.

The above description is that of a routine screening test; no serum blank is included. All sera giving normal results by this technique can be reported as normal straight away. Sera giving raised or borderline results should be retested with a serum blank incorporated.

Serum Glutamic-pyruvic Transaminase Determination.—The technique is similar to that of the SGO-T determination, except that the substrate mixture is incubated with the test serum for only 30 minutes instead of 60, and the step involving aniline-citrate is omitted (in the screening test). The procedure is therefore as follows: 1 ml. of the appropriate substrate mixture is warmed and incubated with 0.20 ml. test serum for exactly 30 minutes at 37° C. Then 1 ml. of the 2-4-dinitrophenylhydrazine reagent is added and mixed. Twenty minutes later 10 ml. of

* Between 0.06 ml. and 0.08 ml.

† The SGP-T reagent blank gives a reading of 23 to 25% of the maximum.

0.4 N.NaOH is added and mixed. Ten minutes later the intensity of colour developed is measured as in the SGO-T technique and compared with a reagent blank.* The difference between the two colorimeter readings is related to the amount of enzyme originally present by application of the SGP-T calibration curve (see below).

The Use of the Serum Blank.—When individual serum blanks are required, they are run in parallel with the tests in the following way:

Two 6 in. by $\frac{5}{8}$ in. test-tubes are set up for each serum to be tested, one labelled T (test) and the other B (blank).

One millilitre of substrate mixture is pipetted into each tube, and they are warmed in the bath at 37° C. Two drops (0.07 ml.) of aniline-citrate reagent are added to tube B only, and 0.20 ml. of the serum to be tested is added to tube T as a stop-watch is started. After this, 0.20 ml. of the same serum is added to tube B. Both tubes are shaken gently to mix their contents. When SGO-T is being measured, exactly 60 minutes later, two drops (0.07 ml.) of aniline-citrate are added to tube T, and both tubes are removed from the water-bath to the bench.

After 20 minutes, both tubes are treated alike, each receiving 1 ml. of 2-4-dinitrophenylhydrazine reagent followed after a further 20 minutes by 10 ml. of 0.4 N.NaOH, as described previously. The colorimeter reading of the serum blank is subtracted from that of the test, and the difference converted into units. No separate reagent blank is necessary. When SGP-T is being measured, aniline-citrate is added to tube B before the test serum is added, and to tube T exactly 30 minutes after adding the test serum; immediately afterwards, the 2-4-dinitrophenylhydrazine reagent is added to both tubes, which are removed to the bench, and, after a further 20 minutes, the 10 ml. 0.4 N.NaOH is added as usual.

Reagents

Serum Glutamic-oxalacetic Transaminase Substrate Mixture.—*l*-Aspartic acid, 0.30 g., and α -keto-glutaric acid, 0.050 g. (weight ratio=6/1), are used.

These acids are dissolved in 20–30 ml. of M/15 Sørensen phosphate buffer (pH 7.5) and 10% NaOH (about 1.1 ml.) added to bring the pH to 7.5. More buffer is added to bring the volume to 100 ml. Chloroform, 2–3 ml., is added, shaken to saturate, and filtered rapidly (Green's 798 $\frac{1}{2}$ 32 cm.) through a large funnel into a screw-capped bottle and kept in the refrigerator. If desired, the bulk can be distributed in small amounts and frozen solid. Unless grossly infected during use, the mixture keeps for several weeks in the refrigerator. The reagent blank value of the substrate mixture rises by about 10% in the first week. After this it remains stable unless grossly infected.

Serum Glutamic-pyruvic Transaminase Substrate Mixture.—*dl*-Alanine, 5.0 g., and α -keto-glutaric acid, 0.020 g. (weight ratio=250/1), are required.

These acids are dissolved in 20 to 30 ml. of buffer (as for SGO-T reagent) and brought to pH 7.5 by adding 10% NaOH (about 0.5 ml.). Buffer is added to 100 ml., and chloroform as above. The mixture is filtered and stored in the refrigerator, where it keeps for several weeks unless grossly infected.

M/15 Sørensen Phosphate Buffer (pH 7.5).—First 840 ml. of 0.947 g.% Na₂HPO₄ (w/v) is added to 160 ml. of 0.910 g.% KH₂PO₄ (w/v), then 5–10 ml. chloroform, and the mixture is shaken, filtered rapidly (Green's 798 $\frac{1}{2}$ 32 cm.), and stored in the refrigerator. Alternatively, the two solutions can be made up in tenfold concentrations and stored separately. If this is done, the solutions may crystallize at low temperatures, so that they must be warmed up before sampling to make the mixture.

Aniline-citrate Reagent.—Citric acid, 50 g., is dissolved in 50 ml. distilled water (gentle warmth is required). Equal volumes of this solution and of aniline (A.R.) are mixed and kept in a bottle with a dropping pipette incorporated in the stopper or housed in a suitable test-tube attached to the bottle with plastic tape. The dropping pipette delivers 25–30 drops of the reagent per millilitre. An ordinary pasteur pipette suffices, but better still is a "clinistest" dropper pipette, which is more robust at the end. The mixture slowly darkens on keeping, but behaves correctly in spite of this change. It keeps for several months.

2-4-Dinitrophenylhydrazine Reagent.—2-4-Dinitrophenylhydrazine, 200 mg., is dissolved in 85 ml. of concentrated HCl, and the volume is made up to 1,000 ml. with distilled water. The final mixture has about 1 N acidity when titrated against 1 N.NaOH using phenolphthalein indicator. It keeps indefinitely if refrigerated or kept cool between tests. A small deposit forms on storing, but this is of no importance.

When first made up, the solid may require some 36 hours to dissolve completely.

0.4 N.NaOH.—NaOH, 16 g., is dissolved in distilled water, and the volume made up to 1,000 ml. with distilled water. The solution must be free from any trace of turbidity for colorimetric use. It should be filtered if necessary.

Pyruvic Acid.—Pyruvic acid of specific gravity 1.260 to 1.267 at 20° C., 0.80 ml., is diluted to 100 ml. in a volumetric flask to give a 1% (w/v) solution in distilled water. This volume should be withdrawn at 20° C. from about 1 ml. previously delivered into a 6 in. by $\frac{5}{8}$ in. test-tube immersed in a large beaker of water at 20° C.; a 1 ml. pipette is placed in the same tube and the contents allowed to reach the temperature of 20° C. before withdrawing 0.80 ml. The solution is made up freshly on the day that the calibration curve is made and discarded thereafter.

Test Sera.—These should be free from haemolysis or bacterial contamination. Kept in the refrigerator at 0° to 4° C., sera retain their activity for several days, sometimes for weeks. Freezing and thawing impair activity in an irregular manner. Slightly

* The SGP-T reagent blank gives a reading of 23 to 25% of the maximum.

TABLE I
COLORIMETER READINGS

1	2	3	4	5	6	7	8	9
Tube No.	Volume of 100 $\mu\text{g.}/\text{ml.}$ Pyruvic Acid Solution (ml.)	Volume of 200 $\mu\text{g.}/\text{ml.}$ Pyruvic Acid Solution (ml.)	Volume of Water (ml.)	Final Concentration of Pyruvic Acid ($\mu\text{g.}/\text{ml.}$)	Colorimeter Readings*	Net Increase of Each Colorimeter Reading over that of Tube (1)	Units of SGO-T (Cabaud) per ml. Serum	Units of SGP-T (Cabaud) per ml. Serum
(1)	3.5	—	6.5	35		0	0	0
(2)	4.0	—	6.0	40			8.5	13.5
(3)	4.5	—	5.5	45			20	31.5
(4)	5.5	—	4.5	55			43	59
(5)	6.5	—	3.5	65			80	96
(6)	7.5	—	2.5	75			135	129
(7)	9.5	—	0.5	95			—	209
(8)	—	6.0	4.0	120			—	300

* To 1.0 ml. of pyruvic acid solution from each of tubes (1) to (8) is added 1 ml. of 2-4-dinitrophenylhydrazine reagent followed 20 minutes later by 10 ml. of 0.4 N.NaOH as described in the text.

haemolysed sera can be tested by running a serum blank simultaneously. (Gross haemolysis partially inhibits the enzyme activity while simultaneously raising the serum blank.) Grossly lipaemic sera can be tested, provided that a serum blank is run.

Calibration Curve

The object of the calibration curve prepared with pyruvic acid solutions is to standardize the electrical response of the colorimeter within a suitable range of galvanometer deflections. In the upper 50% of the galvanometer response range Beer's law is not obeyed by graded solutions of pyruvic acid. The range of the galvanometer scale actually used in the routine tests is approximately 28%–56% for SGO-T tests and approximately 25%–70% for SGP-T tests. Pyruvic acid solutions to cover this range are treated in the manner described below. The series of points thus defined serves to locate SGO-T and SGP-T levels found in sera under standard test conditions in the manner shown in Table I. The amount of colour developed is not related in a simple manner to the amount of enzyme present. If Table I is completed by preparing the pyruvic acid solutions indicated therein, and inserting the colorimeter readings in column 6, then the figures which will thus be inserted in column 7 (increases in optical density of "test" over "blank") can be used as data for the abscissa, and the figures in columns 8 and 9 as data for the ordinates of graphs which show the two calibration curves. It will be found that the curve relating to SGO-T activity is further removed from a straight line than is that relating to SGP-T activity. It must be stressed that the pyruvic acid solutions used in making the calibration curves are arbitrary solutions chosen solely because they give a suitable scatter of fixed points through which to draw the curves. The units of activity are those of Cabaud *et al.*

Procedure

The 1% (w/v) solution of pyruvic acid, freshly made up as described above, is used to make two working solutions, one containing 100 $\mu\text{g.}/\text{ml.}$ (1/100 of the

1%) and the other containing 200 $\mu\text{g.}/\text{ml.}$ (1/50 of the 1%). These are used to make solutions as shown in column 5 of Table I. To 1 ml. of each of these solutions is added 1 ml. of 2-4-dinitrophenylhydrazine solution; 20 minutes later, 10 ml. of 0.4 N.NaOH is added; 10 minutes later, the solutions are read in the colorimeter, as in routine tests. The colorimeter readings are entered in column 6 of Table I.

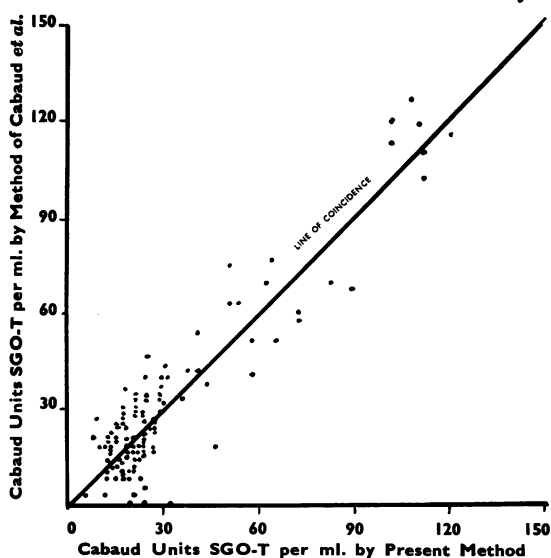


FIG. 3.—The "goodness of fit" of the calibration curve relating the findings on 115 sera whose SGO-T content was measured by the methods of Cabaud *et al.* and of the present authors. Perfect agreement would result in all points lying on the line of coincidence. In fact the points are distributed about the line of coincidence in the following manner:

56 points lie above the line of coincidence (with a standard deviation of 8.0 SGO-T units).

55 points lie below the line of coincidence (with a standard deviation of 7.5 SGO-T units).

4 points lie on the line of coincidence.

Column 7 is constructed by deducting the reading of tube (1) from the readings of the remaining tubes. This is equivalent to deducting the "blank" from the "test" in routine work. The resulting figures in column 7 represent units of SGO-T and SGP-T in the manner shown in Table I. From these data curves are drawn having units as ordinates and the figures from column 7 as abscissae. In this way the curves relate increases of "test" over "blank" to units of SGO-T and SGP-T.

Units of Measurement

Cabaud *et al.* (1956) and Wróblewski and Cabaud (1957) have related their colorimetric units of SGO-T and SGP-T to spectrophotometric units. Therefore, the present results are expressed in the colorimetric units of these authors.

The SGO-T and SGP-T content of sera has been estimated by the techniques of Cabaud *et al.* and by the techniques described in this paper. The colorimeter responses by these techniques were plotted against the units determined by the technique of Cabaud *et al.*, and curves were drawn so that the fit was as good as possible. These curves were then used to relate our results to those obtained by the technique of Cabaud *et al.* The "goodness of fit" is shown in Figs. 3 and 4. In all these estimations,

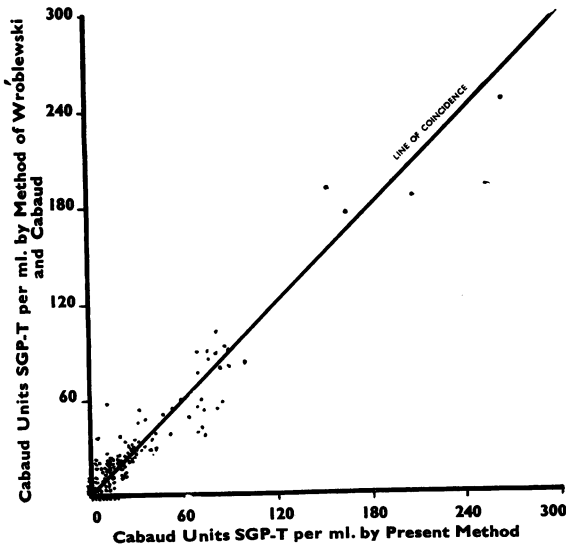


FIG. 4.—The "goodness of fit" of the calibration curve relating the findings on 117 sera whose SGP-T content was measured by the methods of Wróblewski and Cabaud and of the present authors. Perfect agreement would result in all points lying on the line of coincidence. In fact the points are distributed about this line of coincidence in the following manner:

- 58 points lie above the line (with a standard deviation of 10.4 SGP-T units).
- 50 points lie below the line (with a standard deviation of 9.2 SGP-T units).
- 9 points lie on the line.

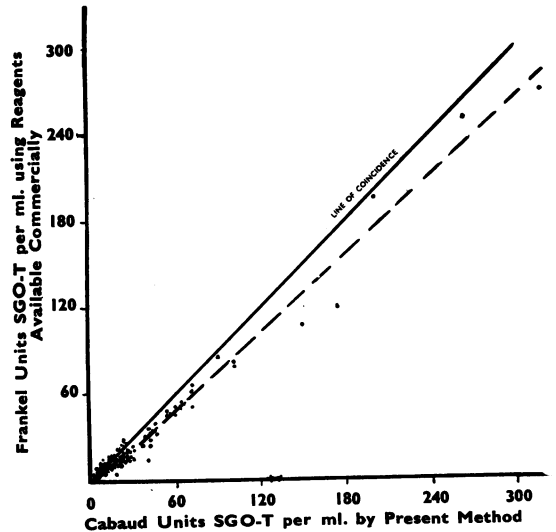


FIG. 5.—Comparison of units of Cabaud *et al.* of SGO-T with those of Frankel and Reitman. One hundred and forty-five sera were tested by the two methods, using the commercially available reagents and those of the present authors, the latter employing the units of Cabaud *et al.* Among the lower values the points cluster too closely to show each one individually; in this area one point may represent more than one reading. Perfect agreement would result in all points lying on the line of coincidence. The dashed line (drawn by visual estimation only) passes approximately through the midst of the array of points, bending at the lower end to do so.

serum blanks were run in order to minimize uncertainty. As a matter of interest, a number of comparisons were made between the SGO-T results (Fig. 5) obtained by the technique described here and by that of Frankel and Reitman (1956).

Reproducibility of Results

The standard deviation of duplicate estimations of the SGO-T content of 33 sera was 17%; that of the SGP-T content of 43 sera was 10%. In both SGO-T and SGP-T estimations, it will be found that the percentage variation between duplicates is highest in the very low ranges, where it is of no significance in practice. This finding applies also to the results by the technique of Cabaud *et al.*

This variability of results in the lower ranges is shown in Figs. 3 and 4.

Normal Ranges of Serum Glutamic-oxalacetic and Serum Glutamic-pyruvic Transaminases

Serum Glutamic-oxalacetic Transaminase.—Cabaud *et al.* (1956) found that 35 normal persons gave values ranging from 4 to 40 units with a mean of 16.4 (± 8.4).

Using the techniques described in this paper, 79 normal persons gave values ranging from 0 to 30 units with a mean of 13.9 (± 7.0). Among hospital patients it is best to take values below 40 as "normal,"

between 40 and 50 as "borderline," and over 50 as "definitely raised."

Serum Glutamic-pyruvic Transaminase.—Wróblewski and Cabaud (1957) found that 50 normal persons gave values ranging from 1 to 45 with a mean of 22.0 (± 11.5).

The present authors found that 73 normal persons gave values ranging from 4 to 38 units with a mean of 17.9 (± 7.6).

Suggested Routine Procedure

In cardiac infarction and some hepatic diseases, serum levels of SGO-T and SGP-T rise and fall to various extents and with varying rapidity in individual cases. Results therefore depend upon accidents of timing in the sampling of the blood. It would seem logical, then, to group results into "normal," "borderline," and "raised" categories, and to pay more attention to the assignment of a given serum to one of these categories than to the "precise" measurement of its enzyme content when this is grossly raised. The screening test is used to establish the normality or otherwise of a serum in terms of SGO-T and SGP-T. If the result is "borderline" or "raised," the test is repeated including a serum blank. If the repeated test is still borderline, a second specimen of blood is requested and tested with inclusion of a serum blank; if the test result is above 140 SGO-T units or 280 SGP-T units, then the test is repeated using a 1 in 5 dilution of the serum in water, and multiplying the result by 5. If this dilution does not contain less than 140 units of SGO-T (or less than 280 units of SGP-T), the final result is reported as "more than 700 units SGO-T" (or "more than 1,400 units SGP-T").

Dilution of sera in water, or buffer, or in normal sera appears to result in some change in the activity of the enzyme apart from that due to the dilution ratio. Thus, if a serum which has a high SGO-T content is diluted 1 in 5, 1 in 10, 1 in 15, 1 in 20, etc., and these dilutions tested for their SGO-T content, it will usually be found that the higher dilutions give higher figures for SGO-T content when allowance is made for the dilution ratios.

It follows that, for routine work, some arbitrary procedure has to be adopted.

Dilution of "high" sera 1 in 5 in water is a convenient procedure.

Cost of Materials

At current prices for suitable quantities of the materials required, the cost of materials to perform 100 SGO-T tests (and a calibration curve) is shown below:

Technique	Cost of Materials for 100 Tests
Cabaud <i>et al.</i>	16s. 7d.
Present authors	2s. 1d.
Reagents already made-up and supplied commercially	£5 18s. plus about 3d. for the NaOH

Summary

Simple techniques are described whereby the glutamic-oxalacetic and glutamic pyruvic transaminase content of sera can be measured by colorimetry in routine laboratories.

Results are expressed in the units of Cabaud *et al.* (1956).

A suggested routine procedure is described.

We are indebted to Dr. Wróblewski for his kindness in sending to us the manuscript of his paper on SGP-T estimation while it was in the press. We wish to thank Dr. J. Carpenter and various members of the nursing staff at New End Hospital for providing some of the specimens of normal sera. Dr. Toohey, senior physician at New End Hospital, gave us a great deal of help in our task of combining this work with that of a busy clinical laboratory.

The substrate acids and other reagents were obtained from Messrs. British Drug Houses Ltd. and from Messrs. L. Light and Co. Ltd., Colnbrook, Bucks.

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