# Statistical Analysis of Twenty Serum Globulin Components by Means of a Computer

—An Aid to Laboratory Diagnosis—

Takaoki MIYAJI, Mitsuko KAMEOKA and Banri TANIGUCHI
*Department of Clinical Pathology, Yamaguchi University School of Medicine*
Reiko FUJII and Keiko FUJISAWA
*School of Medical Technology, Yamaguchi University School of Medicine*
Toshihiro FUKUDA
*Computer Department, Ube College*

Recently, immunodiffusion[1,2] and immunoelectrophoretic methods[3] for the estimation of serum globulin component concentrations have been developed and normal ranges[4] have been defined for a number of these components.

However, statistical analysis of these concentration has never been established satisfactorily enough for use in laboratory diagnosis. Our aim is to establish normal ranges for the twenty serum globulin components and propose a program to be used in the laboratory evaluation of various disorders by means of a computer[5].

## MATERIALS AND METHODS

### Subjects studied

Sera from 326 healthy individuals (142 males and 184 females, age 15 to 70) and about 2400 patients were examined.

### Analytic methods

The immunoglobulin concentrations of immunoglobulin G (Ig G), immunoglobulin A (Ig A), immunoglobulin M (IgM) and immunoglobulin D (Ig D) were estimated by Macini's single radial immunodiffusing procedure.[1] Other globulin component concentrations were estimated by Vaerman's reversed system of single radial immunodiffusion procedure.[6,7] Analytic methods of reversed system of single radial immunodiffusion procedure:

I. Reagents

1. Standard serum

a. Standard human serum*: This serum contains Prealbumin (Pre Alb) (35 mg/dl), Alpha$_1$-Acid Glycoprotein ($\alpha$1AG) (100mg/dl), Group Specific Component (GC) (38 mg/dl), Alpha$_2$-Heat Stable Glycoprotein ($\alpha$2HS) (58 mg/dl), Haptoglobin (HP) (208 mg/dl), Alph$_2$-Macroglobulin ($\alpha$2M) (220 mg/dl), Hemopexin (HX) (94 mg/dl), Beta 1 A/1C (C3) (93 mg/dl), Beta E Globulin (C4) (26 mg/dl) and Transferrin (TF) (386 mg/dl).

b. Protein standard plasma*: This plasma contains Ceruloplasmin (CP) (24 mg/dl), C 4 (24 mg/dl) and Alpha$_1$-Antitrypsin ($\alpha$1AT) (127 mg/dl).

c. Standard control sera for Alpha$_1$B-Glycoprotein ($\alpha$1B), Alpha$_1$-Anti-Chymotrypsin ($\alpha$1X) and Inter Alpha Trypsin Inhibitor (I$\alpha$TI): The control sera were prepared by mixing pooled sera from healthy males and females in a one to one ratio by volume.

d. Beta Lipoprotein standard serum ($\beta$LP) (550 mg/dl)*

2. Specific antisera*

3. Agarose gel (1.2%): 1.2 gm of agarose was added to 100 ml of barbiturate buffer containing 0.1% of Tween 80.

4. Normal saline solution.

5. Amidoblack 10 B solution (0.5%).

6. Acetic acid solution (2%).

II. Procedure

1. Preparation of antigen containing agarose plates: The 1.2% agarose suspension was melted down in a boiling water bath and 6 ml allotments were distributed in test tubes. These test tubes were then cooled to 55°C in a water bath. The five different standard sera and the control were pipeted into the test tubes in 0.2 ml, 0.12 ml, 0.06 ml, 0.04 ml and 0.03 ml allotments to make dilutions corresponding to 30, 50, 100, 150 and 200. 0.12 ml of each sample serum was pipeted into a test tube in the same manner. Antigens containing agarose solution were poured into horizontally placed glass plates and allowed to gelated. After gelation of the antigens containing agarose, circular wells with 2 mm diameters, two with 3 mm diameters for the estimation of Alpha 1 AT and TF, were punched. (Fig. 1)

2. Application of the specific antisera: All wells received 2 $\mu$l of specific antisera (with the exceptions of 6 $\mu$l for Alpha 1 AT and TF) from microsyringes. Diffusion was allowed to proceed for 40 hours at 37°C in a moist chamber.

*Behringwerke (Hoechst)

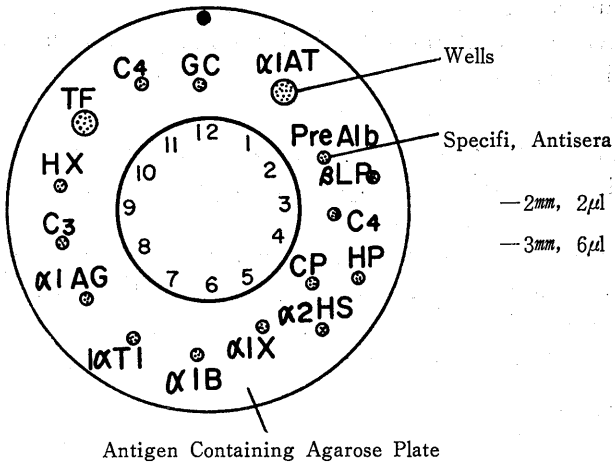


Fig. 1. Antigen containing agarose plate forestimation of globulin components.

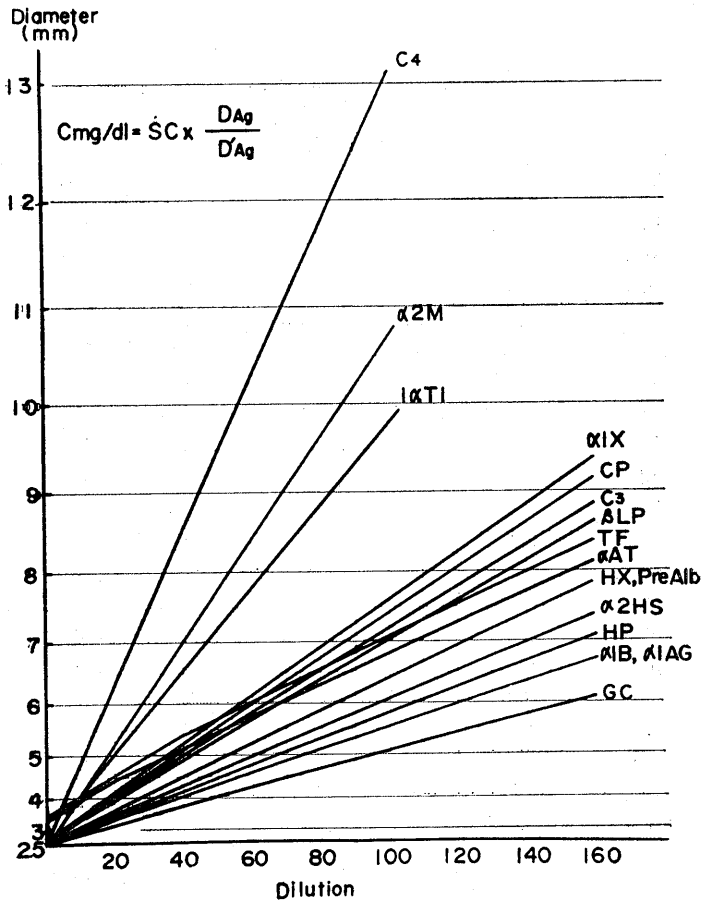


Fig. 2 Calibration Curve

3. Measurement of the size of the precipitation area: When the precipitating areas reached their final size usually after 40 hours, the diameter of each area was measured to 1/10 mm.

4. Calculation: The square of the diameter of the precipitation area was plotted along the ordinate, while the antigen dilution was placed along the abscissa. If a semilogarithmic graph was used, the diameter instead of its square was plotted along the ordinate as shown in Fig. 2. Since each regression line indicates an inverse proportionality between the precipitation area and the concentration of the antigen in the agarose plate, the concentrations of the serum globulin components were calculated by the following equation.

$$C\ \text{mg/dl} = SC \times \frac{DAg}{D'Ag}$$

Where C represents the antigen concentration in a sample, SC the antigen concentration in the standard, DAg the original dilution of the sample and D'Ag the dilution of the sample which is read from the calibration.

Table. 1 Reproducibility

| | Mean | S D | C V | Range | |
|---|---|---|---|---|---|
| | | | | Min. | Max. |
| Pre Alb | 23.5 | 1.2 | 5.1 | 20.3 | 24.0 |
| α1AG | 69.1 | 3.1 | 4.5 | 65.0 | 73.0 |
| α1B | 98.8 | 5.1 | 5.2 | 96.1 | 104 |
| α1AT | 253 | 8.7 | 3.5 | 220 | 290 |
| α1X | 129 | 6.9 | 5.4 | 120 | 120 |
| α2HS | 55.3 | 3.3 | 6.0 | 51.2 | 58.9 |
| CP | 26.2 | 1.9 | 7.3 | 25.3 | 29.0 |
| GC | 32.4 | 1.3 | 4.1 | 30.0 | 34.8 |
| α2M | 208 | 13.8 | 6.7 | 159 | 230 |
| IαTI | 112 | 9.8 | 8.7 | 92 | 127 |
| HP | 151 | 11.2 | 7.4 | 123 | 170 |
| $C_3$ | 54.6 | 5.1 | 9.4 | 51.0 | 59.2 |
| HX | 82.0 | 5.2 | 6.4 | 74.6 | 88.6 |
| TF | 369 | 7.8 | 2.5 | 300 | 340 |
| βLP | 421 | 42.1 | 10.0 | 385 | 439 |
| $C_4$ | 29.6 | 2.4 | 8.1 | 27.0 | 32.3 |
| IgG | 1270 | 55.0 | 4.4 | 1150 | 1350 |
| IgA | 237 | 7.0 | 3.0 | 230 | 250 |
| IgM | 82.0 | 8.5 | 10.9 | 100 | 75 |
| IgD | 10.4 | 1.4 | 13.5 | 10.0 | 11.0 |

n =30

## RESULTS

### Reproducibility

The coefficients of variation, calculated from the results of 30 determinations from the same control sera, ranged from 2.5% to 13.5%, while that from duplicate determinations of 18 different samples ranged from 0.8% to 5.6%, as shown in Table 1 and 2.

### Stability of samples

The globulin component concentrations of two series of sera, which were stored in refrigerator and a deep freezer, were determined every day for two weeks. The concentrations of Alpha 1 AG, Beta LP and Alpha 1Xrevealed decreases of about 20% after 6 days. Concentrations of the other components were virtually stable during the two weeks.

### Diurnal variation

The blood from four healthy individuals was drawn at 2 hrs. intervals from 7.00 a.m. to 7.00 p.m. and concentrations of the twenty globulin components were determined. These results showed no significant variation. (Table 3)

Table. 2 Difference of Duplicate Determination

| | Mean | Difference (%) | $S_1$-$S_2$ (%) |
|---|---|---|---|
| Pre Alb | 97.6 | 2.2 | 4.0 |
| α1AG | 116.3 | 1.3 | 8.0 |
| α1B | 104 | 1.8 | 7.0 |
| α1AT | 113.5 | 1.8 | 5.0 |
| α1X | 131.9 | 4.0 | 13.8 |
| α2HS | 91.4 | 3.6 | 10.0 |
| CP | 140.2 | 3.9 | 9.8 |
| GC | 102.8 | 2.6 | 6.5 |
| α2M | 129.1 | 3.4 | 11.5 |
| IαTI | 119.1 | 3.7 | 11.5 |
| HP | 121.4 | 1.4 | 4.9 |
| $C_3$ | 116.3 | 3.3 | 7.8 |
| HX | 96.7 | 2.0 | 6.0 |
| TF | 102.6 | 2.6 | 5.0 |
| βLP | 115.8 | 3.0 | 7.5 |
| $C_4$ | 116.4 | 5.6 | 13.8 |
| IgG | 1445 | 0.9 | 2.5 |
| IgA | 279 | 0.8 | 5.0 |
| IgM | 106 | 1.2 | 8.0 |
| IgD | 7.8 | 1.9 | 10.0 |

n =18

Table. 3 Diurnal Variation

| | AM 7 | AM 9 | AM 11 | PM 1 | PM 3 | PM 5 | PM 7 |
|---|---|---|---|---|---|---|---|
| Pre Alb | 90 | 98 | 90 | | | | 98 |
| α1AG | 110 | 104 | | 100 | 104 | 106 | 104 |
| α1B | 106 | | | | | | |
| α1AT | 111 | | | | | | 106 |
| α1X | 132 | | | 125 | | 135 | |
| α2HS | 94 | 100 | 94 | 100 | | | |
| CP | 94 | 100 | | | | 108 | |
| GC | 100 | | | 90 | 100 | | |
| α2M | 86 | 88 | 89 | 84 | 90 | 88 | 104 |
| IαTI | 125 | | 110 | | 115 | 125 | 118 |
| HP | 120 | 115 | | | 111 | | 120 |
| $C_3$ | 111 | 119 | 110 | 115 | 106 | 108 | 106 |
| HX | 108 | 100 | 108 | | 100 | | 89 |
| TF | 125 | | | 130 | 125 | | 130 |
| βLP | 100 | 104 | 115 | 106 | 104 | 110 | 115 |
| $C_4$ | 115 | | | 125 | 134 | 120 | 124 |
| IgG | 1100 | 1150 | 1100 | 1150 | 1200 | 1050 | 1000 |
| IgA | 260 | 265 | 260 | 280 | 260 | 255 | 250 |
| IgM | 80 | | 85 | 80 | 75 | | 80 |
| IgD | 5 | | | | | | |

The normal range

The statistical analysis by a "t" test of the twenty serum globulin components from 326 healthy individuals (142 males and 184 females) demonstrated significant sexual differences in the concentration of 8 components, namely Alpha1AG, GC, C3, Beta LP, Alpha2HS, CP, Alpha2M and Ig D. An "F" test demonstrated significant differences among age groups in the concentrations all of components, except Alpha 2HS, GC, Alpha1AT, TF, Alpha 1Xand IAlpha TI. Unfortunately, distribution of many globulin component concentrations did not fit a Gaussian distribution curve when tested by the Chi-square method. (Table 4) By considering sexual and age differences and the non-Gaussian

Table. 4 Tests of Chi-Square Distribution and Differences Between Mean of Sex & Age

| | Chi-Square Distribution | | Differences Between Sex | | Differences Between Age | |
|---|---|---|---|---|---|---|
| | Male | Female | t Values | Remark | F Values | Remark |
| Pre Alb | 0.1 | | 1.57 | | 7.75 | 0.1 |
| α1AG | 1.0 | 0.1 | 2.85 | 1.0 | 15.31 | 0.1 |
| α1B | | 0.1 | 0.67 | | 5.21 | 1.0 |
| α1AT | | 1.0 | 0.84 | | 0.47 | |
| α1X | 0.1 | 0.1 | 0.08 | | 1.02 | |
| α2HS | 1.0 | | 5.21 | 0.1 | 1.34 | |
| CP | | 0.1 | 3.37 | 0.1 | 6.82 | 0.1 |
| GC | 0.1 | 1.0 | 2.94 | 1.0 | 0.98 | |
| α2M | 1.0 | 0.1 | 8.82 | 0.1 | 6.50 | 0.1 |
| IαTI | 0.1 | 0.1 | 0.51 | | 0.18 | |
| HP | | | 0.1 | | 4.77 | 1.0 |
| $C_3$ | | 0.1 | 2.91 | 1.0 | 9.11 | 0.1 |
| HX | | 0.1 | 0.2 | | 13.60 | 0.1 |
| TF | | 0.1 | 0.3 | | 2.43 | |
| βLP | 0.1 | 0.1 | 2.62 | 1.0 | 6.33 | 0.1 |
| $C_4$ | | 0.1 | 0.71 | | 4.12 | 1.0 |
| IgG | | | 1.63 | | 6.93 | 0.1 |
| IgA | 1.0 | | 0.94 | | 3.80 | 5.0 |
| IgM | | 1.0 | 1.87 | | 2.86 | 5.0 |
| IgD | 1.0 | 1.0 | 2.30 | 5.0 | 5.19 | 1.0 |

M142, F184 $n = 277—322$ $n = \frac{3}{322}$

distribution of some of the component concentrations, the data were grouped into eight populations. Although the normal range of some concentrations may deviate from the 96% confidence limits, for convenience, normal ranges of a meanes ±2 standard deviation for the eight populations were calculated for the twenty globulin component concentrations. These normal ranges are indicated in Table 5.

A computer program for laboratory evaluation of various disorders

As parameters for laboratory evaluation of pathological states, the twenty globulin components were subjected to a multivariate discriminant analysis. As shown in Fig. 3, this analysis consist of the following steps. In the first step, the subjects who showed values outside of the mean ±2S.D. for their respective population groups in two components or outside of the mean ±3S.D. in one component, were separated from the normal population. In the second step, everyone in this abnormal popu-

Table. 5 Normal Ranges of the Serum Globulin Components

| AGE / SEX / No | 15 — 20 | | | | 21 — 40 | | | | 41 — 60 | | | | 61 — 99 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M;41 | | F;64 | | M;31 | | F;39 | | M;39 | | F;45 | | M;31 | | F;36 | |
| | M | SD | M | SD | M | SD | M | SD | M | SD | M | SD | M | SD | M | SD |
| SP | 7.3 | 0.3 | 7.6 | 0.4 | 7.4 | 0.4 | 7.5 | 0.3 | 7.5 | 0.4 | 7.7 | 0.4 | 7.4 | 0.5 | 7.7 | 0.3 |
| ALUB. | 64.3 | 2.4 | 62.7 | 2.9 | 64.2 | 2.2 | 60.8 | 3.4 | 60.9 | 2.6 | 58.2 | 2.8 | 59.5 | 3.0 | 59.0 | 2.6 |
| α1 GLOB | 2.6 | 0.4 | 2.7 | 0.4 | 2.7 | 0.3 | 2.8 | 0.4 | 2.6 | 0.3 | 2.8 | 0.5 | 3.0 | 0.5 | 2.7 | 0.4 |
| α2 〃 | 5.9 | 0.7 | 7.3 | 1.9 | 6.1 | 1.9 | 7.3 | 1.9 | 6.7 | 1.2 | 7.5 | 1.6 | 7.3 | 1.2 | 7.6 | 1.7 |
| β 〃 | 9.5 | 1.0 | 9.1 | 1.3 | 9.3 | 1.1 | 8.9 | 1.0 | 9.7 | 1.4 | 9.9 | 1.5 | 9.9 | 1.2 | 10.3 | 1.2 |
| γ 〃 | 16.9 | 2.7 | 17.8 | 2.4 | 16.8 | 1.7 | 19.5 | 2.7 | 19.6 | 2.6 | 20.3 | 3.0 | 19.5 | 2.7 | 20.1 | 2.3 |
| IgG | 1380 | 215 | 1350 | 199 | 1220 | 160 | 1310 | 199 | 1360 | 221 | 1360 | 199 | 1350 | 221 | 1500 | 224 |
| IgA | 259 | 67 | 265 | 63 | 258 | 71 | 280 | 83 | 274 | 77 | 238 | 71 | 306 | 92 | 283 | 69 |
| IgM | 93 | 23 | 93 | 13 | 82 | 20 | 101 | 32 | 87 | 23 | 94 | 30 | 85 | 31 | 80 | 26 |
| IgD | 7.0 | 1.8 | 7.1 | 2.2 | 5.9 | 1.6 | 7.4 | 1.4 | 5.5 | 1.8 | 6.4 | 2.0 | 6.9 | 1.9 | 6.2 | 1.7 |
| PREALUB. | 30.3 | 3.7 | 32.1 | 3.3 | 32.4 | 2.7 | 30.9 | 3.3 | 31.2 | 3.6 | 28.4 | 4.8 | 24.9 | 4.5 | 28.4 | 4.1 |
| α1AG. | 68.6 | 6.1 | 64.4 | 11.4 | 73.4 | 4.1 | 70.0 | 7.4 | 73.4 | 5.1 | 72.0 | 9.3 | 72.7 | 10.2 | 73.4 | 6.4 |
| α1B. | 99.2 | 5.5 | 94.9 | 9.5 | 99.3 | 5.1 | 99.0 | 3.8 | 98.0 | 4.2 | 98.9 | 4.9 | 98.0 | 5.5 | 102 | 5.1 |
| α1AT. | 252 | 20 | 255 | 20 | 254 | 21 | 249 | 24 | 259 | 19 | 251 | 17 | 254 | 31 | 255 | 31 |
| α1X. | 120 | 13 | 122 | 13 | 118 | 17 | 120 | 14 | 123 | 19 | 121 | 18 | 125 | 23 | 124 | 17 |
| α2HS. | 53.4 | 4.8 | 59.1 | 6.7 | 56.6 | 3.4 | 57.3 | 5.4 | 54.6 | 4.1 | 57.8 | 6.9 | 54.4 | 5.4 | 56.1 | 6.3 |
| CP. | 28.8 | 3.1 | 30.8 | 4.8 | 27.5 | 3.8 | 30.5 | 4.8 | 27.7 | 3.8 | 29.4 | 4.5 | 31.9 | 5.0 | 32.1 | 6.0 |
| GC. | 32.6 | 2.0 | 31.6 | 2.9 | 31.6 | 2.3 | 31.9 | 2.0 | 32.2 | 2.6 | 31.1 | 1.8 | 32.3 | 2.7 | 30.9 | 2.0 |
| α2M. | 231 | 35 | 275 | 46 | 211 | 22 | 251 | 31 | 227 | 26 | 253 | 33 | 238 | 37 | 262 | 40 |
| IαTI. | 102 | 11 | 99 | 6 | 103 | 12 | 98 | 11 | 100 | 13 | 99 | 16 | 97 | 16 | 104 | 15 |
| HP. | 142 | 22 | 126 | 27 | 135 | 24 | 131 | 26 | 138 | 27 | 129 | 28 | 134 | 32 | 143 | 30 |
| β1A/β1C. | 59.8 | 5.5 | 56.7 | 7.2 | 57.2 | 5.8 | 52.3 | 8.8 | 55.1 | 6.5 | 51.6 | 6.0 | 52.0 | 8.4 | 54.0 | 8.4 |
| HX. | 83.1 | 6.4 | 78.5 | 6.8 | 83.4 | 6.3 | 85.1 | 10 | 86.0 | 8.2 | 88.5 | 9.3 | 85.5 | 10 | 88.5 | 10 |
| TF. | 346 | 23 | 339 | 42 | 323 | 15 | 336 | 30 | 339 | 22 | 330 | 32 | 330 | 30 | 336 | 30 |
| βLP. | 391 | 35 | 420 | 50 | 428 | 64 | 434 | 57 | 433 | 55 | 441 | 93 | 424 | 81 | 478 | 73 |
| β1E. | 31.2 | 4.3 | 32.1 | 4.5 | 33.9 | 5.3 | 30.6 | 5.7 | 34.2 | 5.7 | 34.5 | 6.3 | 34.2 | 9.2 | 34.2 | 9.2 |

lation was again discriminated from the total patient population. In the third step, the abnormal population was compared with an inflammatory population. The acute and chronic states of the inflammatory population and abnormal population were differentiated from other pathological states by discriminant analysis. In the four step, the abnormal population was compared with and differentiated from malignant states in the same way. In cases of the population with malignancy, the sites of malignancy (lung, stomach, liver and bladder) were discriminated. In the last step, specific states such as collagen disease, nephrotic syndrome and liver cirrhosis, were discriminated from other disorders. We plan to add one more step for the discrimination of defficient and excess states in the

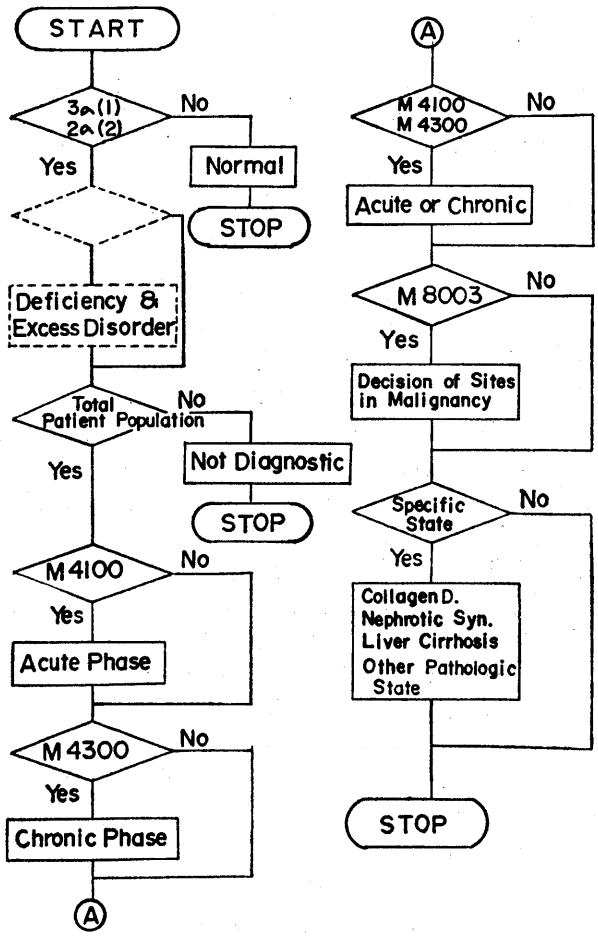


Fig. 3 Flow Chart of Discriminant Analysis

Table. 6 Results of Discriminant Analysis in Health and Disease by Our Flow Diagram

| | Healthy, 286 Cases (M:141, F:145) | Patient, 172 Cases (M:86, F:86) |
|---|---|---|
| Result | Abnormal, 14 Cases (4.9%) | Normal, 21 Cases (12.2%) |
| Details | Pathologic State | Clinical Diagnosis |
| | Inflammation | Acute Inflammation 3(1.7%) |
| | Acute Phase 12(4.2%) | Chronic Inflammation 2(1.1%) |
| | Chronic Phase 12(4.2%) | Malignant State 12(7%) |
| | Malignant State 7(2.5%) | Cervix Uteri 9(5.2%) |
| | | Colon 1(0.6%) |
| | | Mediastinum 1(0.6%) |
| | | Stomach 1(0.6%) |
| | | Liver Cirrhotic Type 1(0.6%) |
| | | Others 3(1.7%) |

concentrations of some globulin components, such as immunodeficiency disease and dysproteinemias.

When this flow diagram was applied to 286 healthy subjects and 172 patients whose diagnosis had been established, 14 of the healthy subjects (4.9%) fell into the abnormal population and 21 of the patients (12.2%) fell into the normal population. Detailed analysis of these cases is indicated in Table 6.

## DISCUSSION

Difficulties in the reversed procedure of single radial immunodiffusion were described in previous reports.[6,7] A major difficulty lies in the relatively poor precision compared to other procedures. One of the reasons for this is that samples and standards are measured in different agarose plates. A second factor is the poor resolution of the precipitate rim because the optimal antigen antibody ratios are not always realized. In spite of these difficulties the reversed procedure is simple and more convenient than other procedures. It is resonably reproducible and especially suitable for the estimation of many globulin component concentrations in serum at the same time. With several precautions such as calibration for every trial, careful application of sample, measuring the diameter after staining the plate and not storing the samples longer than 4 to 5 days, the reversed system of the single radial immunodiffusion method is effective enough for laboratory and clinical use.

Although a Gaussian distribution was not revealed in some globulin component concentrations, cumulative percentage distributions did not indicate large deviation from it. Therefore, the normal ranges of a mean $\pm$ 2 S.D. were defined for practical purposes. To reduce the errors due to sexual and age differences and deviations from Gaussian distributions, a normal for each of the eight population groups was defined and the data were analyzed by a computer aided discriminant analysis.

Since this is the first flow diagram for the screening of pathological sera, there are many problems to be worked out such as the selection of a statistical method, finding a suitable normal range and classifying pathological states. The flow diagram will have to be refined by trial and error.

## REFERENCES

1) Mancini, G., Carbonara, A.O., and Heremans, J.F.: Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem.*, 2 : 235–254, 1965.

2) Fahey, J.L. and Mckelvey, E.: Quantitative determination of serum immunoglobulins in antibody agar plates. *J. Immunol.*, **94** : 84-90, 1965.
3) Grabar, P. and Williams, C.A.: Methode immuno-electrophoretique d'analyse de melanges de substances antigeniques. *Biochim. Biophys. Acta*, **10** : 193-194, 1953.
4) Matsuba, K., Sasaki, M. and Terano, Y.: The normal values of 15 kinds of plasma proteins of the Japanease people. *Jpn. J. Clin. Chem.*, **1** : 70-79, 1971.
5) Fukuda, T., Miyaji, T. and Kameoka, M.: Data processing in hospital laboratories and its applications. *Bull. Coll. Ube*, **12** : 113-121, 1976.
6) Terano, Y., Shibata K. and Ichihashi, H.: Further studies on single radial immunodiffusion in antibody contain gel and antigen contained gel layer. *Jpn. I. Clin. Chem.*, **2** : 28-55, 1973.
7) Nariuchi, H., Usui, M. and Matsuhashi, T.: Studies on the micro quantitatation of antibody by the reversed type single radial immunodiffusion. *Jpn. J. Exp. Med.*, **40** : 15-22, 1970.