Estimation of the Antitrypsin Activity of Serum

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A method for the estimation of serum trypsin inhibitory capacity (TIC) was developed to permit consistent results with various trypsin sources without further standardization. Very small volumes of serum are used and the results are expressed in international enzyme units. Normal women show a slightly higher mean value for TIC than normal men. Sixty-seven patients with pulmonary emphysema were surveyed: one patient homozygous for α1-antitrypsin deficiency was found.

The presence of trypsin inhibitor in the serum was described almost 70 years ago by Camus and Gley (1). Since then, there have been many papers describing diseases and conditions in which the antitrypsin activity of serum is elevated (2–7). In general, serum antitrypsin is elevated in disorders associated with acute or chronic inflammation, malignancy, and pregnancy. There are 2 principal antitryptic proteins in human serum: the α1-antitrypsin accounts for 85–90% of the total activity, and the α2-antitrypsin, the remainder (8, 9). Interest in serum antitrypsin was stimulated recently when Laurell and Eriksson (10) and Eriksson (9, 11) reported cases of hereditary α1-antitrypsin deficiency associated with early onset of pulmonary emphysema.

Many substrates have been used for the assay of trypsin and serum trypsin inhibitor. These have been reviewed by Jacobsson (8) and by Homer et al. (12). The synthetic chromogenic substrate α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) used by Erlanger et al. (13) and by Eriksson (9) has definite advantages in that its hydrolytic product p-nitroaniline (PNA) can be measured directly. Most workers have recorded the results of the inhibition of hydrolysis of this and other substrates in terms of weight of trypsin inhibited by a given volume of serum. Since commercial trypsin preparations are not pure, this requires their standardization, most commonly done with soybean trypsin...
inhibitor (8, 9). The standardization is then dependent on the purity of the soybean trypsin inhibitor and the exactness of the molecular weights and combining ratios assigned to both trypsin and inhibitor. The precision of the results is not affected, even though these assumptions are subject to change, but values from different laboratories may vary 100% (9). A method based on the decrease in moles of substrate hydrolyzed would not require the above assumptions and the results could be reported in terms of international enzyme units. The accuracy and precision of the results would depend only on the conditions used for the assay.

The present paper reports conditions for the reproducible assay of serum trypsin inhibitor. Any one of a number of sources of trypsin can be used without further standardization. The method is based on those described by Eriksson (9), Erlanger et al. (13), and Nagel et al. (14), although modifications were required. The method is adapted for very small volumes of serum.

Materials and Methods

Reagents

Standard  
P-Nitroaniline (PNA) was crystallized from 50% alcohol and dried overnight at 90°. The absorption curve was run both in 4.3% (V/V) acetic acid (pH 3.0) to simulate the acid conditions in the final solution for spectrophotometry, and in Tris buffer (pH 8.2). The absorbance was identical at both pH values. For the assay 400 nm. was chosen because at this wavelength, the change in absorbance due to the hydrolysis of substrate, α-N-benzoyl- DL-arginine-p-nitroanilide (BAPNA), can be neglected, whereas this is not the case at 380 nm., where PNA has its maximum absorptivity. The molar absorptivity of BAPNA at 25°, pH 3.0, and at 400 nm. was 85; that of PNA was 10,500. At 380 nm. the corresponding values were 480 and 12,650, respectively. These values are similar to those reported by Bundy (15).

Buffer  
Tris buffer, 0.1 M, of pH 8.2 at 37° is prepared containing 0.02 M CaCl₂. This consists of 12.11 gm. Tris plus 2.2 gm. CaCl₂ adjusted to pH 8.2 at 37° with HCl and made up to 1 L. at room temperature.

Stock substrate for trypsin  
BAPNA (α-N-benzoyl- DL-arginine-p-nitroanilide hydrochloride,* mol. wt. 434.88), 100 mg., was dissolved in 2.3 ml. dimethyl sulfoxide (DMSO). This solution is stable in the refrigerator for at least 1 week. The DMSO solidifies at refrigerator temperature but slowly melts on warming to room temperature.

*Obtained from Sigma Chemical Co., St. Louis, Mo,
Working substrate, 1 mM One ml. of the stock substrate is made up to 100 ml. with Tris buffer. This solution must be prepared shortly before use as the BAPNA crystallizes on standing.

Soybean trypsin inhibitor (STI) Worthington three times crystallized. A solution containing 1 mg. STI/ml. in 0.001 M HCl was diluted so that 6 μg. could be used per assay.

Trypsin A summary of the trypsin sources with code letters is given in Table 1. Where available, the manufacturer’s assay is given, although the units are not always the same. The table is arranged in descending order of activity as assayed with BAPNA. The various trypsins were further standardized by estimating the amount of STI required to inactivate 1 mg. of each trypsin. This value increases with increasing activity of the trypsin preparation: this can be seen in column 5 of Table 1. When this value is divided into the trypsin activity against BAPNA, a constant should be obtained for all trypsins with a single preparation of STI. This is essentially the case, as shown in column 6 of Table 1. Most of the work was done with trypsins A, F, and G. A stock solution of trypsin was prepared by dissolving 1 mg./ml. in 1 mM HCl. This solution kept well in the refrigerator for several weeks but a fresh stock solution was prepared each week.

For determination of serum trypsin inhibitory capacity (TIC), working solutions of trypsin were prepared so that the increase in absorbance would be approximately 0.4 in 10 min. under the assay conditions. The exact dilution is determined by assay. For example, trypsin G stock solution was diluted 1:25 (40 μg./ml.) with Tris buffer.

Albumin This is conveniently prepared by diluting 25% human

<table>
<thead>
<tr>
<th>Code letter</th>
<th>Source</th>
<th>Trypsin (U./mg.)*</th>
<th>BAPNA† (μmoles/min./mg.)</th>
<th>STI/STI (mg/min./mg. trypsin)</th>
<th>Chymotrypsin‡ (μmoles/min./mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Novo</td>
<td>25</td>
<td>2.04</td>
<td>0.92</td>
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<tr>
<td>B</td>
<td>Mann</td>
<td>10700</td>
<td>2.03</td>
<td>0.94</td>
<td>2.16</td>
</tr>
<tr>
<td>C</td>
<td>Seravac-TPCK</td>
<td>8300</td>
<td>2.02</td>
<td>0.89</td>
<td>2.27</td>
</tr>
<tr>
<td>D</td>
<td>Worthington TRL</td>
<td>10000</td>
<td>1.77</td>
<td>0.84</td>
<td>2.20</td>
</tr>
<tr>
<td>E</td>
<td>Seravac</td>
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<td>1.50</td>
<td>0.61</td>
<td>2.46</td>
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<tr>
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<td>Armour</td>
<td>3160</td>
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<td>0.71</td>
<td>2.20</td>
</tr>
<tr>
<td>G</td>
<td>Worthington TRL</td>
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<td>0.64</td>
<td>2.11</td>
</tr>
<tr>
<td>H</td>
<td>Henley</td>
<td>—</td>
<td>1.27</td>
<td>0.59</td>
<td>2.22</td>
</tr>
</tbody>
</table>

*All figures given in BAEE units except: Novo, Anson units; Armour, Armour units.
†Assays done with 0.2 mg. albumin in control tubes. Values are the average of 3 assays.
‡Values obtained by dividing column 4 by column 5.
§ATEE used as substrate for assay.
serum albumin (used for I.V. injection) to 4% with Tris buffer. It may be prepared from crystalline albumin but the source must be shown to be free of trypsin inhibitor.

Chymotrypsin The manufacturer's assay was 3160 Armour units/gm.

Substrate for chymotrypsin Acetyl-L-tyrosine (AT) had a molar absorptivity of 350 at 237 nm. when 1 mM acetyl-L-tyrosine ethyl ester (ATEE) was used as the blank, which is similar to the findings of Schwert and Takenaka (16).

Procedure

Trypsin

Add, in duplicate, 1 ml. of an appropriate dilution of trypsin to 5 ml. of 1 mM BAPNA at 37°C. The reaction is allowed to proceed for exactly 10 min. and is terminated with 1 ml. of 30% acetic acid. The blank is similar except that the unknown trypsin is added after the acetic acid. The unknown is read against the blank at 400 nm. Absorbance units/min. ÷ 1510 = trypsin activity in μmoles/min. The reaction is linear with time to 20 min., and with trypsin concentration to an absorbance of 0.5.

Serum Trypsin Inhibitory Capacity

As discussed more fully below, it was found that a plot of the serum concentration vs. absorbance was not linear (Fig. 1). The reason for this was found to be related to the absence of protein in the trypsin control tube. Two methods may be used to circumvent this nonlinearity: (1) the assay may be done at 2 serum concentrations, or (2) a single serum concentration is used and an antiprotein-free protein (diluted human-serum albumin) is added to the trypsin control tube. Both procedures are given. For the assay of a large number of samples, the latter method with a single trypsin-albumin control is adequate, but it may be wise to check linearity by using 2 or more serum dilutions of selected samples.

The method has 2 timed steps of 10 min. each. The first is the preincubation of serum with trypsin, and the second, the assay of the trypsin inhibitory capacity. A single operator can conveniently assay 16 serum dilutions and controls.

Preliminary

(1) Prepare the serum dilutions in buffer. If a single assay of each serum is to be run, a serum dilution of 1:100 is prepared. If a second

*Obtained from Armour Pharmaceutical Co., Kankakee, Ill.
†Obtained from Cyclo Chemical Corp., New York, N. Y.
‡Obtained from Mann Research Laboratories, Inc., New York, N. Y.
dilution is used, a 1:250 dilution in buffer is satisfactory and can be prepared from the first dilution.

(2) If a single serum dilution is to be assayed, prepare a 1:100 dilution of 4% human-serum albumin in buffer for the control.

(3) Prepare working BAPNA solution as described under Reagents.

(4) Prepare working trypsin solution as described under Reagents.

Assay

(1) Three tubes are used for each serum dilution and control. Add 5 ml. of diluted substrate to each tube and place the rack in a water bath at 37°.

(2) In other tubes, pipet 2 ml. of each serum dilution. For single serum-dilution control use 2 ml. of diluted albumin; for double serum-dilution control use 2 ml. of buffer.

(3) At room temperature and at 0.5-min. intervals, add 2 ml. diluted trypsin to the serum dilutions and controls; mix.

(4) Exactly 10 min. later, and at 0.5-min. intervals for each set, add 1 ml. of the serum-trypsin mixture to each of 2 substrate tubes of each set; mix.

(5) Exactly 10 min. after addition of the serum-trypsin mixture, add 1 ml. of 30% acetic acid to each of the 3 tubes; mix.

(6) Subsequently, add 1 ml. of the appropriate serum-trypsin mixture or control-trypsin mixture to the third tube of each set for a blank. A separate blank is not necessary for each serum dilution. If the serums are matched for color and turbidity, a single blank may be used for each type.
(7) Read the absorbance of each pair of tubes against its blank at 400 nm in a Beckman DU spectrophotometer.

Calculations

When 2 serum concentrations are assayed, as in Fig. 2, the calculations are based on the difference in absorbance, $\Delta_2$, resulting from the difference in serum volume, 3 $\mu$L, between the 2 assays. When albumin is used in the control tube with a single serum dilution, 5 $\mu$L, the calculation uses the absorbance difference from the control tube, $\Delta_1$. The TIC may be calculated from:

$$\text{TIC} = \frac{7000}{10.5} \cdot \frac{\Delta}{x \cdot y}$$

where 7000 represents the assay volume in $\mu$L, 10.5 the mmolar absorptivity, $y$ the minutes of assay time, $x$ the $\mu$L of serum, and $\Delta$ the absorbance difference, either $\Delta_1$ or $\Delta_2$. As all assays were for 10 min., the equation can be given as:

$$\text{TIC} = \frac{66.6 \Delta}{x}$$

The results are expressed as mmoles of substrate-hydrolysis inhibited/min./L. of serum. A typical example may be taken from Fig. 2 where the absorbance of the control solution (with albumin) is 0.454 and of the solutions containing 2 and 5 $\mu$L. serum, 0.353 and 0.204, respectively.

$$\text{TIC by } \Delta_1 = \frac{66.6 \cdot (0.454 - 0.204)}{5} = 3.33 \text{ mmoles/min./L.}$$

$$\text{TIC by } \Delta_2 = \frac{66.6 \cdot (0.353 - 0.204)}{3} = 3.31 \text{ mmoles/min./L.}$$
Chymotrypsin

The method of Schwert and Takenaka (16) was adopted, using a Beckman DB spectrophotometer with a Sargent recorder to measure the absorbance decrease at 237 nm. at 25°. The material to be assayed (0.2 ml.) is added to 3 ml. of 1 mM ATEE in 5 mM phosphate buffer of pH 7. From the change in absorbance per minute the chymotrypsin activity was calculated as \( \mu \text{moles hydrolysis/min./mg.} \) (\( \mu \text{moles/min./mg.} \times 116 = \text{absorbance units/min./mg.} \)). The purified chymotrypsin preparation assayed 7200 ATEE absorbance units/min./mg. or 62.1 \( \mu \text{moles/min./mg.} \). For assay of the various trypsins, 0.2 ml. of stock solution (1 mg./ml.) was used. The results are given in Table 1.

Results and Discussion

Certain precautions are necessary to achieve reproducible results. In our early work, we frequently found it difficult to obtain the desired precision. The glassware had been washed with detergent in a dishwashing machine, and rinsed with tap and distilled water. We have been unable to determine the exact cause of the poor precision, but the detergent itself was not responsible. All glassware used in the assay was subsequently soaked for 1 hr. in 10% (v/v) HNO\(_3\) before the final rinse with distilled water. With this precaution, good duplication could be achieved. In 50 duplicate runs before the HNO\(_3\) wash was used, the difference between the absorbance of duplicates averaged 0.0059, with differences as great as 0.039. Following the use of the HNO\(_3\) wash, the mean difference was 0.0026 with no difference greater than 0.008.

Tris-buffer concentrations between 0.05 and 0.2 M were tried. The most consistent results were obtained with 0.1 M buffer. It is important that all dilutions of serum and trypsin be made with this buffer.

The effect of varying chymotrypsin content was studied by adding known amounts (w/w) of the purified chymotrypsin preparation to a trypsin, and observing the effect on the TIC assay. An example is shown in Fig. 3. At 1 \( \mu \text{g.-chymotrypsin enrichment/20 } \mu \text{g. trypsin, the curve is parallel to that of the trypsin alone. At 2 } \mu \text{g.-chymotrypsin enrichment, the slope of the line is changed. This effect increases with further increases in chymotrypsin content. For consistent results, then, the trypsin preparation should have a low chymotrypsin content. We would suggest that the trypsin not contain over 116 ATEE-absorbance units/min./mg. (1.00 \( \mu \text{mole/min./mg.} \)). Most commercial recrystallized trypsins meet this requirement. The only trypsin in Table 1 that varied significantly from this requirement was F; this lot was manufactured in 1957.

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As indicated in Fig. 1, a plot of serum concentration vs. absorbance was not linear. The difference between the value obtained by projecting the absorbance from the 2 serum concentrations to the ordinate, and the actual assay value of the trypsin control (Fig. 2), varied with the source of trypsin. The addition of small amounts of serum albumin in the control tube corrected this discrepancy. Various preparations of human and bovine serum albumin, human gamma globulin, and a preparation of a commercial serum control* devoid of antitryptic activity were equally effective.

Initially it was thought that this effect was due to adsorption of the dilute trypsin onto glass, with protection by added protein. However, the same effect was noted with plastic tubes. Furthermore, when a dilute trypsin solution was transferred successively through a series of 5 glass tubes allowing uniform incubation in each, the activity in the first and last tubes were identical. If glass adsorption was an important factor, the activity should have been decreased in the last tube. The amount of chymotrypsin in the trypsin preparation was not responsible because there was no relation between the chymotrypsin content of a given trypsin and the absorbance difference with and without albumin.

Various concentrations of trypsin were preincubated with 0.20 mg. albumin/ml. and the tryptic activity measured. The results are given in Fig. 4. The differences between the curves increased with increasing concentrations of trypsin. A similar experiment was done while re-

*Lab-Trol, Dade Reagents, Inc., Miami, Fla.
cording the change in absorbance: the slopes of the curves were always greater when albumin was present, though only if the trypsin and albumin were premixed before adding to the substrate. If the albumin was added after the trypsin had been added to the substrate, no change in slope resulted. The albumin preparations were assayed for trypsin-like activity and none was found, nor did the trypsin hydrolyze the albumin under the conditions used. All of these results suggest that albumin, and presumably other proteins, when premixed with trypsin, somehow heightens the activity of the trypsin, at least for BAPNA.

Several serums were assayed with all 8 trypsins by both $\Delta_1$ and $\Delta_2$ methods. An example for 1 serum is given in Table 2. The means for the 2 methods are identical. Deviations from each mean were within experimental error, and when calculated as paired experiments, there was no significant difference.

Both methods of assay were used on a group of 17 normal serums using 2 trypsins, one with a low (G) and 1 with a high (F) chymotrypsin content. The results are given in Table 3. When the means were compared, there were no significant differences. When the data were
treated as paired experiments, there were certain significant differences: (1) trypsin F gave lower results than trypsin G for both Δ1 and Δ2; this is possibly due to the high chymotrypsin content of trypsin F; (2) for trypsin G, Δ1 gave a slightly higher value than Δ2.

In another group of 15 normal serums assayed with trypsins A, F, and G by the Δ2 method, the means and the standard deviations were, respectively, 2.72 ± 0.37, 2.68 ± 0.36, and 2.72 ± 0.34. There were no significant differences between the means, and when treated as paired experiments in all combinations, there were no significant differences.

The serums of 30 normal men and 25 normal women were assayed with a trypsin of low chymotrypsin content (G) by the Δ1 method. Excluded from the analysis were 2 women who became ill shortly after blood was drawn and whose results were very high, and 2 men who gave results in the heterozygous range for α1-antitrypsin deficiency. One of these men was later shown, by family study, to be a heterozygote. The

### Table 2. Comparison of TIC-Assay Procedures

<table>
<thead>
<tr>
<th>Trypsin (source)</th>
<th>Δt</th>
<th>Δs</th>
<th>(mmoles/min./L.)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>2.56</td>
<td>2.70</td>
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</tr>
<tr>
<td>B</td>
<td>2.88</td>
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<tr>
<td>C</td>
<td>2.74</td>
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</tr>
<tr>
<td>D</td>
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</tr>
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<td>E</td>
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</tr>
<tr>
<td>F</td>
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</tr>
<tr>
<td>G</td>
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</tr>
<tr>
<td>H</td>
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<td>2.74</td>
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</tr>
<tr>
<td>Mean</td>
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<td>2.72</td>
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<tr>
<td>S.D.</td>
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<td>Coefficient of variation</td>
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### Table 3. Comparison of TIC-Assay Methods with 2 Trypsins*

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<th>Δt</th>
<th>Δs</th>
<th>P1</th>
<th>P4</th>
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</thead>
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<tr>
<td>F</td>
<td>2.75 ± 0.37</td>
<td>2.76 ± 0.37</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>G</td>
<td>2.94 ± 0.37</td>
<td>2.85 ± 0.37</td>
<td>N.S.</td>
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<table>
<thead>
<tr>
<th>Method</th>
<th>F</th>
<th>G</th>
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<th>P4</th>
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<tr>
<td>Δt</td>
<td>2.75 ± 0.37</td>
<td>2.94 ± 0.37</td>
<td>N.S.</td>
<td>0.001</td>
</tr>
<tr>
<td>Δs</td>
<td>2.76 ± 0.38</td>
<td>2.85 ± 0.37</td>
<td>N.S.</td>
<td>0.01</td>
</tr>
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*Assays done on 17 normal serums.
†Mean ± S.D. (mmoles/min./L.)
‡Calculated for differences in means.
§Calculated for paired experiments.
Table 4. TIC of Normal Serum

<table>
<thead>
<tr>
<th>No.</th>
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<tr>
<td>Men</td>
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<tr>
<td>Women</td>
<td>25</td>
<td>2.97</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Fig. 5. Distribution of TIC in normal persons. Assays of 55 normal sera from 30 men and 25 women.

Fig. 6. Distribution of TIC in 67 patients with pulmonary emphysema. Solid portion of bars, sera from 50 men in well-controlled study group; cross-hatched portion of bars, sera from 17 acutely ill patients.

results are given in Table 4. The mean value for women was significantly higher than the mean value for men. Fig. 5 shows the distribution of the values; the values for women are skewed to the right. The cause for the higher value in women is not clear. Perhaps it is hormonal, as in pregnancy (2), or perhaps it is related to the menses.

The TIC was measured in several groups of male patients with pulmonary emphysema. One group comprised 50 subjects who were selected for long term follow-up on the basis of well documented bronchitis and emphysema. Many of them were fairly stable clinically. The other group was smaller and consisted of patients with emphysema, randomly selected from a pulmonary-disease ward; many of them were desperately ill. The distribution of values for both groups is shown in Fig. 6. Both groups have many high values, particularly the randomly drawn group, which is consistent with their poor clinical status. In the stable group there are several patients with low values. One value (TIC = 0.54) is in the range for homozygotes for \( \alpha_1 \)-antitrypsin deficiency given by Eriksson (9). Family study revealed 2 siblings in the
heterozygous range, confirming that the patient is a homozygote for the antitrypsin deficiency. This pedigree with probable genotype is given in Fig. 7.

Adjusting the results of Eriksson (9) to the units of serum TIC presented in this paper, the expected upper limit for homozygotes for \( \alpha_1 \)-antitrypsin deficiency should be 1.00–1.05. The range for heterozygotes should extend from 1.05 to 2.05–2.10. There is bound to be some overlap between heterozygotes and normal subjects since heterozygotes apparently have the ability to increase the serum concentration of \( \alpha_1 \)-antitrypsin under appropriate stimulation. Eriksson (9) suggests that individuals with TIC values in the low normal or borderline range may be suspected of being heterozygotes by finding an increased \( \alpha_2 \)-globulin determined electrophoretically. Elevations of \( \alpha_2 \)-globulin are consistent with an inflammatory disorder, and might indicate that the TIC is elevated due to stimulation. It may be that, in such conditions, the \( \alpha_2 \)-antitrypsin rises and contributes to the elevated TIC, but there is no information available on this point.

References


