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Influence of elastin on the inhibition of leucocyte elastase by α_1-proteinase inhibitor and bronchial inhibitor

Potent inhibition of elastin-bound elastase by bronchial inhibitor

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We have investigated the effect of human lung elastin on the inhibition of human leucocyte elastase by human α_1-proteinase inhibitor and bronchial inhibitor. Elastin was unable to dissociate the elastase–inhibitor complexes during the 150 min of the elastolysis reaction. When elastase was added to mixtures of elastin and α_1-proteinase inhibitor, it was fully bound to the latter. The competition between elastin and bronchial inhibitor was also in favour of the latter, but a 1.5 molar excess of inhibitor over elastase was required to achieve total binding of the enzyme. About 25% of elastin-bound elastase was found to be resistant to the inhibitory effect of α_1-proteinase inhibitor. The major isoenzyme and the mixture of the three minor isoenzymes of elastase exhibited similar behaviour. By contrast, bronchial inhibitor was as efficient in inhibiting the elastin-bound elastase as it was in inhibiting the free enzyme. This inhibitor was also able to inhibit fully the fraction of elastin-bound elastase that was resistant to α_1-proteinase inhibitor. We also describe a rapid procedure for the isolation of gram quantities of α_1-proteinase inhibitor.

INTRODUCTION

Elastin is an important structural protein found in large amounts in mammalian lung and arteries. It is commonly believed that destruction of this macromolecule by neutrophil elastase leads to lung emphysema (Snider, 1981). The alveolar structures of normal individuals are protected from elastolysis by α_1PI, a fast-acting irreversible HLE inhibitor (Beatty *et al.*, 1980). BrI, another potent elastase inhibitor, is present in large amounts in the upper respiratory tract (Hochstrasser *et al.*, 1972; Ohlsson & Tegner, 1976), but has also been demonstrated in small amounts in peripheral airways (Mooren *et al.*, 1983; Abrams *et al.*, 1984; Boudier *et al.*, 1985). Since brI binds so quickly and so tightly to HLE (Gauthier *et al.*, 1982; Smith & Johnson, 1985), it may *a priori* (Bieth, 1980) play a physiological function at the alveolar level, despite its low concentration at that site.

If HLE is released from neutrophils in the lung interstitium, it will be faced with at least three competing macromolecules: α_1PI, brI and elastin. This raises the following questions: (i) is elastin able to dissociate the HLE–inhibitor complexes? (ii) do the inhibitors favourably compete with elastin for the binding of HLE? (iii) are α_1PI and brI able to inhibit elastin-bound HLE? In the present paper we have designed experiments aimed at answering these questons.

MATERIALS AND METHODS

Human elastin was isolated from a non-emphysematous lung by hot alkali treatment as described previously (Boudier *et al.*, 1981). Bovine trypsin came from Worthington and was active-site-titrated with nitrophenyl *p*-guanidinobenzoate (Nutritional Biochemical Co.) (Chase & Shaw, 1967). HLE was isolated from purulent sputum using the procedure of Martodam *et al.* (1979). The preparation was homogeneous as judged by SDS/polyacrylamide-gel electrophoresis and contained 83% of active enzyme as assessed by active-site titration using acetylalanylalanylaza-alanine *p*-nitrophenyl ester (Powers *et al.*, 1984), a gift from Dr. J. C. Powers, Georgia Institute of Technology, Atlanta, GA, U.S.A. The isoenzymes of HLE were partially purified using a fast-protein-liquid-chromatography apparatus and a Mono Q column from Pharmacia. A linear gradient from 0.45 M- to 0.55 M-NaCl in 50 mM-Hepes buffer, pH 7.4, resolved the HLE preparation into two peaks, the first containing isoenzymes E_1, E_2 and E_3 and the second being composed of isoenzyme E_4 (in the nomenclature of Baugh & Travis, 1976). The two peaks represented 61 and 39% of the total elastase activity respectively.

brI was isolated from non-purulent sputum collected from non-infected chronic-bronchitis patients using the procedure of Smith & Johnson (1985). The protein gave one single band on polyacrylamide-gel electrophoresis at pH 4.0 and two major bands and a few minor bands on SDS/polyacrylamide-gel electrophoresis in presence of β-mercaptoethanol, in agreement with a previous report (Smith & Johnson, 1985). brI, as well as dog submandibular-gland inhibitor, a gift from Dr. H. Fritz, University of Munich, were titrated with active-site-titrated HLE and succinyltrialanine *p*-nitroanilide as described in Fig. 2.

Human plasma α_1PI was isolated in gram quantities by using a novel procedure derived from published methods (Pannell *et al.*, 1974; Kurechi *et al.*, 1979). The precipitate obtained between 50 and 80% $(NH_4)_2SO_4$ saturation of 2 litres of fresh plasma was solubilized with 300 ml of 50 mM-Tris/HCl buffer, pH 8.0, and dialysed against the same buffer. This solution was then passed through a 4×80 cm zinc chelate–Sepharose column (Kurechi *et al.*, 1979) previously equilibrated with the above buffer containing 150 mM-NaCl. The column was

Abbreviations used: α_1PI, α_1-proteinase inhibitor (α_1-antitrypsin); brI, bronchial inhibitor; HLE, human leucocyte elastase.

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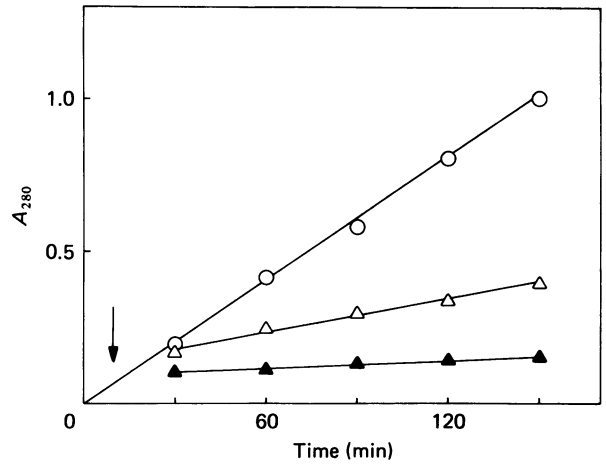

Fig. 1. Kinetics of human lung elastin solubilization by 1 μM-HLE in the absence (○) or the presence of 1.2 μM-α_1PI (△) or 1.2 μM-brI (▲)

HLE was allowed to react for 10 min with elastin before addition of the inhibitors (see the arrow). The solubilization of elastin (5 mg/ml) was performed at pH 8.0 (50 mM-Tris/HCl/150 mM-NaCl) and 37 °C and monitored as described by Boudier *et al.* (1981). Briefly, samples from the continuously stirred suspensions were removed every 30 min, acidified, centrifuged at 4000 *g* for 15 min and read at 280 nm. One absorbance unit corresponds to 15% elastin solubilization.

developed with 50 mM-sodium phosphate/150 mM-NaCl, pH 6.5, at a flow rate of 100 ml/h. The α_1PI-containing fractions were pooled and passed through a 5 cm × 80 cm Cibacron Blue–Sepharose column (Pannell *et al.*, 1974) equilibrated and washed with 50 mM-sodium phosphate, pH 6.9. Under these conditions, α_1PI does not bind to the (matrix and is recovered in the unretarded protein) fraction. At this stage, overloaded SDS/polyacrylamide-gel electrophoresis shows that the inhibitor is at least 95% pure. The minor impurities were removed by using a 4 cm × 50 cm DEAE-Trisacryl column equilibrated with 5 mM-sodium phosphate/30 mM-NaCl, pH 6.5. The inhibitor was eluted from this column with a linear gradient from 30 mM- to 200 mM-NaCl. This simple method yields at least 1 g of electrophoretically and immunologically pure α_1PI. Titration of the inhibitor with active-site-titrated bovine trypsin (Beatty *et al.*, 1980) or HLE (Fig. 2 below) indicated that the preparation was 97% active towards both enzymes.

The binding of HLE to elastin was investigated by adding the enzyme to continuously stirred suspensions of elastin in 50 mM-Tris/HCl/150 mM-NaCl, pH 8.0, 37 °C. The final concentrations of HLE and elastin were 1 μM and 5 mg/ml respectively. After selected periods of time, elastin was removed by sedimentation and the elastase activity was measured with succinyltrialanine *p*-nitroanilide (Bieth *et al.*, 1974). Further experimental details are given in the legends to the Figures.

RESULTS AND DISCUSSION

In an attempt to answer the three questions outlined in the Introduction, we have investigated (i) the effect of elastin on the stability of HLE–α_1PI and HLE–brI complexes, (ii) the competition between elastin and α_1PI or brI for the binding of HLE, and (iii) the inhbiition of elastin-bound HLE by α_1PI and brI. All rates of elastolysis were measured by monitoring the solubilization of human lung elastin with respect to time as shown in Fig. 1. Hence, precise velocity data could be obtained. The kinetic method has also the advantage of detecting elastin-induced dissociation of elastase–inhibitor complexes (upward-concave curves) or assessing inhibitor-induced dissociation of the elastin–elastase complex (downward-concave curves). More than 95% of HLE was bound to elastin within less than 1 min under the experimental conditions described in Fig. 1. The fast and strong binding of HLE to elastin indicates that the latter may be viewed as a potential dissociating agent of HLE–inhibitor complexes or may significantly compete with inhibitors for the binding of HLE.

Fig. 2 reports experiments in which the residual enzymic activity of mixtures formed of various concentrations of inhibitors and constant concentrations of HLE was measured with either succinyltrialanine *p*-nitroanilide or elastin. The time course of the solubilization of the latter was linear in all cases. Whatever the substrate, α_1PI yielded a straight inhibition curve up to 100% inhibition. This is in accord with the irreversible character of the enzyme–inhibitor interaction (Pannell *et al.*, 1974; Beatty *et al.*, 1980). The data obtained with brI were identical with those found with α_1PI, although the former protein is a reversible elastase inhibitor (Ohlsson & Tegner, 1976; Gauthier *et al.*, 1982). The following reasoning may rationalize this finding. The K_i of the HLE–brI complex lies between 12 and 187 pM, depending upon the experimental conditions used (Gauthier *et al.*, 1982; Smith & Johnson, 1985). Even if we take the highest K_i value, the ratio of total enzyme concentration to K_i is 5×10^2 and 5×10^3 in the experiments using succinyltrialanine *p*-nitroanilide and elastin respectively. Theory predicts that, under these conditions, straight inhibition curves are obtained provided the substrate does not significantly dissociate the enzyme–inhibitor complexes during the measurement of residual enzyme activity (Bieth, 1974). Hence, it may be concluded that elastin does not dissociate the HLE–brI complex. To illustrate the importance of the magnitude of K_i on the stability of reversible elastase–inhibitor complexes in presence of elastin, we have used dog submandibular-gland inhibitor, whose K_i for HLE is about 5 nM (Schiessler *et al.*, 1977). Fig. 2 shows that the titration curve obtained with the synthetic substrate is linear up to at least 80% inhibition, in accord with theory. With elastin, however, the inhibition curve is concave, indicating that this substrate efficiently dissociates the elastase–inhibitor complex. The solubilization of elastin was linear with time for all mixtures, suggesting that the dissociation process was fast.

Fig. 3 shows the results of competition experiments in which constant amounts of HLE were added to suspensions formed of various amounts of α_1PI or brI and constant amounts of elastin. Identical results were obtained when HLE and the inhibitors were added simultaneously to elastin (results not shown). The time course of the solubilization of the latter was again linear in all cases. The α_1PI gave an inhibition pattern identical with that shown in Fig. 2. This demonstrates that HLE binds much more quickly to α_1PI than to elastin. The curve obtained with brI was similar to that observed in Fig. 2, except that some deviation from linearity was

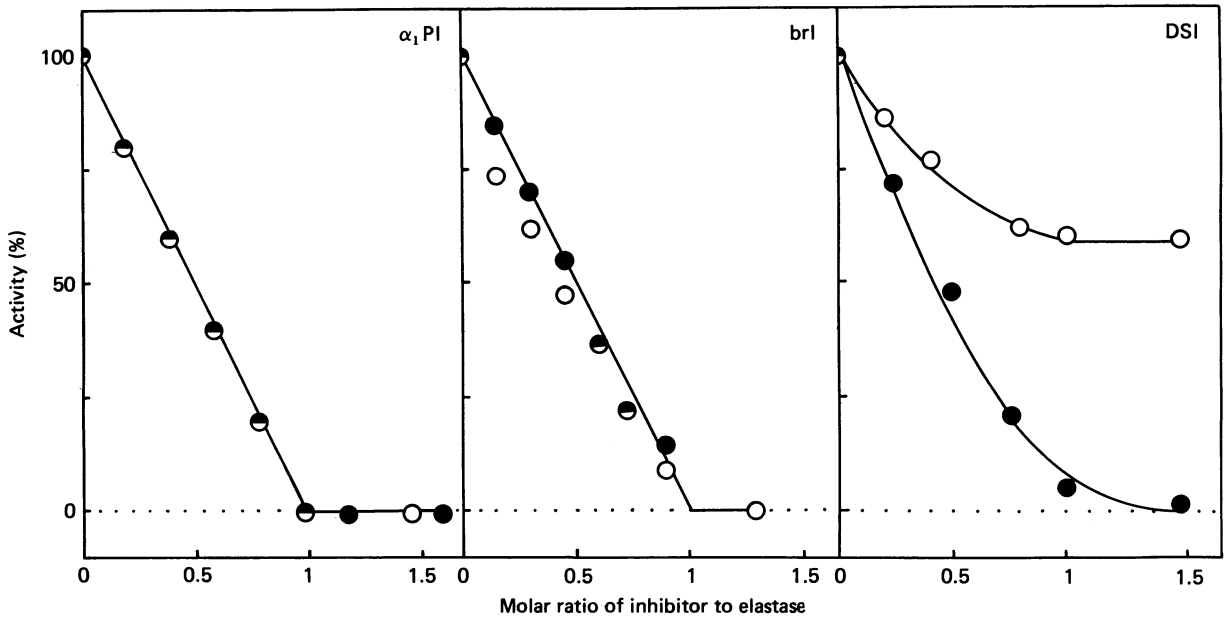

Fig. 2. Stability of elastase–inhibitor complexes in the presence of substrate

The complexes were formed by allowing 2 μM-HLE to react with various concentrations of α_1PI, brI or dog submandibular-gland inhibitor (DSI) in 50 mM-Tris/HCl/150 mM-NaCl, pH 8.0. The preincubation times were 2 min (α_1PI or brI) and 15 min (DSI) at 25 °C. One part of each mixture was diluted tenfold in 0.8 mM-succinyltrialanine *p*-nitroanilide (●) to measure the residual amidolytic activity of elastase (final concn. 0.2 μM) (Bieth *et al.*, 1974). The other part was mixed with an equal volume of lung elastin suspension (○) to assay the residual elastolytic activity of elastase (final concn. 1 μM) (see the legend to Fig. 1).

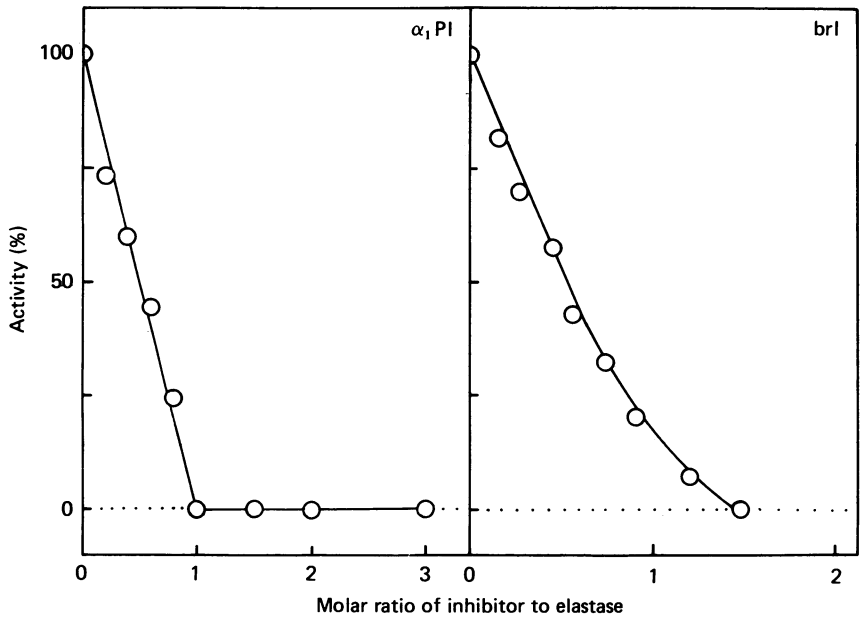

Fig. 3. Competition between elastin and α_1PI or brI for the binding of HLE

Various concentrations of the inhibitors were mixed with lung elastin suspended in 50 mM-Tris/HCl/150 mM-NaCl, pH 8.0. After 10 min of continuous stirring at 37 °C, 1 μM-HLE was added to each medium. The solubilization of elastin was then monitored as described in the legend to Fig. 1.

visible near the equivalence point. Separate experiments confirmed that a 1.5-fold molar excess of brI over HLE was required to get full elastase inhibition in this competition experiment. The slight differences in potencies between α_1PI and brI could arise from the differences in their inhibition mechanisms (irreversible as against reversible) and association rates of HLE [$k_{ass.}$ (α_1PI) < $k_{ass.}$ (brI); Gauthier *et al.*, 1982; Smith & Johnson, 1985]. From these experiments it may be concluded that α_1PI and brI favourably compete with elastin for the binding of HLE and exhibit identical anti-elastase potencies if their concentration exceeds that of HLE, a condition that certainly prevails *in vivo*.

Fig. 4 depicts the action of the two inhibitors on

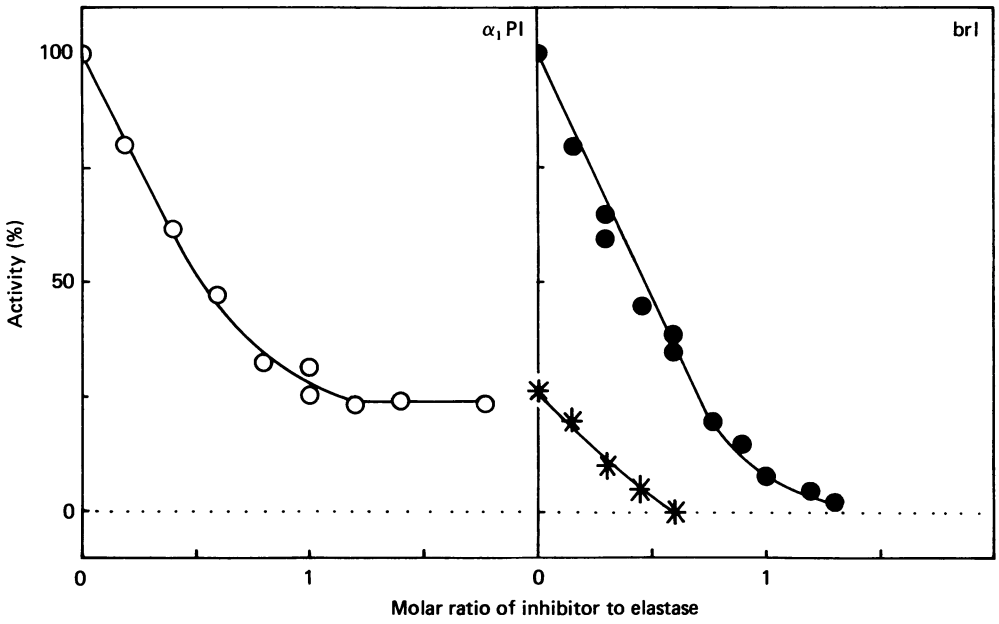

Fig. 4. Inhibition of elastin-bound elastase by α_1PI (○), brI (●) or α_1PI followed by brI (*)

In the last experiment, the concentration of α_1PI was constant (1.2 μM) while that of brI varied from 0.15 to 0.60 μM. The concentration of HLE was 1 μM throughout. Elastin solubilization was monitored as described in the legend to Fig. 1.

elastin-bound elastase. Here we observed significant differences between α_1PI and brI. The latter was as potent on elastin-bound elastase as on the free enzyme. By contrast, the inhibition curve for α_1PI deviated from linearity after about 50% inhibition and levelled off at about 75% inhibition. Higher inhibitor concentrations did not change the residual elastase activity (results not shown). The solubilization of elastin was linear with time for both inhibitors (for an example, see Fig. 1). HLE is isolated as a mixture of four isoenzymes labelled E_1–E_4 (Baugh & Travis, 1976). We have isolated the major isoenzyme, E_4, and repeated the above experiment with this elastase and with the mixture of $E_1+E_2+E_3$. We have obtained results comparable with those shown in Fig. 4 and therefore conclude that the partial resistance of elastin-bound elastase to the inhibitory action of α_1PI is not related to the heterogeneity of the HLE preparation. We have also investigated the effect of sequential addition of α_1PI and brI on the activity of elastin-bound elastase. The former inhibitor was used at a constant concentration, whereas the concentration of the latter was varied. Fig. 4 shows that brI readily inhibited the α_1PI-resistant elastase activity; full inhibition was observed for a molar ratio of brI to HLE of 0.6.

The partial resistance of elastin-bound elastase to the inhibitory action of α_1PI was previously demonstrated by Reilly & Travis (1980) and Hornebeck & Schnebli (1982). These authors observed inhibition patterns similar to that shown here. The property of brI to inhibit elastin-bound elastase fully has not heretofore been reported. However, Hornebeck & Schnebli (1982) have demonstrated that matrix-bound elastase readily reacts with eglin C, another low-molecular-mass proteinase inhibitor. Thus the anti-elastase potential of brI appears to be superior to that of α_1PI.

It has been shown that addition of α_1PI or serum to the HLE–brI complex resulted in a transfer of HLE from brI to α_1PI (Gauthier *et al.*, 1982; Fryksmark *et al.*, 1983). This finding, together with the present data, favours the published hypothesis on the respective roles of α_1PI and brI at the alveolar level (Gauthier *et al.*, 1982): " . . . α_1-proteinase inhibitor inhibits free elastase and maintains bronchial inhbiitor in a free state by continuously removing elastase bound to it; bronchial inhibitor, on the other hand, functons as an inhibitor of elastin-bound elastase."

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REFERENCES

Abrams, W. R., Fein, A. M., Kucich, U., Kueppers, F., Yamada, H., Kuzmowycz, T., Morgan, L., Lippman, M., Goldberg, S. K. & Weinbaum, G. (1984) Am. Rev. Respir. Dis. **129**, 735–741

Baugh, R. J. & Travis, J. (1976) Biochemistry **15**, 836–841

Beatty, K., Bieth, J. & Travis, J. (1980) J. Biol. Chem. **255**, 3931–3934

Bieth, J. (1974) in Proteinase Inhibitors (Fritz, H., Tschesche, H., Greene, L. & Trutscheit, E., eds.), pp 463–469, Springer, Berlin

Bieth, J. G. (1980) Bull. Eur. Physiopathol. Respir. **16** (suppl.), 183–195

Bieth, J., Spess, B. & Wermuth, C. G. (1974) Biochem. Med. **11**, 340–357

Boudier, C., Holle, C. & Bieth, J. G. (1981) J. Biol. Chem. **256**, 10256–10258

Boudier, C., Pelletier, A., Gast, A., Tournier, J. M., Pauli, G. & Bieth, J. G. (1985) Am. Rev. Respir. Dis. **131**, A59

Chase, T. & Shaw, E. (1967) Biochem. Biophys. Res. Commun. **29**, 508–514

Fryksmark, U., Ohlsson, K., Rosengren, M. & Tegner, H. (1983) Hoppe-Seyler's Z. Physiol. Chem. **364**, 793–800

Gauthier, F., Fryksmark, U., Ohlsson, K. & Bieth, J. G. (1982) Biochim. Biophys. Acta **700**, 178–183

Hochstrasser, K., Reichert, R., Schwartz, S. & Werle, E. (1972) Hoppe-Seyler's Z. Physiol. Chem. **353**, 221–226
Hornebeck, W. & Schnebli, H. P. (1982) Hoppe Seyler's Z. Physiol. Chem. **363**, 455–458
Kurechi, T., Kress, L. F. & Laskowski, M., Sr. (1979) Anal. Biochem. **99**, 415–420
Martodam, R. R., Baugh, R. J., Twumasi, D. Y. & Liener, I. E. (1979) Prep. Biochem. **9**, 15–31
Mooren, H. W. D., Kramps, J. A., Franken, C., Meijer, C. J. L. M. & Dijkman, J. A. (1983) Thorax **38**, 180–183
Ohlsson, K. & Tegner, H. (1976) Scand. J. Clin. Lab. Invest. **36**, 437–445
Pannell, R., Johnson, D. A. & Travis, J. (1974) Biochemistry **13**, 5439–5445
Powers, J. C., Boone, R., Carroll, D. L., Gupton, B. F., Kam, C. M., Nishino, N., Sakamoto, M. & Tuhy, P. M. (1984) J. Biol. Chem. **259**, 4288–4294
Reilly, C. F. & Travis, J. (1980) Biochim. Biophys. Acta **621**, 147–157
Schiessler, H., Ohlsson, K., Olsson, I., Arnhold, M., Birk, Y. & Fritz, H. (1977) Hoppe-Seyler's Z. Physiol. Chem. **358**, 53–58
Smith, C. E. & Johnson, D. A. (1985) Biochem. J. **225**, 463–472
Snider, G. L. (1981) Med. Clin. North Am. **65**, 647–665

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