Structural Investigation of Loose Connective Tissue by Using a Series of Dextran Fractions as Non-interacting Macromolecular Probes

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The ability of the uncharged open-coil dextran molecules to penetrate tissue space, without coil-shape change, was utilized to probe (by partitioning experiments) the structural arrangement of the collagen-fibre network and the proteoglycan system. Hyaluronidase digests most of the proteoglycans away and enables the respective contributions to the exclusion volume to be evaluated by using a series of different-molecular-weight dextrans. It appears that the major part of the exclusion volume is due to the collagen-fibril system. The exclusion due to collagen could be understood by regarding the 54 nm collagen fibril as a rod and the dextran coil as an impenetrable sphere. The additional exclusion due to the proteoglycans could be accounted for by a set of points (regions of high proteoglycan-segment density) over which the dextran coils cannot pass. These points are an average of 50 nm apart and are indicative of local extensive entanglement of high-molecular-weight proteoglycans with each other. Reasons are given why these entanglements could not act as cross-links in long-term elastic loading of the tissue.

Connective tissue, in particular loose connective tissue, can be viewed as built up of a set of formed, fixed, structurally interconnected components interspersed with an aqueous solution of various diffusible species, including NaCl, metabolites and most serum proteins. The formed, fixed elements involve in particular the collagen-fibre network. This is the skeletal form-giving structure, essentially macroscopic in dimension, which has a molecularly disperse proteoglycan system tied up into it, in an as yet unknown fashion.

In an earlier investigation of tissue structure and function (Meyer & Silberberg, 1974) we could show that when slices of umbilical cord are exposed to various protein and other solutions *in vitro* swelling almost always occurs and the fibre network becomes expanded. Predigesting by testicular hyaluronidase prevents swelling; treatment of the already swollen tissue slice with the enzyme results in a return of the tissue sample to close to its original volume and shape. Since the enzyme treatment effectively removes most of the proteoglycan system the de-swelling step must occur under the influence of the collagen-fibre network whose distension has become unresisted and which relaxes to its unstressed state.

Electron-microscopic investigation of the tissue in these various states (Meyer & Silberberg, 1974) showed that neither prolonged swelling nor treatment with hyaluronidase changed the appearance of the fibre network. This was seen to be a fibril system with some ten to 100 parallel fibrils composing a fibre. The fibres themselves have no preferred orientation. The fibrils are of 54 nm mean diameter and are on the

average found to be 110 nm apart, fibril cylinder axis to neighbouring fibril cylinder axis.

It was concluded that the use of hyaluronidase allows the effects due to the proteoglycans and collagen systems to be studied separately. It appears that the collagen-fibre network exerts no measurable effect on the chemical potentials of the interstitial components when non-distended; but that the proteoglycan system exercises great influence on these quantities and thus controls tissue swelling.

The present investigation extends this study and uses a series of non-specifically interacting open-chain macromolecular probes.

The present investigation was undertaken to determine the accessibility of the tissue space and the exclusion properties of its various elements. In particular we investigated the structural relationship between the proteoglycan network, and the easily studied and already known build-up of the collagen-fibre network by using treatment with hyaluronidase to separate the various exclusion effects.

As in the earlier investigation (Meyer & Silberberg, 1974), umbilical cord is used as representative of loose connective tissue. Dextran fractions of different molecular weight are the macromolecular probes.

Materials and Methods

Tissue samples

Human umbilical cords were collected under sterile conditions immediately after delivery and stored over gauze moistened with buffer in closed

Table 1. *Characterization of probe macromolecules*
The r_s values were calculated from eqn. (1).

Dextran fraction	$[\eta]$ (dl/g)	$10^{-5} \times M_n$ (g)	r_s (nm)
70	0.25*	0.42	5.5
150	0.37*	0.86*	8.0
500	0.53*	1.95*	11.7
2000	0.70*	3.72†	16.0

* Supplier's specification.

† Estimated by linear extrapolation of a plot of $\log \eta$ against $\log M_n$ for dextran fractions of various molecular weights.

jars at 4°C until use. Experiments were started within 24 h of delivery. Slices about 2 mm thick (200–300 mg) were cut from the cord, washed carefully in buffer to remove blood from the section surface and then superficially blotted. The dry weight of the tissue was determined by heating samples to constant weight at 60°C.

Macromolecular probes

Dextran fractions 70, 150, 500 and 2000 (Pharmacia, Uppsala, Sweden) were used. The size of the dextran molecule is calculated from the intrinsic viscosity and expressed in terms of the radius, r_s , of the equivalent Einstein sphere from the equation

$$[\eta] = 2.5 \left(\frac{4\pi}{3} r_s^3 \right) (N_A/M_n) \times 10^{-23} \quad (1)$$

where $[\eta]$, the intrinsic viscosity, is expressed in dl/g; r_s is expressed in nm; N_A is Avogadro's number and M_n is the number-average molecular weight (see Table 1) of the fraction concerned.

Solutions

Buffer. Phosphate-buffered saline solution (0.127 M-NaCl/0.017 M-Na₂HPO₄, 12H₂O/0.0085 M-HCl), pH 7, was used.

Dextran solutions. Stock solutions of 10% (w/v) dextran in buffer were prepared by dissolving the dry powder at room temperature. The other concentrations used (2.5, 5 and 7.5%) were obtained by dilution with buffer.

Concentration determination of solutions

Concentrations were determined refractometrically by using a hand sugar refractometer (Mitsubishi Scientific Works, Tokyo, Japan). One drop of solution was sufficient for the measurement. Calibration curves were derived from standard solutions of the dextrans prepared by dilution.

Experimental procedure

When tissue samples of known weight are incubated and allowed to equilibrate with known amounts of dextran solution of known concentration, both solvent and dextran can be seen to redistribute; however by monitoring total weight, tissue sample weight and the dextran concentration in the outside medium, all transfers can be calculated and followed.

A problem arises, however, owing to the presence of diffusible protein in the untreated tissue slices. This will also redistribute and if not allowed for, would interfere with the refractometric dextran concentration determination. To circumvent this, the tissue was washed free of protein by preincubating the tissue slices against excess of buffer. This could be done, either by preincubation with unhindered swelling or by preincubation with washing under conditions of fixed tissue volume. Both approaches were tried, although the latter procedure has the advantage that the accuracy of determining the distribution coefficient is higher if tissue volume increases are prevented. It was, however, considered important to determine whether volume changes of the tissue could affect the extent of the volume excluded to the probes.

Specifically the procedures adopted were the following.

Procedure (a): removal of diffusible proteins by dialysis before dextran-distribution determination (no volume restrictions). Weighed tissue samples were mounted on weighed platinum wires, dialysed for several days in excess of buffer to remove diffusible protein, placed in a small plastic bottle of known weight and then reweighed. After that, macromolecular solution of known concentration in buffer was added to cover the sample, the total weight was taken and one drop was immediately removed for an initial concentration determination. The tissue sample was then carefully lifted above the solution on its wire, allowed to drain briefly against the inside of the container and then removed to obtain the initial wet weight. After the sample was restored the whole system was weighed again to measure the loss in weight due to evaporation and due to the drop of sample removed for the concentration measurement.

This procedure was repeated as a function of time till the system reached equilibrium, generally within one week.

Procedure (b): removal of diffusible proteins by dialysis before dextran-distribution determination. The volume was restricted to the original (native) value by the following two methods.

(i) Fixation by glutaraldehyde. Tissue slices were cut, cleaned of blood as described above and placed inside a rubber O-ring of appropriate size placed between two rigid nitrocellulose porous filters. The whole structure was held together by a rubber-band as shown in Fig. 1(a). This system was placed in excess

Fig. 1. Cells for partitioning experiments at constant volume

(a) 1, rigid nitrocellulose porous filter disc; 2, rubber band; 3, rubber O-ring; 4, tissue sample. (b) 1, three-part stainless-steel-cell assembly; 2, stainless-steel-meshwork filters; 3, nut; 4, tissue sample; 5, screw.

of buffer solution for dialysis and then fixed overnight in buffer containing 2% (w/v) glutaraldehyde, the excess of which was afterwards washed out. The tissue was then taken out of the filter, mounted on platinum wire and treated according to procedure (a). Glutaraldehyde-fixed tissue has lost the ability to swell or deswell.

(ii) Confinement to constrained space. To circumvent the use of glutaraldehyde for controlling tissue volume, weighed samples were placed in a stainless-steel cell between two stainless-steel-meshwork filters held together by brass screws (see Fig. 1b). Tissue slices were selected so that the available space in the cell was taken up as much as possible. The system was then placed in excess of buffer for dialysis. After

washing out the protein, the cell was opened, the tissue lightly blotted and reweighed. The tissue was then placed back into the cell, the system put into a plastic bottle of matching size and macromolecular solution added to cover it. The amount added was determined by weighing and the change in concentration followed until equilibrium, as in procedure (a). At that point the cell was opened and the tissue weight retaken.

Infection was minimized by using semi-sterile conditions and by incubating at 4°C in the presence of antibiotics (100 i.u. of penicillin/ml and 100 µg of streptomycin/ml) in all cases.

The concentration of macromolecules inside the tissue was calculated from material balance con-

siderations. Tissue volumes were calculated from tissue weights assuming a density of 1.03 gm/cm³ for unswollen tissue.

Treatment with hyaluronidase

Testicular hyaluronidase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.), which digests tissue glycosaminoglycans was usually added together with the macromolecular solute in an amount of 1200 USP units/tissue section. In some experiments hyaluronidase was added during the preincubation step.

The amount of hyaluronidase added is small enough not to affect the concentration determinations.

Results and Discussion

Dextran molecules are non-interacting probes: calculation of excluded volume

Plots of dextran concentration, γ , in the tissue against concentration, α , in the outside solution at equilibrium were linear. In all cases they followed the relationship

$$\gamma = K\alpha \quad (2)$$

where K , the distribution coefficient, is found to be smaller than 1. This is as expected in the case of non-

interacting probes and K can thus be given a simple geometric interpretation. If the total tissue volume is V_{total} and the volume excluded geometrically is $V_{\text{exc.}}$

$$K = 1 - V_{\text{exc.}}/V_{\text{total}} \quad (3)$$

Since exclusion in these systems is mainly due to the fibrous collagen material, it is convenient to express excluded volume on a per g of collagen basis. If C_{collagen} (g/ml) is the concentration of collagen in the tissue when its total volume is V_{total} , the excluded volume, $V'_{\text{exc.}}$, per g of collagen is given by

$$V'_{\text{exc.}} = V_{\text{exc.}}/V_{\text{total}}C_{\text{collagen}} \quad (4)$$

Of the dry weight of umbilical cord 70% is taken to be collagen, as previously described (Meyer & Silberberg, 1974).

The results obtained are summarized in Tables 2 and 3 for intact and hyaluronidase-treated tissue respectively.

The individual results show considerable scatter but the data, as available, do not show significant differences. This implies that the excluded volume is independent of swelling [the essential difference between procedures (a) and (b) in the case of intact tissue], and that the use of glutaraldehyde [procedure (bi)] is as effective and as non-specific as clamping the tissue mechanically according to procedure (bii).

Table 2. *Dextran-excluded volumes*

Procedure (a) was performed by the removal of diffusible proteins by dialysis before dextran-distribution determination; there were no volume restrictions. Procedure (b) was performed by the removal of diffusible proteins by dialysis before dextran-distribution determination. The volume was restricted to the original (untreated) value by: (i) fixation by glutaraldehyde; (ii) confinement to constrained space. All the results have been equally weighted for the calculating of the mean, since no significant differences could be detected.

Dextran fraction	$V'_{\text{exc.}}$ (ml/g of collagen)			
	Procedure (a)	Procedure (bi)	Procedure (bii)	Mean
70	3.1			3.1
150	2.6	4.2	4.5	3.8
500	4.3, 4.1	4.6, 4.9	5.0, 5.1, 5.1, 6.0	4.9
2000	7.2, 6.6	6.2, 7.2	5.9, 6.6, 7.3, 7.1	6.8

Table 3. *Dextran-excluded volumes in hyaluronidase-treated tissue*

Dextran fraction	$V'_{\text{exc.}}$ (ml/g of collagen)			
	Procedure (a)	Procedure (bi)	Procedure (bii)	Mean
70	2.9			2.9
150	3.0	3.8	3.9	3.6
500	5.0, 4.6, 3.2, 3.7, 3.3	4.2, 4.7	3.8, 3.5	4.0
2000	2.8, 4.2, 4.9, 3.0, 4.8, 4.1, 4.2, 5.1	6.0, 4.5	4.4, 4.7, 4.6, 3.0	4.3

Fig. 2. Excluded volume per g of collagen (V'_{exc}) for different molecular weight dextran probes (M_n)

●, Untreated intact tissue; ○, hyaluronidase-digested tissue. The total volume, V_{total} , ml/g of collagen (based on 84 mg of collagen/ml of tissue) (---), the self volume of 1 g of collagen ($\bar{v}$, ■) and the additional volume in the fibril ($\Delta\bar{v}$, ■) are indicated. The arrow gives the minimum excluded volume if the interfibrillar space within fibre bundles were inaccessible to the probes.

The average values of V'_{exc} are plotted as a function of dextran molecular weight in Fig. 2. In addition, Fig. 2 gives the total tissue volume/g of collagen (about 12 ml, in the case of untreated umbilical cord) as the upper broken line and the partial specific volume $\bar{v}$ of collagen (corresponding to 0.75 ml) (Boedtke & Doty, 1956) as the lower shaded section. This represents the volume occupied by 1 g of collagen, but since collagen in its fibrillar state is structurally non-compact, the actual volume requirements for fibrils per g of collagen are bigger. Katz & Li (1973) showed that an additional $\Delta\bar{v}$ of 1.14 ml is associated for each g of collagen in the case of fibrils assembled from molecular collagen and a slightly larger volume in the case of native fibres. The line at 1.89 ml in Fig. 2 takes account of this additional space requirement. A volume of 1.89 ml may thus be regarded as the volume/g of collagen absolutely inaccessible to any macromolecular entity. The data fit well to these limits and tend towards the point (approx. 2 ml) set by the fibrillar self volume for zero-molecular-weight probes.

Exclusion due to the collagen fibril network alone

The enzyme treatment eliminates hyaluronic acid and chondroitin sulphate, the dominant glycosaminoglycan components in umbilical cord. Hence, the greater part of the exclusion experienced by dextran molecules in the enzyme-treated case is contributed by the collagen fibrils.

Electron microscopy shows that the individual fibrils (average diameter 54 nm) are parallel and some 110 nm apart (cylinder axis to cylinder axis), hence there exists, on the average, a 56 nm gap from fibril surface to fibril surface. Were this interfibrillar space between the 54 nm diameter fibrils inaccessible to dextran the excluded volume/g of collagen (for zero-molecular-weight probes) would correspond to a volume indicated by the arrow in Fig. 2. It is seen to lie well above the exclusion effects measured. There is direct evidence, moreover, that the interfibrillar space is accessible. This is shown by the fact that ferritin (a 10 nm diameter electron-opaque particle) can penetrate it and can be detected there by electron-microscopy (Meyer & Silberberg, 1972).

Excluded volume contributed by the collagen fibrils on a sphere/cylinder model

The exclusion effects on the basis of a cylinder/sphere, purely geometric model are given by

$$V'_{exc} = \pi L(r_F + r_s)^2 \quad (5)$$

where r_F is the cylinder radius in cm ($r_F = 27$ nm), r_s (in cm) is the hydrodynamically estimated coil radius of the dextran molecules listed in Table 1, and L is the length (cm) of fibril that contains 1 g of collagen.

For this estimate to hold, the fibril cylinders of radius r_F may not approach each other closer than by a $2(r_F + r_s)$ cylinder-axis-to-cylinder-axis distance. If this were to occur to any extent, the measured exclusion value would be less than predicted by eqn. (5). In the present case, however, the fibrils within the bundles constituting a fibre are almost everywhere parallel to each other, and 110 nm apart. Hence all the dextran probes fulfill the condition.

Taking the square root of eqn. (5), a linear relationship between $(V'_{exc})^{1/2}$ and r_s is predicted and substantiated by Fig. 3. Remembering that $\pi L r_F^2 = \bar{v} + \Delta\bar{v} = 1.89$ ml and $r_F = 27$ nm, the straight line in Fig. 3 can be drawn from eqn. (5). As can be seen, the experimental data are entirely consistent with the model and the fibril and dextran radii determined by other means.

Exclusion due to the proteoglycan system alone

The difference between the two sets of data in Fig. 2 is due to the proteoglycan system, which is essentially all digested by hyaluronidase. It is instructive to

Fig. 3. Dependence of $(V'_{exc.})^{1/2}$ on the hydrodynamic radius, r_s , of the probes for hyaluronidase-treated tissue. The points were from data in Tables 1 and 3. The line was calculated from eqn. (7) with $\bar{v} = 0.75$ ml/g, $\Delta\bar{v} = 1.14$ ml/g and $r_F = 27$ nm.

express these additional exclusion effects on the basis of 1 g of hyaluronic acid rather than 1 g of collagen. The tissue contains 2.0×10^{-3} g/ml of uronic acid, in its native state (Meyer & Silberberg, 1974). If this is assumed to be entirely due to hyaluronic acid, the ordinate values in Fig. 4 are obtained. As abscissa in Fig. 4 we have chosen the product $[\eta]M_n$, which (see eqn. 1) is a direct measure of the hydrodynamic volume of the dextran molecule. The hatched section in Fig. 4 at 0.66 ml/g represents the partial specific volume of hyaluronic acid (Silpananta *et al.*, 1968). The data are consistent with a linear relationship between the additional exclusion and the volume of the dextran coils.

This result suggests an exclusion model where, in the interstitium, there is a relatively uniform distribution of ν points/unit volume which cannot be penetrated by the dextran coil domains. These 'points' are to be seen as contributed by the intertangled proteoglycan network, and are best visualized as regions of locally high segment density, perhaps some 1–9 nm across. It is not unreasonable that such concentrated but still diffuse junction regions would interact with the diffuse dextran random coils as if, geometrically, the junction were a point (a hard sphere of zero or negligible radius) and the dextran coil a hard sphere of radius r_s .

On this model the volume excluded per g of hyaluronic acid, assuming that the entire uronic acid content of tissue is due to hyaluronic acid, is given by

$$V_{exc.}^{HA} = \nu V^{HA} \left(\frac{4\pi}{3} r_s^3 \right) \quad (6)$$

Fig. 4. Dependence of $V_{exc.}^{HA}$ on effective probe volume $[\eta]M_n$. $V_{exc.}^{HA}$ is the difference between the two curves in Fig. 2 expressed on the basis of 1 g of hyaluronic acid assuming a hyaluronic acid content of 4.1 mg/ml of tissue. Hyaluronic acid is the major proteoglycan component in umbilical cord. ■ Represents the partial specific volume of hyaluronic acid.

where ν is the number of points/unit volume and V^{HA} is the total volume/g of hyaluronic acid. V^{HA} may be identified with the interstitial volume/g of hyaluronic acid.

Since there are approx. 10 ml of interstitial volume/12 ml of tissue (see Fig. 2) and the hyaluronic acid concentration based on the total uronic acid content is 4.1×10^{-3} g/ml of tissue, $V^{HA} = 205$ ml. Combining eqns. (1) and (6), and the result of Fig. 4, we find that $\nu = 1.6 \times 10^{16}$ cross-links/ml, corresponding to a mean distance between effective exclusion points of some 50 nm.

Actually only about 60% of the uronic acid is due to hyaluronic acid (Danishefsky & Bella, 1966). The remainder is contributed by the other glycosaminoglycans about whose structural arrangement, however, very little is known. We have therefore chosen to calculate our results as if hyaluronic acid were the only polysaccharide component and all the uronic acid content were due to it. This does not in any substantial way effect the estimates of the structural parameters to which the model based on ν entanglement regions now leads.

Molecular interpretation of the proteoglycan entanglement regions

The mol.wt. of hyaluronic acid chains between entanglement regions turns out to be about 10^5 . Hence if each hyaluronic acid chain has a mol. wt. of

10^7 it will participate in some 100 entwinements with other chains. Since, in addition, the hyaluronic acid chain is also entangled with the collagen-fibre system, it is very reasonable that it is 'locked' on to the system even if no chemical bonds actually constitute these cross-links (Meyer & Silberberg, 1974).

The tendency of tissue to swell, which, as we have shown, is dependent upon the hyaluronidase-digestible material (Meyer & Silberberg, 1974), is the result of the drive of the molecularly disperse proteoglycan system to dilute itself. In free swelling against Ringer's solution tissue volume almost exactly doubles and the osmotic pressure due to the hyaluronic acid (4.1 mg/ml of original tissue by our convention) can be calculated to fall from 930 Pa (930 dyn/cm²) to a residual 340 Pa (340 dyn/cm²) (Silpananta *et al.*, 1968; Meyer *et al.*, 1969; Wiederhielm, 1972). This residual effect is counterbalanced by the deformation of the collagen-fibre network and swelling is limited.

It is known (Gibbs *et al.*, 1968) that hyaluronic acid systems at the concentrations here involved, although viscoelastic, do not store energy for times much in excess of 1 s. Hence all the original ν correlations in the tissue space will have had ample time, during the week which elapses to reach equilibrium, to relax and reform in the expanded tissue space without any hyaluronic acid chain deformation remaining. It is thus almost certain that the elastic energy has been stored in the distension of the collagen network alone. This is also in keeping with the observation (A. Silberberg & C. A. Wiederhielm, unpublished work) that hyaluronidase applied to tissue samples held under compressive strain does not decrease the stress measured.

Since we know that hyaluronic acid can be extracted from macerated tissue it is most unlikely that chemical bonding to the collagen network is involved. Indeed, if tissue slices are incubated for excessively long periods (beyond 2 weeks) hyaluronic acid content falls and de-swelling occurs.

Early attempts by us (Meyer & Silberberg, 1972) to study interstitial-space accessibility with dextran probes involved a procedure where diffusible proteins were not removed. At high dextran concentrations, however, the error introduced is not particularly large. Indeed, those early results do not qualitatively disagree with the present conclusions, but no attempt was then made to discuss the proteoglycan contribution to exclusion, as was done above. It could be shown, however, that this exclusion was much below what would be expected from a model treating the hyaluronic acid as a rod and the dextran coil as a sphere. The exclusion was much more in line with what simple Flory-Huggins theory (see Flory, 1953) would predict.

There are very few studies with which our present results can be compared. The investigation by Katz & Li (1973) using polyethylene glycol fractions and model fibril systems agree well with our results with respect to fibril exclusion. Hallen (1974) obtained distribution coefficients for a number of tissues using one polyethylene glycol fraction, but the results cannot be recalculated in our terms. Similarly the data of Maroudas (1970) cannot be compared with our results. Apart from the fact that relatively dense connective tissue was used in at least part of these investigations, no attempt was made to digest away the proteoglycan component and thus establish a clear-cut separation of effects.

Conclusions

The arrangement and dimensions of the collagen fibril system determined from the partitioning of dextran in hyaluronidase-treated tissue agreed well with that found by electron-microscopic observations. It was thus demonstrated that dextran can be used as a non-interacting probe to test structural arrangements of connective tissue. In particular the conclusion from our earlier work (Meyer & Silberberg, 1972) that the interfibrillar space is accessible to macromolecules was confirmed.

It was shown that the proteoglycan system is entangled to give rise to some 10^{16} high-segment-density regions/unit volume. These involve predominantly, hyaluronic acid and represent local correlations which form and re-form, but will not carry loads except for very short times.

References

- Boedtker, H. & Doty, P. (1956) *J. Am. Chem. Soc.* **78**, 4267-4280
- Danilshesky, I. & Bella, A. (1966) *J. Biol. Chem.* **241**, 143-146
- Flory, P. J. (1953) *Principles of Polymer Chemistry*, chapter 12, Cornell University Press, Ithaca
- Gibbs, D. A., Merrill, E. W., Smith, K. A. & Balazs, E. A. (1968) *Biopolymers* **6**, 777-791
- Hallen, A. (1974) Ph.D. Thesis, University of Uppsala
- Katz, E. P. & Li, S. T. (1973) *J. Mol. Biol.* **73**, 351-369
- Maroudas, A. (1970) *Biophys. J.* **10**, 365-379
- Meyer, F. A. & Silberberg, A. (1972) *Pflügers Arch.* **336**, S37-S39
- Meyer, F. A. & Silberberg, A. (1974) *Microvasc. Res.* **8**, 263-273
- Meyer, F. A., Preston, B. N. & Lowther, D. A. (1969) *Biochem. J.* **113**, 559-563
- Silpananta, P., Dunstone, J. R. & Ogston, A. G. (1968) *Biochem. J.* **109**, 43-50
- Wiederhielm, C. A. (1972) in *Biomechanics: Its Foundations and Objectives* (Fung, Y. C., Perrone, N. & Anliker, M., eds.), pp. 273-286, Prentice-Hall, Englewood Cliffs