Table II. Serum and urinary lysozyme activity in chronic renal failure

Patient No.	Urea (mg/100 ml)	Cr. clearance ml/min	Serum lysozyme (μg/ml)	Urinary lysozyme (μg/ml)
1	42	60	25.0	0.8
2	63	25.0	50.0	1.5
3	108	10.0	65.0	20.0
4	208	6.0	45.0	5.6
5	171	4.9	44.0	25.0
6	250	3.2	37.5	5.45
7	232	3.0	62.5	30.0
8	261	2.6	45.0	40.0
9	320	2.5	83.0	21.5
10	258	1.4	72.0	100.0

The number of neutrophilic granulocytes were in the normal range in every case. Note the high levels of serum lysozyme in patients with low levels of creatinin clearance. Patients with high urinary lysozyme had other signs of functional tubular impairment.

buffer, resulting in a final concentration of 13.5 mg/100 ml after the addition of 0.2 ml of the enzyme solution. Figure 3 represents the range of the variability of typical standard curves obtained when a bacterial suspension of 15 mg/100 ml was used. However, it is recommended that a standard curve be established for every day on which tests or experiments are to be performed. We found that the addition of NaCl did not improve either the linearity or the reproductibility of the reaction. Therefore, we did not include the addition of salt to the assay system, as Parry and others did[14].

Procedure. A standard curve is established as described above, using 5 different concentrations of enzyme and a bacterial suspension of 15 mg/100 ml. Each assay was recorded for 5 min. (This period of time was chosen because after 5 min the curve changes and tends to approach a plateau.) The tests are now performed in a similar manner, with 0.2 ml of serum being added to the bacterial suspension instead of 0.2 ml of the standard enzyme. The level from the baseline at 5 min is measured and compared to the standard curve. Each test is performed twice and the deviation obtained at 5 min is usually identical.

We tested the serum lysozyme activity of 85 healthy hospital personnel aged between 20 and 60. The mean lysozyme value found was 6.80 μg/ml ± 1.85. These results are in agreement with values reported in the literature for normal serum lysozyme activity, using egg white lysozyme as standard and turbidometric method[3].

We have currently been testing the serum and urinary lysozyme activity of patients suffering from different types of leukemia and renal diseases. The results obtained with the method presently described are within the range of those reported in the literature for the above-mentioned clinical conditions (Tables I and II).

When the serum lysozyme activity was higher than 15 μg/ml, the recorded curve was not linear in the first 5 min; the samples were therefore diluted in these cases with phosphate buffer until a straight line was recorded within the first 5 min, and the dilution was taken into account. In this manner, a simple, rapid and reproducible test is performed using small amounts of serum, which has also the advantage of recorded results that can be added to the protocol or to the case report.

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An Improved Method for Flow Dialysis Studies with Highly Increased Diffusion Rates[1]

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Summary. An improved flow dialysis procedure with highly increased diffusion rates has been developed allowing the study of small changes in the rate of diffusion. The application of the method described with only a few individual experiments, and with the use of small amounts of biological material, gives much information about binding systems.

It is known that for several reasons[2] flow dialysis could be an excellent approach for ligand polymer binding. Some investigations made with this technique have confirmed this thesis, although the sensitivity of the methods used[3–13] was disappointingly low. The same experience was confirmed during the development of our flow dialysis cell. It appears that the drawbacks of the method are caused by an uncontrolled turbulence inside the compartment containing the diffusant. By introducing a laminar flow in both compartments of the dialysis cell, the rate of diffusion increased 200 times and more compared with earlier published results[8].

The high reproducibility, flexibility and sensitivity of this method allows one to study small changes in the rate of diffusion due to parameters such as temperature, pH, ionic strength, viscosity, binding proteins and their various conformations. This allows a rapid screening of

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unknown receptors and other binding structures, the evaluation of binding parameters, characterization of diffusion processes, and if a suitable membrane is available, the self association of any molecules can be studied.

Material and methods. The method is based on measuring the rate of diffusion of a ligand across a membrane. This rate of diffusion is directly proportional to the concentration of the free (i.e., unbound) ligand in the releasing compartment of the flow dialysis cell. Figure 1 shows a diagram of the apparatus developed and used in this study. For continuous flow we used different models of cells adapted from the Dianorm® equilibrium dialysis cell[14]. Finally a channel system was introduced enforcing the laminar flow. The capacity of each half cell is 0.6 ml, including the dead volume of the cell. The membrane has an active area of 11 cm^2 corresponding to an area-volume ratio of about 18 cm^2/ml. The cell unit is composed of the compartments RC and FC (see Figure 1) which are temperature-controlled. The ligand reservoir LR, pump P1 and compartment RC form a closed system whereas fresh buffer is pumped from the buffer reservoir BR through the flow compartment FC. The drive unit used allows flow rates between 0.5 and 16 ml/min with an accuracy better than 1%. The dead space of the complete releasing system (P1, RC, LR), including the connecting teflon tubes, is 0.5 ml. The minimum total volume is not more than 1.2 ml, with no upper limit.

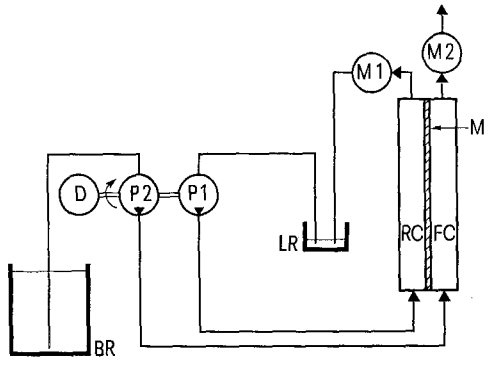

Fig. 1. Diagram of the flow dialysis system used. FC, flow compartment; RC, releasing compartment; M, membrane; LR, ligand reservoir; M1, monitor 1; M2, monitor 2; D, drive; P1, pump head 1; P2, pump head 2; BR, buffer reservoir.

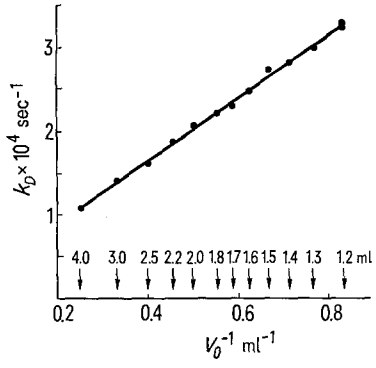

Fig. 2. The volume dependence of k_D for phenylbutazone at an initial concentration of 10^{-2} M in 1/15 M phosphate buffer pH 7.4 at 22 °C continuously monitored by spectrophotometry at 262 nm in the flow compartment (flow rate 11.3 ml/min). Membrane used: Spectrapor T.M. 2.

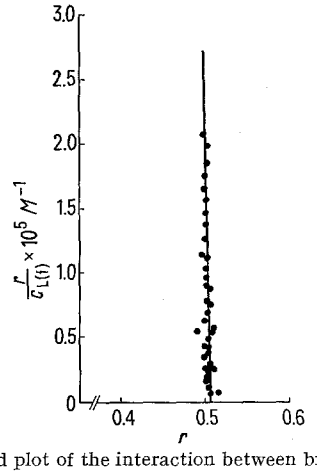

Fig. 3. Scatchard plot of the interaction between biotin-C14 and its binding protein from human serum plasma in 0.01 M phosphate buffer pH 7.4 at 22 °C, membrane Spectrapor T.M. 2. Initial ligand concentration 110 μM, constant protein concentration 22 μM, flow rate 7.7 ml/min (FC).

The dialysis may be monitored in the releasing compartment (monitor 1) as well as in the flow compartment (monitor 2), both including continuous or discontinuous techniques.

A first order process is applied to determine the apparent diffusion constant k_D

$$c_t = c_0 e^{-k_D t'} \tag{1}$$

where c_t is the concentration of the ligand in the compartment RC at time t and c_0 the initial concentration. The first derivative of the releasing curve is the product of the diffusion constant k_D and the free concentration c_f:

$$-\frac{dc}{dt} = k_D c_f \tag{2}$$

Results and discussion. The measured k_D is an apparent constant depending on the initial volume V_o of the ligand solution, the flow rates, and the membrane used. Figure 2 shows a linear correlation between the diffusion constant and the reciprocal volume V_o. This reflects the turnover of the ligand solution. Correspondingly, the k_D values increase with decreasing volumes. The increase of k_D with increasing flow rates reflects the mentioned turnover of the ligand solution. Above a critical flow rate in the releasing compartment of about 0.5 ml/min, a slight decrease in k_D is observed due to the high flux possibly causing microturbulence at the membrane.

As an example, the binding of biotin to a recently isolated[15] human serum protein was studied. The phosphate buffer from the flow compartment was collected in fractions at 2 min intervals for 200 min. The biotin released was determined in aliquots of 1 ml by liquid scintillation counting. The diffusion constant of biotin was determined under identical conditions in the absence of the binding protein. One single set of binding sites was found with a very high affinity constant of at least 10^7 M^{-1} (see Figure 3). In the excess of ligand, the amount bound is equal to the total number of binding sites due to the high affinity constant and the single type of binding sites. This allows one to calculate the free ligand concentration by subtracting the bound from the total concentration. The decrease of the free ligand concentration is a first order process, which is demonstrated in Figure 4, and corresponds to the independently measured diffusion constant of biotin in the absence of the

[14] We thank Diachema Ltd., CH-8803 Rüschlikon/Zürich for having adapted their Dianorm® Equilibrium Dialysis cells to our needs.

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binding protein. This agreement confirms the reality of the binding data.

Binding studies are performed to obtain quantitative and qualitative information about the mechanisms of ligand biopolymer interactions. With our flow dialysis method, the utmost information is gained with only a few individual experiments. Using the Scatchard plot to evaluate binding parameters, the whole saturation range has to be covered as shown earlier[16]. One single flow dialysis experiment leads to the complete Scatchard plot over the covered saturation range (1% of the initial concentration can be reached within 200 min depending on the experimental conditions). In contrast to this, all conventional methods require a set of individual binding experiments, each representing one isolated point of the Scatchard plot which has to be evaluated by a fitting procedure. Further advantages of our method result from the fact that only one experiment has to be performed. The small volume required for this experiment allows one to perform binding studies even with a very small amount of the biopolymer, which is often only available in limited quantities. In order to avoid a possible denaturation or degradation of molecules used, a small series of very short experiments at different initial ligand concentrations is required. The addition of the resulting individual Scatchard plots should give a continuous plot, proving the stability of the system studied under the experimental conditions. Continuous monitoring of the released ligand in the flow compartment allows detection of sensitively small disturbances during the experiment, such as membrane polarization, denaturation and degradation of the binding system and changes of the flow rates. Monitoring the releasing compartment is less sensitive in this respect.

The accuracy of the binding parameters evaluated depends on the accuracy of the diffusion constant used. Consequently the properties of the membrane used have to be held within narrow limits. If a good membrane quality is used and the preparation standardized, reproduceable results can be obtained.

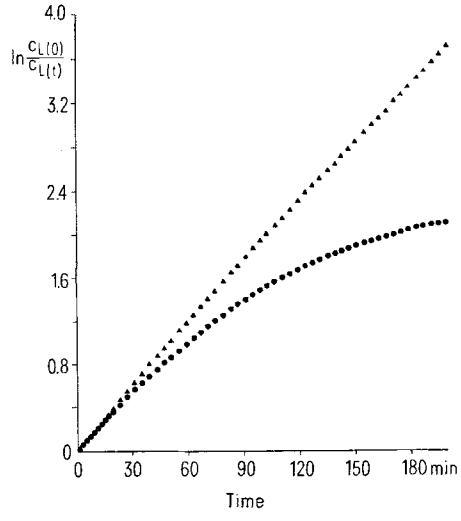

Fig. 4. Release of biotin in the presence of the binding protein. ●, total concentration; ▲, free concentration.

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Contribution to the Primary Tissue Culture Technique. A New Method for Mechanical Cell Dispersion[1]

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Summary. A new technique for preparing primary tissue cultures by mechanical cell dispersion was idealized. The time required to prepare a culture by this procedure was greatly reduced and the cultures obtained are morphologically and physiologically of better quality than that obtained by the classical methods of enzymatic cell dispersion.

A new technique for mechanical cell dispersion was developed which was of special value for primary tissue cultures, in contradistinction to the classical methods of enzymatic cell dispersion.

Proteolytic and chelating agents, among which trypsin and Versene, respectively, are the most commonly used, seem to present certain incompatibilities with perfect success of cell cultures. The action of these agents is based on the removal of Ca^{++} and Mg^{++} ions, the elements mainly responsible for the integrity of the cell matrix[3].

The consecutive posterior contacts between the cell layer in growth and these enzymatic agents seem to produce biochemical intra- and intercellular disturbances, since it becomes more and more difficult to obtain cell lines in vitro from primary cultures.

Concerned with the prevention, or at least a reduction of a close contact between the tissue and such agents, a method has been developed by us for a mechanical cell dispersion suitable to supply viable cells, producing cultures of a much better quality as well as a reduction of

[1] This work was supported by the Conselho Nacional de Pesquisas, Fundo Especial de Despesas do Instituto Butantan and Divisão Nacional do Cancer.

[2] The authors wish to thank Mrs. Sibylle Heller for the translation.

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