Alkaline Phosphatase from Pig Kidney

MICROHETEROGENEITY AND THE ROLE OF NEURAMINIC ACID

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Several alkaline phosphatases (EC 3.1.3.1) could be obtained from pig kidney brush-border membrane on extraction with butan-1-ol. Three of the multiple forms were separated by DEAE-cellulose chromatography and further purified. They form a regular series with different degrees of glycosylation (mainly owing to *N*-acetylneuraminic acid), of charge, of molecular weight, of stability to temperature, to pH and to urea, of minimal requirement for Mg^{2+} and of extractability by butan-1-ol. In contrast, the detectable antigenic sites, the inhibition by amino acids and the pH-dependency of K_m and V_{max} were identical for these multiple forms. On treatment with neuraminidase, the multiple forms became identical in all their properties. It was therefore concluded that the microheterogeneity of alkaline phosphatase is due to different degrees of glycosylation at polypeptide chains which appear to be otherwise identical.

Alkaline phosphatase preparations often exhibit multiple forms as detected by electrophoresis or ion-exchange chromatography. This microheterogeneity is a well known phenomenon (Engström, 1961; Butterworth & Moss, 1966; Butterworth, 1968; Sarawathi & Bachhawat, 1968; Smith *et al.*, 1968), and might be due either to proteins of different primary structure or to different degrees of substitution of the protein by carbohydrate. Further, we do not know whether we are dealing with natural products or with artifacts, which have been produced by solubilization from the membrane or by purification.

Investigations of multiple forms of alkaline phosphatases from serum (Robinson & Pierce, 1964) or crude tissue extracts (Butterworth & Moss, 1966) have demonstrated a change in their electrophoretic mobility on treatment with neuraminidase. Such treatment did not change several kinetic parameters of crude alkaline phosphatases (Butterworth, 1968), but did change some molecular properties of the sheep brain enzymes (Sarawathi & Bachhawat, 1968). Butterworth (1968) suggested that the microheterogeneity is based on different contents of sialic acid. It has been demonstrated (Wachsmuth & Hiwada, 1974) that alkaline phosphatase from pig kidney is a glycoprotein which becomes less negatively charged on neuraminidase treatment without loss of enzymic activity, supporting the suggestion of Butterworth (1968). We therefore decided to determine whether the microheterogeneity is due to the different substitutions of common protein by carbohydrate and whether artifact forms arise during the preparative procedure.

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Experimental

Materials

All reagents used were of analytical grade and several have been previously described (Wachsmuth & Hiwada, 1974; Hiwada & Wachsmuth, 1974). Sodium (meta)periodate, sodium arsenite and thiobarbituric acid were purchased from Merck, Darmstadt, West Germany; *N*-acetylneuraminic acid was from Serva, Heidelberg, West Germany. Complete Freund's adjuvant was from Difco, Detroit, Mich., U.S.A., cyclohexanol from Fluka, Buchs, Switzerland and Ampholine from LKB, Bromma, Sweden.

Methods

Enzyme preparations. Separation of three different alkaline phosphatase fractions (D_1 , D_2 , D_3) from pig kidney was carried out by DEAE-cellulose column chromatography at pH 8.2 as described previously (Wachsmuth & Hiwada, 1974). The D_1 fraction was further purified by precipitating with $(NH_4)_2SO_4$ between 45 and 70% (w/v) saturation at 0°C and by gel filtration on a column (2.5 cm × 86 cm) of Sephadex G-200 in 10 mM-Tris-HCl buffer, pH 8.0, to a specific activity of 121 μ mol/min per mg of protein for *p*-nitrophenyl phosphate hydrolysis. The D_2 fraction was further purified by TEAE-cellulose column chromatography and gel filtration on Sephadex G-200 (Wachsmuth & Hiwada, 1974) to a specific activity of 960 μ mol/min per mg of protein. Fraction D_3 was purified by chromatography on a column (2.5 cm × 18 cm) of DEAE-cellulose in 10 mM-Tris-HCl buffer, pH 8.2, by using a linear gradient of NaCl (25–150 mM) for elution at 75 mM-NaCl, and then by gel filtration on a column

(2.5cm×86cm) of Sephadex G-200 to a specific activity of 50 $\mu\text{mol}/\text{min}$ per mg of protein.

Isoelectric focusing. This was carried out by the method of Latner *et al.*, (1971). Samples were applied to a sucrose-gradient column with Ampholine pH5-8 and run for 48 h at 2000 V. Fractions (1 ml) were eluted and enzymic activity and pH measured.

Treatment with neuraminidase. Fraction D₁ (1.4 mg of protein) was incubated for 3 h at 37°C with neuraminidase (2 mg) in 1.0 ml of 10 mM-Tris-HCl buffer, pH 7.5, containing 30 mM-NaCl and 2 mM-CaCl₂, fraction D₂ (1.5 mg of protein) for 6 h at 37°C with neuraminidase (0.5 mg), and fraction D₃ (30 mg of protein) with neuraminidase (2 mg) for 15 h at 25°C. After digestion with neuraminidase, each incubation mixture was passed through a column (2.5cm×87cm) of Sephadex G-100 in 10 mM-Tris-HCl buffer, pH 8.0, containing 0.1% (w/v) NaN₃ at 15°C. Enzymically active fractions were concentrated by Amicon ultrafiltration with a UM-10 membrane. These fractions (referred to as D_{1N}, D_{2N}, D_{3N} respectively) were used for the studies of molecular and kinetic properties. After treatment with neuraminidase (2 mg/3 ml) for 16 h at 20°C, 7.5 mg of fraction D₃ was freed from sialic acid by passage through a column (4.5cm×90cm) of Sephadex G-100 in 10 mM-Tris-HCl buffer, pH 8.0, and then chromatographed on a column (2.5cm×15cm) of DEAE-cellulose in 10 mM-Tris-HCl buffer, pH 8.2. Most of the applied enzymic activity was eluted with a linear gradient of NaCl at 6 mM-NaCl, the concentration for neuraminidase-treated fraction D₂. No enzymic activity was found in fractions eluted with NaCl up to 0.15 M. The specific activity increased from 50 to 650 $\mu\text{mol}/\text{min}$ per mg of protein.

Determination of N-acetylneuraminic acid. A sample (0.2 ml) of fraction D₁ (0.27 mg), D₂ (0.068 mg), D₃ (0.75 mg) or D_{2N} (0.055 mg) was hydrolysed in 0.05 M-H₂SO₄ at 80°C for 1 h and then analysed as described by Warren (1959). Standard solutions of 2–20 μg of synthetic N-acetylneuraminic acid in 0.2 ml were measured in parallel. The values were corrected by the use of the absorbance at 532 nm.

Measurement of enzymic activity. Unless otherwise stated, assays for enzymic activity were carried out at pH 9.5 by using the procedure of Wachsmuth & Hiwada (1974). The K_m and V_{max} values were measured as described previously (Hiwada & Wachsmuth, 1974).

Protein determination. Protein was measured with biuret reagent (Gornall *et al.*, 1949) with bovine serum albumin as standard.

Antisera. Anti-(fraction D₂) sera were prepared by injecting a goat with purified fraction D₂ (approx. 0.6 mg) in complete Freund's adjuvant intradermally, bleeding the animal at intervals of 2 weeks (six times), and then each time injecting the same amount of

antigen intradermally. The sera were then pooled. For the experiments, a fraction precipitated from serum at 50% (NH₄)₂SO₄ saturation at 0°C was used. Anti-(fraction D₃) sera were prepared by two injections of purified fraction D₃ (each 13 mg), containing no other detectable alkaline phosphatase activity, into the foot pads of a rabbit within 2 weeks and then bleeding after 4 weeks, followed by an injection into the knee-joints and another bleeding 2 weeks later. The sera were pooled.

Determination of antigenicity. Immuno-electrophoretic analysis and staining of slides was carried out as described previously (Wachsmuth & Hiwada, 1974); 1.5% (w/v) agar was used for the double-diffusion technique, the diffusion time being 48 h.

Results

Electrophoretic mobilities

On electrophoresis (Plate 1), fraction D₁ migrated most towards the cathode and fraction D₃ only slightly. This correlated with the elution pattern of the three enzymes from DEAE-cellulose (Wachsmuth & Hiwada, 1974). After treatment of the three fractions with neuraminidase, however, their electrophoretic mobilities became identical, indicating that the net negative charge of fractions D₁, D₂ and D₃ had decreased (Plate 1). These observations correlate well with the neuraminic acid content of the fractions found by neuraminidase hydrolysis (Table 1). The neuraminic acid contents of fractions D₁, D₂ and D₃ were obtained by normalizing the specific activity to 1320 $\mu\text{mol}/\text{min}$ per mg of protein. This procedure is justified since the V_{max} and therefore the specific activity appear independent of the degree of glycosylation (see below). However, the values of sialic acid content for fractions D₃ and D₁ could be artificially high if glycoprotein impurities are present. Table 1 demonstrates that neuraminidase treatment has removed practically all the N-acetylneuraminic acid from the enzyme.

Table 1. *N*-Acetylneuraminic acid content of the multiple forms of alkaline phosphatase

The values for *N*-acetylneuraminic acid on a molar basis are normalized to a specific activity of 1320 $\mu\text{mol}/\text{min}$ per mg of enzyme and the apparent molecular weights of the multiple forms are used (see the Results section).

Protein preparation	Specific activity ($\mu\text{mol}/\text{min}$ per mg)	<i>N</i> -Acetylneuraminic acid ($\mu\text{g}/\text{mg}$ of protein)	<i>N</i> -Acetylneuraminic acid (mol/mol of enzyme)
D ₁	121	1.7	9.5
D ₂	960	11.1	9.0
D ₃	50	4.4	76.0
D _{2N}	1320	<0.2	<0.1

EXPLANATION OF PLATE I

Electrophoretic mobility of the multiple forms of alkaline phosphatase

Electrophoresis was done in 1.5% (w/v) agar gel, in 60mm-veronal-HCl buffer, pH 8.6, at 13 mA/slide, at 700 V for 4 h at 0°C. In each case the enzyme solution applied could hydrolyse 0.6 μ mol of *p*-nitrophenyl phosphate/min. Enzymes were localized by staining with α -naphthyl phosphate (Wachsmuth & Hiwada, 1974). (a) Fraction D₁ and the neuraminidase-treated fractions D_{1N}, D_{2N} and D_{3N}. (b) Fractions D₁-D₃ after separation by DEAE-cellulose chromatography and further purification (for details see under 'Methods').

EXPLANATION OF PLATE 2

Electrophoretogram of membrane extracts

A pH5 sediment from pig kidney homogenate was adjusted to the desired pH and extracted with 0.5 vol. of butan-1-ol. After centrifugation, the water phase was concentrated to about $10\mu\text{mol/min}$ per ml and $1\mu\text{l}$ of this solution was applied to 1.5% (w/v) agar in 60mm-veronal buffer, pH8.6, and electrophoresis carried out (6mA/slide for 3 h, approx. 45V/cm). The values on the abscissa give the pH at which the membrane was extracted. The fractions marked with N were treated for 14h with neuraminidase ($100\mu\text{g/ml}$) at room temperature. The pH9 fraction was then dialysed and treated again with neuraminidase, concentrated and dialysed ($9_{\text{N}2}$), and the same procedure repeated ($9_{\text{N}3}$). The enzyme was localized by staining with α -naphthyl phosphate (Wachsmuth & Hiwada, 1974).

Apparent molecular weights

The apparent molecular weights of fractions D₁, D₂ and D₃ were determined by gel filtration on Sephadex G-200 columns in 0.01M-Tris-HCl (pH8.0)-0.1% (w/v) NaN₃ at 15°C as described previously (Wachsmuth & Hiwada, 1974) and elution was followed by measurement of enzymic activity. The molecular weights were estimated to be 155000 for fraction D₁, 180000 for fraction D₂ and 200000 for fraction D₃. After incubation with neuraminidase, molecular weights of fractions D_{1N}, D_{2N} and D_{3N} were identical with that of fraction D₁ (155000). The apparent molecular weight of fraction D₁ did not change on neuraminidase treatment.

Temperature stability

The enzyme solutions (4 µmol/min) were incubated at 55°C in 50mM-Tris-HCl buffer (pH8.0)-5mM-MgCl₂ and samples removed after given times; 50% inactivation of fraction D_{1N}, D_{2N} or D_{3N} occurred in 24min, of fraction D₁ in 25min, of fraction D₂ in 29min and of fraction D₃ in 37min. The neuraminidase-treated enzymes were the most heat-sensitive proteins, whereas fraction D₃, with the largest amount of neuraminic acid, was the most insensitive.

pH stability

The enzyme solutions (4 µmol/min per ml) were incubated at 20°C in 50mM-veronal-acetate-5mM-MgCl₂, pH5.5, and samples removed after given times; 50% inactivation occurred within 36min for fractions D_{1N}, D_{2N} and D_{3N}, 40min for fraction D₁, 62min for fraction D₂ and 210min for fraction D₃. The sensitivity to the H⁺ concentration increased with decreasing enzyme content of neuraminic acid.

Inactivation by urea

Fractions D_{1N}, D_{2N} and D_{3N} were the most rapidly inactivated enzymes (50% within 20min) when incubated in 3M-urea, and fraction D₃ the most stable one (50% within 29min), the other enzymes behaving according to their sialic acid content (Fig. 1). This demonstrated a protective effect of the sugar residue at the enzyme site.

Immunochemical analysis

In the double-diffusion test against goat anti-(fraction D₂) serum and rabbit anti-(fraction D₃) serum, the three enzymes and the neuraminidase-treated derivatives produced single precipitation lines as demonstrated by enzymic-activity stain. A protein stain was not used, since the enzyme preparation contained contaminant proteins. No crossing or weakening of the enzymically stained lines was

detectable between the fractions D₁, D₂ or D₃ and with the neuraminidase-treated enzymes. This was also the case when antiserum raised with the most carbohydrate-containing enzyme, fraction D₃, was used. This indicated antigenic identity, independent of the carbohydrate or sialic acid content of the enzymes.

Measurement of K_m and V_{max} .

Fractions D_{1N}, D_{2N}, D_{3N}, D₁, D₂ and D₃ were analysed for *p*-nitrophenyl phosphate hydrolysis activity over the pH range 7.5-10.0. As for highly purified fraction D_{2N} (Hiwada & Wachsmuth, 1974), all the enzymes were increasingly inhibited by substrate as the pH decreased. The K_m values of the multiple forms were identical (Fig. 2). Since the enzymes were of different stages of purity, i.e. had different specific activity, a real V_{max} value could not be measured. However, the ratios of enzyme activity over the pH range were constant, i.e. the ratio V_{max} at pH9.5 to V_{max} at pH7.5 was for fraction D₁ 15.2, for fraction D₂ 15.2, for fraction

Fig. 1. Inactivation of alkaline phosphatase by urea

The enzyme solutions (4 µmol/min per ml) were incubated at 37°C in 3M-urea-10mM-Tris-HCl-5mM-MgCl₂, pH8.0, and samples subsequently removed into 50mM-Tris-HCl-5mM-MgCl₂, pH9.5. The enzyme activity was estimated after a preincubation time of 5min in this buffer by the addition of substrate. O, Fraction D_{2N}; ●, fraction D₂; ■, fraction D₃.

Fig. 2. pH dependence of the K_m value

The hydrolysis of *p*-nitrophenyl phosphate in 50mM-Tris-HCl-10mM-MgCl₂ at 37°C was measured for 0.27 μg of fraction D₁ (▲), 0.034 μg of fraction D₂ (●) or 0.65 μg of fraction D₃ (■).

D₃ 14.9. The Arrhenius plot between 13° and 45°C gave no differences between fractions D₁, D₂ and D₃ for the activation energy, with *p*-nitrophenyl phosphate as substrate at pH9.5. Treatment with neuraminidase did not affect the enzymic activity at all and did not alter the K_m value.

The K_m values of fractions D₁, D₂ and D₃ for the hydrolysis of β-glycerophosphate were 1×10^{-3} M in all cases, when measured at pH9.5 (Fig. 3). The ratio of V_{max} at pH9.5 for β-glycerophosphate hydrolysis and V_{max} for *p*-nitrophenyl phosphate hydrolysis was for fraction D₁ 0.96, for fraction D₂ 0.98 and for fraction D₃ 0.97. Thus no differences for substrate binding, catalytic activity or specificity were detected among the different forms.

Inhibition by amino acids

Fractions D₁, D₂ and D₃ were inhibited to the same degree and in the same fashion by the amino acids tested (Table 2). Treatment of the enzymes with neuraminidase did not alter this inhibition.

Enzyme activation by Mg²⁺

Fractions D_{1N}, D_{2N} and D_{3N} could be activated by Mg²⁺ to the highest extent, and fraction D₃ to the lowest, the membrane preparation showing the

Fig. 3. Lineweaver & Burk (1934) plots for the hydrolysis of β-glycerophosphate

Hydrolysis rates in 12.5ml of reaction mixture by 1.8 μg of fraction D₁ (▲), 0.43 μg of fraction D₂ (●), or 5 μg of fraction D₃ (■) were measured in 50mM-Tris-HCl-10mM-MgCl₂, pH9.5, at 37°C.

smallest effect (Fig. 4). However, it must be kept in mind that the original enzymic activity of all the enzymes before dialysis was the same and that fraction D₃ did not lose activity on neuraminidase treatment. Therefore at 1 μM-MgCl₂ fractions D_{1N}, D_{2N} and D_{3N} had 25% of their maximal activity, fraction D₁ had 26%, fraction D₂ had 28%, fraction D₃ had 32% and the membrane preparation had 45%. Maximal activation by Mg²⁺ decreases with increasing amounts of sugar at the protein site, but the same concentration of MgCl₂ (10 μM) is required for half-maximal activation for all the enzymes.

Extraction of the multiple forms from the membrane

The different charges of the enzymes suggested a possible role of the charge when extraction of a pH5 sediment from homogenate supernatants, i.e. a membrane preparation (Wachsmuth & Hiwada, 1974), was carried out. Most of the enzyme, as detected by activity determination, was extracted at pH7.0 (Wachsmuth & Hiwada, 1974). The extracts obtained at different pH values were analysed for their enzyme distribution. At neutral and acid pH the enzymes with the lowest negative charge were extracted, whereas extraction in the alkaline range yielded those with more negative charge (Plate 2). The latter enzymes became less negatively charged on treatment with neuraminidase and eventually became fraction D_{1N}. The pattern of enzyme distribution did not change when the membrane fractions were

Table 2. Inhibition of the multiple forms of alkaline phosphatase by amino acids

The hydrolysis of 1 mM-*p*-nitrophenyl phosphate was measured in 50 mM-Tris-HCl-10 mM-MgCl₂, pH 9.5, at 37°C in the presence of 10 mM-amino acids. The enzyme amount used hydrolysed the substrate at a rate of 0.03 μmol/min.

Amino acids (10 mM)	Inhibition (%)					
	Fraction D ₁	Fraction D _{1N}	Fraction D ₂	Fraction D _{2N}	Fraction D ₃	Fraction D _{3N}
L-Leucine	46	48	48	50	52	50
L-Phenylalanine	47	47	47	47.5	48	46
L-Histidine	64	64.5	64	64	64	64
DL-Serine	0	0	0	0	0	0

Fig. 4. Effect of Mg²⁺ on the enzymic activity

All enzyme preparations were dialysed against 2 × 2 litres of 1 μM-MgCl₂-50 mM-Tris-HCl buffer, pH 9.5, for 24 h at 4°C. For determination of the enzymic activity the enzymes were incubated at 37°C for 5 min in 50 mM-Tris-HCl buffer, pH 9.5, containing given amounts of MgCl₂. The hydrolysis was measured after addition of 1 mM-*p*-nitrophenyl phosphate. ○, Fraction D_{2N}; ▲, fraction D₁; ●, fraction D₂; ■, fraction D₃; □, membrane fraction. The enzymic activity was expressed as a percentage of the activity determined with 1 μM-MgCl₂.

extracted for different lengths of time with butan-1-ol or were kept after extraction at pH 7.0 for at least 4 days at 4°C. The isoelectric points of such extracts differed as was expected from the electrophoresis at pH 8.6. The alkaline phosphatase extracted at pH 9.0 contained mostly enzymes with pI 5.45. Alkaline phosphatase extracted at pH 7.0 consisted of a major fraction with pI 6.4 and minor fractions with pI 6.15 and 6.0.

Discussion

When alkaline phosphatase was solubilized from pig kidney, at least three well distinguishable enzyme fractions could be eluted from the DEAE-cellulose column (Wachsmuth & Hiwada, 1974). This was puzzling but agreed with findings of multiple forms in serum (Robinson & Pierce, 1964), extracts of kidney (Butterworth & Moss, 1966), liver (Latner & Skillen, 1969) and brain (Sarawathi & Bachhawat, 1968).

Several questions require elucidation.

1. Do multiple forms of alkaline phosphatase proteins exist or are they separation artifacts? At least three bands are electrophoretically detectable in the butan-1-ol extract at pH 7.0. The fractions from DEAE-cellulose chromatography corresponded to these electrophoretic bands, indicating different degrees of negative charge. The apparent molecular weight, the temperature and the pH stability increased with an increase in negative charge and so did the stability in urea. From the data of Smith *et al.* (1968) a similar correlation between charge and urea inactivation can be determined. These properties were independent of the degree of purity of individual forms and therefore demonstrate proteins of different charge, size and conformation having alkaline phosphatase activity.

2. Do the multiple forms differ in their catalytic properties? Estimation of the Michaelis constants and ratios of *V*_{max} at pH 7.5 and 9.5 showed identical values for the hydrolysis of two different substrates by the three isolated forms of alkaline phosphatase. No difference in amino acid inhibition was observed. Thus the catalytic properties of the enzymes appeared to be identical. Butterworth's (1968) measurements at 5 mM-MgCl₂ suggested a higher degree of activation for the neuraminidase-treated enzyme than for the untreated enzyme. Our titration experiments show that in fact neuraminidase-treated enzymes are more activated by Mg²⁺ than the untreated enzymes. However, this is due to a requirement for Mg²⁺ indirectly proportional to size or charge, the large

molecules losing less activity in the absence of Mg^{2+} . Therefore all the differences of the multiple forms were found in molecular and not in catalytic properties. Since the only measurable effect of Mg^{2+} was on V_{max} . (Wachsmuth & Hiwada, 1974; Hiwada & Wachsmuth, 1974), this phenomenon may be explained by an effect occurring near the catalytic centre.

3. Are the multiple forms composed of different protein units, as is the case for isoenzymes (Latner & Skillen, 1969)? From the data of Ghosh *et al.* (1967) on human placental alkaline phosphatase, we have calculated 20 sialic acid residues/molecule of enzyme with mol. wt. 70000 or 10 for mol.wt. 140000 (Ghosh *et al.*, 1967). These data would agree with ours for fraction D₂, which gave 8–12 residues. On treatment with neuraminidase the carbohydrate (Wachsmuth & Hiwada, 1974) and the neuraminic acid content per fraction of pig kidney alkaline phosphatase decreased and the three forms (D₁, D₂ and D₃) became identical. This suggested that the main difference resided in the carbohydrate content, which could not be detected immunochemically, and that the protein core was identical in the multiple forms.

4. Are the multiple forms of alkaline phosphatase extraction artifacts? The highest yield of alkaline phosphatase was achieved by extraction with butan-1-ol at pH7 (Wachsmuth & Hiwada, 1974). The electrophoretic pattern of the enzymes in the extract did not change on prolonged incubation with butan-1-ol at pH7. Electrophoretic analysis of extracts obtained at different pH values yielded different multiple forms of the enzyme: an inactivation cannot account for this, because the enzymes are stable up to 47°C and between pH11.3 and pH6.0. Different forms of the enzyme are extracted at different pH values. There was always residual activity in the membrane fraction, which increased with increasing pH. Therefore the solubilization of alkaline phosphatase forms from the membrane depends on neuraminic acid and carbohydrate content and on the reciprocal of the H^+ ion concentration, indicating that the multiple forms are not extraction artifacts. One explanation is that the forms

are in differently charged membrane environments. Protonation would allow solubilization depending on the amount of sialic acid in the enzyme, providing the enzyme with a minimum of ionic bonds to interact with neighbouring groups. Whether this is due to localization of the enzyme in different environments in the membrane or in different cells remains unanswered. The multiple forms are not intermediates in the synthesis of enzymically active alkaline phosphatase, since they possess similar catalytic properties and since the presence of an enzymically inactive cytoplasmic form of the enzyme has been demonstrated (Wachsmuth & Torhorst, 1974). The fact that *N*-acetylneuraminic acid stabilizes the enzyme could mean that the enzymes with large amounts of *N*-acetylneuraminic acid and sugar are intermediate forms to transfer the enzyme into and through the membrane.

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