Phase Transitions of the Purple Membranes of *Halobacterium halobium*†

Meyer B. Jackson and Julian M. Sturtevant*

ABSTRACT: Purple membranes of *Halobacterium halobium* were studied by differential scanning calorimetry. No transition was detected at temperatures below 70 °C. A small endothermic transition was seen at about 80 °C and a larger one at 100 °C. The larger transition is the irreversible denaturation of bacteriorhodopsin. The smaller transition is accompanied by a change in the visible absorption spectrum and is believed to be reversible, involving a cooperative change in crystalline structure of the membrane.

The organism *Halobacterium halobium* is a halophilic bacterium which is found in extremely salty waters. This organism has two highly pigmented membranes, one red and one purple. The purple membrane exists as segregated patches contiguous with the red membrane. The color thus distinguishes two specialized regions within the membrane (Blaurock & Stoeckenius, 1971).

The purple membrane has been shown to be crystalline with a P3 hexagonal protein lattice (Unwin & Henderson, 1975). There is only one protein in the purple membrane which owes its color to a retinal moiety covalently bound to the protein. For this reason it has been named bacteriorhodopsin (Oesterhelt & Stoeckenius, 1971). It has been demonstrated that this protein can act as a light-driven proton pump providing photosynthetic energy for the cell (Oesterhelt & Stoeckenius, 1973). The red membrane has many proteins (Kushwaha et al., 1975).

H. halobium grows optimally in approximately 4 M NaCl and in the presence of smaller amounts of other salts. The cells undergo lysis when the salt concentration is lowered, due to a fragmentation of the membrane into separate red and purple particles. The fragmentation has been used as the basis for separation and purification of the purple and red membrane fractions (Oesterhelt & Stoeckenius, 1974).

The crystallinity of the purple membrane, the segregation of the purple and red membranes as separate domains, and the stability of the membrane in the presence of high salt concentrations are all thermodynamically interesting phenomena. For this reason, we have undertaken an analysis of the membranes and lipids of *H. halobium* using differential scanning calorimetry (DSC).[1]

Methods

Growth of H. halobium. Growth conditions and medium are described by Oesterhelt & Stoeckenius (1974). Strain *H. halobium* R (kindly supplied by both Drs. W. Stoeckenius and R. Henderson) was grown in 8-L batches in a 10-L glass carboy. Aeration was achieved with fritted glass gas dispersion tubes and was monitored with a gas flow meter. Illumination was achieved with eight "cool white" 2-ft fluorescent tubes supplemented with several incandescent flood lamps. The temperature was regulated at 39 °C with a regulatory thermometer by means of a bridge circuit and knife heater. Growth was initiated with a 0.5-L stationary starting culture grown in a shaker under constant illumination at 39 °C. The aeration rate was 4 L per min during the first 24 h and was then lowered to 2 L per min for 3 days. The absorbance at 560 nm was approximately 3.0 when the cells were harvested.

Membrane Preparation. Purple membranes were prepared either according to the procedure described by Kushwaha et al. (1975) or Becher & Cassim (1975) except that a 30–50% sucrose gradient was used as described by Oesterhelt & Stoeckenius (1974).

Red membranes were isolated as described by Kushwaha et al. (1975) and whole membrane envelopes were isolated according to the method described by Kanner & Racker (1975).

Total bacterial lipids were extracted by the method given by Kates et al. (1965). Lipids were extracted from the isolated purple and red membranes by a simple chloroform–methanol extraction (Kushwaha et al., 1975).

Bacteriorhodopsin was incorporated into synthetic phospholipids by sonication. The method was introduced by Racker & Stoeckenius (1974) and further developed by Hwang & Stoeckenius (1977). The details for these experiments are given in the appropriate figure legends.

The membrane samples were suspended in either distilled water, 0.1 M potassium phosphate (pH 6.6), or basal salts (25% NaCl, 2% $MgSO_4 \cdot 7H_2O$, 0.2% KCl) and dialyzed against the same solution as a reference before calorimetric experiments. DSC was performed in a Privalov calorimeter (Privalov et al., 1975). Scans past 100 °C were made possible by pressurizing the cells with 1 atm of excess pressure. Scans below the freezing point of the solution being studied are not possible in the Privalov calorimeter. Enthalpies were determined from the areas under the specific heat curves. Maxima in the specific heat of a transition were read directly from the plotted specific heat data.

The concentration of bacteriorhodopsin was determined spectrophotometrically using a molar extinction coefficient of 63 000 at 568 nm (Oesterhelt & Hess, 1973) and a molecular weight of 25 000 (Bridgen & Walker, 1976). Occasional Lowry protein determinations (Lowry et al., 1951) gave excellent agreement with the protein concentration as determined from the molar extinction coefficient.

All spectrophotometric measurements were done in a Cary 14 spectrophotometer.

α-L-Dielaidoylphosphatidylcholine (DEPC) was synthesized by the method of Cubero-Robles & van den Berg (1969).

Results

Figure 1 shows the DSC results obtained with purple membranes suspended in water, in basal salts, and in 0.1 M

† From the Departments of Chemistry and Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520. *Received August 19, 1977.* Supported by Grants GM-04725 from the National Institutes of Health and PCM76-81012 from the National Science Foundation.

[1] Abbreviations used: DSC, differential scanning calorimetry; DEPC, α-L-dielaidoylphosphatidylcholine.

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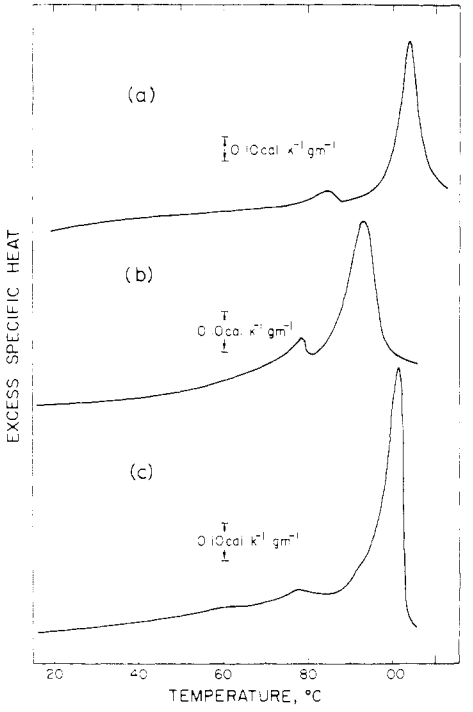

FIGURE 1: DSC scans of purple membranes in (a) basal salt; (b) water; and (c) 0.1 M potassium phosphate (pH 6.6). Scan rates were 1 °C per min.

TABLE I: Calorimetric Data.

	80 °C transition		100 °C transition	
	ΔH (kcal/mol)[a]	T (°C)	ΔH (kcal/mol)[a]	T (°C)
Basal salt	7.83 ± 0.52	82.7 ± 0.7	101.1 ± 0.3	103.1 ± 0.7
Water	6.50 ± 0.63	79.4 ± 0.8	79.1 ± 2.0	95.0 ± 0.7
Phosphate		76.8 ± 0.5	103.5 ± 5.1	101.1 ± 0.2

[a] Refers to moles of bacteriorhodopsin monomer.

potassium phosphate (pH 6.6). There are two transitions, one at about 80 °C and the other at about 100 °C, with the exact temperatures depending on the ionic conditions as seen in the figure. These transitions shall be referred to as the 80 and 100 °C transitions in the text for the sake of convenience. The temperatures and enthalpies of the transitions under various conditions are given in Table I. In phosphate the 80 °C transition was too broad to permit accurate enthalpy measurement so none is given. The 100 °C transition in water or in basal salt is nearly symmetrical, but in phosphate buffer for unknown reasons it is very unsymmetrical.

In some preparations, the purple membrane was impure as was evidenced by a shoulder in the visible absorption spectrum due to red membrane contaminants (Becher & Cassim, 1975). DSC of these membranes in water or phosphate showed a lowering and broadening of the 80 °C transition but the 100 °C transition was unchanged. This indicates that the 80 °C transition is not due to small amounts of impurities. A sample of purple membranes prepared from *H. cutirubrum* generously supplied by Dr. Morris Kates showed the same thermal behavior as our *H. halobium* preparations.

Membranes prepared by suddenly adding water to pelleted cells (Becher & Cassim, 1975) gave cleaner and more reproducible results than membranes prepared by dialysing out the salt (Oesterhelt & Stoeckenius, 1974; Kushwaha et al., 1975).

No transition was seen in any purple membrane preparation

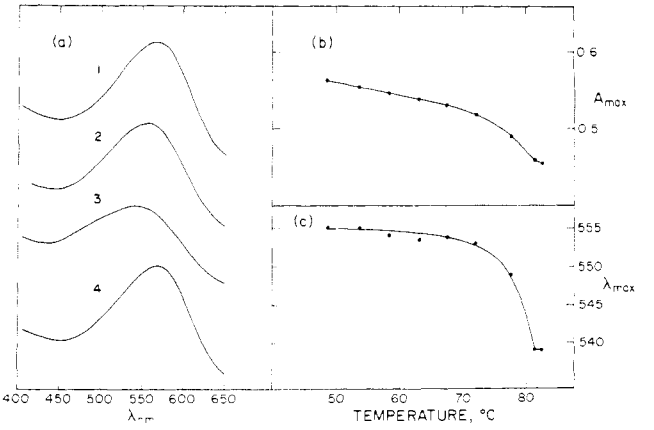

FIGURE 2: (a) Absorbance spectra (1) at 25 °C illuminated; (2) at 58 °C; (3) at 81 °C; (4) after cooling to 25 °C and illuminating. (b) Plot of absorbance at λ_{max} vs. temperature. (c) Plot of λ_{max} vs. temperature. All data here are from purple membranes in water.

from 0 to 75 °C. Thus we did not detect a transition at 30 °C which has been reported previously (Chignell & Chignell, 1975; Sherman et al., 1976).

Heating purple membranes through the 80 °C transition does not visibly alter the purple color. Heating through the 100 °C transition results in an irreversible change in color to orange, with a shift of the absorption maximum to about 370 nm, indicating that the environment of the chromophore has changed considerably and that the protein is no longer in the native state.

A study of the visible absorption spectrum of purple membranes in water (aggregation occurred in basal salt) as a function of temperature was undertaken. Representative spectra are shown in Figure 2a, and plots of absorbance at λ_{max} and of λ_{max} as a function of temperature are shown in Figures 2b and 2c. The purple membranes were first light-adapted (Becher & Cassim, 1976), but the spectrum changed to dark-adapted as the temperature was raised. There was a slight lowering in λ_{max} and in the absorbance at λ_{max} with increase of temperature up to 75 °C. A small but sudden further decrease in λ_{max} and absorbance at λ_{max} was seen to result from the thermal transition at 80 °C. Upon cooling back to room temperature, the original dark-adapted spectrum was obtained which could be converted to the light-adapted form by illumination. There was, however, some loss in absorbance as compared with the spectrum before heating. We attribute this to a small amount of decomposition at elevated temperatures.

When the purple membranes are heated in water through the 80 °C transition, cooled, and reheated, a smaller amount of heat uptake is seen again at about 80 °C (Figure 3a). In salt, however, the transition is not seen in the second heating (Figure 3b), and exposure to temperatures higher than 50 or 60 °C for more than half an hour results in a smaller amount of heat uptake at 80 °C. Neither of the transitions was seen in a reheat after heating through the 100 °C transition. Variation of the scan rate between 0.5 and 2 K min^{-1} indicated that neither transition is kinetically limited under these conditions.

The results of two experiments in which purple membranes were incorporated into synthetic lipids by sonication are shown in Figure 4. In these experiments purple membranes in water (Figure 4a) or in basal salt (Figure 4b) were incorporated into DEPC. Because this lipid melts at 12 °C the sonication could be done at room temperature. In addition the lipids were first sonicated alone to make vesicles. This allowed us to sonicate the purple membrane only 6 min; sonication for no more than

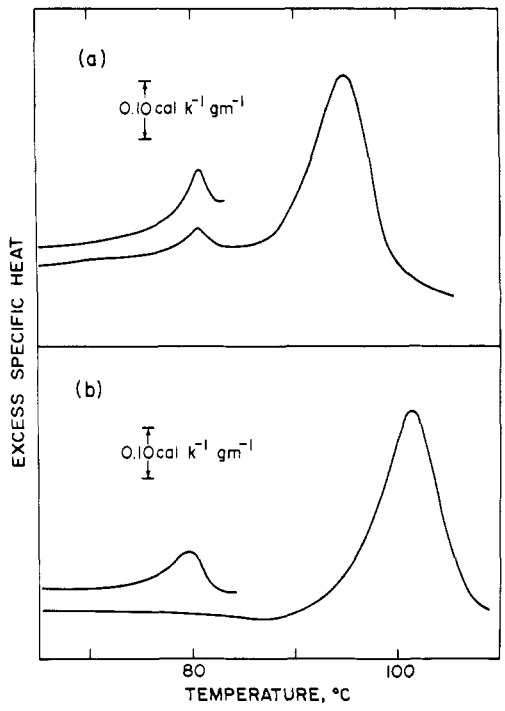

FIGURE 3: Heating purple membranes through the 80 °C transition, cooling, and reheating past the 100 °C transition in the DSC. (a) In water; (b) in basal salt.

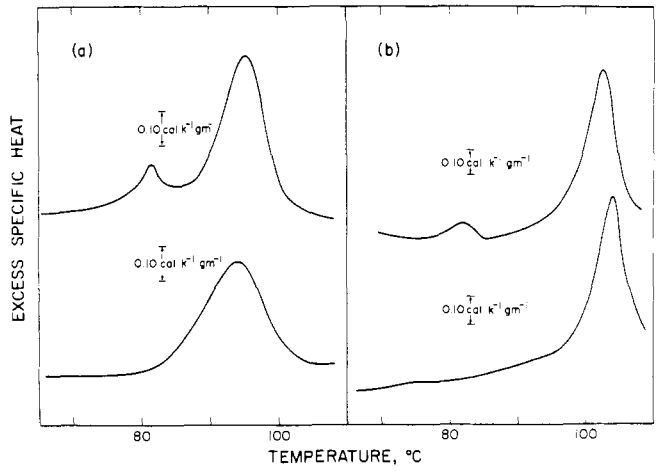

FIGURE 4: DSC of purple membranes sonicated (upper scans) and incorporated into synthetic phospholipids (lower scans). (a) Purple membranes in water were sonicated for 6 min at room temperature. The bacteriorhodopsin concentration in both scans was 3.51 mg/mL. The DEPC concentration in the lower scan was 10.3 mg/mL. (b) Purple membranes in basal salt were sonicated for 6 min at room temperature. In the upper scan the purple membrane concentration was 2.20 mg/mL. In the lower scan the concentration of DEPC was 12.0 mg/mL and of bacteriorhodopsin was 2.42 mg/mL.

6 min does not damage bacteriorhodopsin (Hwang & Stoeckenius, 1977). Sonication alone had no effect on either of the two transitions, but incorporation into DEPC removed the 80° transition in either water or in basal salt with only small changes in the 100 °C transition. Purple membranes were incorporated into dipalmitoylphosphatidylcholine with the same result.

Red membranes and whole membrane envelopes were also studied by DSC. Above 55 °C (the maximum growth temperature is 57 °C (Oesterhelt & Stoeckenius, 1974)) several endothermic peaks were seen but, since it was not possible to identify or interpret them, these scans are not shown.

The lipids of the red and purple membranes have different chemical compositions (Kushwaha et al., 1975). It would therefore be interesting to know if they have different physical properties. Suspensions of red membrane lipids, purple membrane lipids, and total *H. halobium* lipids in water could be made by vortexing and briefly sonicating. None of these suspensions showed any phase transition from 5 to 90 °C. The transitions seen by Chen et al. (1974) occur below 0 °C and are thus in a temperature range which is inaccessible to the Privalov calorimeter.

We were unable to make stable suspensions, suitable for DSC, of any of these lipids in a basal salt solution. Working the lipids with a glass homogenizer while immersed in a bath sonicator produced an unstable turbid suspension from which the lipids rapidly precipitated. If a more concentrated salt solution was added to a lipid suspension in distilled water to a final salt concentration equal to that of basal salt, as was done by Lanyi (1974), the lipids precipitated within minutes.

Discussion

With purple membranes, our basic results are the detection of two regions of heat absorption at rather high temperatures, and the absence of a previously reported transition at ~30 °C (Chignell & Chignell, 1975; Sherman et al., 1976).

All of the previous reports of a phase transition at 30 °C were based on a slope change in a plot of either an electron spin resonance splitting parameter (Chignell & Chignell, 1975), vs. the temperature or the logarithm of the rate of a phototransient decay (Sherman et al., 1976) vs. the reciprocal of the absolute temperature. In these types of plots, it is frequently impossible to distinguish between a break in slope at a definite temperature and a broad curvature in the plot throughout the temperature range studied. Although it is clear that these properties show a temperature dependence, our inability to detect a phase transition between 0° and 75° with a sensitive instrument leads us to the conclusion that no unique transition temperature is associated with these properties. It should be noted that curved van't Hoff or Arrhenius plots for processes involving macromolecules can in general be at least as well interpreted in terms of nonvanishing heat capacities of reaction or of activation as in terms of conformational or phase transitions.

Having identified two transitions not previously reported, the problem which remains is to characterize them. The 100 °C transition is in all probability an irreversible denaturation of the protein bacteriorhodopsin. The change in color to orange is due to a change in the environment of the retinal as the protein unfolds. The loss of the absorbance maximum at 560 nm concomitant with the appearance of an absorbance maximum at about 370 nm is also observed when the retinal is extracted with hydroxylamine under intense illumination (Oesterhelt et al., 1974). Schiff base hydrolysis may be occurring during this denaturation but we have no direct evidence of this.

The enthalpy of thermal denaturation of several typical globular proteins is about 12.5 cal/g at at 100 °C (Privalov & Khechinashvili, 1974) which is more than three times as large as the enthalpy of denaturation of bacteriorhodopsin. According to this criterion, the enthalpy of denaturation of a Na/K ATPase (~1.3 cal/g at 55 °C) is also low (Halsey et al., 1977). These low values may be reflections of the vastly different, nonpolar environment of membrane proteins as compared with a protein in aqueous solution, or an indication that a membrane protein retains much structure after denaturation due to the constraints imposed by the lipid bilayer.

Effective enthalpies of thermally induced conformational changes are often determined by applying the van't Hoff

equation. With typical globular proteins it has been shown that the calorimetric and van't Hoff enthalpies are approximately equal, indicating that denaturation is a cooperative two-state process (Tsong et al., 1970; Privalov & Khechinashvili, 1974). Although it is strictly correct to apply the van't Hoff equation only to two state reversible processes, one can define a van't Hoff enthalpy by the expression $\Delta H_{vH} = 4RT_m^2(c_{max}/\Delta q)$ and make interesting comparisons of this quantity with true calorimetric enthalpies (Mabrey & Sturtevant, 1978). In the above expression R is the gas constant, T_m the transition temperature, c_{max} the maximum excess specific heat observed at T_m, and Δq is the enthalpy of the transition obtained by integration of the curve showing the excess specific heat as a function of temperature. It may be noted that the ratio $c_{max}/\Delta q$ has the dimension K^{-1}, and that it may be evaluated without any knowledge of the amount or nature of the material undergoing the transition. For the 100° transition we obtain a van't Hoff enthalpy which is nearly twice as large as the calorimetric enthalpy in Table I regardless of ionic conditions. This may be a consequence of the irreversibility of the transition, or, as seems more likely, it may be evidence for intermolecular cooperativity in the denaturation of bacteriorhodopsin. Such an indication of intermolecular cooperativity is of interest in view of the membrane crystallinity and the existence of a protein trimer in the unit cell (Unwin & Henderson, 1975).

The results in Table I show that there is an increase in the temperature and enthalpy of this denaturation with ion concentration. The native state is apparently stabilized by ions. There are many polar amino acids in bacteriorhodopsin (Oesterhelt & Stoeckenius, 1971) and many titratable acid groups in the membranes of *H. halobium* (Brown, 1965). In addition the lipids are acidic and charged (Kates, 1972). It is thus not surprising that electrostatic interaction between the ions in solution and the charges on the membrane should be of significance.

The smaller transition at about 80 °C is more difficult to understand. The spectroscopic studies mentioned above clearly show that a spectral change accompanies this transition, suggesting that a conformational transition of the protein is taking place. At 50 °C or higher, but below the transition at 80 °C, the absorption maximum occurs at 555 nm. This could be due to a variety of causes such as a slight change in the chromophore orientation with an alteration of the exitonic interaction (Bauer et al., 1976; Becher & Ebrey, 1976; Kriebel & Albrecht, 1976), or a change in the environment of the chromophore within the protein. This change may be similar to that which occurs during dark adaption.

Upon cooling, the original spectrum is recovered which can be light adapted but which shows some loss in absorbance. The loss in absorbance as well as the broadening and lowering of the transition after heating slightly past 80 °C indicate that slow decomposition takes place at high temperatures but that the transition itself is reversible. Resonance Raman studies of purple membranes also show that decomposition takes place at higher temperatures (Mendelsohn, 1976), but the rate of decomposition under various conditions is not known. In basal salt the decomposition is more rapid and seems to start very slowly even below 80 °C. The characteristics of the transition are independent of scan rate, which indicates that equilibrium thermodynamics are applicable.

Application of the van't Hoff equation to calculate the effective enthalpy for this transition and evaluation of the ratio $\Delta H_{vH}/\Delta H_{cal}$ provides an estimate for the size of the cooperative unit for the transition. This ratio turns out to be 30.8 ± 3.8 if ΔH_{cal} is calculated per mole of bacteriorhodopsin, and 7.6 if the weight of the unit cell of the purple membrane crystal is used in calculating ΔH_{cal}. The transition thus appears to involve a large degree of intermolecular cooperation and probably a restructuring of the crystal lattice.

When the purple membrane is incorporated into synthetic phosphatidylcholine by sonication, the crystal lattice is destroyed (Hwang & Stoeckenius, 1977). Calorimetric scans of bacteriorhodopsin incorporated into synthetic bilayers by sonication show no 80 °C transition, again supporting the idea that this transition involves a cooperative change in the crystal lattice. The transition itself could, however, be primarily a protein conformational transition with strong intermolecular cooperativity. If the transition still occurred in reconstituted phosphatidylcholine–protein bilayers and was broadened by as much as 10-fold, it would be very difficult to detect by calorimetry.

This small transition at 80 °C is a novel type of membrane phase transition characteristic of the crystallinity of the membrane and requiring further study by a variety of techniques. Both of these transitions should be useful in providing more information concerning the membranes of this organism.

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Dr. Morris Kates is to be thanked for sending a sample of purple membranes isolated from *H. cutirubrum* and for some helpful discussions. Drs. W. Stoeckenius and R. Henderson supplied the strain *H. halobium* R. We would like to thank Michael Hogan and James Terner for arousing our interest in this problem and for many helpful discussions.

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Alkaline Phosphatase: Affinity Chromatography and Inhibition by Phosphonic Acids†

Michael Landt, Steven C. Boltz, and Larry G. Butler*

ABSTRACT: Five phosphonic acid derivatives were synthesized, coupled to agarose, and tested for affinity chromatographic binding of alkaline phosphatase from bovine intestine. Agarose coupled to L-histidyldiazobenzylphosphonic acid was found to be a highly effective adsorbent. In order to understand the large differences in binding capacity observed with derivatized agaroses, inhibition of alkaline phosphatase by phosphonic acid ligands, and related phosphonic acids, was measured. The results of affinity chromatography and inhibition studies were in good agreement, demonstrating that phosphonic acids with large aromatic/hydrophobic, carboxylate substituents bind strongly and competitively to the enzyme active site.

Alkaline phosphatase and 5′-nucleotide phosphodiesterase from bovine intestine are similar in many characteristics important in purification, causing these enzymes to purify together (Dardinger, 1974; Kelly et al., 1975). A persistent difficulty in purification of 5′-nucleotide phosphodiesterase has been removal of contaminating alkaline phosphatase. Previously a large fraction of the phosphodiesterase activity eluted from columns of DEAE-cellulose was sacrificed due to overlapping elution with alkaline phosphatase (Kelly et al., 1975).

The most promising avenue explored to effectively separate these enzymes was affinity chromatography. Because the amount of alkaline phosphatase protein present at the final stage of purification was considerably less than the amount of phosphodiesterase protein, the most efficient resolution was removal of alkaline phosphatase by affinity chromatography. Doellgast & Fishman (1974) employed phenylalanyl agarose in conjunction with high salt concentration to obtain threefold purification of human placental alkaline phosphatase. However, comparison of phenylalanyl agarose to agarose derivatized with other amino acids indicated that binding of alkaline phosphatase to these materials, including phenylalanyl agarose, was due to hydrophobic interactions.

Recently Brenna et al. (1975) have reported the synthesis and use of agarose derivatives incorporating arsonic acid moieties, which are analogues of phosphates, as affinity ligands for bovine intestinal alkaline phosphatase. Best results were achieved with tyraminyl agarose coupled via diazotization to 4-(4-aminophenylazo)phenylarsonic acid. Alkaline phosphatase from bovine intestine was purified 45-fold to apparent homogeneity. The capacity of tyraminyl-4-(4-diazophenylazo)phenylarsonic acid agarose for binding alkaline phosphatase is rather low. From the data of Brenna et al. (1975), approximately 20 mL of gel would be required to bind 1 mg of alkaline phosphatase. Our preliminary experiments with tyraminyl agarose and 4-(4-aminophenylazo)phenylarsonic acid indicated that poor solubility of the arsonic acid in conditions for derivatization and the considerable hydrophobicity of these materials which favors nonspecific protein binding (Shaltiel, 1974) made development of alternative affinity media desirable.

This report presents methods of synthesis and the results of affinity binding tests for a series of affinity chromatography

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