Functional Interactions of Lipids and Proteins in Rat Intestinal Microvillus Membranes†

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ABSTRACT: Interactions of lipids and proteins in isolated rat intestinal microvillus membranes were examined by studying the temperature dependence of enzyme activities and of D-glucose transport in relation to the membrane lipid thermotropic transition observed by fluorescence polarization (26 ± 2 °C) and differential scanning calorimetry (23–39 °C). Two groups of activities were defined. Enzymes of the first group, comprising lactase, maltase, sucrase, leucine aminopeptidase, and γ-glutamyl transpeptidase, all yielded a single slope on the Arrhenius plot in the range 10–40 °C and did not appear to experience functionally the effects of the lipid thermotropic transition. Each activity of the second group, comprising calcium- and magnesium-dependent adenosine triphosphatases, *p*-nitrophenylphosphatase, and D-glucose transport, showed a change in the slope of the Arrhenius plot in the range 25–30 °C, corresponding to the lower region of the lipid transition. The terms "extrinsic" and "intrinsic" activities could be applied to these groups. Delipidation of the particulate *p*-nitrophenylphosphatase removed the discontinuity in the Arrhenius plot. Subsequent relipidation with a variety of lipids restored a break point, but the temperature corresponded to the original discontinuity (25–29 °C) rather than to the phase transition temperature of the exogenous lipid added.

The intestinal microvillus membrane is highly differentiated to perform a variety of digestive and transport functions, and there is considerable information concerning the membrane proteins responsible for many of these discrete mechanisms (Kenny & Booth, 1978). The interactions of the proteins and lipids of the microvillus membrane, however, are less well documented, although it is increasingly recognized that such interactions can influence protein functions in many membrane types (Fox, 1975; Lee, 1975; Razin, 1975; Melchior & Steim, 1976). We recently reported (Schachter & Shinitzky, 1977; Brasitus et al., 1979) that rat microvillus membrane lipid has relatively low fluidity and exhibits a characteristic thermotropic transition[1] at approximately 26 ± 2 °C as determined by fluorescence polarization studies with the hydrocarbon fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH).[2] Differential scanning calorimetry of the microvillus lipid indicates that the transition is broad and occurs over the approximate range 23–39 °C (Brasitus et al., 1979).[3] In *Escherichia coli* and *Mycoplasma* membranes with well-defined lipid thermotropic transitions, it has been possible to demonstrate that a temperature-dependent change in the physical state of the lipids can influence certain membrane activities dependent on proteins, in particular, transmembrane transport mechanisms (Overath et al., 1970; Wilson et al., 1970; Rosen & Hackette, 1972; Tourtellotte, 1972; Linden et al., 1973; Linden & Fox, 1973; Rottem et al., 1973; Shechter et al., 1974). Accordingly, rat microvillus membrane enzyme activities and D-glucose transport were examined at various temperatures, and Arrhenius plots were constructed. The results described below provide an operational criterion for subdivision of the membrane activities into two groups. Each enzyme of the first group, comprising lactase, maltase, sucrase, leucine aminopeptidase, and γ-glutamyl transpeptidase, yields a single slope on the Arrhenius plot in the range 10–40 °C and thus does not appear to experience functionally the effects of the lipid thermotropic transition. Each member of the second group, including Ca-dependent adenosine triphosphatase (Ca-ATPase), Mg-dependent adenosine triphosphatase (Mg-ATPase), *p*-nitrophenylphosphatase, and D-glucose transport, shows a change in the slope of the Arrhenius plot, i.e., a change in energy of activation, in the range 25–30 °C, corresponding thereby to the lower region of the lipid transition. The terms "extrinsic" and "intrinsic" membrane activities could appropriately be applied to the respective groups defined by this operational criterion.

Experimental Procedures

Membrane Preparations. Albino male rats of the Sherman strain weighing 250–300 g were fasted 18 h with water ad libitum before removal of the small intestine. Microvillus membranes were prepared as previously described (Schachter & Shinitzky, 1977) by using procedures reported by Schmitz et al. (1973) and Hopfer et al. (1973). Preparations were maintained at 2–5 °C throughout. In a typical experiment mucosal scrapings of the proximal half of the small intestine of each of 6–12 rats were pooled, homogenized, and treated with 10 mM $CaCl_2$ (Schmitz et al., 1973) and the brush border particulate fraction was obtained by differential centrifugation. After homogenization at high speed (Hopfer et al., 1973), the microvillus membranes were isolated by differential centrifugation and suspended in a 13 mM Tris buffer of pH 7.4. When not tested immediately, the membranes were stored frozen at −15 °C. The purity and comparability of the various preparations were assessed by estimations of sucrase and *p*-nitrophenylphosphatase specific activities. All preparations

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[1] The term "thermotropic transition" is used in a general sense throughout this report to denote a thermally induced change in physical state of the membrane lipids. The change might involve order–disorder transitions of the liquid-crystalline to gel type, lateral phase separations, and other mechanisms [cf. Lee (1977a,b)].

[2] Abbreviations used: CaATPase, calcium-dependent adenosine triphosphatase; MgATPase, magnesium-dependent adenosine triphosphatase; DPH, 1,6-diphenyl-1,3,5-hexatriene.

[3] Thermotropic transitions in membranes may be characterized in terms of upper and lower critical temperatures (Fox, 1975). The transition temperatures observed by fluorescence polarization studies of microvillus membranes (26 ± 2 °C) thus correspond approximately to the lower critical temperature determined by differential scanning calorimetry.

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were purified from 10- to 20-fold as compared to the original homogenates.

Membrane Vesicle Preparations. For glucose transport studies membrane vesicles were prepared by minor modification of the method of Schmitz et al. (1973). The brush border particulate fraction obtained on treatment of the homogenates with 10 mM $CaCl_2$, as described above, was suspended in 100 mM mannitol and homogenized with 10 strokes in a Potter homogenizer. $CaCl_2$ was added to the suspension to a final concentration of 10 mM, and the mixture was stirred slowly for 15 min at 2 °C and then centrifuged at 10000*g* for 15 min in a Sorvall centrifuge (Dupont Instruments; Model RC-5B) at 5 °C. The supernatant solution was retained and centrifuged at 16000*g* for 30 min. The resulting pellet was suspended in 100 mM mannitol, and the vesicle preparation was tested immediately for glucose transport. These preparations were also assayed for sucrase and *p*-nitrophenylphosphatase activities and were consistently 10- to 15-fold purified as compared to the original homogenates.

Enzyme Assays. Assay conditions were chosen to assure linear kinetics and excess of substrate, i.e., maximal velocity, throughout the test period. In general, no more than 5% of a given substrate was utilized in any reaction period. Sucrase, maltase, and lactase were assayed by the method of Dahlqvist (1964), using a glucose oxidase method (Kowarski & Schachter, 1973) to quantify the D-glucose liberated. The *p*-nitrophenylphosphatase activity was estimated as described previously (Kowarski & Schachter, 1973) by using as substrate 5 mM *p*-nitrophenyl phosphate (Sigma Chemicals) in 34 mM glycine buffer of pH 9.5 containing 3.4 mM $MgCl_2$ plus 0.34 mM $ZnCl_2$. When appropriate, the glycine buffer was replaced by 34 mM Tris buffer of pH 7.4. CaATPase and MgATPase were estimated as described previously (Kowarski & Schachter, 1973) in a 1.0-mL reaction mixture containing 5 mM Tris–ATP (Sigma Chemicals), 25 mM Tris buffer of pH 7.4, 0.25 mM Tris–EDTA, and membranes equivalent to approximately 50 μg of membrane protein. Appropriate tubes contained either 2 mM $CaCl_2$ plus 0.5 mM $MgCl_2$, 0.5 mM $MgCl_2$, or no divalent cation. The inorganic phosphate released in the presence of calcium plus magnesium minus that released in the presence of magnesium alone was quantified to determine CaATPase activity. MgATPase was calculated from the increment in phosphate released in the presence of Mg^{2+} as compared to the absence of divalent cation. γ-Glutamyl transpeptidase was assayed by a modification of the method of Naftalin et al. (1969), using γ-L-glutamyl-*p*-nitroanilide (Sigma Chemicals) as the donor substrate and glycylglycine as the acceptor (Sigma Tech Bulletin No. 545). Leucine aminopeptidase was assayed with L-leucyl-β-naphthylamide (Sigma Chemicals) as the substrate by using a modification of the Bratton–Marshall reaction (Sigma Tech Bulletin No. 251) to quantify released β-naphthylamine. All enzyme specific activities were expressed in micromoles per minute per milligram of protein, and for Arrhenius plots the log specific activity was plotted against 1/K.

Glucose Transport. Stereospecific uptake of D-glucose by microvillus membrane vesicles was estimated by a modification of the method of Kessler et al. (1978). All assays were in triplicate. The test reaction mixture had the following composition: 2.0 mM D- and L-glucose, 1 mM Tris–Hepes buffer of pH 7.5, 100 mM mannitol, and sufficient [^{3}H]-D-glucose (sp radioact. 18 mCi/mmol; New England Nuclear) and [^{14}C]-L-glucose (specific radioactivity 55 mCi/mmol, New England Nuclear) to yield approximately 10^6 cpm/mL of ^{3}H and 2.5×10^5 cpm/mL of ^{14}C. In addition, each sample was tested with the same reaction mixture containing 100 mM NaSCN. An aliquot of 100 μL of the appropriate reaction mixture was equilibrated to the desired temperature for 10–15 min, and 5–20 μL of vesicle suspension containing approximately 20 μg of protein was added rapidly to initiate the influx. Uptake was terminated by the very rapid injection of 2 mL of ice-cold 0.2 mM phloridzin in 145 mM NaCl containing 10 mM Tris buffer of pH 7.4. Injection was sufficiently rapid to permit assay over an influx period of 3 s. The mixture was then filtered within 10 s through a Millipore filter (pore size 0.45 μm), and the filter was washed twice with 2 mL of the ice-cold phloridzin solution. Filters were placed in 10 mL of Aquasol (New England Nuclear) and counted in a Beckman LS230 liquid scintillation spectrometer. Aliquots of reaction mixture minus the vesicle suspension were filtered identically, and the filters were counted to correct for nonspecific binding; corrections amounted to 25–52% of the total counts. Stereospecific uptake was calculated as the difference between the uptake (micromoles per minute per milligram of protein) of D-glucose and that of L-glucose.

To distinguish between D-glucose transport to the intravesicular space and possible glucose binding to the membrane, we used the method of Hopfer et al. (1973). Vesicles prepared in 100 mM cellobiose were assayed for glucose uptake at 25 °C by using extravesicular reaction mixtures of various cellobiose concentrations (i.e., osmolarities). Uptake was quantified at 3 s and 20 min, and the values were plotted against 1/osmolarity. Increasing osmolarity markedly diminished the uptake, and extrapolation to infinite osmolarity (origin of the plot) corresponded to negligible uptake. Thus, uptake in these experiments represents primarily transport to the intravesicular space.

Delipidation and Relipidation. Microvillus membranes were delipidated by treatment with 1-butanol at 4 °C. The suspension of membranes (1–3 mg/mL protein) was mixed with butanol to a final concentration of 30% (v/v), shaken very vigorously for 1 h, and centrifuged at 39000*g* for 30 min. The resulting upper (butanol) layer was removed, and the lower (aqueous) layer was retained for assay of protein, lipid, and enzyme activity. Further butanol treatments as described above were used to delipidate progressively the solubilized material in the aqueous layer. Microvillus membranes were also solubilized and delipidated by suspension in 65% (v/v) 2,2,2-trifluoroethanol (Eastman Kodak Co.). Suspensions were shaken for 1 h at 4 °C, and small amounts of insoluble material were removed by centrifugation.

The butanol-solubilized and -delipidated membrane proteins prepared as described above were relipidated by the procedure of Racker (1972). The following lipids were used: 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (98%; synthetic; Sigma Chemicals); 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (98%; synthetic; Sigma Chemicals); 3-*sn*-phosphatidylethanolamine (Sigma Chemicals; Type III; 98%; from egg yolk); 3-*sn*-phosphatidylinositol (Sigma Chemicals; 98%; from soybean); oleic acid (Sigma Chemicals; 99%); glycerol trioleate (Nuchek Prep, Inc., Elysian, MN; >99%); microvillus membrane lipid, prepared as described previously (Schachter & Shinitzky, 1977) by extraction of membranes according to the method of Folch et al. (1957). In a typical relipidation procedure approximately 2–5 mg of lipid was added to 1 mL of 0.15 M KCl containing 5 mM Tris buffer of pH 7.5 and 40 mM sodium cholate. The mixture was sonicated at 4 °C, under N_2, for periods up to 10 min. (Dipalmitoylphosphatidylcholine suspensions were sonicated at approximately 41 °C.)

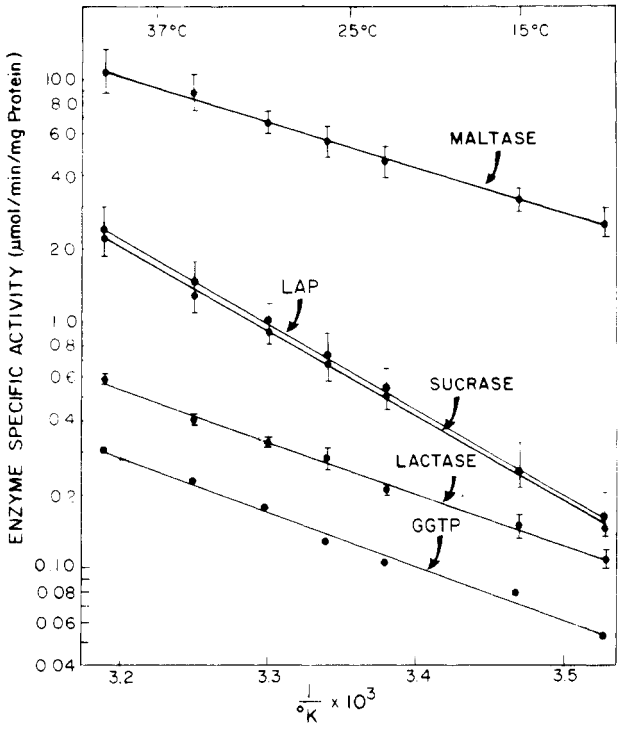

FIGURE 1: Arrhenius plots of three disaccharidase enzymes, leucine aminopeptidase (LAP), and γ-glutamyl transpeptidase (GGTP) of rat microvillus membranes. Table I lists the apparent energies of activation and the number of preparations.

Thereafter, approximately 100 μg of delipidated protein was added, and the mixture was dialyzed for 16–20 h, at 4 °C, against 500 volumes of 0.15 M KCl and 5 mM Tris of pH 7.5.

Fluorescence Polarization Studies. The fluorescence polarization of DPH in sonicated dispersions of lipid (liposomes) was quantified as described previously (Schachter & Shinitzky, 1977). To prepare the liposomes, suspensions containing 0.3 mg/mL lipid in phosphate-buffered saline were sonicated for 10 min, under N_2, at 5 °C. Thereafter, the preparations were centrifuged at 10000*g* for 10 min, and the supernatant liposome suspensions were treated with DPH and examined in a SLM polarization spectrofluorometer. The polarization of fluorescence was expressed as the fluorescence anisotropy, *r*, and the parameter $[(r_0/r) - 1]^{-1}$ was calculated by using a value of $r_0 = 0.362$ for DPH (Shinitzky & Barenholz, 1974). Plots of log $[(r_0/r) - 1]^{-1}$ against 1/K were constructed to detect lipid transition temperatures (Schachter & Shinitzky, 1977).

Other Methods. Protein was estimated by the method of Lowry et al. (1951), phospholipid was estimated by the procedure of Ames & Dubin (1960), and cholesterol was estimated by the method of Zlatkis et al. (1953).

Results

Temperature Dependence of Enzyme Activities. Suspensions of isolated microvillus membranes were assayed for lactase, maltase, sucrase, leucine aminopeptidase, and γ-glutamyl transpeptidase over the temperature range 10–40 °C, and the resulting Arrhenius plots are shown in Figure 1. Linear plots with a single slope were observed for each of the foregoing activities, and the apparent energies of activation ranged from approximately 9.0 kcal/mol for lactase to 16.1 kcal/mol for leucine aminopeptidase (Table I). Comparable studies of microvillus membrane *p*-nitrophenylphosphatase activity at pH 9.5 and 7.4, on the other hand, yielded Arrhenius plots with two slopes (Figure 2) and a break point at approximately 29 °C (Table II), i.e., close to the lipid thermotropic transition at 26 ± 2 °C previously observed by fluorescence polarization (Schachter & Shinitzky, 1977). The break-point temperature was similar at both pH values, although the enzyme specific activity at pH 7.4 was only approximately 1/3 to 1/2 that at pH 9.5, in accord with the known pH dependence of the activity (Fernley, 1971).

Inasmuch as an apparent break in the Arrhenius plot of an enzyme activity may result from temperature-dependent changes in the half-saturation constant (K_m) of the substrate (Silvius et al., 1978), the membrane *p*-nitrophenylphosphatase activity at each temperature was also estimated at five different substrate concentrations. The maximal velocity (V_{max}) and

Table I: Temperature Dependence of Microvillus Membrane Enzymes with No Break in the Arrhenius Plot[a]

enzyme	no. of prepn	apparent energy of activation, ΔE (kcal/mol)
lactase	3	9.0 ± 0.7
maltase	3	9.4 ± 2.0
sucrase	3	15.3 ± 0.8
γ-glutamyl transpeptidase	2	10.1, 10.4
leucine aminopeptidase	3	16.1 ± 1.5

[a] Values are means ± SE.

Table II: Temperature Dependence of Microvillus Membrane Activities with a Break in the Arrhenius Plot[a]

act.	no. of prepn	apparent break point (°C)	apparent energy of activation, ΔE (kcal/mol) below break point	above break point
p-nitrophenyl-phosphatase (pH 9.5)	8	29.1 ± 0.7	11.6 ± 1.1	6.9 ± 1.1
p-nitrophenyl-phosphatase (pH 7.4)	4	29.1 ± 1.8	8.7 ± 1.5	2.3 ± 0.4
CaATPase	3	26.2 ± 0.1	6.5 ± 1.6	0.9 ± 0.5
MgATPase	3	27.6 ± 1.5	7.8 ± 1.6	3.0 ± 0.3
glucose transport, with Na^+	3	27.2 ± 1.3	15.2 ± 6.1	4.1 ± 2.4
glucose transport, without Na^+	3	28.8 ± 2.1	18.1 ± 1.9	4.7 ± 0.3

[a] Values are means ± SE.

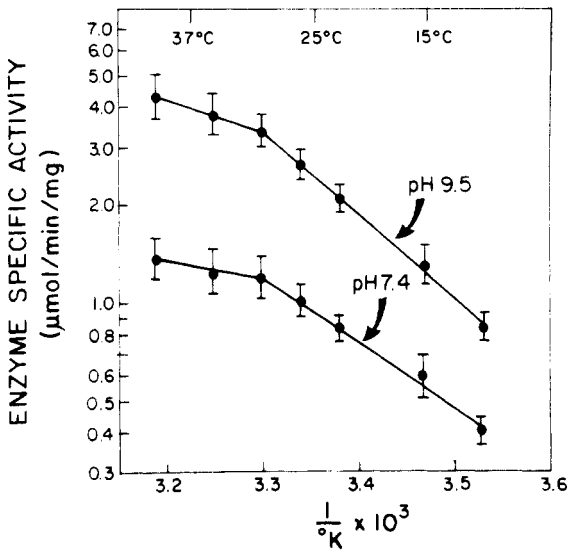

FIGURE 2: Arrhenius plots of rat microvillus membrane *p*-nitrophenylphosphatase studied at pH 9.5 and pH 7.4. Table II lists the number of preparations, the apparent energies of activation, and the temperature of the break points.

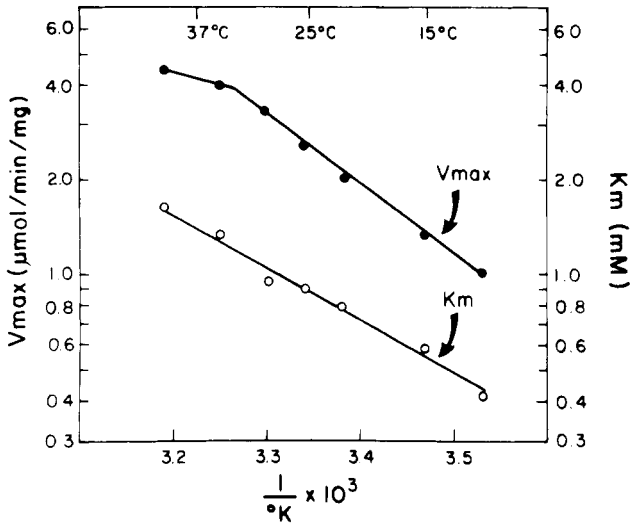

FIGURE 3: Arrhenius plot of microvillus membrane *p*-nitrophenylphosphatase (pH 9.5) kinetic parameters, maximal velocity (V_{max}) and half-saturation concentration (K_m). The method is described in the text.

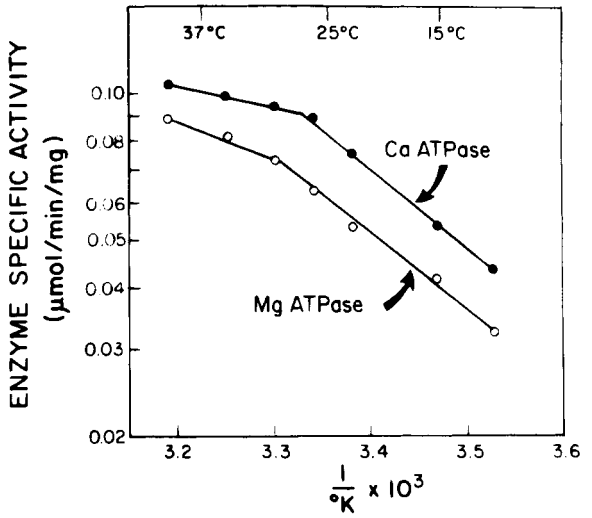

FIGURE 4: Arrhenius plots of rat microvillus membrane calcium- and magnesium-dependent adenosine triphosphatases. Mean values are plotted. Apparent energies of activation and break points are listed in Table II.

K_m parameters were evaluated from double-reciprocal plots (Lineweaver & Burk, 1934), and Arrhenius plots were constructed for each parameter (Figure 3). The V_{max} values again showed a break point at approximately 33 °C, whereas the K_m values did not. The V_{max} values in Figure 3 are almost identical (average deviation 3.0%) with the corresponding activities in Figure 2 (pH 9.5) estimated at one substrate concentration. Hence, the enzyme activities in Figure 2, assayed at substrate concentration/K_m ratios of 3–12, are, in fact, V_{max} values.

In addition to the membrane-bound *p*-nitrophenylphosphatase studied above, homogenates of rat intestinal mucosa contain a "soluble" *p*-nitrophenylphosphatase activity which does not sediment on centrifugation at 106000*g* for 60 min. Four preparations of the soluble enzyme activity were studied at various temperatures, and Arrhenius plots were constructed. In contrast to the microvillus membrane activity, the soluble enzyme shows no break point in Arrhenius plot, and the single slope corresponds to an apparent energy of activation of 9.8 kcal/mol.

Suspensions of microvillus membranes were also used to study CaATPase and MgATPase as functions of temperature, and the results are summarized in Figure 4 and Table II. The Ca- and Mg-dependent activities each yielded a two-slope plot with a break point respectively at approximately 26 and 28 °C.

Temperature Dependence of Glucose Transport. The intestinal absorption of D-glucose and related hexoses occurs via an active transport mechanism which is dependent on sodium in the lumenal medium (Crane, 1965). The active transfer mechanism has been localized to the microvillus membrane, and it can be studied with suspensions of isolated microvillus membrane vesicles (Murer & Hopfer, 1974; Kessler et al., 1978). Accordingly, vesicle preparations were tested for D-glucose uptake at various temperatures and in the presence and absence of added Na^+. Figure 5 shows the Arrhenius plots, and Table II lists the break points and apparent energies of activation. The plots with and without Na^+ were each characterized by two slopes and by break points respectively at approximately 27 and 29 °C. In accord with prior reports (Hopfer et al., 1973; Murer & Hopfer, 1974; Kessler et al., 1978), the rates of transport were considerably higher in the presence of Na^+ at all temperatures tested. Despite the striking

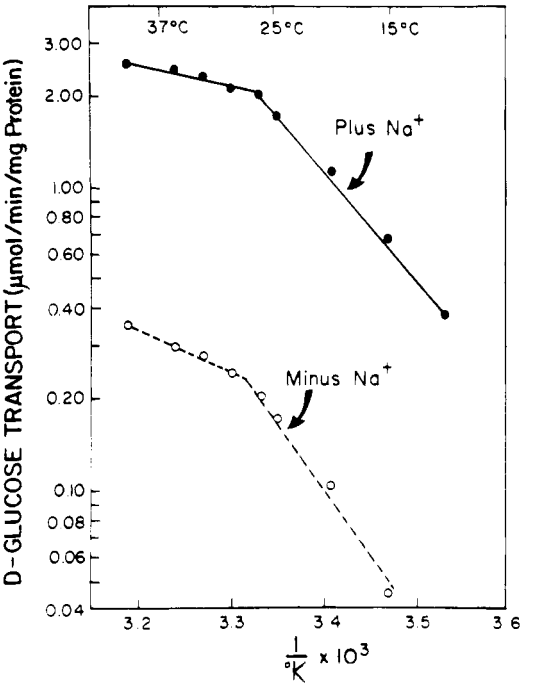

FIGURE 5: Arrhenius plots of D-glucose transport into vesicles of rat intestinal microvillus membranes in the presence and absence of external sodium ion. Uptake of D-glucose was determined as described under Experimental Procedures, and mean values are plotted. Table II lists the apparent energies of activation and break point temperatures.

effect of Na^+ on the transport rate, the apparent energies of activation in the presence and absence of the cation were not significantly different (Table II).

Delipidation and Relipidation Studies. Arrhenius plots of the various enzyme and transport activities summarized in Table II all yielded similar break points which ranged from 26 to 29 °C and corresponded closely, therefore, to the lipid transition temperature. To explore more directly the influence of membrane lipid on the *p*-nitrophenylphosphatase activity, we performed a series of delipidation and relipidation studies. Microvillus membranes were treated with 1-butanol (Experimental Procedures) to remove membrane lipids, a procedure which also solubilizes the *p*-nitrophenylphosphatase (Saini & Done, 1972). One 1-butanol treatment removed

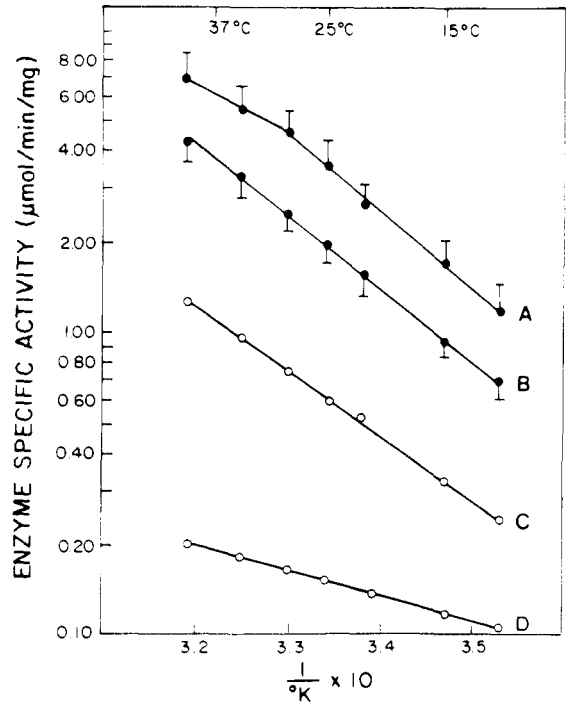

FIGURE 6: Arrhenius plots of *p*-nitrophenylphosphatase activity (pH 9.5) in delipidated preparations of rat microvillus membranes. Mean ± SE values of enzyme specific activity are shown. Results of eight microvillus preparations solubilized and partially delipidated by one treatment with 1-butanol (Experimental Procedures) are plotted in (A). Two successive treatments with 1-butanol (seven preparations) yielded the results in (B). Assay of the interfacial, precipitated membrane proteins obtained on treatment with 1-butanol (three preparations) gave the results in (C), and solubilization of microvillus membranes in 65% trifluoroethanol yielded the values in (D) (two preparations).

approximately 77% of the membrane phospholipids and 86% of the cholesterol. Arrhenius plots of the activity of the resulting partially delipidated and solubilized enzyme are summarized in Figure 6 for eight preparations. In these preparations a break point was still observed at approximately 31 °C (Figure 6A) although the apparent energies of activation above and below the break point, 7.6 and 11.9 kcal/mol, respectively, appeared to differ less than the corresponding values for the untreated membranes, 6.9 and 11.6 kcal/mol (Table II). When these solubilized preparations were retreated with 1-butanol, additional lipid was removed, and the resulting preparations contained less than approximately 5% of the membrane phospholipids and less than 7% of the cholesterol. Figure 6B shows that the enzyme activity of these more thoroughly delipidated preparations now gave an Arrhenius plot with a single slope and an apparent energy of activation of 11.3 kcal/mol (Table III).

As noted by prior investigators (Saini & Done, 1972), treatment of the membranes with 1-butanol to solubilize the enzyme yielded preparations of higher specific activity (Figure 6A as compared to Figure 3). The total enzyme activity recovered in this step, however, averaged 68%, indicating that the increase in specific activity resulted mainly from the removal of inactive proteins. Reextraction with 1-butanol lowered the specific activity (Figure 6B), and the final recovery of total enzyme activity averaged 40%.

The 1-butanol treatment of membrane suspensions described above resulted in an interfacial fluff containing delipidated, precipitated proteins. This material retained sufficient *p*-nitrophenylphosphatase activity to permit temperature studies, and the resulting Arrhenius plot was characterized by a single

Table III: Temperature Dependence of *p*-Nitrophenylphosphatase in Delipidated and Relipidated Preparations[a]

prepn	no.[c]	app break pt (°C)	app energy of activation, ΔE (kcal/mol) below break pt	app energy of activation, ΔE (kcal/mol) above break pt
delipidated[b]	7	none	11.3 ± 1.1	same
relipidated				
endogenous microvillus membrane lipid	4	27.5 ± 1.3	13.0 ± 2.0	6.9 ± 0.4
dipalmitoyllecithin	5	27.5 ± 1.1	11.1 ± 1.1	5.5 ± 1.0
dimyristoyllecithin	5	24.8 ± 1.6	11.9 ± 1.5	5.2 ± 0.8
phosphatidylethanolamine	3	26.7 ± 1.6	11.5 ± 0.7	2.9 ± 1.3
phosphatidylinositol	3	26.7 ± 1.8	13.3 ± 1.7	5.2 ± 1.3
glyceryl trioleate	2	28.2	12.4	8.1
sodium oleate	2	25.6	10.4	6.9

[a] Values are means ± SE. [b] Delipidation was carried out by two successive treatments with 1-butanol (Experimental Procedures). [c] Number of determinations.

slope (Figure 6C). Finally, microvillus membranes were dissolved in 65% (v/v) trifluoroethanol (Shinitzky & Rivnay, 1977) as an alternative method of delipidating the membrane proteins. Although the specific activity of the *p*-nitrophenylphosphatase was quite low in these solutions, Arrhenius plots could be obtained and again showed a single slope (Figure 6D).

Microvillus membrane suspensions were solubilized and delipidated by two treatments with 1-butanol, and the resulting preparations were relipidated by the procedure of Racker (1972). Arrhenius plots of the *p*-nitrophenylphosphatase activities are shown for representative experiments in Figure 7, and the appropriate parameters are listed in Table III. Relipidation with seven different lipids uniformly decreased the specific activity of the *p*-nitrophenylphosphatase. As a percentage of the initial value of the delipidated preparation, the final specific activities ranged from approximately 37% (dimyristoyllecithin) to 58% (endogenous microvillus membrane lipids). Moreover, each of the lipids tested restored a break to the Arrhenius plot, and the apparent break-point temperatures, ranging from 25 to 29 °C, were similar for all (Table III) and not significantly different from the break-point temperatures characteristic of the intact membrane (Table II). In view of the foregoing, additional relipidation experiments were performed as described above, except that the cholate was excluded in some experiments and the added lipid was excluded in others. In the absence of cholate, added lipid again restored the break in the Arrhenius plot, and the break-point temperatures were not significantly different from those in Table III. In the absence of exogenous lipid, however, the procedure itself did not induce a break in the Arrhenius plot.

It is noteworthy that the break point for *p*-nitrophenylphosphatase activity induced by relipidation did not necessarily correspond to the thermotropic transition temperature of the lipid used. This lack of concordance has also been observed for certain other membrane enzymes (Anzai et al., 1978; Ceuterick et al., 1978). To explore this point further, we studied sonicated dispersions of the dipalmitoyllecithin, dimyristoyllecithin, and phosphatidylinositol used in these experiments, as well as the relipidated enzyme preparations containing these lipids, by fluorescence polarization with DPH to detect thermotropic transitions (Experimental Procedures). In accord with prior results (Shinitzky & Barenholz, 1978) the dipalmitoyllecithin and dimyristoyllecithin preparations showed major transition temperatures of 40–43 and 23–26 °C,

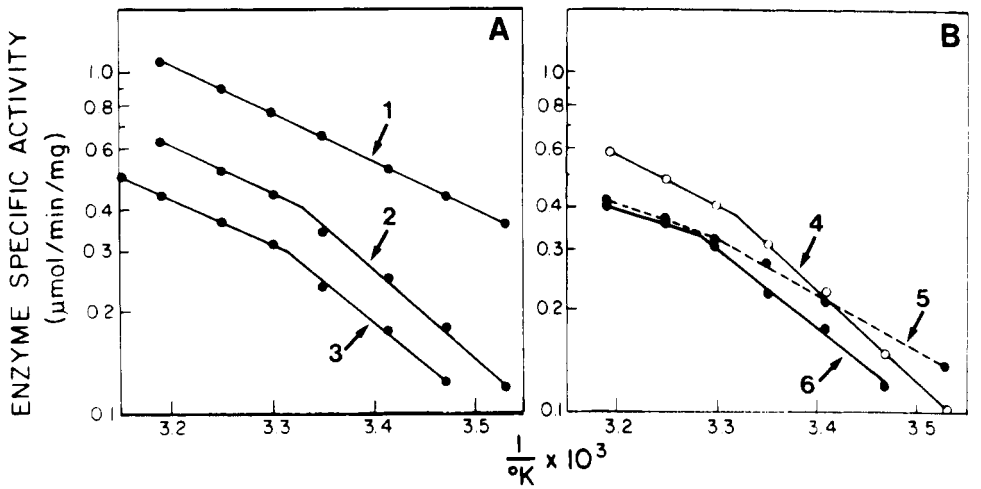

FIGURE 7: Arrhenius plots of *p*-nitrophenylphosphatase activity (pH 9.5) in delipidated and relipidated preparations. A preparation of rat microvillus membranes was delipidated and solubilized by two successive treatments with 1-butanol (A1) and subsequently relipidated (Experimental Procedures) with either endogenous microvillus membrane lipid (A2), synthetic dipalmitoyllecithin (A3), phosphatidylinositol (B4), dimyristoyllecithin (B5), or phosphatidylethanolamine (B6). Apparent energies of activation and break points are listed in Table III.

respectively, and the phosphatidylinositol preparations showed no clear transition in the range 2–40 °C. Yet all three phospholipids, as well as phosphatidylethanolamine, glyceryl trioleate, and sodium oleate, yielded a break in the *p*-nitrophenylphosphatase Arrhenius plot at approximately 25–29 °C.

Inasmuch as Arrhenius plots of the soluble mucosal *p*-nitrophenylphosphatase showed only one slope, an attempt was made to lipidate the soluble enzyme with endogenous microvillus membrane lipid, using the procedure of Racker (1972). In contrast to the results above with the microvillus membrane enzyme, neither the specific activity nor the Arrhenius plot of the soluble enzyme was altered by the procedure.

Discussion

Proteins of the highly differentiated intestinal microvillus membrane mediate a variety of digestive and absorptive functions. The results above demonstrate that the membrane lipids can influence certain of the protein-dependent activities, namely, *p*-nitrophenylphosphatase, CaATPase, MgATPase, and transport of D-glucose. Arrhenius plots of each of these activities show a break in slope in the range 25–30 °C (Table II), thereby corresponding to the thermotropic transition of the membrane lipid (Schachter & Shinitzky, 1977; Brasitus et al., 1979). It is reasonable to conclude that proteins of this group are intimately associated with the hydrophobic core of the membrane. In support of this conclusion, it is noteworthy that the enzyme activities in this group, in addition to the D-glucose transfer mechanism, may be involved in membrane transport. There is considerable evidence that membrane transport mechanisms in *E. coli* (Schairer & Overath, 1969; Wilson et al., 1970; Rosen & Hackette, 1972; Linden & Fox, 1973; Linden et al., 1973; Overath & Traüble, 1973; Shechter et al., 1974), *Mycoplasma* (Tourtellotte, 1972; Rottem et al., 1973), human erythrocyte membranes (Cabantchik & Rothstein, 1974; Rothstein et al., 1976; Marchesi et al., 1976; Guidotti, 1977), sarcoplasmic reticulum (Warren et al., 1974a,b; MacLennan & Holland, 1975; Tada et al., 1978), and microsomal membranes (Dahl & Hokin, 1974; Kimelberg & Papahadjopoulos, 1974; Glynn & Karlish, 1975) are mediated by proteins which are intimately associated with membrane lipids, often span the hydrophobic region of the membrane, and generally require lipids for their function. Both the CaATPase and the *p*-nitrophenylphosphatase studied here are dependent on vitamin D in the diet (Melancon & Deluca, 1970; Haussler et al., 1970; Kowarski & Schachter, 1973, 1975), and it is well established that the vitamin is required for normal absorption of calcium across the intestinal mucosal cell. There is indirect evidence that both of these activities may reside in a single enzyme complex (Haussler et al., 1970; Kowarski & Schachter, 1973); the microvillus membrane MgATPase, however, is not dependent on vitamin D and is probably a separate enzyme (Kowarski & Schachter, 1973).

In contrast to the activities above, a second group of microvillus membrane enzymes is characterized by a single slope on the Arrhenius plot in the range 10–40 °C (Table I). Lactase, maltase, sucrase, leucine aminopeptidase, and γ-glutamyl transpeptidase, mainly hydrolytic enzymes required for digestion, are apparently less intimately associated with the membrane lipids involved in the thermotropic transition. Additional evidence also supports the conclusion that these enzymes function in an aqueous environment, probably at the lumenal surface of the membrane. Treatments of intestinal microvillus membranes or brush borders with papain or detergents, e.g., Triton X-100 or sodium deoxycholate, solubilize the enzymes of this group much more rapidly than they release *p*-nitrophenylphosphatase. Louvard et al. (1975) reported rapid release of maltase, sucrase, and aminopeptidase but much slower release of *p*-nitrophenylphosphatase from rat, rabbit, and pig microvillus membrane vesicles treated with papain; Triton X-100 (1%) released 80–100% of the porcine maltase and aminopeptidase but only 30–50% of the *p*-nitrophenylphosphatase. Boedeker et al. (1976) observed that guinea pig microvillus membranes treated with 0.04% Triton X-100 released 62% of the maltase and sucrase but only 3.9% of the "alkaline phosphatase". Papain treatment of human microvillus membranes released much more lactase, maltase, sucrase, leucine aminopeptidase, and γ-glutamyl transpeptidase than it did *p*-nitrophenylphosphatase (Maestracci, 1976). Haase et al. (1978) concluded that rat "aminopeptidase M" is localized at the lumenal surface of the microvillus membrane and is accessible there to inhibition by specific antibodies. Finally, there is electron microscopic evidence that digestive enzymes of this group are organized in "knoblike" structures visible on the lumenal surface of the microvillus membrane (Johnson, 1966; Nishi et al., 1968; Takesue & Sato, 1968; Maestracci, 1976).

The foregoing results suggest that the presence or absence of a break in Arrhenius plot in the range 25–30 °C provides an operational criterion for classifying intestinal microvillus

membrane activities as intrinsic or extrinsic, respectively. Overath & Traüble (1973) suggested a similar criterion for categorizing membrane activities of an *E. coli* fatty acid auxotroph. Guidotti (1977) has proposed that intrinsic membrane proteins, defined as proteins which span the membrane and require detergent treatment for solubilization, can be classified further into two groups on the basis of the location of the main mass of the protein, either in the cytosol or in the aqueous environment at the external surface. The heuristic value of the operational criterion proposed in the present report, on the other hand, is that it focuses specifically on whether the rate-limiting step of a given membrane function is subject to modulation by the lipids; there is no necessary implication concerning the location of the protein. For example, in *E. coli* (Shechter et al., 1974) and in *Mycoplasma* (Rottem et al., 1973), sugar transport via the phosphotransferase system, a mechanism which necessarily involves transit across the nonpolar domain of the bilayer, did not show breaks in the Arrhenius plot corresponding to the transition temperature of the membrane lipids. In these cases the rate-limiting step in transit via the phosphotransferase system may occur in an aqueous domain; alternatively, the lipids in immediate association with the enzyme complex ("annular" lipids) may differ in composition from the bulk membrane lipid. Similar considerations apply to the results of Esfahani et al. (1971) and Mavis & Vagelos (1972), who reported that certain membrane-bound enzymes in auxotrophs of *E. coli* did not show breaks in Arrhenius plot corresponding to the lipid transition temperature. Many other membrane activities, however, do exhibit breaks in Arrhenius plot concordant with the lipid transition temperature: in *E. coli*, β-galactoside transport (Schairer & Overath, 1969; Wilson et al., 1970; Overath & Traüble, 1973), β-glucoside transport (Linden & Fox, 1973; Linden et al., 1973), proline transport (Shechter et al., 1974), and arginine and glycine transport (Rosen & Hackette, 1972); in *Mycoplasma*, α-methylglucoside efflux (Rottem et al., 1973) and MgATPase (Rottem et al., 1973) and 2-deoxyglucose transfer (Tourtellotte, 1972).

More direct evidence that the intestinal microvillus membrane lipids can modulate the activity of a membrane protein comes from an examination of *p*-nitrophenylphosphatase. Delipidation with either 1-butanol or trifluoroethanol abolished the break in Arrhenius plot (Figure 6B–D), although the resulting preparations differed considerably in enzyme specific activity. Relipidation with either endogenous microvillus membrane lipid or with a number of synthetic lipids (Table III and Figure 7) restored the break in the Arrhenius plot and in each instance the resulting break point occurred in the range 25–29 °C; i.e., it corresponded to the transition temperature of the original membrane lipid rather than to the phase transition temperature of the exogenous synthetic lipid. Relipidation of a number of other enzymes, including *Azotobacter* nitrogenase (Ceuterick et al., 1978), rabbit kidney sodium-plus-potassium ATPase (Kimelberg & Papahadjopoulos, 1974), and CaATPase of sarcoplasmic reticulum (Anzai et al., 1978), also resulted in break points which did not correspond completely to the phase transition temperature of the lipid used. The exact explanation for this behavior is unknown and reflects the more general lack of an accepted picture of how thermally induced changes in the physical state of the lipids influence membrane enzymes (Lee, 1977a,b). Some authors (Thorneley et al., 1975; Anzai et al.,1978; Gómez-Fernández et al., 1979) emphasize the importance of temperature-induced conformational changes or alterations in aggregation of the proteins, processes which presumably require a particular lipid environment. Others (Ceuterick et al., 1978) stress the role of the annular lipids and the strength of their interaction with the protein as a determinant of the break-point temperature. These factors may be incorporated into a working hypothesis to explain our results. In the intact microvillus membrane, the bulk and annular lipids appear to be similar in composition, inasmuch as quite different activities, e.g., divalent cation ATPases and glucose transport, show a similar break point in the Arrhenius plot and the break point corresponds to the thermotropic transition of the lipid. Treatment of the membrane with 1-butanol yields a solubilized *p*-nitrophenylphosphatase which retains a small fraction of the original annular lipid. The discontinuity in the Arrhenius plot, however, is lost, because it apparently requires a critical mass of lipid. On reconstitution with a variety of lipids, the discontinuity is restored, with the temperature of the break point determined in each instance by the endogenous annular lipid.

Examination of the temperature dependence of the *p*-nitrophenylphosphatase kinetic parameters indicated that only the V_{max}, and not the K_m, showed the characteristic break point in the Arrhenius plot (Figure 3). Apparently, therefore, the rate-limiting step influenced by the membrane lipids occurs subsequent to the formation of the enzyme–substrate complex. It is further noteworthy that rat intestinal mucosa contains at least two *p*-nitrophenylphosphatases: the microvillus membrane bound activity described above and a soluble enzyme, which does not show a break in the Arrhenius plot either before or after a lipidation procedure. Prior studies have also indicated that the particulate enzyme, but not the soluble activity, is dependent on vitamin D (Kowarski and Schachter, unpublished experiments). Recent purifications of rat intestinal alkaline phosphatase (Saini & Done, 1972; Malik & Butterworth, 1976) have used whole intestine as the starting material, and it is not clear to what extent the purified preparation contains the particulate vs. the soluble enzyme.

The intestinal microvillus membrane is particularly useful for examining lipid–protein interactions in a mammalian preparation, inasmuch as the lipids undergo a broad thermotropic transition, in the range 23–39 °C, as determined by differential scanning calorimetry (Brasitus et al., 1979). Fluorescence polarization studies with the DPH probe detect the lower end of the transition (Schachter & Shinitzky, 1977; Brasitus et al., 1979), and in this lower zone, approximately 25–30 °C, the break points in the Arrhenius plot of a number of membrane protein activities are observed. The correspondence of the lipid thermotropic transition to the break points is similar to well documented observations of bacterial membranes [cf. Razin (1975) and Melchior & Steim (1976)], although the enthalpy of the lipid transition is lower in the microvillus as compared to the bacterial membranes. By contrast, in other well studied mammalian membranes, e.g., sarcoplasmic reticulum (Martonosi, 1974; Davis et al., 1976; Mitsui, 1978) and rat liver mitochondria and microsomes (Blazyk & Steim, 1972), lipid thermotropic transitions were detected by calorimetry only at low temperatures, in the range 0–5 °C. Spin-label studies of the membrane lipids and Arrhenius plots of a number of protein activities, however, have shown concordant breaks in the range 18–25 °C in sarcoplasmic reticulum (Eletr & Inesi, 1972; Lee et al., 1974) and rat liver microsomes (Eletr et al., 1973) and at 23–26 °C in rat liver mitochondria (Tinberg et al., 1972; Williams et al., 1972). Lee (1975, 1977a,b) has discussed possible mechanisms, including lateral phase separation (Linden et al., 1973) and lipid cluster formation (Lee et al., 1974), to account for

the thermally induced change observed with spin-labels in the absence of transitions detected by calorimetry. No definitive interpretation is yet available, although there is agreement that a liquid-crystalline to gel, order–disorder transition is apparently not involved (Martonosi, 1974; Davis et al., 1976; Mitsui, 1978).

Linden et al. (1973) have pointed out that thermotropic transitions of membrane lipids may be characterized by upper and lower critical temperatures. The break points in Arrhenius plots observed in our studies occur near the lower critical temperature of the lipid transition. To date we have not detected additional break points near the upper limit of the lipid transition, perhaps because temperatures in excess of approximately 39–40 °C would be required.

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In Vivo Studies on the Incorporation of Microinjected Acidic Proteins of the Large Ribosomal Subunit from *Escherichia coli* and *Artemia salina* into Oocyte Ribosomes from *Xenopus laevis*†

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ABSTRACT: Tritium-labeled acidic proteins from the large ribosomal subunit of *Artemia salina* or *Escherichia coli* were microinjected into the cytoplasm of stage IV/V oocytes from *Xenopus laevis*. eL12 from the large ribosomal subunit of *A. salina* but not L7/L12 or L7/L12–L10 from *E. coli* is specifically incorporated into 60S ribosomal subunits of oocytes. This incorporation is not significantly inhibited by actinomycin D. Incorporation of eL12 into the 60S subunits occurs in enucleated oocytes, suggesting that active ribosomal ribonucleic acid synthesis and ribosome assembly as well are not prerequired for this reaction. Apparently the incorporation proceeds via an exchange reaction between a free cytoplasmic pool of eL12 and ribosomal eL12.

It is well established that procaryotic and eucaryotic ribosomes and their associated factors differ in their biochemical and physical properties, while the mechanism of protein synthesis is essentially similar in both classes [for a review, see Brimacombe et al. (1978)]. Structural differences may explain the observation that, in general, factors and ribosomes from the two classes are not freely interchangeable. There are only a few exceptions to the class specificity: bacterial type of elongation factor Tu can interact with 80S eucaryotic ribosomes (Krisko et al., 1969; Richter & Lipmann, 1970). Hybrid ribosomes consisting of *Escherichia coli* 50S and eucaryotic 40S subunits but not of 30S and 60S subunits show limited function in protein synthesis (Klein & Ochoa, 1972; Richter, 1973; Boublik et al., 1979). Functional interchangeability has also been found between the two acidic ribosomal proteins L7/L12 from *E. coli* and eL7/eL12 from yeast (Richter & Möller, 1974; Wool & Stöffler, 1974) or *Artemia salina* (Möller et al., 1975). Sequence (Amons et al., 1978) and immunological analyses (Wool & Stöffler, 1974; Stöffler et al., 1974; Howard et al., 1976; Leader & Coia, 1978) imply that eL7 and eL12 are the eucaryotic counterparts of *E. coli* proteins L7 and L12. So far little is known about formation of hybrid ribosomes under physiological conditions. Therefore, we used the oocyte system from *Xenopus laevis* as an in vivo model system for studying the incorporation of microinjected ribosomal proteins into ribosomes. As it turned out, this system may also be a promising model for investigating the ribosomal assembly process in eucaryotes. In the present communication we studied the in vivo incorporation of microinjected radioactively labeled acidic proteins L7/L12 from *E. coli* and eL12 from *A. salina* into ribosomes from oocytes of *X. laevis*.

Materials and Methods

Female frogs of *X. laevis* were obtained from South African Snake Farm (Fish Hoek, South Africa). Sodium boro-[^{3}H]hydride (sp act. 5–20 Ci/mmol) was purchased from Amersham-Buchler. Ribosomal proteins eL12 from *A. salina* (Möller et al., 1975) and L7/L12 from *E. coli* (Möller et al., 1972) were kindly provided by W. Möller, Leiden, and L7/L12–L10 from *E. coli* were provided by J. Dijk, Berlin (Dijk et al., 1977). Ribosomal proteins were labeled by the reductive methylation method using tritium-labeled sodium borohydride. Lyophilized proteins were dissolved in (0.5–1.0 mg/mL) and dialyzed against 20 mM Hepes buffer, pH 8.5, containing 10 mM $MgCl_2$, 20 mM KCl, and 6 mM 2-mercaptoethanol and methylated in the presence of 4 mM formaldehyde and 10 mCi of sodium boro[^{3}H]hydride at the time intervals indicated in published procedures (Means & Feeney, 1968; Kleinert & Richter, 1975). Aliquots were precipitated with 10% trichloroacetic acid, collected on filters (0.45-μm pore size, Sartorius), and analyzed in a Packard sample oxidizer and liquid scintillation counter to calculate the specificity of the labeled proteins. On the average 250–300 cpm/ng of protein

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