Use of a Monoclonal Antibody to Quantify (Na\(^+\),K\(^+\))-ATPase Activity and Sites in Normal and Regenerating Rat Liver*

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Quantitative measurements of (Na\(^+\),K\(^+\))-ATPase activity and numbers of (Na\(^+\),K\(^+\))-ATPase sites in membranes from quiescent and regenerating rat liver have been made using an anticytotoxic monoclonal antibody (9-A5) that binds to α subunits of the sodium pump (Schenk, D. B., and Leffert, H. L. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5281–5285). To validate the measurements, kinetic properties of 9-A5 binding to plasma membrane sodium pumps, specificity and requirements of the reactions, and mechanisms by which 9-A5 inhibits (Na\(^+\),K\(^+\))-ATPase were analyzed. \(^{125}\)I-9-A5 binding is saturable and reversible (\(k_1 = 1.8 \times 10^{-4}\) M\(^{-1}\) s\(^{-1}\); \(k_2 = 2.7 \times 10^{-4}\) s\(^{-1}\)). At equilibrium, 9-A5 binds to a single class of sites revealed by Scatchard plots (\(K_{D,9-A5} = 0.64\) nM, \(B_{max} = 29.3\) pmol/mg of protein; = 238,000 sites-cell\(^{-1}\)). This binding requires monovalent cations (sodium, potassium, or lithium); is blocked by purified (Na\(^+\),K\(^+\))-ATPase; is inhibited noncompetitively by ATP (\(K_{D,ATP} = 0.5\) mM); and is unaffected by ouabain. 9-A5 inhibits ATP-stimulated (Na\(^+\),K\(^+\))-ATPase noncompetitively by blocking sodium-dependent phosphorylation of α subunits of liver or kidney membrane (Na\(^+\),K\(^+\))-ATPase. Twelve hours after 67% hepatectomy, maximal \(^{125}\)I-9-A5 binding to plasma membranes from regenerating liver falls 30 ± 7% compared to sham-operated controls (\(p < 0.01\)). In contrast, (Na\(^+\),K\(^+\))-ATPase activity in regenerating liver membranes rises 58 ± 12% compared to controls (\(p < 0.05\)). Similar experiments with particulate fractions from regenerating liver show insignificant decreases in maximal \(^{125}\)I-9-A5 binding (22 ± 12%) but large increases in (Na\(^+\),K\(^+\))-ATPase activity (325 ± 14%) compared to controls (\(p < 0.001\)). No differences among groups are seen in \(K_P\) values for 9-A5 binding or in the activities of plasma membrane 5′-nucleotidase (EC 3.1.3.5). Thus, stimulation of the sodium pump during the late prereplicative phase of liver regeneration is not accompanied by increases in the numbers of (Na\(^+\),K\(^+\))-ATPase sites. Instead, it appears that pre-existing (Na\(^+\),K\(^+\))-ATPases are activated specifically before DNA replication starts.

Several events occurring at the cell surface are thought to regulate the initiation of animal cell proliferation. Some of these include accelerated rates of amiloride-sensitive sodium (2, 3) and ouabain-sensitive \(^{86}\)Rb\(^+\) influx (3–7), membrane potential hyperpolarization (8, 9), and the stimulation of Na\(^+\)-dependent amino acid transport (2, 10, 11). All are influenced by asymmetric sodium and potassium gradients across the plasma membrane. These gradients are generated, in part, by (Na\(^+\),K\(^+\))-ATPase (EC 3.6.1.3) (12).

To understand how sodium pump-mediated changes in cation transport rates might influence the initiation of DNA replication, particularly during liver regeneration, it is important to learn how hepatic (Na\(^+\),K\(^+\))-ATPase activity is regulated during the prereplicative phase of the “cell cycle.” Previous investigations with isolated (7, 13, 14) and cultured (3) hepatocytes, and regenerating liver (9, 15–17), suggest that ouabain-sensitive \(^{86}\)Rb\(^+\) influx (a reflection of sodium pump-dependent potassium influx (18)) and (Na\(^+\),K\(^+\))-ATPase activity rise during transitions from quiescent to proliferating states. Since (Na\(^+\),K\(^+\))-ATPase sites were not quantitated in these studies, the molecular basis for such increases in pump activity, for example, increases in the numbers of sodium pump sites as opposed to increases in enzyme turnover number, remains unknown.

Various approaches have been employed to quantitate (Na\(^+\),K\(^+\))-ATPase sites. In binding studies unrelated to mitogenesis, \(^{3}H\)ouabain has been used since it inhibits sodium pump activity by binding to the (Na\(^+\),K\(^+\))-ATPase catalytic α subunit (19). Detailed binding experiments are difficult to make in mitogen-related work, however, since animal cell growth control systems often consist of rodent cells whose sodium pump activity is relatively insensitive to ouabain (\(K_{D,ouabain} = 0.2\) mM; Ref. 20). In addition, studies with \(^{3}H\) ouabain frequently generate curvilinear Scatchard plots (21, 22) and are hampered by high nonspecific binding (23), problems that complicate interpretation of results. Another approach, based upon the quantitation of ouabain-dependent uptake of \(^{32}\)P into α subunits, has several advantages (22) but potentially underestimates the numbers of (Na\(^+\),K\(^+\))-ATPase sites, since it requires the detection of alkali labile acylphosphate bonds after gel electrophoresis (22). Many of these problems might be circumvented with a new (Na\(^+\),K\(^+\))-ATPase specific ligand.

In this report, the binding characteristics and anticytotoxic properties of such a ligand, a high-affinity mouse monoclonal antibody (9-A5) directed against the α subunit of rat (Na\(^+\),K\(^+\))-ATPase (1), are described. By using 9-A5 as a specific ligand to (Na\(^+\),K\(^+\))-ATPase, we find that sodium pump activation in regenerating liver occurs without detectable increases in the numbers of (Na\(^+\),K\(^+\))-ATPase sites.

MATERIALS AND METHODS

Reagents and Chemicals—The following reagents were purchased from Sigma: lactoperoxidase (EC 1.11.1.7), Type V glucose oxidase

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Activation of (Na⁺,K⁺)-ATPase during Rat Liver Regeneration

(EC 1.1.2.4), bovine serum albumin (Fraction V), Type V pyruvate kinase (EC 2.7.1.40), Type II lactate dehydrogenase (EC 1.1.2.7), phosphoenolpyruvate, vanadate-free ATP, and trichloroacetic acid. NaH[32P]ATP was obtained from ICN (Irvine, CA). [35S]Methionine and [γ-32P]ATP were obtained from Amersham Corp. C₆H₄O₇ was obtained from Nikko Chemicals (Tokyo, Japan). Luciferin-luciferase preparations were obtained from Analytical Vanguard, Inc. (San Diego, CA). Molecular weight protein standards were supplied by Bio-Rad. Kodak X-1 film and X-OMAT intensifying screens were obtained from Merry X-ray Products (San Diego, CA).

Preparation and Purification of Monoclonal Antibody 9-A₅—9-A₅, an IgG₁, was purified from stationary phase hybridoma culture media by immunoaffinity chromatography (26) in the presence of 1% (v/v) p-mercaptoethanol, revealed two bands of M₅₀ and M₃₅ as determined by standard assays (24). Analyses of these preparations by SDS-PAGE (1) revealed Coomassie Blue-stained bands of 15-18 pCi.pg of proteins (5.7 pmol antibody degradation (see below)) was unaltered after 6-8 weeks at -70 °C. Under these conditions, 9-A₅ retained its ability to inhibit (Na⁺,K⁺)-ATPase activity (Kᵢₑ₈ was = 5 μg of 9-A₅/ml = 30 nM) in crude particulate liver fractions about 6 months, as determined by ATPase assays (24).

Radiolabeling of Antibody 9-A₅—9-A₅ was labeled either enzymatically with Na[32P] or metabolically with [35S]methionine. Enzymatic radiiodination of 9-A₅ was performed with lactoperoxidase and glucose oxidase (25). All buffers and reagents used for iodination reactions and [125I]-9-A₅ purification were passed through 0.45-μm NAAC filter (Rochester, NY). Labelled antibody was separated from other reaction mixture components by Sephadex G-100 chromatography at 21 °C in a buffer containing 50 mM sodium phosphate (adjusted to pH 7.5) in H₂O containing 0.15 M sodium chloride (1 mg of 9-A₅/ml) at -70 °C. Under these conditions, 9-A₅ retained its ability to inhibit (Na⁺,K⁺)-ATPase activity (Kᵢₑ₈ was = 5 μg of 9-A₅/ml = 30 nM) in crude particulate liver fractions about 6 months, as determined by ATPase assays (24).

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Enzyme Activity Determinations—(Na⁺,K⁺)-ATPase activity was measured in various fractions (30 μg of proteins·ml⁻¹, or as noted) with an NADH-coupled enzyme assay (24) employing NADH, pyruvate kinase, lactate dehydrogenase, and unless otherwise noted, 3 mM ATP, 100 mM sodium chloride, 20 mM potassium chloride, and 5 mM magnesium chloride. Linear initial rates of ATP hydrolysis were monitored for 3-4 min by following decreases in A₅₇₅ with a Beckman DU-8 spectrophotometer equipped with a Kinetics II Compuares. 5'-Nucleotidase activity was measured in liver membrane or crude particulate fractions (2-20 μg of proteins·ml⁻¹) at 37 °C for 30 min in a buffer containing 50 mm Tris-Cl (pH 7.5), 10 mM MgCl₂, and 5 mM AMP. The reactions were terminated by the addition of ice-cold 10% (w/v) trichloroacetic acid. Samples were centrifuged at 2000 X g for 10 min at 4 °C. Chen's (32) reagent was added and the resulting precipitate was allowed to stand for 15 min at 4 °C. The supernatant was quantitated by readings at 550 nm.

ATP and Protein Measurements—Liver membranes (50-100 μg of proteins) were precipitated with 0.6 N HClO₄ and centrifuged at 2000 X g for 10 min at 4 °C. The pellets were resuspended (at 4 mg of proteins·ml⁻¹) and stored at -70 °C.

The abbreviations used are: C₁₂E₄, octaethylene glycoldecyl ether; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)piperazineethanesulfonic acid.

Preparation of Rat Kidney (Na⁺,K⁺)-ATPase—Purified enzyme was obtained from outer kidney medullas of male Sprague-Dawley rats (300-400 g) by the method of Jørgensen (27). Specific activities of these preparations were 15-24 μmol of ATP hydrolyzed per mg·min⁻¹ as determined by standard assays (24). Analyses of these preparations by SDS-PAGE (1) revealed Coomassie Blue-stained bands of M₅₀, 34,000 (highly resolved) and 45,000-55,000 (diffuse). Active (Na⁺,K⁺)-ATPase was solubilized in C₁₂E₄, using detergent protein ratios of 2.3 (w/w) (28).

Preparation of Rat Liver Membranes—Livers were obtained from male Fischer/344 rats (250-300 g). Neville's procedure was followed through step 11 (Ref. 29). The membranes were stored at -70 °C in "histidine/sucrose" buffer (25 mM histidine-HCl, 0.25 M sucrose, and 1.0 mM EDTA (adjusted to pH 7.5 with 1 N sodium hydroxide)) at 15 mg of proteins·ml⁻¹ and thawed immediately before use.

Preparation of Crude Rat Liver Particulate Fractions—Livers from male Fischer/344 rats (250-300 g) were perfused via the portal vein with 20 ml of 0.15 M sodium chloride (10 ml·min⁻¹) at 37 °C. The remaining steps were performed at 4 °C. The livers were excised and minced in histidine/sucrose buffer. Minced tissue was homogenized with 10 up and down strokes of a loose fitting Dounce homogenizer (clearance = 0.1 mm) in a 5-fold volume of buffer. The homogenate was filtered through four layers of buffer soaked gauze (U.S.F. type VII, Johnson and Johnson, New Brunswick, NJ) and centrifuged at 280 X g for 10 min to remove nuclei and intact cells. The supernatant fraction was retained. The pellet was rehomogenized and centrifuged to obtain a second supernatant. The two supernatant fractions were pooled and centrifuged at 40,000 X g for 30 min. The final pellets were resuspended (at 15 mg of proteins·ml⁻¹) and stored at -70 °C. Ouabain-sensitive ATPase activity in these preparations was stable for at least 8 weeks.

Hepatocytes from 13-day-old quiescent primary cultures were generated by standard procedures (30). The viability, yields, and differentiated functions of such cell preparations have been described elsewhere (30, 31). Particular fractions of cultured cells were used instead of freshly isolated cells (to avoid artefactual proteolysis of membrane proteins) and were prepared as follows. Culture media were aspirated from the dishes and the monolayers were washed twice at 4 °C with 2 ml of Rat Ringer's buffer (140 mM sodium chloride, 5 mM potassium chloride, 10 mM sodium bicarbonate, and 10 mM d-glucose) and stored at -70 °C. Under these conditions, 9-A₅ retained its ability to inhibit (Na⁺,K⁺)-ATPase during Rat Liver Regeneration.
Activation of (Na\(^{+}, K^{+}\))-ATPase during Rat Liver Regeneration

14943

Effects of purified (Na\(^{+}, K^{+}\))-ATPase preparations on specific \(^{125}\text{I}-9\text{A5}\) binding to liver plasma membranes

Binding assays were performed under equilibrium conditions for 90 min at 37 °C in the presence of 100 nM sodium chloride and 20 mM potassium chloride. \(^{125}\text{I}-9\text{A5}\) and liver membranes were present at 2 nM and 40 μg of proteins/ml, respectively. Under these conditions, 20 pmol of \(^{125}\text{I}-9\text{A5}\)-mg of proteins−1 represented maximal specific binding (100%). Effects of membrane-bound purified kidney (Na\(^{+}, K^{+}\))-ATPase were studied by preincubation of excess kidney enzyme for 1 h at 25 °C with 2 nM \(^{125}\text{I}-9\text{A5}\) followed by centrifugation at 4 °C for 10 min at 12,000 X g to remove antibody-enzyme complexes from the supernatant. The supernatant was then assayed for \(^{125}\text{I}-9\text{A5}\) available for binding by incubation with liver membranes under standard binding conditions. Kidney (Na\(^{+}, K^{+}\))-ATPase, solubilized with C\(_{12}\)E\(_{6}\) to achieve maximal enzyme activity (1, 28), was added directly to liver membrane binding reaction mixtures. The data are average values of three separate experiments (S.E. ≤10% in all cases).

### TABLE I

| Competitor | Concentration Specific \(^{125}\text{I}-9\text{A5}\) binding |
|------------|--------------------------|-------------------|
|            | μg proteins−1 | % maximal |
| Membrane-bound kidney | | |
| (Na\(^{+}, K^{+}\))-ATPase | 10 | 0.0 |
| C\(_{12}\)E\(_{6}\)-solubilized kidney | 10 | 8.2 |
| (Na\(^{+}, K^{+}\))-ATPase | | |
| C\(_{12}\)E\(_{6}\) | 0.65* | 101.2 |

* Micrograms of C\(_{12}\)E\(_{6}\)-ml−1.

FIG. 1. Specific \(^{125}\text{I}-9\text{A5}\) binding to rat liver plasma membranes as a function of membrane protein concentration. \(^{125}\text{I}-9\text{A5}\) (1.5 nM; specific activity = 73 cpm fmol−1) was incubated in the absence or presence of 200 nM unlabeled 9-A5, with the indicated amounts of membrane proteins for 90 min at 37 °C under standard conditions. Specific \(^{125}\text{I}-9\text{A5}\) binding was calculated as described under “Materials and Methods.” The data are average values of three separate experiments (S.E. ≤7% in all cases); the curve was determined by linear regression analysis (p < 0.01).

**RESULTS**

**Binding and Biochemical Properties of Monoclonal Antibody 9-A5**

**Effects of (Na\(^{+}, K^{+}\))-ATPase Preparations on \(^{125}\text{I}-9\text{A5}\) Binding to Liver Membranes—**Two different kinds of highly purified preparations of rat kidney (Na\(^{+}, K^{+}\))-ATPase blocked \(^{125}\text{I}-9\text{A5}\) specific binding to membranes (Table I). These effects were obtained by either (a) preincubating \(^{125}\text{I}-9\text{A5}\) with excess particulate kidney (Na\(^{+}, K^{+}\))-ATPase, followed by measuring the residual unbound antibody available for binding to liver membranes, or (b) directly competing for \(^{125}\text{I}-9\text{A5}\) binding to membranes by adding C\(_{12}\)E\(_{6}\)-solubilized (Na\(^{+}, K^{+}\))-ATPase to reaction mixtures. Control experiments indicated that \(^{125}\text{I}-9\text{A5}\) binding was unaffected by C\(_{12}\)E\(_{6}\).

### Dependence of \(^{125}\text{I}-9\text{A5}\) Binding on Liver Membrane Protein Concentration—**FIG. 1 shows the amount of specific \(^{125}\text{I}-9\text{A5}\) binding as a function of membrane protein concentration. Binding was directly proportional to protein concentrations between 4 and 40 μg of proteins−1 (slope = 1.0, p < 0.05). Nonlinear binding occurred above 60 μg of proteins−1. Linear curves, with reduced specific binding, also were obtained with crude liver particulate fractions (data not shown). To maintain experimental conditions of antibody excess (required for Scatchard analyses, Ref. 36) within a linear range of membrane binding site concentrations, 5–20 μg of proteins−1 were used in further studies (or 50–100 μg of crude particulate proteins−1).

### Association Kinetics—**FIG. 2A shows the kinetics of association of 1.5 nM \(^{125}\text{I}-9\text{A5}\) with specific membrane sites at 37 °C. The association was time-dependent and saturable. Half-maximal specific binding occurred at 5 min; apparent equilibrium occurred within 45 min. The data were plotted according to the equation

\[
\ln(B_m/[B_m - B_0]) = (k_1^{125}\text{I}-9\text{A5}) + k_2 t
\]

gels were stained with Coomassie Blue, dried, and subjected to autoradiography for 1–2 days at −70 °C. The developed films were scanned under white light with a densitometer to determine relative proportions of phosphoprotein species (1).

**Measurements of Radiolabeled 9-A5 Binding to Liver Membrane or Particulate Frac-**

tions—Unless stated otherwise, standard binding reactions were performed as follows. To measure total \(^{125}\text{I}-9\text{A5}\) binding, varying concentrations of \(^{125}\text{I}-9\text{A5} (0.10-30.0 \text{ nM}) \) were added to one set of polystyrene tubes (12 × 75 mm) in buffer containing 100 mM NaCl, 20 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, adjusted to pH 7.5 (with 1 N HCl), and 2% (w/v) bovine serum albumin. Under these conditions, adsorption of radioligand to the tubes was negligible. To measure nonspecific \(^{125}\text{I}-9\text{A5} \) binding, a 100-fold excess of unlabeled 9-A5 was added to a second set of identical tubes (both sets of tubes were prepared in duplicate). Binding reactions were initiated by adding either purified liver membranes (10 μg of proteins·tube−1) or crude liver particulate fractions (180 μg of proteins·tube−1) to the mixtures (final total volume = 250 μl) and allowed to proceed for 90 min at 37 °C. The reactions were stopped by adding 2 ml of ice-cold binding buffer. Unbound (“free”) \(^{125}\text{I}-9\text{A5} \) was separated from membranes-bound \(^{125}\text{I}-9\text{A5} \) by centrifugation at 2000 X g for 1–2 days at −70 °C. The developed films were scanned for 1–2 days at −70 °C. The developed films were scanned under white light with a densitometer to determine relative proportions of phosphoprotein species (1).

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### Dependence of \(^{125}\text{I}-9\text{A5}\) Binding on Liver Membrane Protein Concentration—**FIG. 1 shows the amount of specific \(^{125}\text{I}-9\text{A5}\) binding as a function of membrane protein concentration. Binding was directly proportional to protein concentrations between 4 and 40 μg of proteins−1 (slope = 1.0, p < 0.05). Nonlinear binding occurred above 60 μg of proteins−1. Linear curves, with reduced specific binding, also were obtained with crude liver particulate fractions (data not shown). To maintain experimental conditions of antibody excess (required for Scatchard analyses, Ref. 36) within a linear range of membrane binding site concentrations, 5–20 μg of proteins−1 were used in further studies (or 50–100 μg of crude particulate proteins−1).

### Association Kinetics—**FIG. 2A shows the kinetics of association of 1.5 nM \(^{125}\text{I}-9\text{A5}\) with specific membrane sites at 37 °C. The association was time-dependent and saturable. Half-maximal specific binding occurred at 5 min; apparent equilibrium occurred within 45 min. The data were plotted according to the equation

\[
\ln(B_m/[B_m - B_0]) = (k_1^{125}\text{I}-9\text{A5}) + k_2 t
\]

was plotted as a function of time after dilution and addition of


dilution of bound \( ^{125}\text{I}-9\text{-A5} \) was followed at the indicated times by removal of specifically bound radioligand (total counts/min to calculate rate constant, \( k_2 \)). Membranes (10 pg of proteins/tube) were incubated at 37 °C for 90 min at 37 °C (to see if binding site degradation occurred). Inset, semilogarithmic transformation of the data (fit to a linear curve (p < 0.02) described by the equation \( \ln(B/\theta) = k_a \Delta t \) (Ref. 38). A plot of \( \ln(B/\theta) \) versus time gave a linear curve with slope (a dissociation rate constant, \( k_2 \)) of 2.85 \( \times 10^{-4} \) s\(^{-1} \) (Fig. 2B, inset).

Using experimentally determined values of \( k_a \) and \( k_b \), the association rate constant (\( k_b \)) was calculated from the equation

\[
k_b = k_a \frac{\theta}{k_b} + k_d \quad \text{(Ref. 38)},
\]

where \( k_a = 2.85 \times 10^{-4} \) s\(^{-1} \), \( [^{125}\text{I}-9\text{-A5}] = 1.5 \times 10^{-9} \) nM, and \( k_d = 2.4 \times 10^{-4} \) s\(^{-1} \). A value of 1.8 \( \times 10^6 \) M\(^{-1} \) s\(^{-1} \) was obtained for \( k_d \). Thus, the kinetic derived dissociation constant (\( k_d/\theta \)) for the \( ^{125}\text{I}-9\text{-A5}\) or \( [\text{Na}^+,\text{K}^+]\text{-ATPase complex is 0.15 nM.}

Equilibrium Binding of \( ^{125}\text{I}-9\text{-A5} \) to Liver Membranes—Fig. 3 shows the amount of specific \( ^{125}\text{I}-9\text{-A5} \) binding at equilibrium as a function of \( ^{125}\text{I}-9\text{-A5} \) concentration. The binding curve was hyperbolic, suggesting that the radioligand bound to a single class of sites. This interpretation was supported by Scatchard analysis (Fig. 3, inset), which generated a linear curve from which an apparent dissociation constant (\( K_{D_{app}} \)) of 0.64 nM was derived. Maximal \( ^{125}\text{I}-9\text{-A5} \) binding (\( B_{max} \)) was 29.3 pmol of \( ^{125}\text{I}-9\text{-A5} \) bound per mg of proteins (Fig. 3, inset). Using a different liver plasma membrane sample derived from caudate and accessory lobes (as opposed to the whole liver), similar experiments with \( [\text{S,S}]\text{-ATPase activity, and was seen when either \( [\text{S,S}]\text{-A5} \) or \( ^{125}\text{I}-9\text{-A5} \) were used to quantify \( \text{(Na}^+,\text{K}^+)\text{-ATPase bind-

FIG. 2. Kinetic studies of specific \( ^{125}\text{I}-9\text{-A5} \) binding to rat liver plasma membranes. A, time course of association of \( ^{125}\text{I}-9\text{-A5} \). Membranes (10 pg of proteins/tube) were incubated at 37 °C with 1.5 nM \( ^{125}\text{I}-9\text{-A5} \) (73 cpm/fmol) in the absence (total binding) or presence ("nonspecific" binding) of 200 nM unlabeled 9-A5. Aliquots (250 µl) were removed at the indicated times and the amount of specifically bound radioligand (total counts/min – nonspecific counts/min) was determined (O). The data are average values of three separate experiments (S.E. ≤ 8% in all cases). \( ^{125}\text{I}-9\text{-A5} \) specifically bound to membranes that were preincubated in binding buffer for 90 min at 37 °C (to see if binding site degradation occurred) (O). Inset, semilogarithmic transformation of the data (fit to a linear curve (p < 0.02) described by the equation \( \ln(B/\theta) = k_a \Delta t \) (Ref. 38). B, time course of dissociation of bound \( ^{125}\text{I}-9\text{-A5} \). \( ^{125}\text{I}-9\text{-A5} \) (1.5 nM; 73 cpm/fmol) was preincubated for 90 min at 37 °C in the absence or presence of 200 nM unlabeled 9-A5. Both samples (200 µg of membrane proteins/tube) were centrifuged, washed, and resuspended in 10 ml of assay buffer containing 200 nM unlabeled 9-A5 at 37 °C (time \( t_0 = \theta \)). The dissociation of bound \( ^{125}\text{I}-9\text{-A5} \) was followed at the indicated times by removing 250-µl aliquots (5 µg of proteins) from the two sets of samples and washing as described under "Materials and Methods." Specifically bound radioligand (total counts/min – nonspecific counts/min) was plotted as a function of time after dilution and addition of unlabeled 9-A5. The data are average values of three separate experiments (S.E. ≤ 10% in all cases). Inset, semilogarithmic transformation of the data, fit to a linear curve (p < 0.04) described by the equation \( \ln(B/\theta) = k_b \Delta t \) (Ref. 38). The decreased \( k_a \) and \( k_b \) concentrations were 29.3 and 13 pmol/mg proteins for \( ^{125}\text{I}-9\text{-A5} \) and \( [\text{S,S}]\text{-A5} \) uptake. Aligned with 30 min. Almost full dissociation occurred within 120 min, indicating reversibility of the reaction. Based on the equation

\[
\ln(B/\theta) = k_d \quad \text{(Ref. 38),}
\]

where \( k_d = 2.85 \times 10^{-4} \) s\(^{-1} \), \( [^{125}\text{I}-9\text{-A5}] = 1.5 \times 10^{-9} \) nM, and \( k_d = 2.4 \times 10^{-4} \) s\(^{-1} \). A value of 1.8 \( \times 10^6 \) M\(^{-1} \) s\(^{-1} \) was obtained for \( k_d \). Thus, the kinetic derived dissociation constant (\( k_d/\theta \)) for the \( ^{125}\text{I}-9\text{-A5}\) or \( [\text{Na}^+,\text{K}^+]\text{-ATPase complex is 0.15 nM.}

Equilibrium Binding of \( ^{125}\text{I}-9\text{-A5} \) to Liver Membranes—Fig. 3 shows the amount of specific \( ^{125}\text{I}-9\text{-A5} \) binding at equilibrium as a function of \( ^{125}\text{I}-9\text{-A5} \) concentration. The binding curve was hyperbolic, suggesting that the radioligand bound to a single class of sites. This interpretation was supported by Scatchard analysis (Fig. 3, inset), which generated a linear curve from which an apparent dissociation constant (\( K_{D_{app}} \)) of 0.64 nM was derived. Maximal \( ^{125}\text{I}-9\text{-A5} \) binding (\( B_{max} \)) was 29.3 pmol of \( ^{125}\text{I}-9\text{-A5} \) bound per mg of proteins (Fig. 3, inset). Using a different liver plasma membrane sample derived from caudate and accessory lobes (as opposed to the whole liver), similar experiments with \( [\text{S,S}]\text{-ATPase activity, and was seen when either \( [\text{S,S}]\text{-A5} \) or \( ^{125}\text{I}-9\text{-A5} \) were used to quantify \( \text{(Na}^+,\text{K}^+)\text{-ATPase bind-

FIG. 3. Equilibrium specific \( ^{125}\text{I}-9\text{-A5} \) or \( [\text{S,S}]\text{-A5} \) binding to rat liver plasma membranes as a function of radioligand concentration. Membranes (10 µg of proteins/tube) were incubated at 37 °C for 90 min with the indicated concentrations of \( ^{125}\text{I}-9\text{-A5} \) (88 cpm/fmol; O, left y axis) or \( [\text{S,S}]\text{-A5} \) (20 cpm/fmol; ■, right y axis) in the absence or presence of 200 nM unlabeled 9-A5. The data (specific binding) are average values of 2–5 separate experiments (S.E. ≤ 10% in all cases). Inset, Scatchard analyses were plotted according to the equation \( ^{125}\text{I}-9\text{-A5} \) or \( [\text{S,S}]\text{-A5} \) bound = \( K_{D_{app}} \) (\( B_{max} \) \times [\( ^{125}\text{I}-9\text{-A5} \) or \( [\text{S,S}]\text{-A5} \) bound]). Least-squares analyses yielded a straight line for each ligand (p < 0.02 (O) and p < 0.07 (■)). Receptor concentrations (\( B_{max} \)) were 29.3 and 13 pmol/mg proteins for \( ^{125}\text{I}-9\text{-A5} \) and \( [\text{S,S}]\text{-A5} \), respectively. Dissociation constants (\( K_{D_{app}} \)) were 0.64 and 0.89 nM for \( ^{125}\text{I}-9\text{-A5} \) and \( [\text{S,S}]\text{-A5} \), respectively.
ing sites (see below). The equality of $^{[35]S}$9-A5 and $^{125}$I-9-A5 dissociation constants suggests that radioiodination of 9-A5 had little or no effect on its interaction with (Na$^+$,K$^+$)-ATPase.

Stability of $^{125}$I-9-A5 and Plasma Membrane (Na$^+$,K$^+$)-ATPase during the Binding Reaction—To see if $^{125}$I-9-A5 degradation occurred during the binding reaction, samples containing $^{125}$I-9-A5 were withdrawn at the beginning ($t = 0$) and end ($t = 90$ min) and analyzed by SDS-PAGE. Autoradiographs of electrophoresed radioligands showed only bands corresponding to the light and heavy chain of 9-A5 ($M_r = 31,000$ and 55,000). No $^{125}$I-labeled proteolytic fragments were detected. Similar results were seen on autoradiographs from gels containing $^{125}$I-9-A5 that had been allowed to bind, and then eluted from membranes with 0.1 M glycine, adjusted to pH 2.8 with 6 N HCl (data not shown).

Evidence of binding site stability during the reaction was obtained from two observations. First, liver membranes incubated for 90 min at 37 °C were capable of binding similar amounts of $^{125}$I-9-A5 in subsequent rounds of binding (Fig. 2A). Second, (Na$^+$,K$^+$)-ATPase activity was only slightly reduced (15%) after the 90 min preincubation at 37 °C (data not shown).

Effects of (Na$^+$,K$^+$)-ATPase Substrates and Inhibitors on $^{125}$I-9-A5 Binding to Liver Membranes—Table II shows that three monovalent cation substrates of the sodium pump, sodium, potassium, or lithium, supported maximal $^{125}$I-9-A5 binding. Choline*, a sodium substitute which does not bind to (Na$^+$,K$^+$)-ATPase, reduced $^{125}$I-9-A5 binding by more than 90%. Ouabain, a sodium pump inhibitor that binds extracellularly (39), had no effect on 9-A5 binding.

In contrast to these results, monovalent cation-dependent $^{125}$I-9-A5 binding was blocked 99% by 3 mM ATP (Table II). Fig. 4A shows that this effect was ATP concentration-dependent, with an apparent $K_I$ of 0.5 mM (= $K_{iATP}$) for enzyme activity (see Fig. 5). Fig. 4B shows the concentration dependence of $^{125}$I-9-A5 binding to membranes in the absence or presence of 0.6 mM ATP. As expected (see Fig. 3), both binding curves were hyperbolic. The $B_{max}$ values obtained in the absence or presence of ATP were 29 or 9 pmol of $^{125}$I-9-A5 of proteins, respectively. For both curves, Scatchard analyses gave $K_{iATP}$ values = 0.9 mM (data not shown).

The inhibitory effects of ATP were not due to degradation products like adenosine or AMP, or to ATP-regenerating system components (phosphoenolpyruvate or pyruvate kinase) since none of these molecules inhibited specific binding (Table II). However, ADP partially blocked $^{125}$I-9-A5 binding (51% at 3 mM ADP). This effect was not due to ATP contamination of the ADP preparation or to ATP contamination of the membranes (which contained less than 1.0 nmol of ATP/mg of proteins). The addition of 25 mM inorganic phosphate reduced the inhibitory effect of 3.0 mM ATP on antibody binding by 30% but had no effect on ADP inhibition of binding. These observations are consistent with the ability of inorganic phosphate to bind to (Na$^+$,K$^+$)-ATPase and block ATP but not ADP binding (40).

Effect of 9-A5 on the ATP Dependence of (Na$^+$,K$^+$)-ATPase Activity—Fig. 5 shows that 30 nM 9-A5 lowered the $V_{max}$ value.

![Figure 4](image-url)
preincubated without 90 min at 37 °C. ATP was added at the indicated concentrations to initiate the enzymatic reaction, and ouabain-sensitive activity (micromoles of ATP hydrolyzed per mg−1 min−1) was determined during (Na+,K+)-ATPase activity in the absence or presence of 9-A5. Purified rat kidney (Na+,K+)-ATPase (2 µg of proteins·tube−1) was incubated with 30 µM Tris[γ-32P]ATP for 20 s at 4 °C, in the presence of 100 mM sodium chloride, 50 mM sodium phosphate, 5 mM magnesium chloride (lane 1); 100 mM potassium chloride, 50 mM potassium phosphate (lane 2); or, 50 mM sodium phosphate, 5 mM magnesium chloride, and 1 mM ouabain (following a 1-h preincubation with the drug at 37 °C, lane 5). After terminating the reactions, 20 µg of proteins·lane−1 were analyzed by acidic SDS-PAGE. Purified (Na+,K+)-ATPase preincubated for 1 h at 37 °C with 100 µg of 9-A5·ml−1 was incubated with [γ-32P]ATP, as above, in the presence of (a) 100 mM sodium chloride, 50 mM sodium phosphate, 5 mM magnesium chloride (lane 3), or (b) 100 mM potassium chloride and 50 mM potassium phosphate (lane 4); both preparations were analyzed under similar conditions. Arrows (at left) show gel positions of (Na+,K+)-ATPase α and β subunits and (at right) protein standards of varying Mr. Membranes were incubated with [γ-32P]ATP, as above, in the presence of 100 mM sodium chloride, 50 mM sodium phosphate, and 5 mM magnesium chloride without (lane 1) or with (lane 2) prior incubation with 9-A5 (as described under "Materials and Methods"). Reactions performed in the presence of 100 mM potassium chloride and 50 mM potassium phosphate are shown in lane 3. Acidic SDS-PAGE (40 µg of proteins·lane−1) was performed as above. For experimental incubations in both panels (A and B), the pH values were adjusted as described under "Materials and Methods."

$M_1 = 12,000$ phosphoprotein (lane 2). The origin of the $M_1 = 12,000$ phosphoprotein is unclear but it may have arisen from α subunit degradation (41). As seen with the kidney enzyme (Fig. 6A), phosphorylation of the $M_1 = 110,000$ liver protein, which co-migrated with (Na+,K+)-ATPase α subunit, was blocked by potassium chloride (Fig. 6B, lane 3). These results show that 9-A5 inhibited sodium-dependent phosphorylation of the liver membrane (Na+,K+)-ATPase α subunit.

Comparisons between 125I-9-A5 Binding to Liver Membranes and Crude Particulate Fractions—To exclude the possibility that artifacts of liver membrane isolation (e.g. preferential sodium pump losses or recoveries) might influence binding of 125I-9-A5 to (Na+,K+)-ATPase, $B_{\text{max}}$ and $K_{\text{D(app)}}$ values obtained with liver plasma membrane and total crude particulate fractions (whole liver or cultured hepatocytes) were compared. The results are given in Table III.

No differences in the $K_{\text{D(app)}}$ of 125I-9-A5 binding to the three different “receptor” (sodium pump) sources were found (0.4-0.89 nM). As seen with membranes, both cultured hepatocyte or liver tissue particulate fractions yielded linear Scatchard plots (see below). However, with respect to liver tissue

The use of kidney enzyme in these studies was indicated because of its high specific activity. In addition, no structural differences between kidney and liver α subunits (purified by affinity chromatography over 9-A5-Sepharose columns) have been detected by peptide mapping.

![FIG. 5. Lineweaver-Burk plot of ATP dependence of (Na+,K+)-ATPase activity in the absence or presence of 9-A5.](image)

![FIG. 6. Effects of 9-A5 on sodium-dependent phosphorylation of kidney and liver (Na+,K+)-ATPase. A, purified kidney (Na+,K+)-ATPase proteins phosphorylated by [γ-32P]ATP and analyzed by acidic SDS-PAGE and autoradiography. Purified enzyme was incubated with 30 µM Tris[γ-32P]ATP for 20 s at 4 °C, in the presence of 100 mM sodium chloride, 50 mM sodium phosphate, 5 mM magnesium chloride (lane 1); 100 mM potassium chloride, 50 mM potassium phosphate (lane 2); or, 50 mM sodium phosphate, 5 mM magnesium chloride, and 1 mM ouabain (following a 1-h preincubation with the drug at 37 °C, lane 5). After terminating the reactions, 20 µg of proteins·lane−1 were analyzed by acidic SDS-PAGE. Purified (Na+,K+)-ATPase preincubated for 1 h at 37 °C with 100 µg of 9-A5·ml−1 was incubated with [γ-32P]ATP, as above, in the presence of (a) 100 mM sodium chloride, 50 mM sodium phosphate, 5 mM magnesium chloride (lane 3), or (b) 100 mM potassium chloride and 50 mM potassium phosphate (lane 4); both preparations were analyzed under similar conditions. Arrows (at left) show gel positions of (Na+,K+)-ATPase α and β subunits and (at right) protein standards of varying Mr. Membranes were incubated with [γ-32P]ATP, as above, in the presence of 100 mM sodium chloride, 50 mM sodium phosphate, and 5 mM magnesium chloride without (lane 1) or with (lane 2) prior incubation with 9-A5 (as described under "Materials and Methods"). Reactions performed in the presence of 100 mM potassium chloride and 50 mM potassium phosphate are shown in lane 3. Acidic SDS-PAGE (40 µg of proteins·lane−1) was performed as above. For experimental incubations in both panels (A and B), the pH values were adjusted as described under "Materials and Methods."](image)
Table III

Comparison among 125I-9-A5 equilibrium binding constants, (Na⁺,K⁺)-ATPase activity, and (Na⁺,K⁺)-ATPase sites-hepatocyte⁻¹ in liver plasma membranes and crude particulate fractions

| Receptor source                          | (Na⁺,K⁺)-ATPase activity | (Na⁺,K⁺) ATPase |
|-----------------------------------------|--------------------------|----------------|
|                                         | Kᵦ (nM)                  | Bmax (pmol/mg proteins) | 9-A5- sensitive  |
| (a) Liver plasma membranes (intact tissue) | 0.64                      | 29.3                  | 500                  | 319                  | 264,000                  |
| (b) Crude particulate fraction (intact tissue) | 0.40                      | 1.2                   | 8.5                  | 9.2                  | 217,000                  |
| (c) Crude particulate fraction (cultured hepatocytes) | 0.89                      | 2.4                   | 42.0                 | 37.0                 | 232,000                  |
| Ratio a:b                               | 24.4                     | 35.3                  | 34.7                 |                      |                          |

Preparations, 125I-9-A5 Bmax values were 24-fold higher in membrane (29.3 pmol of 125I-9-A5/mg of proteins) than in particulate fractions (1.2 pmol of 125I-9-A5/mg of proteins). This difference was paralleled by a 35-fold enrichment² of ouabain or 9-A5-sensitive ATPase activity in membranes compared to crude particulate fractions (Table III), in agreement with findings that ouabain and 9-A5 both inhibited equal proportions of total ATPase activity in both kidney and liver preparations (1).

Based upon these results, and conversion factors for proportions of liver membrane and particulate proteins that constitute hepatocyte plasma membrane proteins (see legend to Table III), the numbers of (Na⁺,K⁺)-ATPase sites-hepatocyte⁻¹ were estimated to be 264,000 (plasma membranes), 217,000 (tissue crude particulate fractions), and 232,000 (cultured hepatocyte crude particulate fractions).

Quantitation of (Na⁺,K⁺)-ATPase Activity and Binding Sites in Tissue Preparations from Normal and Regenerating Rat Liver

Plasma Membrane Preparations—Fig. 7A shows that 12 h after surgery, 9-A5-sensitive Vmax ATPase activity rose 58 ± 12% in membranes from regenerating liver, compared to

³ Similar enrichment ratios of enzyme activity and Bmax values have not been determined for crude particulate fractions prepared from 12-day-old cultured hepatocytes (whose specific enzyme activities were consistently 4-5-fold higher than adult liver tissue levels) because of technical difficulties in preparing large enough quantities of plasma membranes from such samples.

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membrane (Na⁺,K⁺)-ATPase recovery and/or stability, similar experiments were performed with crude particulate fractions of liver caudate and accessory lobes. Particulate fractions were chosen since their yields of (Na⁺,K⁺)-ATPase activity are greater than 80% (where activity of starting homogenates = 100%), compared to 1–16% yields for liver plasma membranes (29, 42). The results are shown in Fig. 8.

The same trends were found: 1) (Na⁺,K⁺)-ATPase activity in regenerating tissue was elevated (325 ± 14%) compared to sham-operated rats (9.0 nmol of ATP hydrolyzed per mg⁻¹ min⁻¹; p < 0.001); 2) regenerating and quiescent tissues had similar levels of 5'-nucleotidase activity (61 and 69 nmol of inorganic phosphate released per mg⁻¹ min⁻¹, respectively); 3) decreased B_max values were seen in regenerating tissue fractions (22 ± 12%) compared to B_max values of fractions from sham-operated rats (1.0 pmol of ¹²⁵I-9-A5 bound per mg⁻¹ of proteins; p = 0.1); and 4) no differences between the two groups were seen in Kₐ₅ₑ₅₅ values for ¹²⁵I-9-A5-(Na⁺,K⁺)-ATPase complexes (Kₐ₅ₑ₅₅ = 0.88 ± 0.2 nm).

**DISCUSSION**

Biphasic increases in sodium pump activities are implicated in the repertoire of “early” and “late” prereplicative events that initiate animal cell proliferation (2, 3). Mitogen-stimulated passive sodium influxes account for much of this early pump activation (2, 3, 5–7). Less is known about mechanisms which regulate the later changes. In an attempt to understand their molecular basis, quantitative measurements of (Na⁺,K⁺)-ATPase activity and numbers of (Na⁺,K⁺)-ATPase sites have been made in regenerating liver, 12 h after partial heptectomy, with an anticatalytic mouse monoclonal antibody (9-A5) directed against the sodium pump (1). The results of these experiments, using plasma membranes or crude particulate fractions from normal and regenerating liver, show that (Na⁺,K⁺)-ATPase is stimulated in regenerating liver without detectable increases in the numbers of 9-A5 binding sites. This indicates that late prereplicative increases in (Na⁺,K⁺)-ATPase activity are due, directly and/or indirectly, to specific activation of pre-existing pump molecules. This activation is apparently manifested by increases in (Na⁺,K⁺)-ATPase turnover number.

To validate the measurements, initial studies were designed to elucidate the specificity and nature of interactions between 9-A5 and its putative (Na⁺,K⁺)-ATPase binding sites in native membranes. Six kinds of investigations indicated that 9-A5 binding, which is time-dependent, saturable, and reversible, occurs with absolute specificity for a single class of (Na⁺,K⁺)-ATPase binding sites.

First, purified (Na⁺,K⁺)-ATPase, from solubilized or intact kidney membrane preparations, blocked ¹²⁵I-9-A5 specific binding to liver membranes. These results are consistent with previous observations that 9-A5: (a) immunobLOTS specifically to α subunits of (Na⁺,K⁺)-ATPase, (b) directly inhibits liver membrane (Na⁺,K⁺)-ATPase V₅₈ activity (Kₐ₅ₑ₅₅ = 30 nm), and (c) localizes to hepatocyte bile canalicular (1) and kidney epithelium basolateral membranes as revealed by immunofluorescent staining.⁷

Second, the kinetics of association and dissociation of radioligand binding to liver membranes were pseudo first-order and zero-order, respectively, with a low Kₐ₅ₑ₅₅ (0.15 nm). Third, Scatchard plots from equilibrium binding studies, made over a broad ¹²⁵I-9-A5 concentration range (0.10–100 nm), showed strictly linear curves. In both cases, this behavior

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⁴The reasons for the absolute decreases in B_max values obtained in control tissue preparations in experiments shown in Fig. 7B, compared to B_max values shown in Fig. 3, are unclear. Similar differences were found in membrane preparations from anesthesia and diurnal variation controls. Contributing factors might be intrinsic binding site concentration differences with respect to lobular structure (e.g. caudate plus accessory lobes (Fig. 7B) versus whole liver (Fig. 3)).

⁷H. L. Leffert, M. Schumacher, D. B. Schenk, G. Keller, K. Miyai, X. Yang, R. Ariyasu, and M. Ellisman, manuscript in preparation.
is expected for ligand interactions with homogeneous classes of binding sites (36). These results are unlikely to have been affected by 9-A5 or (Na+,K+)ATPase binding site degradation because (a) autoradiographs showed that [32P]9-A5 (analyzed by SDS-PAGE) was intact after prolonged incubation in binding reaction mixtures, and (b) binding activity levels and (Na+,K+)ATPase binding site numbers remained constant throughout the binding reactions. In addition, the binding properties of [32P]9-A5 were probably equivalent to unlaabeled 9-A5, inasmuch as [32P]9-A5 showed identical properties with respect to its $K_d$ and $B_{max}$ values. Although the $K_d$ from Scatchard analysis (0.64 nM) was higher than the $K_u$ from nonequilibrium kinetic data ($k_1/k_i = 0.15$ nM), this “discrepancy” has been seen in other antibody binding studies (43) and discussed elsewhere (44).

Fourth, (Na+,K+)ATPase substrates significantly influenced [32P]9-A5 binding to liver membranes. Monovalent cations that bind to the sodium pump (sodium, lithium, or potassium) (18) were required for maximal 9-A5 binding. Antibody binding did not seem to be affected by different enzyme conformations, since either sodium or potassium supported maximal binding yet each cation stabilizes distinct conformations (sodium $= E_1$, potassium $= E_2$; Ref. 40). The cation requirement was specific, however, since choline did not replace the cations tested. In contrast to these stimulatory effects, ATP, a substrate required for active potassium and sodium transport (45), blocked $K_u$ (0.5 nM) ion-dependent [32P]9-A5 binding (as did ADP, although less potently). Since ATP affected only [32P]9-A5 maximal binding and not its apparent affinity this blockade was noncompetitive. The large positive Hill coefficient seen for inhibition of [32P]9-A5 binding by ATP ($n_H = 3.2$; Fig. 4A, inset) suggests that several ATP molecules were involved in this interaction. These findings are consistent with 9-A5’s ability to block (Na+,K+)ATPase activity in the presence of ATP, since anticalytic studies were performed with enzyme preparations preincubated with 9-A5 (before adding ATP) and negligible amounts of antibody dissociated from 9-A5 enzyme complexes under these conditions. In addition, both kinetic constants ($K_u$; $n_H$) were similar to those obtained in the presence of potassium for low-affinity ATP binding sites on (Na+,K+)ATPase (46).

These kinetic observations (suggesting cooperative binding of ATP to the enzyme) and other results (suggesting (Na+,K+)ATPase activation as a hyperbolic function of ATP concentration (Fig. 5)) have been reported by previous investigators (18,46). One interpretation of these findings in light of the effects of ATP on [32P]9-A5 binding to the purified enzyme (Fig. 4B and Table II) is that ATP and [32P]9-A5 bind to distinct sites on different enzyme intermediates. This interpretation is supported (in a fifth kind of investigation) by preliminary findings that 9-A5 behaved like a noncompetitive inhibitor of ATP-dependent stimulation of (Na+,K+)ATPase, as revealed by Lineweaver-Burk analyses (Fig. 5). However, further kinetic studies are needed to substantiate this conclusion since simple noncompetitive inhibition cannot arise from removal from the incubation system of active enzyme species and, therefore, is not necessarily indicative of “distinct” sites. In addition, the interpretation is further complicated because (i) there is some uncertainty in the $K_u$ and $V_{max}$ values, since appropriate weighting factors (47) were not used to make these calculations from the curves (Fig. 5), and (ii) the fact that ATP inhibits [32P]9-A5 binding to the enzyme in a cooperative manner.

Sixth, direct biochemical investigations of its anticalytic properties showed that 9-A5 specifically blocked sodium-dependent phosphorylation of α subunits of (Na+,K+)ATPase (in contrast, ouabain stabilized phosphoenzyme intermediates). Notably, with regard to their $M$, after acidic SDS-PAGE, their ion requirements of phosphorylation, and their response to 9-A5, phosphorylated α subunits were indistinguishable in liver and kidney preparations. These and other results suggest a high degree of structural homology between α subunits from these different tissues; and, they validate the assumption (see e.g. Fig. 5) that the mechanisms of inhibitory effects of 9-A5 on kidney (Na+,K+)ATPase are similar with respect to liver (Na+,K+)ATPase.

The ability to quantitate the numbers of (Na+,K+)ATPase sites in hepatic tissue extracts provides a way to estimate the numbers of sodium pumps hepatocyte–1. Calculations along these lines yield ≈238,000 pumps·cell–1. It is reasonable to equate 9-A5 binding sites with sodium pumps (i.e. at least, αβ dimers (41)) since 9-A5 binds specifically to α subunits; electrically charged cations, by this protein requires both catalytic functions that 9-A5 inhibits (ATPase activity and sodium-dependent phosphorylation of the α subunit); and, the numbers of 9-A5 binding sites, in any single tissue preparation examined, are strictly proportional to 9-A5- and ouabain-sensitive (Na+,K+)ATPase activity. Furthermore, 9-A5 also inhibits ATP-driven cation fluxes across membranes of everted heart vesicles, and (b) co-localizes with “hybridoma 24” (a mouse monoclonal antibody directed against β subunits of chicken (Na+,K+)ATPase (48)) at basolateral membrane sites in chicken kidney tubular epithelial cells, as revealed by immunofluorescent light microscopic studies. The interme-

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8 D. B. Schenk, J. J. Hubert, and H. L. Leffert, unpublished results.
9 R. Grosse, D. B. Schenk, and H. L. Leffert, unpublished results.
10 D. Fambrough, personal communication.
this high pump site density to bile canalicul ar fluid transport is unknown.

Hepatocyte sodium pump concentrations are within the range (1 to 10 sites-cell^-1) obtained from [H] ouabain binding studies in several other cell systems (e.g. HeLa (53, 54), muscle (55, 56), and neuronal (57)). However, similar determinations with [H] ouabain have not been reported for liver cells. Apart from the fact that liver contains >10-fold less (Na^+)-ATPase activity than kidney, measurements with [H] ouabain are technically difficult (Refs. 20-23). Work in this laboratory indicates that specific [H] ouabain binding to liver plasma membranes is undetectable from 0.1-100 μM ouabain. Furthermore, in contrast to [H] ouabain (which is thought to bind only to the phosphorylated enzyme intermediate E_2-P (58), which might lead to underestimations of binding site numbers in studies with this ligand), the inhibition of (Na^+,K^+)-ATPase activity by 9-A5 does not require a phosphorylated enzyme intermediate (see Fig. 6). Thus, [32P] 9-A5 has both practical and theoretical advantages over [H] ouabain as a radioligand for (Na^+,K^+)-ATPase, especially for site quantitation studies in tissues with low levels of sodium pumps.

Several lines of evidence support the conclusion that, during liver regeneration, direct and/or indirect sodium pump activation accounts for late prereplicative phase increases in (Na^+,K^+)-ATPase. First, Scatchard analyses of binding of [3H] 9-A5 to regenerating liver membranes show that a small, statistically significant decrease occurs in B_max, values, in comparison to sham-hepatectomized controls. The increases in (Na^+,K^+)-ATPase activity and concomitant decreases in the numbers of enzyme sites cannot be attributed to generalized nonspecific changes in membrane enzymes since 5' nucleotidase activities are similar in membrane preparations isolated from either 67% or sham-hepatectomized rats. In addition, the sites available for inhibition of enzyme activity by 9-A5 are the same population of sites that are quantitated in the binding studies (see above). Second, in agreement with other reports (15-17), the increases (≥58%) in ouabain-sensitive ATPase activity in regenerating liver membranes are quantitatively identical to the increases in (Na^+,K^+)-ATPase activity seen when 9-A5 is used as the specific inhibitor. Since ouabain and 9-A5 bind to distinct (Na^+,K^+)-ATPase sites, which probably exist on contralateral membrane surfaces, the increases in enzyme activity must reflect intrinsic membrane alterations and not artifactual differences in inhibitor accessibility to (Na^+,K^+)-ATPase binding sites. Third, similar trends, increases in (Na^+,K^+)-ATPase activity (325%) and small decreases in numbers of sodium pump sites (22%), are seen when crude particulate fractions are used as the enzyme source. These results exclude the possibility that the observed differences are due to membrane isolation artifacts (e.g. enrichment or exclusion of a subpopulation of enzyme molecules) since crude particulate fractions contain almost all (≥80%) of the enzymatic activity present in total rat liver homogenates. This latter point is important because the yields of plasma membrane and membrane (Na^+,K^+)-ATPase activity are typically ≤2% and ≤16%, respectively (29, 42). Underlying causes of the larger relative increases in regenerating liver (Na^+,K^+)-ATPase activity seen in crude particulate fractions compared to membranes (325 versus 58%, respectively) are as yet unknown. Either endogenous (Na^+,K^+)-ATPase activators, present in crude particulate fractions, are lost during membrane isolation, or "activated" forms of (Na^+,K^+)-ATPase are labile under current isolation procedures. Further work is needed to distinguish between these or other alternatives.

Our studies and earlier ones (15-17) do not exclude the possibility that increases in (Na^+,K^+)-ATPase activity in regenerating liver tissues arise from alterations in the K_m for one or more (Na^+,K^+)-ATPase substrate (ATP, sodium, or potassium). This possibility seems unlikely since in several cases where increases in (Na^+,K^+)-ATPase activity have been reported, without corresponding increases in the numbers of sodium pump "sites," no differences in substrate K_m values were detected (21, 54, 59). The experiments needed to address this problem, however, are difficult to perform accurately, especially under sub-V_max conditions, because liver tissue preparations contain very low levels of enzyme activity. Once the hepatic enzyme is purified and concentrated, this problem seems like to be fairly simple to resolve. The present experiments also do not directly exclude the possibility that the observed (Na^+,K^+)-ATPase activation, without corresponding increases in the numbers of enzyme sites, might be due to subtle changes in the sodium pump itself (see below), reflected by alterations in rate constants of binding of 125I-9-A5 to the enzyme. This seems unlikely because measurements of the apparent K_D values of 125I-9-A5 binding, at equilibrium, which reflect the ratio of these rate constants (k off/k on), gave identical results with liver tissues from both sham- and 67%-hepatectomized rats (Figs. 7 and 8). Moreover, these K_D values were in excellent agreement with those determined directly (in normal tissues) from kinetic studies (Fig. 2).

Calculations of the apparent turnover number of hepatic (Na^+,K^+)-ATPase show that it increases 4-fold, from a value of 150 ATP molecules hydrolyzed per 125I-9-A5 binding site^-1 s^-1 in crude particulate fractions from quiescent liver to 625 ATP molecules hydrolyzed per 125I-9-A5 binding site^-1 s^-1 in similar fractions from 67% hepatectomized liver (see Fig. 8). The findings of increased (Na^+,K^+)-ATPase enzyme turnover number during rat liver regeneration support the hypothesis that this might be a general mechanism responsible for increases in ouabain-sensitive 86Rb+ influx, membrane hyperpolarization, and ATPase activity in other proliferating cells (60) midway to late in the prereplicative phase (6-14 h post-stimulus).

The structural modifications that cause late G_1 sodium pump activation are unknown. If the activation mechanism is direct, one possibility is that the enzyme is phosphorylated by a cAMP-dependent (37) or -independent protein kinase, or dephosphorylated by a specific phosphatase. Recent studies have shown that cAMP-elevating agents lead to increases in (Na^+,K^+)-ATPase activity (61, 62), and during transitions to growth arrest in cultured Friend erythroleukemia cells, the α subunit of (Na^+,K^+)-ATPase appears to be phosphorylated on a threonine residue as (Na^+,K^+)-ATPase activity subsides (63). Alternatively, if the activation mechanism is indirect, mitogen-dependent increases in membrane fluidity, which increase membrane (Na^+,K^+)-ATPase activity (17), might be involved. The latter possibility seems especially intriguing since decreases in plasma membrane cholesterol (that occur during liver regeneration (17)) and in lipid acyl chain order parameters have been shown to stimulate (Na^+,K^+)-ATPase activity in a cholesterol-biosynthetic mutant of Chinese hamster ovary cells (64).

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