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Research Article

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Stimulation of Hepatic Sodium and Potassium-Activated Adenosine Triphosphatase Activity by Phenobarbital

ITS POSSIBLE ROLE IN REGULATION OF BILE FLOW

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A B S T R A C T Since phenobarbital administration produces a profound increase in bile flow without changing bile acid secretion, we examined whether this drug increases the activity of hepatic sodium-potassium-activated ATPase [Na^+-K^+]-ATPase], the postulated regulating enzyme in the secretion of bile salt-independent bile flow. After freeze-thawing to increase substrate accessibility, [Na^+-K^+]-ATPase activity was determined by ouabain inhibition of total ATPase activity. Its activity was highest in isolated liver surface membrane fractions enriched in bile canaliculi. Phenobarbital administration significantly increased [Na^+-K^+]-ATPase activity in both liver surface membrane fractions as well as liver homogenates. This enhanced activity is apparently selective for other membrane phosphatases and the enzyme activity in other tissues is either unaltered or decreased.

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thesis since the apparent degradation rates were similar with or without phenobarbital treatment. The apparent half-life for [Na^+-K^+]-ATPase was estimated to be approximately 2.5 days, consistent with liver surface membrane protein turnover.

The correlation of changes in bile flow with [Na^+-K^+]-ATPase was examined under several experimental situations. Phenobarbital caused a parallel increase in each during the 1st 2 days of treatment; thereafter other factors become rate limiting for flow, since enzyme activity doesn't reach a new steady state until 4 days. Consistent with increased sodium-potassium exchange, bile sodium was unchanged while potassium concentrations were significantly reduced. Changes in both bile flow and [Na^+-K^+]-ATPase induced by phenobarbital are independent of thyroid hormone. These studies support the postulate that [Na^+-K^+]-ATPase is an important factor in regulation of bile flow. In addition, phenobarbital enhancement of both bile flow and [Na^+-K^+]-ATPase is dependent upon de novo protein synthesis.

INTRODUCTION

Two major processes presumably located in the canalicular membrane initiate and regulate hepatocytic bile flow (1, 2). In addition to active secretion of bile acids, there is a quantitatively important fraction of bile flow which may be dependent upon the active transport of sodium across the canalicular membrane (3-6). Since in mammalian cells a major proportion of sodium transport is regulated by sodium-potassium-activated ATPase [Na^+-K^+]-ATPase],¹ it has been

¹Abbreviations used in this paper: EGTA, ethylene glycol-bis (β -aminoethyl ether)- N,N' -tetraacetic acid; IDPase, inosine diphosphatase; K_m , half-maximum activation constant; [Na^+-K^+]-ATPase, sodium-potassium activated adenosine triphosphatase; PEP, phospho(enol) pyruvate; V_{max} , maximum velocity.

hypothesized that activity of this enzyme controls bile salt-independent bile flow (7, 8).

Presently it is not possible to measure transport of sodium across the canalicular membrane because of the inaccessibility of this structure to direct examination. Although indirect studies suggest that alterations in bile salt-independent bile flow are due to changes in $(\text{Na}^+ \text{-K}^+)$ -ATPase activity, conclusive evidence is lacking. Studies of the effects of cardiac glycosides and ethacrynic acid, inhibitors of $(\text{Na}^+ \text{-K}^+)$ -ATPase, are conflicting in both intact animals and the isolated perfused rat liver (8-11).

A useful technique for elucidating the possible physiological role of rate-limiting enzymes is to correlate changes in enzyme activity with changes in the function in question (12). Correlation of changes in $(\text{Na}^+ \text{-K}^+)$ -ATPase activity with bile flow would support (but not prove) a role for the sodium pump in regulating bile flow. Since phenobarbital produces a marked increase in bile salt-independent flow, in addition to its well-known effects on drug metabolism (13-16), we studied its effect on $(\text{Na}^+ \text{-K}^+)$ -ATPase. The present investigation was designed to answer three questions: (a) whether phenobarbital administration increases $(\text{Na}^+ \text{-K}^+)$ -ATPase activity; (b) if $(\text{Na}^+ \text{-K}^+)$ -ATPase activity is increased, whether de novo protein synthesis is required; and (c) to ascertain the correlation, if any, between $(\text{Na}^+ \text{-K}^+)$ -ATPase activity and bile flow. The results indicate that an adaptive increase in $(\text{Na}^+ \text{-K}^+)$ -ATPase is induced by phenobarbital which depends upon new protein synthesis. Furthermore, correlation of changes in $(\text{Na}^+ \text{-K}^+)$ -ATPase activity with alterations in bile flow supports the concept that the sodium pump is involved at least in part in regulation of bile flow.

METHODS

Animals and materials. Male Sprague-Dawley rats weighing 180-250 g (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) allowed free access to Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and water were used in all experiments. Rats were kept in wire-mesh drop-through cages with aspen wood chip bedding (American Exelsior Co., Arlington, Tex.) and alternating 12 h of light and darkness. Insecticides were not used in the animal quarters. One group of rats was made hypothyroid by thyroidectomy at Charles River Breeding Laboratories and injection of 1 Ci of carrier-free ^{131}Na -iodide (Abbott Laboratories, South Pasadena, Calif.) given intraperitoneally. Thyroidectomized rats were maintained on drinking water which contained 1.0% calcium gluconate and were studied 4-6 wk later.

Liver surface membranes were prepared according to Neville (17) through step 12 as described by Pohl et al. (18). Mitochondrial, lysosomal, and microsomal fractions were prepared by modification of the procedure of Evans and Gurd (19) as previously described (20). Kidney homogenates were prepared in 1 mM NaHCO_3 after removal of the capsule. Homogenates from the proximal small intestine were prepared from the first 20 cm of small intestine and distal small intestinal homogenates were obtained from the last

20 cm of small intestine. The entire small intestine was removed, perfused with 1 mM NaHCO_3 to remove contents, and after opening the lumen the mucosa was gently scraped with a glass slide. Both kidney and small intestinal mucosa were homogenized with the Dounce glass homogenizer (Kontes Co., Vineland, N. J.) and filtered through two layers of gauze. Skeletal muscle samples were obtained from the diaphragm, homogenized in the Virtis homogenizer model 45 (The Virtis Co., Inc., Gardiner, N. Y.), and filtered through gauze.

Double-glass distilled water was used. Albumin (bovine), adenosine 5-monophosphate, adenosine 5'-triphosphate, cytochrome *c*, ouabain, inosine diphosphate (IDPase), azide, and ethylene glycol-bis β -aminoethyl ether-*N,N'*-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co., St. Louis, Mo., and hydroxysteroid dehydrogenase from Worthington Biochemical Corp., Freehold, N. J.

Enzyme assays. $(\text{Na}^+ \text{-K}^+)$ -ATPase (ATP phosphohydrolase, EC 3.6.1.3) was determined in liver subcellular fractions as well as whole homogenates from liver, small intestinal mucosa, kidney, and skeletal muscle according to the method of Ismail-Beigi and Edelman (21) with minor modifications. The incubation media were warmed to 37°C for 5 min before initiation of the enzyme reaction by the addition of 0.1 ml of tissue sample. Total ATPase was determined at pH 7.4 in a final volume of 2 ml and contained (all in millimolars) ATP, 5.0; Mg^{++} , 5.0; Na^+ , 120; K^+ , 12.5; Tris, 25; Cl^- , 137.5, azide, 5.0, and EGTA, 1.0. $(\text{Na}^+ \text{-K}^+)$ -ATPase activity was determined by the difference between total ATPase and the activity remaining after addition of ouabain (1 mM), a specific inhibitor of $(\text{Na}^+ \text{-K}^+)$ -ATPase (22, 23). Correction was made for the spontaneous, nonenzymatic breakdown of ATP, measured as inorganic phosphate. The effect of phenobarbital on the half-maximum activation constant (K_m) and maximum velocity (V_{max}) for ATP of liver surface membrane fractions was determined in an ATP regenerating system consisting of phospho(enol) pyruvate (PEP) and pyruvate kinase (24), both obtained from Sigma Chemical Co.

Specificity of ouabain inhibition of $(\text{Na}^+ \text{-K}^+)$ -ATPase activity was confirmed by omission of potassium from the incubation mixtures of both normal and phenobarbital-treated rats. In each instance $(\text{Na}^+ \text{-K}^+)$ -ATPase activity was similar to the value obtained with ouabain inhibition. Ouabain inhibition was preferred to potassium removal because the K_m for potassium is only 1.5 mM (25) and results may vary with homogenate potassium concentrations. The reactions were terminated by addition of 0.5 ml of ice-cold 30% TCA to the reaction mixtures and the liberated inorganic phosphate was determined by the method of Fiske and Subbarow (26).

Glucose-6-phosphatase (EC 3.1.3.9) was measured by the method of de Duve et al. (27), 5'-nucleotidase activity (EC 3.1.3.5) by the method of Song and Bodansky (28); Mg^{++} -ATPase (EC 3.6.1.3) by the method of Emmelot et al. (29); and alkaline phosphatase (EC 3.1.3.1) according to Bodansky (20). IDPase (EC 3.6.1.6) was determined without the addition of deoxycholate, which inhibits the liver surface membrane isoenzyme, according to Novikoff and Heus (31, 32). Liver surface membrane samples were frozen in 0.01 M Tris buffer, pH 7.4, for later measurement of the activities of enzymes other than $(\text{Na}^+ \text{-K}^+)$ -ATPase which were stable for at least 48 h. Enzyme activities were determined by the initial rate of release of phosphate from appropriate substrates at 37°C and expressed as micromoles of inorganic phosphate released per milligram protein per hour (33). Cytochrome *c* oxidase activity (EC 1.9.3.1) was measured by the method of Straus (34) and expressed as micrograms of amino-hydrochloride formed by milligram protein per hour.

Determination of bile flow. Under pentobarbital anes-

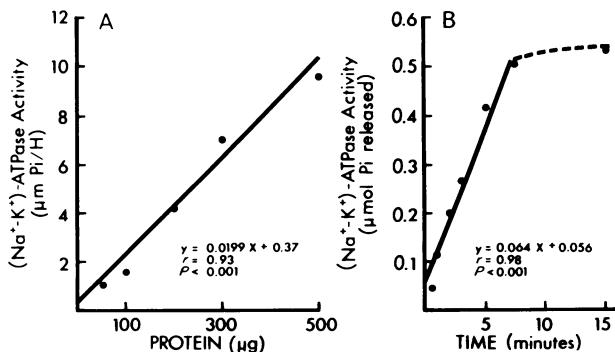


FIGURE 1 Liver surface membrane (Na⁺-K⁺)-ATPase activity as a function of protein (A) and of time (B). Liver surface membrane fractions from control rats were isolated and (Na⁺-K⁺)-ATPase activity was measured as described under Methods.

thesia (4 mg/100 g body wt i.p.) the abdomen of fed control and experimental rats was exposed and the common bile duct cannulated with polyethylene tubing (Clay Adams PE 10, Clay Adams, Inc., Parsippany, N. J.). Hypothyroid rats were anesthetized with only one-half the dose of pentobarbital (2 mg/100 g body wt). After a 5-min stabilization period, two consecutive 20-min samples were collected in pre-weighed tubes. During bile collections the abdomen was closed and animals were maintained under light anesthesia. The rectal temperature was maintained at 37±0.5°C with a heating lamp.

Mathematical and statistical analysis. Values of the rate constant of degradation were calculated from the formula described by Berlin and Schimke (35):

$$\frac{E}{E_0} = \frac{S'}{k'E_0} - \left(\frac{S'}{k'E_0} \right)^{3-k't},$$

where E = basal content of enzyme per unit weight, S = the rate constant for synthesis, and k = the first order rate constant for enzyme degradation. The steady-state level is denoted by primes. Half-lives ($t_{1/2}$) were calculated with the equation: $t_{1/2} = \ln 2/k$.

Rate constants of degradation and Michaelis-Menton constants were determined by a nonlinear least-squares regression program on a CDC series 6000 computer (Control Data Corp., Minneapolis, Minn.). Variances of best-fit lines obtained by nonlinear least-squares regression were compared by an F test (36). Significance of differences among estimated parameters was determined by a two-sample test or a Z score (37). P values equal to or less than 0.05 were considered significant.

Chemical analysis. Bile salts were determined by the enzymatic method of Talalay (38). Serum thyroxine concentration and thyroxine binding was determined by the displacement method (Thiolute, Ames Co., Elkhart, Ind.). Appropriate bile samples were analyzed for sodium and potassium by flame photometry.

Measurement of protein synthesis. L-[U-¹⁴C]leucine, 5 μCi, (324 mCi/mmol Amersham/Searle Corp., Arlington Heights, Ill.) was administered i.p. and incorporation into liver protein was determined 30 min later. The effect of cycloheximide (150 μg/100 g body weight, i.p.) was examined 1 and 12 h later and compared to controls (three rats in each group). After sacrifice, livers were perfused, homogenized in 1 mM NaHCO₃, and approximately 1.5 mg of protein was precipitated with an equal volume of ice-cold 10%

TCA and collected on glass fiber disks (Whatman Inc., Clifton, N. J.). Samples were serially washed with 5 ml each of ethanol, ether, acetone, and TCA. The disks were transferred to scintillation vials and protein was solubilized with 0.5 ml protosol (New England Nuclear, Boston, Mass.). After 12 h, 10 ml of toluene-omnifluor (New England Nuclear) containing 98% 2,5-diphenyloxazole and 2% p-bis-(0-methylstyryl benzene, 4 g/liter of toluene) was added to each vial, and the radioactivity of the samples was determined in a Packard 2425 Tri-carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) at 80% efficiency. Quench was determined by automatic external standardization. Results are reported as disintegrations per minute per milligram of protein.

RESULTS

Characteristics and distribution of (Na⁺-K⁺)-ATPase in rat liver. Hepatic (Na⁺-K⁺)-ATPase activity is linear with increasing protein concentrations to at least 500 μg (Fig. 1A) but for only 7.5 min with time (Fig. 1B) under the conditions described in Methods. Therefore, since (Na⁺-K⁺)-ATPase is a heat-labile enzyme, 5-min incubation times were used to obtain reliable results. The apparent half-maximum activation constant (K_m) with ATP as substrate is 1.2±0.2 mM (Table I), a value close to that described recently for rat skeletal muscle (39). Since freeze-thawing in 1 mM NaHCO₃ results in significantly increased (Na⁺-K⁺)-ATPase activity compared to the original assay, we examined whether this modification altered the affinity of the membrane-bound enzyme for substrate or rather permits increased substrate availability. The results shown in Table I demonstrate that the Vmax for (Na⁺-K⁺)-ATPase is significantly increased by freeze-thawing, but the apparent K_m is unaltered,

TABLE I
Effect of Storage Conditions on (Na⁺-K⁺)-ATPase Enzyme Kinetics

Conditions	Apparent K_m		Vmax
	μM ATP	μmol Pi/mg protein/h	
Tris (Fresh)*	2.0±1.6 (1)	21.9±5.9 (1)	
NaHCO ₃ (Freeze-thaw)†	1.2±0.2 (3)	32.3±3.6 (3)	
Significance	NS*	<0.05	

Liver surface membrane fractions prepared according to Neville (17), were washed with ice-cold normal saline before storage. Values are mean±SEM. The number in parentheses is the number of individual liver surface membrane preparations studied. The best-fit line of activity was identified by nonlinear least-squares regression (36).

* Samples were stored in 0.01 M Tris-HCl, pH 7.4, and determined on day of preparation.

† Samples were stored in 1 mM NaHCO₃ at -20°C for 18 h and thawed rapidly at 37°C.

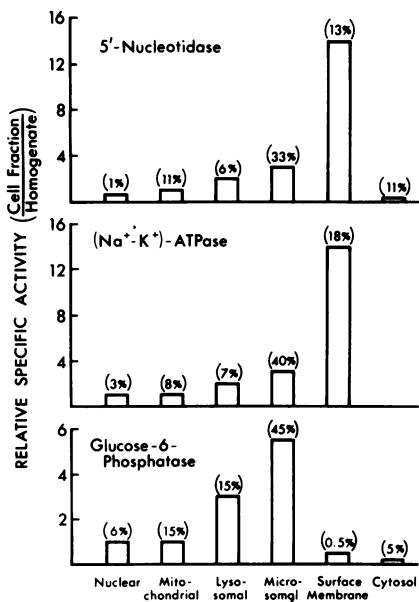


FIGURE 2 Relative activities of 5'-nucleotidase, $(\text{Na}^+\text{-K}^+)$ -ATPase, and glucose-6-phosphatase in liver subcellular fractions. Subcellular fractions were prepared and the enzyme activities measured as described under Methods. The values in parentheses represent percent recovery of enzymes in each fraction.

consistent with the suggestion that activation is due to increased access of substrate to reactive sites on the enzyme (40).

Since physiological studies suggest a role for $(\text{Na}^+\text{-K}^+)$ -ATPase at the surface membrane, the subcellular distribution of the enzyme was examined. The purity of hepatic subcellular fractions was monitored by measuring 5'-nucleotidase and glucose-6-phosphatase activities (Fig. 2), two well-established markers for the liver surface membrane and endo-

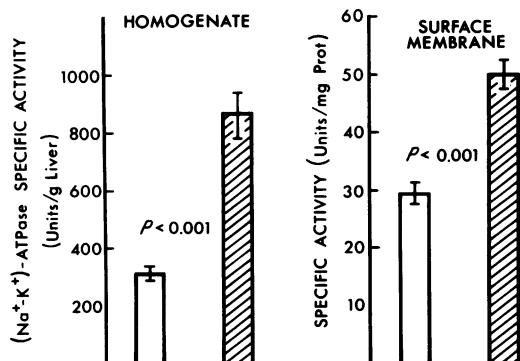


FIGURE 3 Effect of phenobarbital administration on hepatic $(\text{Na}^+\text{-K}^+)$ -ATPase activity. Phenobarbital (80 mg/kg) was administered for 5 days. The bars represent control (saline, □) and phenobarbital (▨) rats and are shown as the mean \pm SEM. Prot, protein.

plasmic reticulum, respectively (27, 41). Liver surface membrane fractions are similarly enriched in both $(\text{Na}^+\text{-K}^+)$ -ATPase and 5'-nucleotidase activities. In contrast, intracellular membrane fractions containing nuclei, mitochondria, lysosomes, and microsomes show less $(\text{Na}^+\text{-K}^+)$ -ATPase activity than surface membranes (Fig. 2). Total recovery of hepatic $(\text{Na}^+\text{-K}^+)$ -ATPase is highest in the microsomal fraction similar to results obtained in other tissues (42, 43). However, this probably represents sedimentation of surface membrane elements in this fraction rather than its primary location, for $(\text{Na}^+\text{-K}^+)$ -ATPase activity is high in the surface membrane fraction when glucose-6-phosphatase activity is very low. Thus, these studies support a primary surface membrane location for $(\text{Na}^+\text{-K}^+)$ -ATPase.

Effect of phenobarbital administration on hepatic $(\text{Na}^+\text{-K}^+)$ -ATPase activity. Administration of phenobarbital (8 mg/100 g body wt for 5 days significantly increases $(\text{Na}^+\text{-K}^+)$ -ATPase specific activity in liver homogenates compared to saline-injected controls (Fig. 3). Furthermore, this increased activity is preserved in the surface membrane fraction, but to a lesser degree because of lability of the induced enzyme (rather than altered distribution). Lability of the enzyme is shown by its reduced recovery in membrane pellets prepared by centrifugation of homogenates at 100,000 g for 1 h in phenobarbital-treated rats (62 \pm 5%) compared to controls (90 \pm 7%) ($P < 0.001$).

Since phenobarbital may induce many changes in the liver surface membrane which might lead to an apparent *in vitro* stimulation of $(\text{Na}^+\text{-K}^+)$ -ATPase, we compared the relative change in enzyme activities of freshly prepared liver surface membrane fractions

TABLE II
Effect of Phenobarbital Administration on $(\text{Na}^+\text{-K}^+)$ -ATPase Activity under Different Storage Conditions

	$(\text{Na}^+\text{-K}^+)$ -ATPase activity	
	Fresh	Freeze-thaw
$\mu\text{M P/mg P/h}$		
Control	19.3 \pm 1.6 (8)	29.6 \pm 1.5 (9)
Phenobarbital	33.1 \pm 2.9 (9)	50.0 \pm 2.5 (9)
Difference, %	+72	+69
P value	<0.001	<0.001

Liver surface membrane fractions prepared according to Neville (17), washed in cold saline, and were either assayed day of preparation (fresh) or stored in 1 mM NaHCO_3 at -20°C for 18 h and thawed rapidly at 37°C . Values are mean \pm SEM. Number in parentheses is the number of individual liver surface membrane preparations studied.

TABLE III
Effect of Phenobarbital on Liver Surface Membrane Protein Recovery
and Subcellular Organelle Enzyme Markers

	LSM protein recovery <i>mg protein/g liver</i>	Relative specific activity (LSM/homogenate)		
		Glucose-6- phosphatase	Cytochrome c oxidase	Mg ⁺⁺ -ATPase
		NS	NS	NS
Control	1.45±0.48 (10)	0.54±0.06 (6)	1.9±0.1 (6)	13±1 (6)
Phenobarbital	1.45±0.24 (10)	0.46±0.1 (6)	1.7±0.2 (6)	12±2 (6)
Significance	NS	NS	NS	NS

Liver surface membrane (LSM) fractions prepared according to Neville (17) and washed with ice-cold normal saline. Enzymatic activity was measured as described in Methods, within 48 h of membrane preparation. Values are means ±SEM. Values in parentheses are the number of individual LSM preparations studied.

to that observed after freeze-thaw. The results shown in Table II indicate that although specific activities are greater after freeze-thawing, the percentage of change is comparable.

To determine whether phenobarbital administration alters recovery of the liver surface membrane fraction, recovery of protein and the relative enzyme activities for representative marker enzymes from the endoplasmic reticulum (glucose-6-phosphatase), mitochondria (cytochrome c oxidase), and the canalicular surface membrane (Mg⁺⁺-ATPase) were compared to controls (Table III). These studies demonstrate that phenobarbital does not alter the recovery or apparent purity of liver surface membrane fractions.

Effect of phenobarbital on liver surface membrane enzymes and (Na⁺-K⁺)-ATPase activity. To investigate whether phenobarbital administration selectively increases (Na⁺-K⁺)-ATPase or whether it also increases other surface membrane-bound enzymes, the activities of several phosphatase enzymes in isolated liver surface membrane fractions from control and phenobarbital-treated rats were examined. Phenobarbital did not significantly alter Mg⁺⁺-ATPase but it reduced IDPase, alkaline phosphatase, and 5'-nucleotidase activities each to a different extent (Fig. 4). Thus, phenobarbital treatment increases (Na⁺-K⁺)-ATPase activity while other surface membrane phosphatase activities are either unaltered or decreased.

(Na⁺-K⁺)-ATPase is present in the surface membrane of most cells (25) and has been shown to adapt to increased sodium loads (43, 44), glucocorticoids (45, 46), and thyroid hormone (21, 47). The tissue specificity of phenobarbital induction was therefore examined in the small intestine, kidney, and skeletal muscle. Fig. 5 demonstrates that phenobarbital administration for 5 days did not increase (Na⁺-K⁺)-ATPase in these

tissues and, in fact, significantly reduced activity in skeletal muscle and the proximal small intestine.

Investigation of phenobarbital induction of (Na⁺-K⁺)-ATPase activity. Increased (Na⁺-K⁺)-ATPase activity may result from either changes in the proportion of latent enzyme present or changes in the total number of enzyme molecules. Activation of a fixed

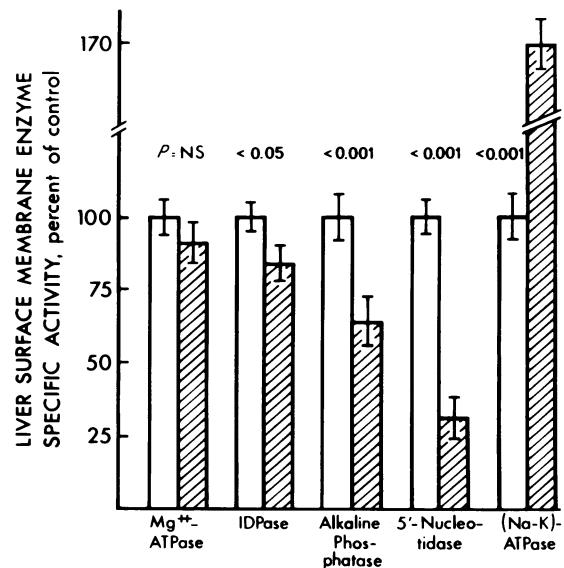


FIGURE 4 Effect of phenobarbital administration on enzyme activities in liver surface membrane fractions. Bars represent the mean ± SEM for control (□) and phenobarbital-treated (▨) animals. The mean ± SEM for control enzyme specific activities (all in $\mu\text{m P}/\text{mg P per h}$) are: Mg⁺⁺-ATPase, 88.8±5.8 (11); IDPase, 45.5±2.2 (11); alkaline phosphatase, 6.1±0.5 (20); 5'-nucleotidase, 92.2±6.1 (15); and (Na⁺-K⁺)-ATPase, 29.6±1.5 (8). The numbers in parentheses indicate the number of experiments.

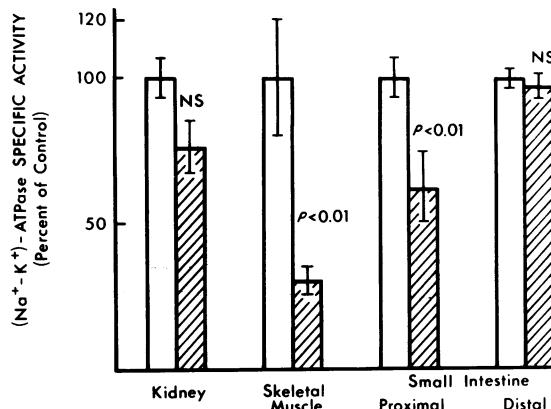


FIGURE 5 Effect of phenobarbital administration on $(\text{Na}^+ - \text{K}^+)$ -ATPase activities ($\mu\text{m Pi}/\text{mg P per h}$ in kidney, skeletal muscle, and small intestinal homogenates. Phenobarbital (80 mg/kg) was administered for 5 days. The bars represent mean \pm SEM for control (□) and phenobarbital-treated (▨) animals. The mean \pm SEM for control enzyme values are: kidney, 7.3 ± 1.1 (5); muscle, 3.0 ± 0.6 (3); small intestines, proximal, 8.7 ± 0.3 (5) and distal, 7.3 ± 0.22 (5). The numbers in parentheses indicate the number of experiments.

number of sites may be revealed by a decreased apparent K_m alone or in combination with increased V_{max} , while an increase in the total number of enzyme sites would result in an increased V_{max} . In liver surface membranes from phenobarbital-treated rats, V_{max} was increased 2.5-fold ($P < 0.001$), and the apparent K_m was not significantly changed (Fig. 6). The increased V_{max} is probably due to an increased number of enzyme molecules rather than activation, a conclusion supported by the finding that mixing the liver cytosol and surface membranes from phenobarbital-treated and normal animals or the addition of 0.5 or 1 μM phenobarbital directly to the assay did not significantly alter $(\text{Na}^+ - \text{K}^+)$ -ATPase activity.

Effect of cycloheximide on phenobarbital-enhanced $(\text{Na}^+ - \text{K}^+)$ -ATPase activity. The mechanism of phenobarbital-enhanced $(\text{Na}^+ - \text{K}^+)$ -ATPase activity was further investigated by examining the effect of cycloheximide, a potent inhibitor of protein synthesis (48, 49) on the changes in enzyme activity (Fig. 7). Cycloheximide prevented the rise in hepatic $(\text{Na}^+ - \text{K}^+)$ -ATPase. Furthermore, cycloheximide apparently prevents phenobarbital-enhanced activity by inhibiting protein synthesis. Addition of 0.01, 0.1, and 1 μM cycloheximide to the assay *in vitro* did not alter $(\text{Na}^+ - \text{K}^+)$ -ATPase activity and its *in vivo* administration inhibited protein synthesis by 93% at 1 h and 47% at 12 h. As previously shown (50), cycloheximide also prevents phenobarbital-enhanced bile flow, suggesting that phenobarbital increases bile flow and $(\text{Na}^+ - \text{K}^+)$ -ATPase by mechanisms which require *de novo* protein synthesis. However, $(\text{Na}^+ - \text{K}^+)$ -ATPase, bile flow, and bile acid excretion are not significantly

reduced when cycloheximide is given alone, suggesting that its prevention of the phenobarbital effects does not result from generalized hepatotoxicity.

Time course of changing $(\text{Na}^+ - \text{K}^+)$ -ATPase activity. Since the extent of change in amount of enzyme present is dependent upon both synthesis and degradation and since both processes require continuous protein synthesis, it is not possible to define specific alterations in enzyme turnover by protein inhibition experiments (51). However, it is possible to estimate the half-life of a protein by determining the time course of its change from a basal state to another level. This requires the assumption that the rate of synthesis is either increased or decreased rapidly and maintained at a constant rate until the new steady state becomes a function of the rate of degradation (35, 51-53). Therefore, if phenobarbital affects only the rate of synthesis without altering degradation, the half-life of the enzyme in the presence or absence of drug should be similar. The full time course of phenobarbital-enhanced $(\text{Na}^+ - \text{K}^+)$ -ATPase was examined (Fig. 8) to determine whether synthesis, degradation, or both were altered.

$(\text{Na}^+ - \text{K}^+)$ -ATPase activity was significantly increased as early as 14 h after phenobarbital administration

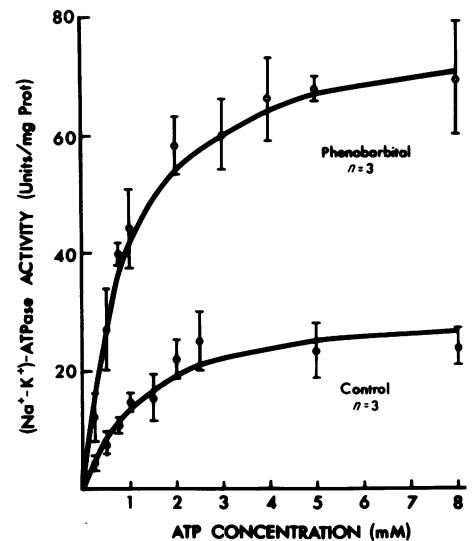


FIGURE 6 Activity of $(\text{Na}^+ - \text{K}^+)$ -ATPase in liver surface membrane fractions as a function of ATP concentration. Membranes were isolated from saline- (●—●) and phenobarbital-treated (○—○) rats. $(\text{Na}^+ - \text{K}^+)$ -ATPase activity was determined in an ATP regenerating system as described in Methods for each of three separate experiments in each group. The points and vertical lines represent the mean \pm SD. The best-fit line of activity was identified by nonlinear least-squares regression. The apparent $K_m \pm \text{SE}$ for control and phenobarbital-treated animals are 1.2 ± 0.2 and 0.9 ± 0.2 mM (NS); and the $V_{max} \pm \text{SE}$ are: 32.3 ± 3.6 and 78.4 ± 3.4 ($P < 0.001$), respectively. n denotes the number of rats. Prot, protein.

and continued to rise for 4 days when its activity reached a new induced steady state and remained relatively unchanged in spite of continued treatment. After discontinuation of phenobarbital there was a lag of a day before the logarithmic decay of $(\text{Na}^+ \text{-K}^+)$ -ATPase occurs reaching the original steady state on the 5th post-treatment day. The first-order rate constant in the presence of phenobarbital was determined from the linear slope of logarithmic plots of enzyme activity. Because cessation of phenobarbital effects was delayed 24 h, which may reflect persistence of drug in the animal due to the high dose of phenobarbital, the degradation rate constant was derived from the logarithmic plots of decay activities between 2 and 5 days post-treatment. The half-life of the increase in $(\text{Na}^+ \text{-K}^+)$ -ATPase to its new phenobarbital-induced steady state ($t_{1/2} = 69$ h) was not significantly different from the decay rate calculated for its return to the original basal level in the absence of drug ($t_{1/2} = 58$ h).

Effect of hypothyroidism on hepatic $(\text{Na}^+ \text{-K}^+)$ -ATPase activity and bile flow and their response to phenobarbital administration. Thyroid hormone is an important regulator of hepatic $(\text{Na}^+ \text{-K}^+)$ -ATPase activity and bile flow (21, 54, 55). Since phenobarbital administration increases hepatic accumulation and metabolism of thyroxine (56, 57) it seemed possible that the selective induction of sodium transport sites may be mediated through thyroid hormone. Fig. 9 demonstrates that thyroidectomy reduces $(\text{Na}^+ \text{-K}^+)$ -ATPase and bile flow 41 and 49% of control, respectively. Despite persistence of the hypothyroid state, shown by unmeasurable serum thyroxine con-

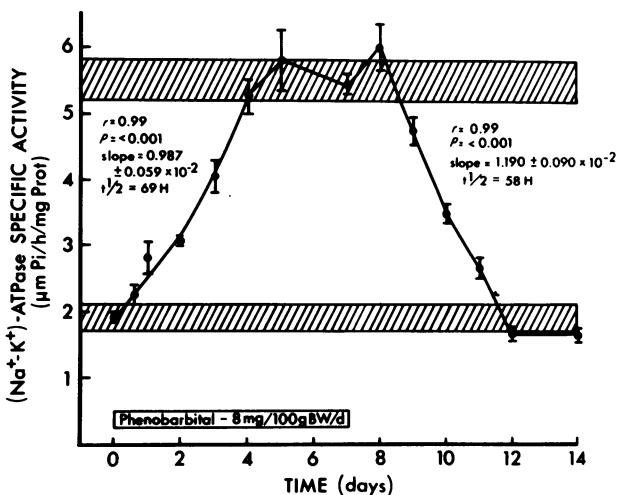


FIGURE 8 Effect of phenobarbital administration on hepatic $(\text{Na}^+ \text{-K}^+)$ -ATPase activity. Phenobarbital was administered for 7 days and six animals were sacrificed at each time point. $(\text{Na}^+ \text{-K}^+)$ -ATPase is expressed as the mean \pm SEM. The lower hatched area indicates the basal $(\text{Na}^+ \text{-K}^+)$ -ATPase activity mean \pm SD, and the upper hatched area indicates the new drug-enhanced steady state determined as the mean \pm SD of values obtained on days 4, 5, and 7 of phenobarbital treatment. The slope was determined as described under Methods. Prot, protein; BW, body weight.

centration and T_4 -normalized values (Table IV), during phenobarbital administration both liver surface membrane $(\text{Na}^+ \text{-K}^+)$ -ATPase activity and basal bile flow were doubled ($P < 0.001$) and were now not significantly different from control.

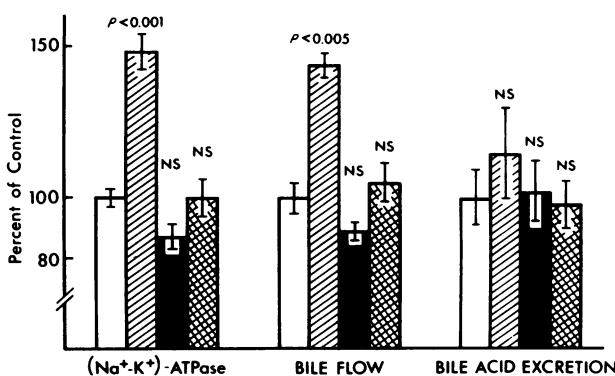


FIGURE 7 Effect of phenobarbital and cycloheximide on $(\text{Na}^+ \text{-K}^+)$ -ATPase activity, bile flow, and bile acid excretion. Homogenate $(\text{Na}^+ \text{-K}^+)$ -ATPase activity, bile flow, and bile acid excretion was determined 24 h after administration of saline (□), phenobarbital (80 mg/kg) (▨), phenobarbital plus cycloheximide (150 $\mu\text{g}/100$ g, ▨), and cycloheximide alone (▨). The bars represent mean \pm SEM. Control values are $(\text{Na}^+ \text{-K}^+)$ -ATPase, $1.9 \pm 0.1 \mu\text{m}/\text{mg P per h}$ (8), bile flow, $7.6 \pm 0.7 \mu\text{l}/\text{min per 100 g body wt}$ (4); and bile acid excretion, $88.7 \pm 7.1 \mu\text{g}/\text{min per 100 g body wt}$ (10). The numbers in parentheses represent number of determinations.

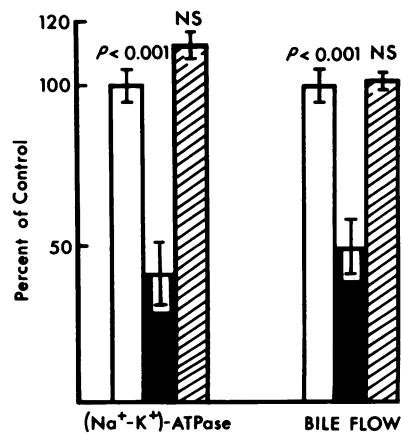


FIGURE 9 Effect of thyroidectomy and thyroidectomy plus phenobarbital on hepatic liver surface membrane $(\text{Na}^+ \text{-K}^+)$ -ATPase activity and bile flow. The bars represent the mean \pm SEM for control (□), thyroidectomy (▨), and thyroidectomy plus phenobarbital (▨). Statistical comparisons refer to comparison of experimental groups to controls. Control values for $(\text{Na}^+ \text{-K}^+)$ -ATPase is $29.6 \pm 1.5 \mu\text{m Pi}/\text{mg P per h}$ and bile flow is $7.6 \pm 0.7 \mu\text{l}/\text{min per 100 g body wt}$.

TABLE IV
Serum Thyroxine Levels in Untreated and Phenobarbital-Treated Thyroidectomized Rats

Experimental status	No.	T ₄ concentration	T ₄ normalized
Control	11	3.4±0.42	0.88±0.05
Thyroidectomy	6	ND	0.40±0.07*
Thyroidectomy + phenobarbital	6	ND	0.43±0.02*

Rats were surgically thyroidectomized and given 1 μ Ci ^{131}I i.p. and studied 1 mo later. Either phenobarbital (8 mg/100 mg/per day) or saline was administered for 5 days. Results are expressed as mean±SD. ND, not detected.

* $P < 0.001$.

Time course of phenobarbital-induced changes in hepatic (Na⁺-K⁺)-ATPase activity, bile flow, electrolyte and bile acid excretion, and liver weight. To examine further the relationship of hepatic (Na⁺-K⁺)-ATPase to bile flow the sequence of changes in enzyme activity, bile flow, and biliary electrolytes was determined during 5 days of daily phenobarbital administration (Table V). Bile flow, as well as hepatic (Na⁺-K⁺)-ATPase, is increased as early as 14 h after administration of phenobarbital and increase proportionally for 2 days. As previously shown, bile flow peaks at 48 h and does not increase further despite continued administration of phenobarbital (14). In contrast, (Na⁺-K⁺)-ATPase activity continues to rise for 5 days. Consistent with increased canalicular (Na⁺-K⁺)-

ATPase activity, biliary sodium concentrations were not significantly altered, but potassium concentrations were lower than control at all time periods ($P < 0.05$). Changes in (Na⁺-K⁺)-ATPase and bile flow are apparently independent of bile acid excretion, which was not significantly altered. Relative liver weight neither increased to the same degree nor in the same time sequence as (Na⁺-K⁺)-ATPase and bile flow.

DISCUSSION

(Na⁺-K⁺)-ATPase, which is the enzymatic expression of the sodium pump (22, 25, 58), is responsible for maintenance of Na⁺ and K⁺ gradients and cell volume (59, 60). The activity of this enzyme in a number of tissues adapts to varying cellular requirements for transport of Na⁺ and K⁺, indicating that it is an important factor in determining cation and water transport. If the hypothesis that sodium is actively transported from hepatocytes into bile is valid, bile secretion should depend on the activity of (Na⁺-K⁺)-ATPase. The present study demonstrates that phenobarbital selectively induces hepatic (Na⁺-K⁺)-ATPase activity, which correlates closely with bile flow.

Previous studies with known inhibitors of (Na⁺-K⁺)-ATPase such as cardiac glycosides and ethacrynic acid have shown conflicting effects on bile flow. Such results may result from the rat's large capacity to excrete these drugs (61, 62), its resistance to inhibition of (Na⁺-K⁺)-ATPase (63), or lastly to the possible location of cardiac glycoside binding sites on the biliary rather than the sinusoidal surface where they are not

TABLE V
Effect of Phenobarbital on Hepatic (Na⁺-K⁺)-ATPase, Bile Flow, Biliary Electrolyte Concentration, Bile Acid Excretion, and Percent Liver Weight/Body Weight

	Duration of phenobarbital treatment, h					
	0	14	24	48	72	120
(Na ⁺ -K ⁺)-ATPase, $\mu\text{mol Pi/mg Prot/h}$	1.9±0.2 (12)	2.4±0.3† (6)	2.8±0.6* (6)	3.1±0.2* (6)	4.1±0.6* (6)	5.8±1.7* (14)
Bile flow, $\mu\text{l/min}/100 \text{ g body wt}$	7.4±0.9 (10)	9.1±1.7† (4)	11.39±0.8* (3)	13.8±1.2* (4)	11.5±2.6* (4)	10.6±2.0* (4)
Na ⁺ concentration	152.5±3.1 (4)	146.0±1.0 (4)	145.0±2.2 (3)	156.5±6.8 (4)	151.7±1.2 (4)	146.5±3.0 (4)
K ⁺ concentration	7.8±0.4* (4)	6.1±0.1* (4)	6.4±0.2* (3)	5.8±0.3* (4)	6.5±0.1† (4)	5.5±0.3* (4)
Bile acid excretion, $\mu\text{g/min}/100 \text{ g body wt}$	88.7±15.3 (10)	103.6±11.7 (4)	93.7±4.8 (3)	96.8±20.1 (4)	96.9±15.3 (4)	104.3±4.4 (4)
Liver wt $\times 100$ Body wt	3.9±0.3 (6)	4.1±0.4 (6)	4.5±0.3† (6)	5.0±0.1* (6)	5.3±0.1* (6)	5.9±0.1* (6)

Hepatic (Na⁺-K⁺)-ATPase, bile flow, sodium and potassium, and bile acids were determined as described under Methods. Results are expressed as mean±SEM. Values in parentheses are the number of individual animals studied.

* $P < 0.01$.

† $P < 0.05$.

available to sufficient concentrations of inhibitors. Therefore, we examined whether phenobarbital, a powerful inducer of bile salt-independent flow and biliary sodium excretion, is also associated with increased $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity.

Determination of $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase in liver homogenates is difficult because of the high background of Mg^{++} -ATPase and the relatively low $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity. Bakkeren and Bonting (64) incubated liver homogenates with urea to inhibit Mg^{++} -ATPase to distinguish changes in $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase under different experimental conditions. Since the enzyme system is localized in membranes, deoxycholate has been successfully used to increase $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity, permitting access of substrate to the enzyme site (65). But, in the liver this detergent inhibits activity (66), and therefore we used the technique of freeze-thawing samples in hypotonic NaHCO_3 to increase activity. Since V_{max} increases, indicating an increased number of transport sites, and the apparent K_m is unaltered, this method apparently increases activity without altering the structure of the enzyme.

The highest $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase specific activity was obtained in liver surface membrane fractions enriched in bile canaliculi. Both specific activity and percent recovery co-isolate with 5'-nucleotidase, an established liver surface membrane enzyme. In addition, the distribution of $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase is different than the microsomal enzyme glucose-6-phosphatase consistent with sedimentation of liver surface membrane fragments with the microsomal fraction (42, 67). Although these studies do not clarify whether $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase is located on the sinusoidal, canalicular, or on both poles of the hepatic parenchyma cell, at the very least the finding of greatly increased $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity in liver surface membrane fractions enriched in bile canaliculi suggests that the sodium pump is located at a site where it can regulate biliary excretion of sodium (23).

Development of an assay with threefold increased activity permits re-examination of the question whether phenobarbital increases hepatic $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity. In contrast to findings of previous studies (68-70), administration of phenobarbital significantly increased $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity almost twofold in liver surface membrane fractions. Increased $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase is not due to isolation of different surface membrane fractions in the treated animals, as shown by the threefold increase in total liver homogenates $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity and the unaltered recovery of surface membrane protein and relative enrichment of membrane marker enzymes (Table II).

Increased hepatic $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity is selective. Other membrane phosphatases as well as sodium pump activity in the small intestines, kidney, and skele-

tal muscle are either unaltered or reduced. Although the mechanism is not clear, heterogeneous tissue responses of $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase after administration of thyroid hormone and mineralocorticoids has been reported for colon and brain (21, 46). In addition, the differential increase in hepatic $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase compared to Mg^{++} -ATPase and other phosphatases suggests phenobarbital may induce new sodium transport sites per unit of surface membrane protein.

Enhanced $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity induced by phenobarbital administration may result from changes in enzyme kinetic properties, activation of a fixed number of enzyme molecules, or induction of enzyme protein. Since isolation, purification, and immunological quantitation of hepatic $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase has not been achieved, studies of kinetic analysis from partially purified surface membrane fractions was utilized to examine whether phenobarbital altered kinetic properties or increased the number of active molecules. If phenobarbital increased the total number of sites, an increase in V_{max} of the enzyme would be observed while activation will decrease K_m . Utilizing ATP as the variable in both control and phenobarbital-treated animals, hyperbolic kinetics were obtained. The K_m for ATP was not significantly altered, but V_{max} was increased, consistent with an increased number of sodium transport sites.

An apparent increased number of $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase sites can result from activation of latent enzyme as well as induction of new sodium transport sites. One way to distinguish these alternatives is to examine whether inhibition of protein synthesis required for induction blocks the increase in $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase. Cycloheximide, which inhibits polypeptide elongation (49), decreases hepatic protein synthesis and prevents phenobarbital enhancement of $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase and bile flow. Significantly, cycloheximide when given alone does not alter basal $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity, bile flow, or bile acid excretion. Further evidence against activation of latent sites was obtained from the experiments showing that phenobarbital added *in vitro* failed to change activity and that liver cytosol and membrane fractions from phenobarbital-treated animals failed to activate enzyme activity in control liver surface membrane. Thus, although further studies are needed to confirm an increased number of sodium transport sites utilizing such methods as binding of [^3H]ouabain and the sodium-dependent incorporation of [^{32}P] from [$\gamma^{32}\text{P}$]ATP, these studies support the conclusion that phenobarbital induces *de novo* protein synthesis of $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase macromolecules.

To determine whether phenobarbital increases new synthesis or rather stabilizes preformed $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase molecules, degradation rate constants in the presence and absence of drug were estimated. Consistent with previous studies (53, 71), phenobarbital

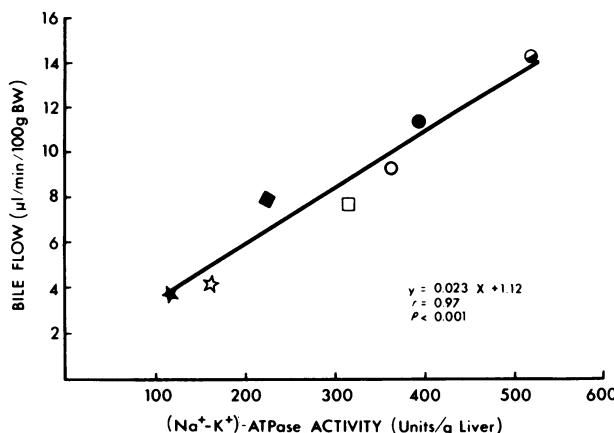


FIGURE 10 Correlation of bile flow with hepatic ($\text{Na}^+ \text{-K}^+$)-ATPase activity. Bile flow and ($\text{Na}^+ \text{-K}^+$)-ATPase activity are determined as described under Methods. The symbols represent the mean of at least four to six determinations of enzyme activity and bile flow in separate animals. Ethynodiol (5 mg/kg body wt for 5 days [BW] (★), thyroidectomy (☆), thyroidectomy plus phenobarbital (■), control (□), and phenobarbital treatment for 14 h (○), 24 h (●), and 48 h (○).

appeared to increase enzyme synthesis within 14 h and the rate of synthesis continued to increase along an exponential time course to a new steady level. After a delay of 1 day, enzyme activity decayed according to exponential kinetics. Although the theoretical model used to determine enzyme half-lives is over simplified as emphasized by Chee and Swick (72), it is useful where isotope-labeling techniques are not yet possible. Thus, within the methodologic limitations of these experiments the half-life of ($\text{Na}^+ \text{-K}^+$)-ATPase is approximately 2.5 days, both during and after cessation of phenobarbital administration. The mean half-life for ($\text{Na}^+ \text{-K}^+$)-ATPase is similar to the bulk turnover for liver surface membrane proteins determined by isotopic decay techniques (71), but faster than the 18 days estimated for NAD(P)^+ glycohydrolase (EC 3.2.2.6) another liver surface membrane protein (73). Thus, similar to the endoplasmic reticulum surface membrane proteins also undergo heterogeneous turnover (74). Furthermore, these studies demonstrate that liver surface membrane proteins also undergo adaptive changes in response to drugs. Both in intact animals (75) and in mammalian liver cell cultures (76) phenobarbital increases microsomal membrane proteins primarily at the level of transcription. The present studies are consistent with, but do not prove, a similar sequence of events for induction of a liver surface membrane protein.

Phenobarbital administration causes many diverse effects including hepatic accumulation and metabolism of thyroid hormone (56). Ismail-Beigi and Edelman (21)

have demonstrated that thyroid hormone increases ($\text{Na}^+ \text{-K}^+$)-ATPase and Layden and Boyer (55) recently have shown that this altered enzyme activity is associated with changes in bile salt independent flow. Since thyroid hormone has also been implicated in the increased hepatic ($\text{Na}^+ \text{-K}^+$)-ATPase activity of chronic alcohol ingestion in rats (77, 78), we investigated whether thyroxine may also mediate the hepatic response to phenobarbital. Hypothyroidism was confirmed by unmeasurable serum thyroxine binding capacity in untreated and phenobarbital-treated rats. Thyroidectomy proportionally reduced both ($\text{Na}^+ \text{-K}^+$)-ATPase and bile flow to 50% of control as reported by others (55). In this setting of thyroid deficiency, phenobarbital doubled ($\text{Na}^+ \text{-K}^+$)-ATPase activity and basal bile flow, indicating that the pathways mediating phenobarbital enhancement are intact in the hypothyroid rat. However, phenobarbital does not increase either ($\text{Na}^+ \text{-K}^+$)-ATPase or bile flow to levels found in phenobarbital-treated euthyroid animals, suggesting that thyroid hormone may play a permissive role in phenobarbital induction.

The present studies do not clarify whether phenobarbital has a specific effect on ($\text{Na}^+ \text{-K}^+$)-ATPase or whether changes in activity are mediated by changes in intracellular sodium and (or) potassium concentrations due to increased passive movements of these cations or, alternatively, by an increase in the ATP:ADP ratio, an effect of phenobarbital on mitochondrial oxidative phosphorylation. Studies measuring the temporal relationship of intracellular cations and nucleotide concentrations to sodium pump activity are needed to answer these questions.

If activation of the sodium pump mediates bile salt-independent flow, changes in bile flow should be temporally related to changes in ($\text{Na}^+ \text{-K}^+$)-ATPase activity. Analyses of basal bile flow with daily administration of phenobarbital were similar to previous studies revealing a latent period of 14 h and an increase in flow to a maximum value at 2 days which remained relatively unchanged in spite of continued treatment (14, 50). The time course in hepatic ($\text{Na}^+ \text{-K}^+$)-ATPase was in phase for only the first 2 days, but continued to rise reaching a plateau level at 4 days. Thus, both ($\text{Na}^+ \text{-K}^+$)-ATPase and basal bile flow are proportional for 48 h, supporting the inference that basal ($\text{Na}^+ \text{-K}^+$)-ATPase activity mediates an important part of bile salt-independent flow by placing a ceiling on the rate of sodium excretion. However, when basal sodium pump activity is increased greater than twofold, other factors apparently become rate limiting for sodium excretion. The lack of correlation after 2 days of phenobarbital treatment may be due to decreasing efficiency of the sodium pump (i.e., the coupling ratio of $\text{Na}^+ \text{:K}^+ \text{:ATP}$ is changed) or increased passive permeability of the canalicular

membrane to Na^+ so that less osmotic activity is present. It has been suggested that the dissociation of increased $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase and radiolabeled rubidium transport observed in experimental chronic alcohol ingestion in rats may be due to alterations in both of these mechanisms (78).

Fig. 10 summarizes the relationship in the experimental situations described in the present paper between basal bile flow and $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity per gram of wet liver weight. These results demonstrate that a quantitative correlation ($r = 0.97$, $P < 0.001$) exists between bile flow and the activity of the putative sodium pump. It should be emphasized however that although the correlation between $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase activity and bile flow, under many experimental conditions, is strong, it does not necessarily indicate an identity. These studies are consistent with previous studies demonstrating an association between bile flow and $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase for hepatic regeneration (79, 80), selective biliary obstruction (81), rose bengal (69), icterogenin (82), hypo- and hyperthyroidism (55), chronic alcohol administration (77, 83), ethinyl estradiol administration (50, 84), and chlorpramazine (85-87). Furthermore, recent findings from this laboratory indicate that other drugs known to alter bile flow such as 6-methyl prednisolone, cortisone acetate, and pregnenolone-16-carbonitrile also increase $(\text{Na}^+ \text{-} \text{K}^+)$ -ATPase.²

In conclusion, these studies demonstrate that the hepatic sodium pump adapts to phenobarbital therapy and other agents in accordance with its postulated role in transport of sodium across the bile canalicular membrane and regulation of bile salt-independent bile flow.

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