cortical low voltage fast activity on the recruiting response was first described by Moruzzi and Magoun(9). Recruiting responses that are abolished by "alerting" stimuli(10), have been observed in the awake, unanesthetized relaxed animal(11). Recently recruiting responses in awake, alert preparations have been illustrated with what appears to be a sustained response(12). In the present experiments, care was taken to distinguish between recruiting and augmenting responses in the oscilloscopically monitored recording. The sustained responses we have described are related to the long latency recruiting cortical evoked potential and were observed after cortical ablation, which has been shown to abolish the augmenting response(13).

Although the recruiting and sustained responses had a similar waveform, latency and distribution, they were affected differently by brainstem stimulation and barbiturate anesthesia.

The similarities of waveform, latency and distribution suggest that the same thalamocortical pathways are involved for recruiting and sustained responses. However, the differences in the effect of "alerting" stimuli or barbiturate anesthesia would indicate that only a portion of the diffuse thalamic projection system is altered by afferent fibers from the reticular formation and hypothalamus, or by barbiturate anesthetics.

Summary. When certain intralaminar thalamic nuclei are stimulated at intensities slightly above threshold for the cortical recruiting response, a response of similar wave form, latency and cortical distribution maintains a sustained amplitude between epochs of recruitment. This sustained response differs from the recruiting response in its reaction to reticular activating system stimulation and barbiturate anesthesia.

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Effect of Digitoxin, Aldosterone, and Dietary Sodium Chloride on Incorporation of Inorganic P^{32} into Liver and Kidney Nuclear and Mitochondrial Phospholipids.* (29213)

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There is evidence linking phospholipid metabolism with active transport. An increased turnover of phospholipids is known to be associated with the movement of secretory products out of cells stimulated by acetyl-

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choline or by certain hormones. Glands stimulated to release amylase(1,2), mucin(2,3), pepsin(3), epinephrine(4), adrenacorticotropic hormone(5), and thyroxine(6) have an increased incorporation of P_i^{32} into phosphatidic acid and phosphatidyl inositol. Acetylcholine stimulates the incorporation of P_i^{32} into phosphatidic acid and phosphatidyl inositol in brain(7) and increases the turnover of phosphatidic acid in slices of the avian salt gland at the same time that sodium chloride is secreted(8,9). As a result of these experiments, Hokin and Hokin(10) have suggested that phosphatidic acid and phosphatidyl inositol might serve as a carrier in the active transport of sodium through cell membranes.

On the other hand, cardiac glycosides are known to inhibit the active transport of cations in a number of tissues, including brain slices(11) and kidney(12) and therefore might be expected to inhibit the incorporation of P_i^{32} into phosphatidic acid. However, Yoshida *et al*(13) have shown that ouabain, a cardiac glycoside which inhibits sodium transport, stimulates the incorporation of P_i^{32} into phosphatidic acid of brain and of the avian salt gland. The stimulatory effect of ouabain is not limited to the phospholipid fraction as there is also an increased incorporation of P_i^{32} into the sugar phosphate fraction of rabbit cortex slices(14). Pitressin and acetylcholine also stimulate, but not as markedly as does ouabain. Ouabain decreases ATP levels in brain slices but is without effect on the specific activity of nucleotide phosphorus(15). Marinetti *et al*(16) have demonstrated that the cardiac glycoside, digitoxin, increased the incorporation of P_i^{32} into lecithin and phosphatidyl ethanolamine of rat whole heart ventricle. The other heart muscle lipids or cellular fractions were not investigated. In view of the incomplete and contradictory evidence for a role of phospholipids in sodium transport, experiments were undertaken to study the effects of digitoxin, aldosterone and sodium chloride feeding on the incorporation of P_i^{32} into the individual phosphatides of heart, liver, and kidney nuclear and mitochondrial phospholipids.

Experimental procedure. Male Sprague-Dawley rats weighing 160 to 180 g were used in the digitoxin and aldosterone experiments and rats weighing 55-65 g were used in the salt experiment. Rats were individually caged and fed Purina Laboratory Chow Pellets. The animals in the digitoxin group were each treated intraperitoneally with 1 μg/g of digitoxin in 49% ethanol 24 hours prior to sacrifice. Control animals received the same volume of 49% ethanol. The animals in the aldosterone experiment were each treated intraperitoneally with 20 μg d-aldosterone at 28, 16, and 6 hours before sacrifice. Control animals received saline. The animals used in the salt experiments were individually caged and maintained by pair feeding the Purina Laboratory Chow, *i.e.*, the amount of food given to the control animals was determined by weighing daily the amount of food consumed from the experimental animal's feeding cup. The animal's drinking water contained 0.5% NaCl for 3 days followed by 1% NaCl for 3 days, 1.5% for 4 days and 1.7% for 4 days. Control animals received distilled water.

Phospholipids. Following the experimental period each animal was injected intraperitoneally with 200 μc of P_i^{32} and sacrificed by decapitation 4 hours later. This time was chosen so as to be on the ascending portion of the P_i^{32} uptake curve, where incorporation of the isotope into the phospholipids occurs to a greater degree than breakdown(17,18). The heart, liver, and kidneys were quickly removed, rinsed with cold water and placed on cracked ice. The organs were immediately weighed and homogenized in cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with a teflon pestle. The heart and kidney were diluted to 25 ml with the cold sucrose solution and the liver to a concentration of 1 g per 8 ml. Aliquots of 3 ml were pipetted into 10 ml of a cold solution of 10% trichloroacetic acid plus 0.4 M $MgCl_2 \cdot 6H_2O$(19) and kept at 3°C overnight and inorganic phosphorus(20) and radioactivity(18) analysis performed on the supernatant.

The remaining homogenates were centrifuged(21) in the cold for 15 minutes at 800 $\times$ *g* to remove the nuclei. The nuclear pellet was rehomogenized in sucrose and centrifuged

and the supernatants combined. Mitochondria were separated from the combined supernatants by centrifugation at 10,000 × *g* for 15 minutes. The mitochondrial pellet was twice resuspended in sucrose and centrifuged to wash out contaminating microsomes. This procedure removed over 99% of the microsomes as determined by assaying for microsomal glucose 6-phosphatase(22).

The lipids were extracted from the mitochondria by extracting once with 8 ml of methanol and twice with 8 ml portions of $CHCl_3$-CH_3OH (2:1) at room temperature. This was followed by 4 extractions with 8 ml portions of $CHCl_3$-CH_3OH (2:1) at 55°C. Each extraction was for 15 minutes in a shaking water bath followed by centrifugation for 3 minutes at 1,600 × *g* to facilitate removal of the solvent. The organic solvents of the pooled extracts were evaporated on a Rinco Rotating Vacuum Evaporator (Rinco Instrument Co.) at 35° and the water removed by lyophilization in Freeze-Dryer (VirTis Co., Inc.). The lipids were taken up in $CHCl_3$-CH_3OH and the phospholipids obtained by chromatography on silicic acid impregnated glass filter paper(23). The solvent system was diisobutyl ketone:acetic acid:water:benzene (160:50:8:7, v/v). The chromatograms were dried, stained with Rhodamine 6G and the phospholipids identified with ultraviolet light(24). The individual phosphatides were extracted with 1 N methanolic HCl. The hydrolysate was evaporated to dryness and phosphorus and radioactivity determined.

Phosphorus and radioactivity. For organic phosphorus analysis each sample was digested for 30 minutes with 0.2 ml of H_2SO_4-HNO_3 (1:1), allowed to cool, and 3 drops of HNO_3 added and the digestion continued for an additional 15 minutes. The samples were then neutralized to pH 6-7 with NH_4OH with phenol red as internal indicator. Inorganic phosphorus was determined by the method of Fiske and Subbarow(20). An aliquot of the colored solution of the phosphate analysis was pipetted into a steel planchet containing a drop of concentrated NaOH solution and dried under a heat lamp. The radioactivity was determined by placing the planchet under an ultrathin window, gas flow Geiger tube (Tracerlab TGC-14) and correcting for decay. From the phosphorus and radioactivity analysis the relative specific activity was calculated(25). The relative specific activity is the ratio of the specific activity of organic fraction to specific activity of inorganic fraction and expresses the relationship between the isotopic concentration in a compound and in its precursor(26). Under the conditions of our experiments an increase in formation of phospholipids may be reasonably assumed when higher values for relative specific activities are found. The specific activity is defined as counts per minute/mg of phosphorus. To evaluate the significance of results the *t* test of significance(27) was applied to the difference between the means of the control and experimental groups.

Results. Table I shows the composition of the individual phospholipids of rat liver, kidney and heart mitochondria and nuclei. The percentages of total phospholipid phosphorus for the various phosphatides of liver, kidney and heart mitochondria are similar to those of whole liver(28,29), kidney and heart(28). Table II shows the effect of digitoxin, a cardiac glycoside, on uptake of radioactive phosphorus into the various phospholipids of liver, kidney, and heart mitochondria and nuclei. The data show that administration of digitoxin produces a statistically significant increase in phospholipid synthesis of phosphatidyl inositol, sphingomyelin, phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine of liver and heart mitochondria and in all fractions of kidney mitochondria except phosphatidyl inositol. Phosphatidyl choline and phosphatidyl ethanolamine synthesis is stimulated significantly in nuclei of liver, kidney and heart. Phosphatidyl serine synthesis is stimulated significantly only in the kidney nuclei and phosphatidyl inositol in kidney and heart nuclei. Digitoxin has been shown to increase synthesis of phosphatidyl choline and phosphatidyl ethanolamine in total heart ventricle(16), however the other muscle lipids were not investigated. These experiments support the hypothesis that in part, the action *in vivo* of digitoxin is on the turnover of phospholipids. The glycoside ouabain has been shown to stimulate the total phospholi-

pid synthesis in rabbit-brain cerebral cortex (14). Phospholipids are found in cellular membranes(30), and membranes of mitochondria, nuclei and microsomes(31,32) of the cell. Liver mitochondria are composed of about 20% phospholipid and nuclei 16% on a dry weight basis with the phospholipids accounting for about 90% of the total lipids present(32). Mitochondria(33) and enzymes (34,35) involved in electron transport are inactivated if phospholipids are removed; however, activity is restored with the addition of phospholipids. The postulation that phospholipids are involved in electrolyte transport across membranes of the cell is not substantiated by the data in Tables III and IV. Aldosterone, the most active mineralocorticoid of the adrenal cortex stimulates only the synthesis of phosphatidyl choline of kidney mitochondria and nuclei and phospha-

TABLE I. Composition of Individual Phospholipids of Rat Liver, Kidney and Heart Mitochondria and Nuclei.*

Fraction	No. of rats	Phospholipid fractions (% of total lipid P)				
		Phosphatidyl inositol	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
Liver:						
Mitochondria	20	7.8 ± 1.7	3.3 ± .8	50.6 ± 2.0	4.0 ± 1.1	26.9 ± 2.9
Nuclei	12	7.7 ± 1.5	8.0 ± 2.6	49.4 ± 1.9	6.0 ± 1.3	23.8 ± 1.4
Kidney:						
Mitochondria	16	5.5 ± .3	8.8 ± 1.2	39.6 ± 2.5	5.9 ± .3	31.0 ± 2.2
Nuclei	11	5.2 ± 1.1	12.7 ± 1.1	41.5 ± 3.1	7.1 ± 1.7	26.2 ± 2.3
Heart:						
Mitochondria	15	3.9 ± 1.5	4.9 ± 1.8	41.7 ± 2.1	4.0 ± 1.3	33.3 ± 3.2
Nuclei	11	3.8 ± 1.2	5.1 ± 1.1	47.0 ± 2.3	4.6 ± 1.9	29.6 ± 3.1

* Mean ± standard deviation is given.

TABLE II. Relative Specific Activity (×100) of Mitochondrial and Nuclear Phospholipids of Liver, Kidney, and Heart Following Single Dose of Digitoxin.

Material administered	No. of animals	Phospholipid fractions				
		Phosphatidyl inositol	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
Liver mitochondria						
Control	5	2.94 ± .51	2.38 ± .73	4.69 ± 1.36	3.66 ± .48	5.10 ± 1.61
Digitoxin	5	5.77 ± 1.31*	5.09 ±1.41*	9.12 ± 1.14*	8.65 ±1.22*	11.10 ± 2.31*
Kidney mitochondria						
Control	5	7.44 ± .71	1.91 ± .35	10.70 ± 2.34	1.74 ± .63	2.04 ± .52
Digitoxin	5	9.55 ± 3.95	3.42 ±1.05†	17.90 ± 2.35*	4.20 ± .76*	3.50 ± .42*
Heart mitochondria						
Control	5	7.10 ± 1.80	1.46 ± .26	1.50 ± .39	.74 ± .11	.62 ± .17
Digitoxin	6	10.30 ± 1.60*	2.12 ± .44†	2.54 ± .64*	1.38 ± .34*	.90 ± .16†
Liver nuclei						
Control	5	3.40 ± 1.07	1.36 ± .34	4.78 ± 1.21	3.12 ± .86	5.13 ± 1.09
Digitoxin	5	4.04 ± .54	2.91 ± .63*	10.14 ± 1.21*	4.19 ±2.39	11.94 ± 1.06*
Kidney nuclei						
Control	5	9.17 ± .82	1.68 ± .33	11.98 ± 1.34	2.60 ± .73	2.98 ± .35
Digitoxin	5	13.00 ± 2.37*	1.98 ± .17	15.80 ± 2.30†	3.46 ± .45†	4.41 ± .53*
Heart nuclei						
Control	5	8.80 ± 1.91	.53 ± .22	1.88 ± .19	1.05 ± .23	.91 ± .08
Digitoxin	6	11.77 ± 1.28*	.87 ± .12*	2.61 ± .28*	1.23 ± .17	1.07 ± .12†

The animals received intraperitoneally digitoxin 1 μg/g in 49% ethanol and were sacrificed 24 hr later. Four hours before sacrifice 200 μc of P_i^{32} were injected intraperitoneally. Controls received same volume of 49% ethanol. Numbers preceded by ± are standard deviations. Test of significance was applied to difference between mean of control and experimental values. The P probability for chance occurrence of this difference was: * <.01. † <.05.

tidyl ethanolamine of mitochondria. Another hormone, Pitressin which effects cell membrane permeability increases the uptake of P_i into phospholipids of rabbit-brain cerebral cortex(14). It is apparent from the data of Table IV that the feeding of sodium chloride or loading the body with excess sodium chloride failed to demonstrate any increase in turnover of phospholipids of the kidney or liver except a decreased turnover occurred in phosphatidyl choline and ethanolamine of kidney mitochondria. This observation would agree with the work of Tinker *et al*(36) who has demonstrated that the addition of diphenylhydantoin, a potent stimulator of sodium transport in kidney slices has no effect on the incorporation of P_i^{32} into phospholipid fractions. It would appear from the results

TABLE III. Relative Specific Activity (×100) of Mitochondrial and Nuclear Phospholipids of Liver and Kidney Following Administration of 3 Doses of Aldosterone.

Material administered	No. of animals	Phospholipid fractions: Phosphatidyl inositol	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
		Liver mitochondria				
Control	6	4.36 ± .93	3.92 ±1.02	7.63 ± 1.56	6.68 ±1.38	10.80 ± 2.28
Aldosterone	6	4.60 ± .83	3.94 ±1.12	8.96 ± .80	6.95 ±1.20	11.81 ± 1.21
		Kidney mitochondria				
Control	7	11.59 ± 1.73	3.06 ± .62	14.90 ± 1.61	3.59 ± .60	3.63 ± .48
Aldosterone	6	11.90 ± 1.74	2.66 ± .23	17.55 ± 1.62*	3.74 ± .39	4.84 ± .40*
		Liver nuclei				
Control	6	4.01 ± .51	2.82 ± .48	7.54 ± 2.01	6.31 ±1.75	10.70 ± 1.79
Aldosterone	6	5.10 ± 1.14	2.89 ± .53	8.92 ± .42	5.99 ± .78	11.12 ± 1.37
		Kidney nuclei				
Control	7	13.21 ± 2.02	2.50 ± .29	12.33 ± 1.86	3.33 ± .45	4.65 ± .86
Aldosterone	6	14.82 ± 2.51	2.66 ± .52	16.00 ± .74*	2.93 ± .53	4.80 ± .54

The animals received intraperitoneally 20 μg d-aldosterone at 28, 16 and 6 hr before sacrifice. Four hours before sacrifice 200 μc of P_i^{32} was administered. Control animals received saline. Numbers preceded by ± are standard deviations. Test of significance was applied to difference between mean of control and experimental values. The P probability for chance occurrence of this difference was: * <.01.

TABLE IV. Relative Specific Activities (×100) of Mitochondrial and Nuclear Phospholipids of Liver and Kidney Following Ingestion of Sodium Chloride for 14 Days.

Material administered	No. of animals	Phospholipid fractions: Phosphatidyl inositol	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
		Liver mitochondria				
Control	4	4.83 ± 1.11	3.62 ±1.16	7.00 ± .59	3.84 ± 1.35	6.53 ± 1.47
Salt	4	4.02 ± 1.18	4.13 ±1.84	6.20 ± 1.57	4.20 ± 1.66	5.47 ± 1.07
		Kidney mitochondria				
Control	4	9.87 ± 1.73	3.86 ± .47	15.30 ± .90	5.03 ± 1.25	3.87 ± .24
Salt	4	11.36 ± 1.66	3.64 ±1.07	12.55 ± .68*	4.10 ± 1.42	2.40 ± .22*
		Liver nuclei				
Control	4	4.31 ± .23	2.57 ± .33	7.02 ± .76	4.42 ± 1.20	6.83 ± .20
Salt	4	4.92 ± 1.50	2.40 ± .48	7.18 ± 2.19	4.13 ± 1.36	7.59 ± 2.27
		Kidney nuclei				
Control	4	12.61 ± 4.02	3.41 ± .59	13.20 ± 2.59	3.24 ± .41	4.49 ± 1.42
Salt	4	14.80 ± 2.42	2.50 ± .47	12.10 ± .47	3.06 ± .78	3.71 ± .91

Animals were pair fed and received in drinking water 0.5% NaCl for 3 days, 1% for 3 days, 1.5% for 4 days and 1.7% for 4 days. Four hours before sacrifice 200 μc of P_i^{32} was injected intraperitoneally. Numbers preceded by ± are standard deviations. Test of significance was applied to difference between mean of control and experimental values. The P probability for chance occurrence of this difference was: * <.01.

that the participation of phosphorus turnover in phospholipid seems to be excluded from a role in sodium transport in the *in vivo* system studied, but compounds which effect membrane permeability do stimulate phospholipid synthesis.

Summary. 1. The effect of digitoxin, aldosterone and sodium chloride feeding on synthesis of mitochondrial and nuclear phospholipids of liver, kidney and heart was studied. 2. Administration of digitoxin stimulated incorporation of the isotope into phosphatidyl inositol, sphingomyelin, phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine of liver, heart mitochondria and in all fractions of kidney mitochondria except phosphatidyl inositol. Phosphatidyl choline and phosphatidyl ethanolamine synthesis was stimulated in nuclei of liver, kidney and heart. Phosphatidyl serine synthesis was stimulated only in kidney nuclei and phosphatidyl inositol in heart and kidney nuclei. 3. Administration of aldosterone only stimulates the synthesis of phosphatidyl choline of kidney mitochondria and nuclei and phosphatidyl ethanolamine of mitochondria. 4. The feeding of excess sodium chloride failed to increase the incorporation of the isotope into the mitochondrial or nuclear phospholipids of liver and kidney.

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