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Endoplasmic reticulum: the major contributor to the PDE peak in hepatic ^{31}P-NMR spectra at low magnetic field strengths

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^{31}P-NMR spectra of liver in vivo, subcellular fractions and model systems were acquired in order to characterise further the hepatic phosphodiester peak seen at low magnetic field strengths previously shown to be predominantly due to phospholipid bilayers. The data obtained in this study in vitro suggested that the phospholipid membranes of the endoplasmic reticulum provide the dominant contribution to this phosphodiester peak. Support for this hypothesis was provided by experiments on rats. Phenobarbitone, which is known to induce proliferation of the endoplasmic reticulum produced a considerable increase in intensity of the phosphodiester peak in liver spectra in vivo.

Introduction

^{31}P-NMR spectra of brain [1] kidney [2] and liver [3,4] have a large contribution in the phosphodiester (PDE) region from a broad peak which is more prominent at magnetic fields below 2.5 T than above. Spectra of perchloric acid extracts of brain and liver have shown that the signal from low-molecular-weight compounds, such as glycerophosphorylcholine and glycerophosphorylethanolamine contribute less than 25% of the PDE signal observed in vivo at low field-strengths [5].

In a previous study, we showed that this field-dependent PDE signal in vivo, accounting for as much as 45% of the total observed signal, is due primarily to phospholipid bilayers [4]. Observation of hepatic ^{31}P signals in vivo at 1.9 T whilst proton decoupling, thereby removing the effects of strong proton-phosphorus dipolar coupling, changed the shape of the broad PDE peak to that typical of a bilayer spectrum. At higher fields dipolar coupling constants, which are field independent, become insignificant and chemical shift anisotropy (CSA) effects dominate the lineshape. This leads to a very broad signal and the dramatic reduction of the PDE peak in vivo seen at higher magnetic fields [4]. Low-field ^{31}P spectra obtained in vivo with a selective off-resonance saturation pulse revealed that in addition to the signal due to phospholipid bilayer there was also a small contribution to the PDE signal from a saturable, motionally averaged macromolecule. Several possibilities were proposed as the source of this signal [4].

In this study, we provide further evidence that phospholipid bilayers are the primary source of the field-dependent PDE resonance. We present studies of subcellular structures in vitro that suggest that the membranes forming the endoplasmic reticulum (ER) are responsible for the bilayer signal in liver spectra in vivo. In addition, we show that treating rats with phenobarbitone in their drinking water leads to a dramatic increase in the PDE peak compared to control rats. As phenobarbitone is known to cause a large increase in the amount of smooth ER in hepatocytes [6], these results suggest that the membranes in the ER are the cellular structures primarily responsible for the field-dependent PDE peak in rat liver in vivo.

Materials and Methods

Model solutions

All reagents (analytical grade) and enzymes used were supplied by Sigma (St. Louis, MO). Several forms of nucleic acid were studied: RNA (type IV from calf liver) as a 1 mg/ml solution, single-stranded DNA

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(from herring sperm, degraded free acid) in a 1.7 mg/ml solution and double-stranded DNA (type I; sodium salt, highly polymerised from calf thymus) in a 1 mg/ml solution. Three phospholipid systems were investigated. Multilamellar bilayers were prepared from a L-α-phosphatidylcholine (from egg yolk, approx. 60%) dispersion (approx. 40 mg/ml) in a 50 mM Hepes, 15 mM EDTA solution. The solution was mixed by gentle inversion and allowed to settle at room temperature before use. Phospholipid vesicles were prepared by sonicating the above multilamellar bilayers for a minimum of 30 min in a sonicating water bath. Lastly, human low density lipoprotein (LDL) (22% protein and 78% lipid) was investigated in a 0.15 M NaCl, 0.01% EDTA buffer solution.

Homogenate preparation

Livers from male Wistar rats (200–250 g) were homogenised in 5 volumes of a buffer solution of 0.25 M sucrose, 50 mM Tris, 25 mM KCl and 5 mM $MgCl_2 \cdot 6H_2O$. The mixture was dialysed at 4°C in 3×5 volumes of buffer solution to remove small low-molecular-weight phosphorus compounds, such as inorganic phosphate (P_i) that might contribute to the NMR spectrum. The dialysed homogenate was studied before and 1 h after treatment with phospholipase C (type XI from *Bacillus cereus*, suspended in 3.2 M $(NH_4)_2SO_4$ solution at pH 6). Phospholipase C was added to hydrolyse phospholipid into phosphomonoesters (PME) and diacylglycerol.

Cellular fractions

Differential centrifugation, producing enriched rather than pure fractions, was used for the separation of mitochondria, lysosomes and microsomes. Livers from four ether-anaesthetised male Wistar rats (200–275 g) were removed, chopped with scissors and washed in a medium containing 0.25 M sucrose, 0.01 M Tris-HCl, 0.05 mM EDTA and 5 mM $MgCl_2$ (pH 7.2). The liver was resuspended in 9 volumes of medium and homogenised in a Potter-Elvehjem homogeniser. The homogenate was fractionated by differential centrifugation using a modification of the procedures of Williams and Wilson [7] and Chambers and Rickwood [8]. Smooth microsomes, rough microsomes and free ribosomes were further isolated using sucrose gradients. Rough microsomes were stripped of ribosomes with puromycin according to the method of Adelman and colleagues [9]. The purity of the fractions was assessed by determining enzyme activities for the following marker enzymes: lactate dehydrogenase (cytosol), citrate synthase (mitochondria), acid phosphatase (lysosomes) and glucose-6-phosphatase (microsomes). The enzyme assays indicated that enriched fractions were obtained though there was cross contamination between the lysosomal and microsomal fractions. This resulted in a very significant microsomal content in the lysosomal fraction. Microsomal subfractions were examined by electron microscopy (courtesy of Dr. D. Ferguson, John Radcliffe Hospital, Oxford) which showed no visible cross-contamination between the three fractions.

Phenobarbitone treatment

Two groups of male Wistar rats ($n = 5$) weighing 250 g were used for these experiments. The test group were allowed a phenobarbitone/water solution (350 mg/l) as their sole source of drinking water for seven days prior to the NMR experiment. The control group had no phenobarbitone added to their drinking water. After the NMR experiments were completed, liver samples were taken and processed for electron microscopy. The animals on phenobarbitone solution showed evidence of large-scale proliferation of ER, whilst the normal animals exhibited normal hepatocellular architecture.

NMR spectroscopy

In vitro. ^{31}P spectra were obtained at 121.5 MHz in a 7.0 T Oxford Instruments magnet interfaced to a Bruker AM 300 spectrometer using a 60° pulse, 8K data points, an interpulse delay of 2.4 s and a spectral width of 6 kHz. ^{31}P-NMR spectroscopy was also performed at 32.5 MHz in a 1.9 T Oxford Instruments magnet interfaced to a Bruker Biospec I spectrometer using a 50° pulse, 2K data points, an interpulse delay of 2.26 s and a spectral width of 4 kHz. All spectra were obtained at 37° C and a methylenediphosphonic acid (MDP) capillary was used as a chemical shift reference set to correspond to glycerophosphorylcholine at 2.85 ppm. ^1H decoupling was achieved using WALTZ-16 composite pulse broadband decoupling [10] during acquisition. Presaturation, used in experiments at 1.9 T, was achieved using a selective, low-power 2 s ^{31}P pulse at a frequency 900 Hz (28 ppm) downfield from the PDE peak for all samples, including stripped rough ER, with the exception of the microsomal subfractions, which were irradiated at 15.5 ppm. There were no differences in the effects of irradiation at this range of frequency offsets. Spectra of these subfractions were obtained with the presaturation pulse 500 Hz (15.5 ppm) downfield from the PDE resonance. Control irradiation was performed at an offset of 10000 Hz.

In vivo. Animals were anaesthetized with halothane/oxygen and the liver surgically exposed. A three-turn 1.5-cm diameter radiofrequency coil was placed over the exposed liver separated from it by a thin sheet of polyethene. The coil was then tuned to the ^{31}P frequency (40.5 MHz). Tuning to the ^1H frequency (100.1 MHz) was acheived by transmission-line tuning [11]. ECG, respiratory rate and core temperature were monitored throughout the experiments and

the core temperature was kept close to 37°C by warm air. ^{31}P spectra were obtained in a 2.35 T Oxford Instruments magnet interfaced to a Bruker AM 100 spectrometer. The field homogeneity was adjusted using the proton signal from water within the liver. Typical water linewidths of 40–60 Hz were obtained. Pulses of approx. 45° at the centre of the coil were applied every 0.6 s and 200 scan spectra were acquired. The spectra were processed by convolution difference to remove the broad underlying hump using line broadenings of 15 Hz and 500 Hz. Saturation factors were determined by comparing the PDE resonance intensities at 0.6 s and 16.1 s repetition times.

Results

The chemical shifts and linewidths of ^{31}P peaks of model solutions at 1.9 T and 7.0 T are summarised in Table I together with data from our previous study in vivo [4]. Also reported is whether or not a selective off resonance presaturation pulse produces a dramatic reduction in signal intensity as has been shown for the PDE peak in vivo [4]. These data indicate that of the macromolecular systems studied only double-stranded DNA and phospholipid bilayers have NMR characteristics similar to those seen in the PDE peak in vivo. The signals from both are dramatically affected by selective presaturation and both experience extreme broadening and a significant decrease in intensity at higher fields, as can be seen in Fig. 1. RNA, single-stranded DNA, phospholipid vesicles and LDLs all exhibited a slight increase in linewidth at higher field, but none were affected by selective presaturation.

In order to assess the effects of dipolar relaxation on the low-field ^{31}P spectrum, these interactions were removed using proton decoupling (Fig. 1). The effect on the spectrum of DNA is a narrowing of the signal. The effect on the bilayer is much more dramatic and involves a reduction in signal in the PDE region and a

TABLE I

Characterestics of the PDE peak in model compounds and in vivo

ds, double-stranded; ss, single-stranded; linewidth, full width at half maximum height. σ values and presaturation effects were determined at 1.9 T.

	σ [a] (ppm)	Linewidth (Hz)		Presaturation effects
		7.0 T	1.9 T	
RNA	2.0	110	30	no
DNA (ss) [b]	1.8	45	19	no
DNA (ds)	1.1–1.9	500	80	yes
LDL [b]	2.0–2.7	63	32	no
Bilayer	2.3	7000 [a]	210	yes
Vesicles	2.2	60	40	no
In vivo [c]	2.3		320	yes

[a] Because of the asymetrical lineshape of the bilayer spectrum at 7.0 T, this value represents the CSA chemical shift difference. $\Delta\sigma$, rather than the actual linewidth.

[b] The chemical shift ranges reported are due to separate resonances visible with resolution enhancement.

[c] Data from Murphy et al. [4].

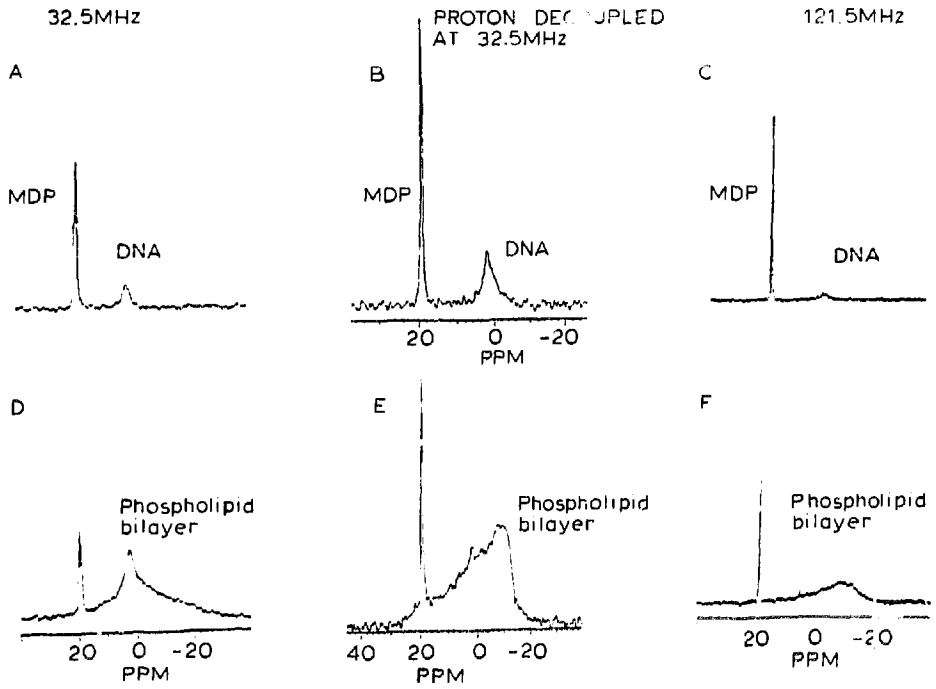
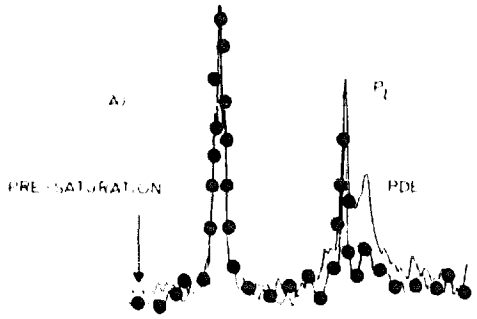


Fig. 1. ^{31}P-NMR spectra of double-stranded DNA at different field strengths: (A) at 1.9 T (32.5 MHz; 8192 scans processed with 15 Hz exponential line broadening); proton decoupled at 1.9 T (32.5 MHz) (B) at 7.0 T (121.5 MHz): (C) ^{31}P-NMR spectra of phospholipid bilayes at different field strengths: (D) spectrum of phospholipid bilayer at 1.9 T (32.5 MHz; 2560 scans processed with 15 Hz exponential line broadening); proton decoupled at at 1.9 T (32.5 MHz) (E), at 7.0 T (121.5 MHz) (F).

complete rearrangement of the broad component to a characteristic bilayer lineshape with a sharp upfield peak and a sloping downfield shoulder extending through 40 ppm, as is seen for a significant portion of the PDE peak in vivo. It should also be noted that this spectrum is similar to that obtained at 7.0 T, where dipolar interactions have an insignificant contribution to the lineshape.

Dialyzed liver homogenate produced ^{31}P spectra with a very pronounced PDE peak that was affected by selective presaturation (Fig. 2A), Addition of phospholipase C resulted in a virtual disappearance of the PDE peak; a corresponding peak appeared in the phosphomonoester region of the spectrum consistent with the enzymatic hydrolysis of phospholipid to phosphomonoester and diacylglycerol (Fig. 2B). There was also an increase in the P_i peak probably derived from nucleotide phosphates and hydrolysis of compounds released by phospholipase C from PDE. As with the homogenate, all subcellular fractions examined produced spectra with a PDE peak that was present at lower magnetic fields and disappeared or broadened at higher magnetic fields. However, the PDE peak from these subcellular fractions was more pronounced and more significantly affected by selective presaturation in the lysosomal and microsomal fractions. The spectrum from free ribosomes (Fig. 3A), consisting of signal principally from nucleic acid, showed no effect with presaturation. Spectra from microsomes further fractionated into smooth and rough microsomes showed an approx. 35 and 50% reduction in signal in the two subfractions, respectively (Fig. 3B and 3C).



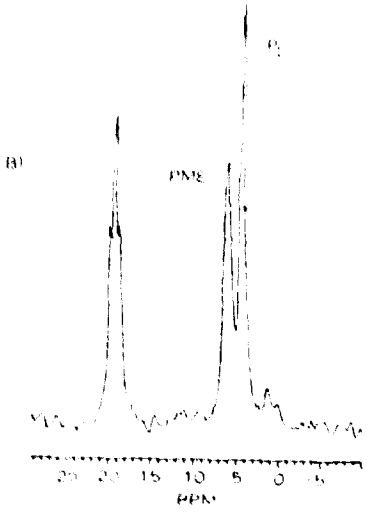


Fig. 2. ^{31}P-NMR spectra at 1.9 T (32.5 MHz) of dialyzed rat liver homogenate. Spectra represent 1024 scans processed with 15 Hz exponential linebroadening. (A) Control spectrum (solid), and control spectrum with presaturation at arrow (presaturated spectrum is shown as a continous line with superimposed dots). (B) Spectrum 1 h after addition of phospholipase C. Note the disappearance of the PDE peak. The increase in P_i is due to general breakdown of phosphates as the spectrum was obtained 1 h after phospholipase treatment.

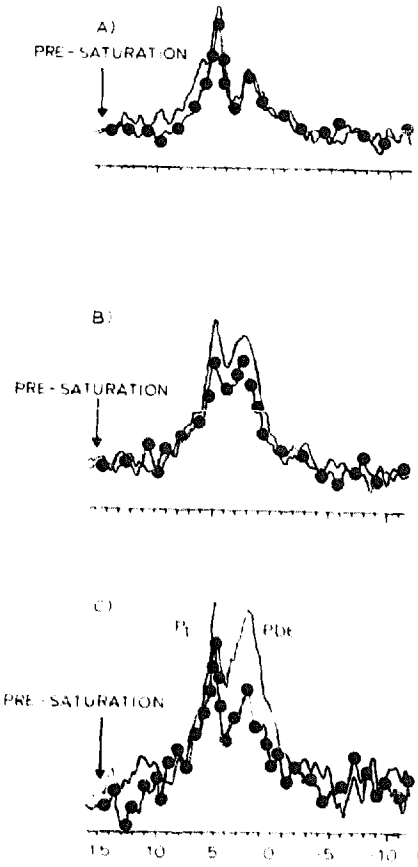


Fig. 3. ^{31}P-NMR spectra at 1.9 T (32.5 MHz) of rat liver microsomes with (continuous line with superimposed dots) or without (continuous line) selective presaturation at 15.5 ppm. Spectra represent 512 scans processed with 15 Hz exponential linebroadening. (A) Free ribosomes, (B) smooth microsomes and (C) rough microsomes.

In order to assess the contribution of ribososmes to the spectra more accurately, spectra of rough ER were obtained before and after being stripped of ribososmes. Spectra of rough ER showed reduction in signal after presaturation both before and after removal of ribosomes (Fig. 4). Ribososmes and smooth ER obtained from the same livers were also studied and the effects of presaturation on the spectra were similar to those shown in Fig. 3.

Fig. 5 shows summed liver spectra obtained in vivo from control rats and from phenobarbitone-treated

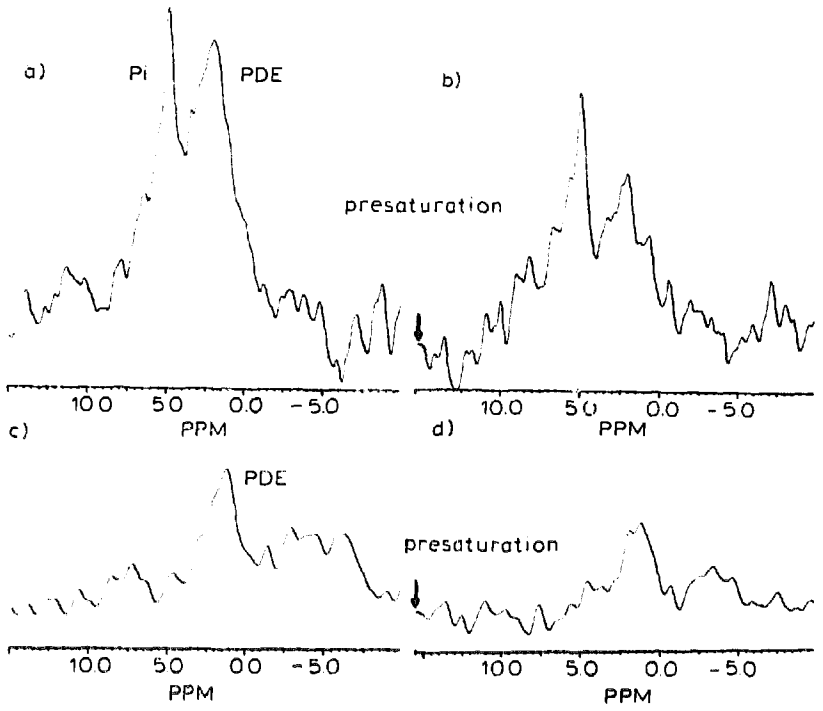


Fig. 4. ^{31}P-NMR spectra of rough ER before and after removal of ribosomes at 1.9 T. Line broadening of 25 Hz. (A) spectrum from rough ER before stripping of ribososmes without presaturation, (B) spectrum of same sample with presturation, (C) spectrum of rough ER after removal of ribosomes, without presaturation, (D) spectrum of rough ER after removal of ribosomes with presaturation.

animals. All the spectra were normalised to the same intensity of the β-ATP peak before summation. Most apparent is the large increase in the intensity of the resonance in the PDE region in the phenobarbitone-treated animals. Electron micrographs of the livers of control and phenobarbitone-treated animals are shown in Fig. 6. These demonstrate increased amounts of bilayers from ER in the livers of phenobarbitone

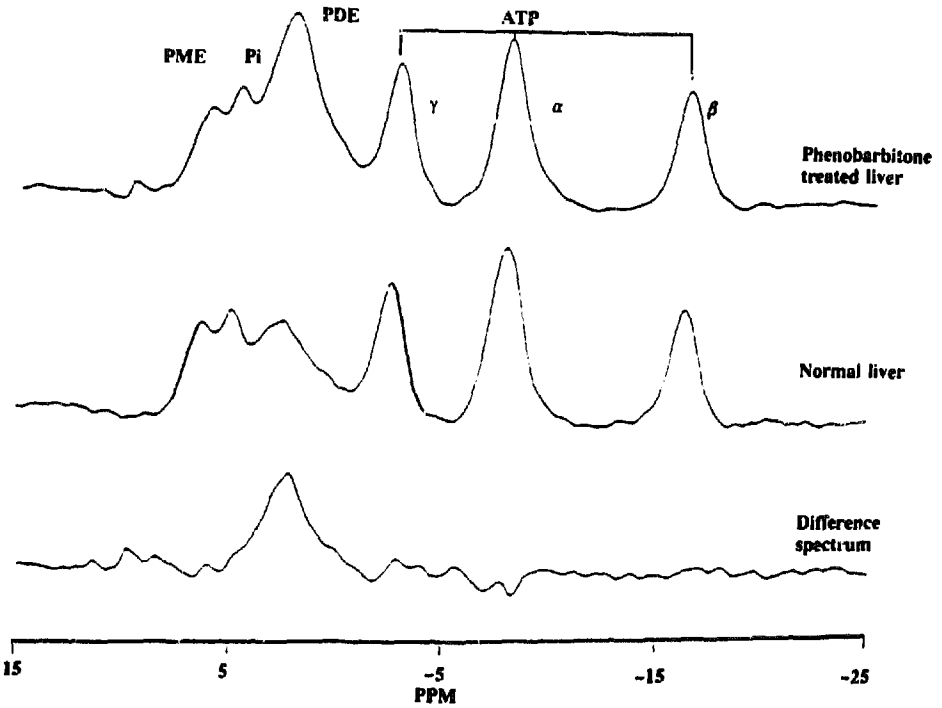


Fig. 5. In vivo liver spectra of normal and phenobarbitone-treated rats: summed spectra from five normal and five phenobarbitone-treated animals and difference spectrum to show the change in the diester peak. The spectra have been normalised to the height of the β-ATP resonance

treated animals. The intracellular pH and the ATP/P_i ratios were not significantly different. Previous data from different sets of animals indicated that the ATP concentration determined from freeze clamped perchloric acid extracts was not significantly different between control (1.92 ± 0.17 mmol/kg wet wt, $n = 3$, Williams, S.R., unpublished results) and phenobarbitone-treated animals (2.09 ± 0.33 mmol/kg wet wt, $n = 5$ [12]).

At the repetition rate used, the PDE peak in the control animals was saturated to about $64\% \pm 6\%$ of its fully relaxed intensity. The PDE peak in the phenobarbitone-treated animals was saturated to $61\% \pm 4\%$ of its fully relaxed intensity.

Discussion

These studies indicate that a major portion of the PDE peak in ^{31}P liver spectra at low magnetic fields is derived from the endoplasmic reticulum. This has contributions from the lipid bilayer (which is saturable with off-resonance irradiation) and probably from ribosomal RNA (which is non-saturable). The PDE peak increases under conditions in which the ER content of hepatocytes is increased.

These results extend the observations reported in an earlier paper [4] that the PDE peak at 1.9 T contained significant contributions from phospholipid bilayers. In that paper [4], we showed that selective off-resonance presaturation almost completely eliminates the broad component and most of the PDE signal in the ^{31}P spectra leaving P_i and a small residual PDE peak. Computer integration showed that this broad saturable PDE signal constituted 45% of the control spectrum. The shape of this signal was also modified by proton decoupling which caused an upfield shift of the signal. Thus, the PDE peak is clearly shown to have a large component of phospholipid bilayer. Other components that could produce broad peaks in this region include nucleic acid, small vesicles, or phospholipids in the hexagonal type II phase [4]. As shown here, none of these components would produce the spectral changes observed in vivo with proton decoupling. Further, spectra of nucleic acids in vitro show no changes with presaturation.

Results from the fractionation experiments indicate that the majority of the broad PDE signal arises from the microsomal fraction. The lysosomal fraction also produced spectra with a pronounced PDE peak but, recalling the microsomal contamination of this frac-

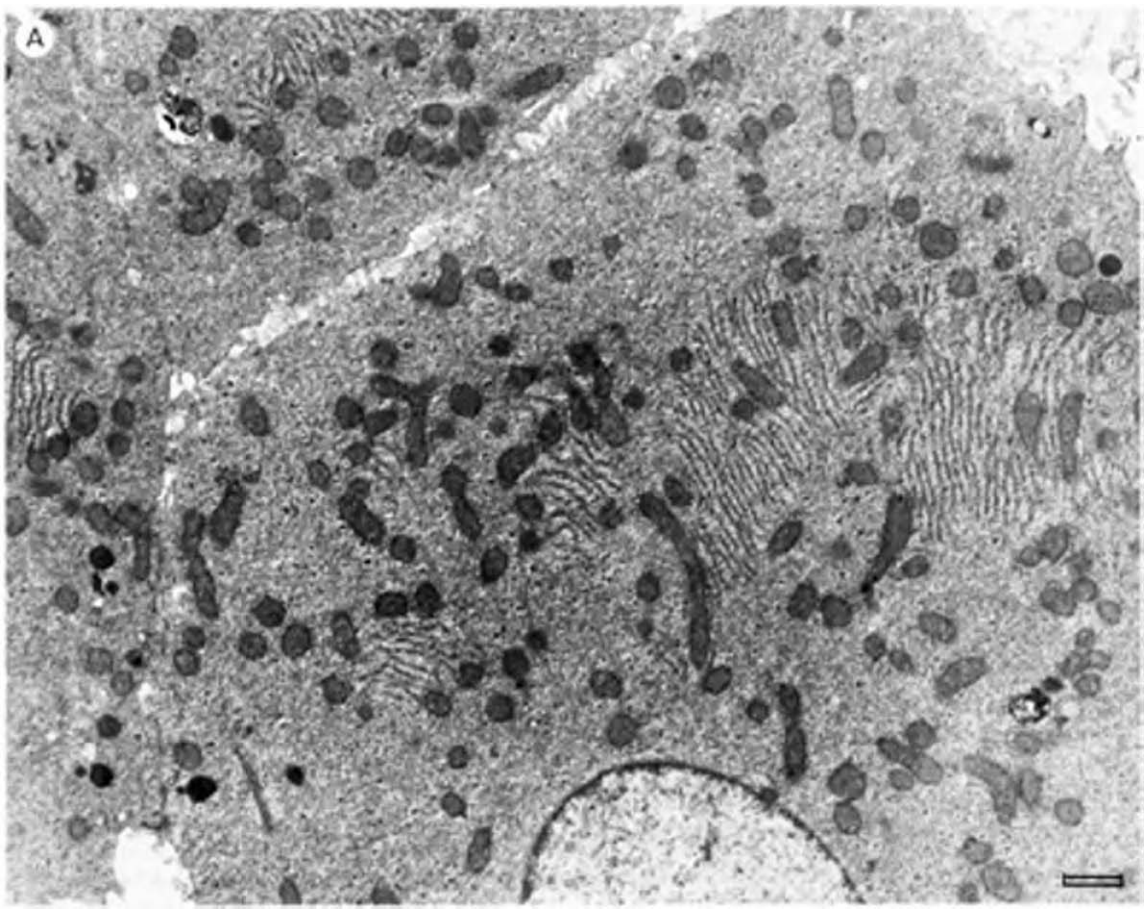


Fig. 6. Electron micrographs of rat livers: (A) control, (B) phenobarbitone-treated. Bar represents 0.1 mm. The original magnification was 12000×. Note the increase in membranes in the micrograph from the phenobarbitone-treated rat.

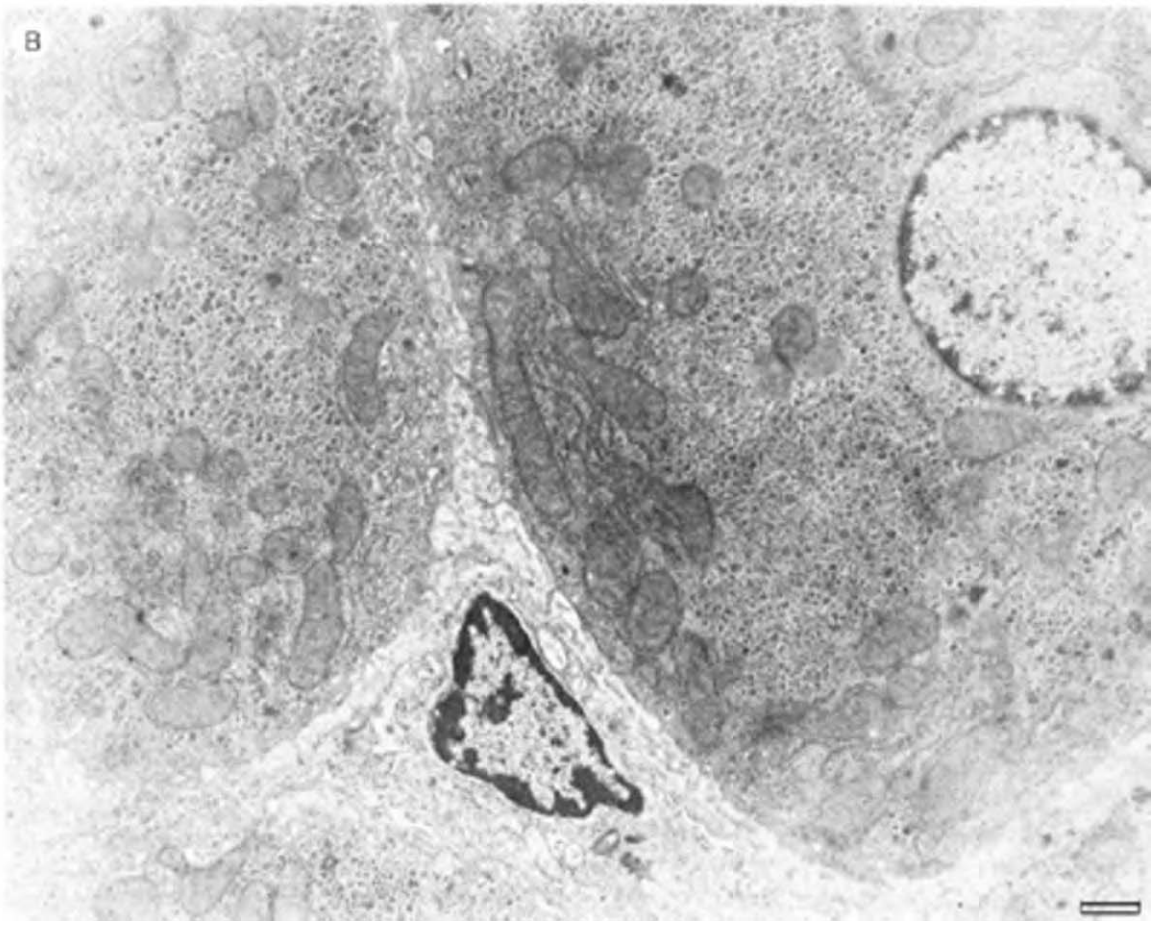


Fig. 6 (continued).

tion, that peak also can be attributed to microsomes. Since ER constitutes over 50% of the membrane in the cell [13], it is not surprising that the majority of the saturable-field-dependent PDE signal arises from the microsomal fraction. Nevertheless, limitations to these results should be kept in mind. The lamellar and cisternal structures of the smooth and rough ER are damaged in the homogenisation process. Therefore, the microsomes which are formed after homogenisation only bear limited resemblance to the structure of the intact ER. Thus, in vivo, the larger membranes present might produce extremely broad asymmetric lineshapes, while after homogenisation, the size of some of the microsomal vesicles formed may well be small enough for their NMR signal to be narrow.

Though the prestripped rough ER, stripped rough ER and smooth ER showed reduction in signal intensity with presaturation, free ribosomes did not. This supports our hypothesis that though both bilayers and nucleic acids can produce a broad component in the PDE region only the signal from bilayers is affected by presaturation. Since, in our previous paper [4], we showed that the majority of the PDE peak is saturable, the contribution of nucleic acid in quantitative terms is likely to be small. It is also important to note that the signal from ER in vitro does not disappear entirely with presaturation. Thus, the minimum contribution of ER to the spectra is at least 45% of the total signal in vivo.

A relationship between the microsomal fraction and the ER in vivo was established by the experiments in phenobarbitone treated rats. A wide range of drugs are known to induce enzymes in the liver and EM studies have shown an increase in cellular content of ER. Barbiturates in particular have been shown to induce a proliferation of smooth ER and a shortening and vesiculation of rough ER cisternae in hepatocytes [6,14]. The large increase in the PDE peak seen in the spectra from phenobarbitone-treated rats can be attributed to a large increase in the phospholipid bilayers of the smooth ER. Phenobarbitone may have many effects on cellular metabolism and is known to induce liver enzymes. However, of macromolecular components that could contribute to the PDE region, only the ER has been shown to increase significantly in the rat after phenobarbitone treatment. Obstructive jaundice is also known to increase the ER content of hepatocytes and liver spectra of patients with cholestatic jaundice due to primary biliary cirrhosis show an increase in the intensity of the PDE peak [15].

In conclusion, the saturable-field-sensitive PDE peak present in the ^{31}P spectrum obtained from liver in vivo at low field strengths has been demonstrated to contain substantial contributions from phospholipid bilayers of the endoplasmic reticulum. In this study, we have shown that it is possible to alter the PDE peak by altering the ER content of liver by drug administration. Previous studies of liver metabolism in patients with established disease states have used the PDE peak as an index of membrane breakdown, as intracellular phosphodiesters, such as glycerophosphorylcholine and glycerophophorylethanolamine are on the pathway of membrane breakdown. Clearly, the finding that membrane synthesis can also change the PDE peak in the liver spectrum obtained at low field strengths has major implications for clinical liver spectrocopy. ^{31}P-NMR will offer the possibility to study directly the effects of drugs which are known to influence ER formation, while at the same time effects on ER must be borne in mind in the interpretation of liver spectra obtained from patients undergoing drug therapy.

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