Mapping Organic Molecules in Biological Space by High Resolution N.M.R. Spectroscopy and Electron Microscopy

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Summary Studies using high resolution n.m.r. spectroscopy and electron microscopy methods are described which permit the mapping of organic molecules in biological space; inorganic probes are used to correlate observations by the two methods.

Two chemical techniques are available at present for mapping biological systems. The electron microscope (E.M.) can be used in the analytical mode to provide the distribution in space of elements heavier than sodium. It is a destructive technique; it requires dissected and prepared samples and it cannot be used to discover the location

of organic molecules made from C, H, N, and O. Zeugmatography, an n.m.r. method, maps biological space by utilising an externally imposed magnetic field gradient to place in space zones of water of different relaxation times.[1] It cannot identify organic molecules or many inorganic materials as it is a low field, low sensitivity method, but it is non-destructive and can be used to follow changes in living systems. We describe here an approach which relates E.M. studies to high resolution (high field) n.m.r. studies and is an illustration of the way in which it may well be possible to map organic molecules within living systems. The method depends upon the introduction locally of susceptibility probes which show, through the perturbation of the n.m.r. spectrum, those organic molecules which lie in the vicinity of the probes. The probes can be located in space in the electron microscope image using *X*-ray emission since they are chosen to contain atoms heavier than sodium. The probes must be shift or broadening n.m.r. reagents based on molecular or macroscopic inserts which are restricted to particular parts of space.

As a trial 'biological' object we have chosen to examine vesicles made by sonicating mixtures of lecithin (phosphatidylcholine) and water.[2] The vesicles so formed are known to have an inner aqueous space of some 200 Å diameter. The vesicle membrane is a bilayer and roughly one third of the lecithin choline headgroups point into the inner space and two thirds point outwards. The system simulates a biological specimen with two compartments and we must arrange to put probes into one of the compartments. Through selective n.m.r. perturbation of one region, and knowledge of the location of the probe, we can then map amounts of choline (headgroup) in one part of space relative to another. The trial experiments proceeded as follows.

Phosphatidylcholine vesicles of some 250 Å diameter were prepared in a conventional manner by the sonication of egg yolk lecithin.[2] The vesicles are difficult to see by electron microscopy but have been imaged when stained externally by molybdate stains. However such staining may introduce artefacts. In order to introduce new probes the sonication of the phosphatidylcholine (17 mM) was carried out in the presence of a paramagnetic reagent, *e.g.* $CoCl_2$ (210 mM). Subsequent chromatographic fractionation on Sephadex removed the metal cations from the external aqueous phase of the vesicles. Analysis for phospholipid and the trapped reagent gave the elemental content of the internal solution of the vesicles. Additional reagents were added externally to vesicles with or without $CoCl_2$. Two types of external addition were studied: (i) a reagent, *e.g.* ammonium molybdate, which cannot permeate the vesicle membrane but binds to it and which therefore acts as an external phase marker, and (ii) a reagent, *e.g.* ammonium sulphide, which can permeate the vesicle membrane and react with the $CoCl_2$ inside the vesicle.[3] In the first case there is an obvious external marker, molybdenum. In the second case a new marker for the internal space of the vesicle, sulphide, has been introduced, while the paramagnetic marker, free Co^{2+} ions, is removed. The vesicles containing the precipitate were separated from excess of $(NH_4)_2S$ in the external phase by dialysis. In this way a series of vesicles have been prepared as shown in Table 1. These vesicles are characterised by the presence or absence of internal n.m.r. (paramagnetic) markers, and by marker elements, internally or occasionally bound externally, which are readily studied analytically

TABLE 1. N.m.r. and E.M. markers for vesicular phases.[a]

N.m.r. reagent Phases		E.M. analysis
External	Internal	
None	$CoCl_2$	Co, Cl, P
None	$CoCl_2 + (NH_4)_2S \rightarrow CoS$	Co, S, P
Ammonium molybdate	$CoCl_2 + (NH_4)_2S \rightarrow CoS$	Co, S, P, Mo
Ln_3^+	$K_3Fe(CN)_6$	K, Fe, P, Ln

[a] The concentrations of the chemicals are not stated since different values were used in different experiments.

in the electron microscope and provide sufficient contrast for the vesicles to be imaged[3] (Figure). For n.m.r. purposes the vesicles were examined using the Bruker 90 n.m.r. spectrometers as described elsewhere.[2] The electron microscope used is a JEOL JEM 100CX with an ASID attachment and a Link energy dispersive *X*-ray analysis system.

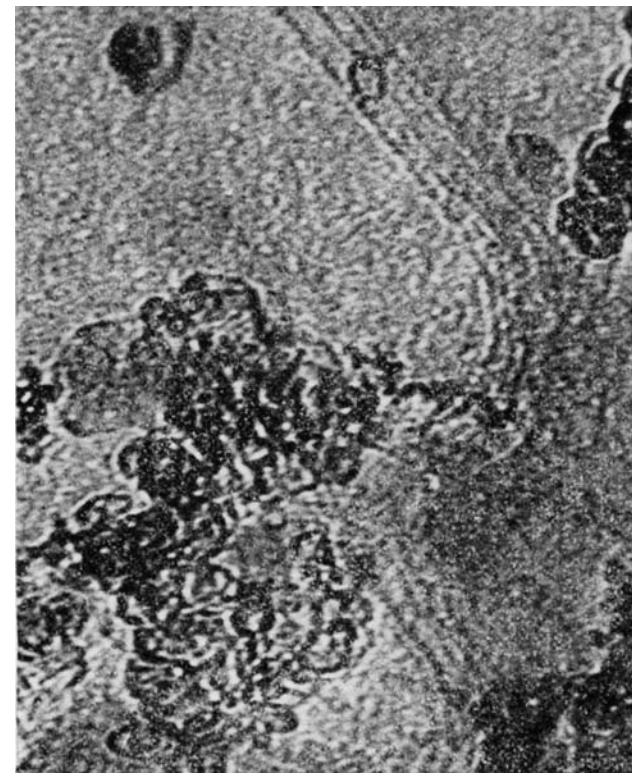

FIGURE. This plate shows a T.E.M. image of phosphatidylcholine vesicles. Clusters of vesicles as well as a single vesicle can be seen. The vesicles are *ca.* 25 nm (= 7 mm in the plate) in diameter as expected. The vesicles were analysed in thin (single layers of vesicles) and dense regions (Table 2).

The n.m.r. spectra in the absence of probe and in the presence of internal cobalt sulphide showed one set of choline resonances but in the presence of Co^{2+} ions two sets of resonances were observed. Two parts of space are clearly distinguished by the effect of Co^{2+} on the resonances and are represented by the headgroup choline both inside and outside the vesicle which differ quantitatively in the ratio 1 : 2. The definition of the phases is possible because

the location of the probe follows directly from the chromatographic separation procedures. In a real biological experiment this would be impossible and we would only know that they were two compartments containing choline.

TABLE 2. E.M. analysis of vesicles

Area Analysed/nm²	Counts in 100 s per unit area			
	P	S	Co	
10,000	3410	905	602	Sample
Dense[a]	157	91	86	Background
	3253	814	516	Counts above background
400	387	197	103	Sample
Thin[a]	280	175	65	Background
	107	22	38	Counts above background

[a] 'Dense' and 'thin' refer to the regions of high and low vesicle content. The ratio of P : S : Co by chemical analysis and known composition is 5 : 1 : 1. The imaging and analyses of vesicles were carried out as follows. A carbon-coated copper grid was prepared by depositing carbon on a copper grid previously treated with Formvar. (We are grateful to Mr. Wakely, Botany Department, University of Oxford, for his directions.) The grid was dipped into a solution containing vesicles and excessive liquid was removed by placing the grid on a filter paper. After 30 s drying in air the grid was introduced into the microscope. Analyses by *X*-ray emission took 100 s.

We then turn to the electron microscope to find the location of the probe chemicals. Firstly the internal and external probe atoms, Co and Mo, are sufficiently electron dense for direct imaging of the vesicles (Figure). In all experiments the phase boundary could be located and vesicles of the correct size 250 Å diameter were seen. (It is desirable to precipitate the probe *in situ*, here as CoS, to prevent diffusion during sample preparation.) The precipitation reaction was followed by the n.m.r. signals (Table 1). The concentration of cobalt and sulphur in the vesicles was now determined by detection of the characteristic *X*-rays and checked by reference to a chemical component of the phase system, here the phosphorus of the phosphatidylcholine. Typical analyses are given in Table 2.

By perturbation of the internal and external choline n.m.r. signals by a probe, and then location of the probe in space by precipitation followed by E.M. imaging and *X*-ray analysis, we were able to locate this organic chemical. This procedure could be extended to determine the whereabouts of any organic chemical in a real biological material. Alternative stains can be used (see Table 1).

The above precipitation procedure can also be used for the study of growth of microscopic solids within vesicles, *e.g.* the formation of calcite or apatite, or to make catalysts. It is believed at present that shell and bone are made from vesicular deposits transported to particular sites.

Here we have used chemical probes but elsewhere we have discussed the use of physical methods of changing susceptibility in order to uncover the distribution of chemicals in biological compartments.[4]

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