Table 1. RELAXATION TIMES DETERMINED BY THE FREE INDUCTION DECAY (FID) AND CARR–PURCELL (CP) METHODS

	T_2 (ms)	$\Delta\nu$ (Hz)
Ghost lipids	0·56 ± 0·3 (FID)	1,100
	110 ± 10 (CP)	5·8
Ghosts	1·4 ± 0·2 (FID)	460
	75 ± 1 (CP)	8·4

inhomogeneities occurs with both membranes and extracted lipids. The transverse relaxation times obtained with the spin–echo technique are long, suggesting a high degree of molecular mobility.

Erythrocyte ghosts were prepared from fresh citrated beef blood by lysis in phosphate buffer[17], and were then lyophilized from distilled water and resuspended in deuterium oxide containing 0·1 M NaCl. Lipids were extracted from lyophilized ghosts with chloroform–methanol (2 : 1), lyophilized from benzene, and also suspended in 0·1 M NaCl-^2H_2O. For both systems the concentrations were made approximately 25 per cent w/v. Measurements were made at 60 MHz and room temperature with a 'Magneon SE-30' high-power spin–echo spectrometer; 5 mm tubes were used for free induction decay, and 12 mm tubes for Carr–Purcell runs. Oscilloscope traces were photographed on 'Polaroid' film, digitized with an Autotrol Corp. Model 3400 digitizer, recorded on paper tape, and fitted by a computerized least-squares routine. One dominant relaxation time was present in each case. Relaxation times determined by the two methods are listed in Table 1, together with equivalent full linewidths at half-height calculated from the relation $\Delta\nu = 1/\pi T_2$.

As expected, linewidths calculated from free induction decay experiments are several hundred Hz, in agreement with published steady-state absorption spectra. Those obtained from the Carr–Purcell programme, however, are only a few cycles and are typical of liquid-like systems. These narrow lines are similar to those obtained by extrapolation of phospholipid absorption spectra to zero field. The true correlation times appear to be short and are approximately the same in both lipid dispersions and membranes. We interpret these results to mean that in both systems absorption lines are inhomogeneously broadened by magnetic field gradients. Interpreted in terms of molecular motion, broad lines obtained by absorption NMR are misleading.

Preliminary experiments with intact ghosts which were not lyophilized and with sonicated ghosts also show disparities between free induction decay and Carr–Purcell results. Minor changes are caused by freeze-drying; and these are under investigation. The phenomena described are not confined to erythrocyte ghosts; membranes of *Mycoplasma laidlawii* and *Escherichia coli* also appear to exhibit broadening due to internal magnetic field inhomogeneity.

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Formation of Epithelial Basement Membrane is restricted by Scurvy *in vitro* and is stimulated by Vitamin C

ALTHOUGH scurvy was probably the first clearly defined nutritional disease and active antiscorbutic material was the first of all the vitamins to be crystallized and synthesized, the biochemical function of ascorbic acid in synthesis of collagen has only recently been identified. Some of the proline residues of the collagenous polypeptide are hydroxylated to form hydroxyproline, and ascorbic acid is a cofactor for the hydroxylating enzyme[1]. Most pathological findings in scurvy can be attributed to a restriction in ability to synthesize collagen of bone, teeth and other connective tissues. Some manifestations occasionally found in infantile scurvy are more prominent in adult scurvy and are difficult to explain by a restriction in ability to synthesize collagen of connective tissues. For example, the cutaneous haemorrhages or "scorbutic spots" appearing spontaneously about hair follicles are one of the cardinal signs of adult scurvy[2].

Epithelial and vascular basement membrane contains a protein similar or identical in composition to collagen of bone and connective tissue[3]. The purpose of this report is to present evidence that the synthesis of epithelial basement membrane is dependent on ascorbic acid and to suggest that some of the manifestations of scurvy may be attributed to a restriction in synthesis of basement membranes.

Cell cultures were initiated from explants of a transplantable parietal yolk sac carcinoma of the mouse, LS402A VI, shown to synthesize epithelial basement membrane both *in vivo* and in cell culture[4,5]. The cultures were grown at 37° C in Dulbecco and Vogt's modification of Eagle's medium supplemented with 10 per cent foetal bovine serum and equilibrated with 10 per cent CO_2 in air. In these conditions the cells do not form a confluent monolayer in the culture vessel, but grow as separated colonies on the glass surface. After the cells within a colony proliferate and become densely packed, spherical masses of cells approaching a millimetre in diameter detach and float in the nutrient medium. These cellular masses enlarge further while floating. A "stationary phase" of growth is difficult to identify.

From histological evidence, epithelial basement membrane is more abundant in the cellular masses that are detached or nearly detached than in the cells attached to the glass substrate. It was therefore decided to examine the influence of ascorbic acid on the ability of the detached cellular masses to synthesize hydroxyproline of protein.

The cell masses were removed from the nutrient medium by centrifugation, were resuspended in fresh medium and two aliquots were reincubated at 37° C in the presence of 5 μCi/ml. of ^{3}H-L-proline (304 mCi/mmole, obtained from Nuclear Chicago, Chicago, Illinois). One aliquot was incubated with 125 μM sodium ascorbate and the other aliquot without. After incubation for 3 h the cells were again separated by centrifugation, washed with 0·01 M L-proline in ethanol, and an aliquot taken for analysis of cellular protein[6]. The remainder was hydrolysed for determination of radioactivity in proline and hydroxyproline in the cells[7]. In order to estimate the amount of radioactively labelled proline and hydroxyproline secreted

Table 1. EFFECT OF ASCORBIC ACID ON APPEARANCE OF RADIOACTIVITY IN PROLINE AND HYDROXYPROLINE OF PROTEIN IN CELLS AND MEDIUM OF CULTURED PARIETAL YOLK SAC CARCINOMA

		Without ascorbate	With ascorbate
Cells	mg protein	32·1	31·5
	DPM in proline	$8{\cdot}6\times10^6$	$7{\cdot}9\times10^6$
	DPM in hydroxyproline	$1{\cdot}2\times10^5$	$1{\cdot}8\times10^5$
Medium	DPM in proline	$2{\cdot}7\times10^4$	$2{\cdot}8\times10^4$
		$3{\cdot}3\times10^4$	$2{\cdot}8\times10^4$
	DPM in hydroxyproline	$1{\cdot}5\times10^3$	$2{\cdot}6\times10^3$
		$1{\cdot}5\times10^3$	$3{\cdot}0\times10^3$

by the cells into the nutrient medium, protein was precipitated from aliquots of nutrient medium by adding 4 volumes of absolute ethanol. The precipitate was washed with 0·01 M L-proline in ethanol and was hydrolysed for determination of radioactivity in proline and hydroxyproline[7].

The results of this experiment (see Table 1) indicate that ascorbic acid stimulates the appearance of hydroxyproline in cellular protein by about 50 per cent. Hydroxyproline secreted by cells into the nutrient medium was stimulated nearly two-fold by the vitamin. Ascorbic acid did not, however, affect the amount of radioactive proline in either cells or medium. Additional experiments indicated that the effects of the vitamin were reproducible. Thus ascorbic acid enhances the synthesis of hydroxyproline by cultured parietal yolk sac carcinoma cells making basement membrane. The enhancement by ascorbic acid is quantitatively similar to that reported for cultured fibroblastlike cells during collagen production[8]. Only about 1·5 per cent of the radioactive proline incorporated into protein of parietal yolk sac carcinoma is, however, hydroxylated to form hydroxyproline, whereas a much larger fraction is reported to be hydroxylated by fibroblast-like cells.

In order to determine whether the antigenic components of basement membrane are increased by ascorbic acid, the tumour cells were grown on 22 × 40 mm coverslips in medium devoid of ascorbic acid or supplemented with 125 μM sodium ascorbate. The medium was changed on alternate days. One week later the coverslips were removed and rinsed in phosphate-buffered saline. They were then exposed for 30 min at room temperature to antibasement membrane rabbit globulin conjugated with fluorescein isothiocyanate, were again rinsed with saline and were then mounted. These conjugated antibodies were prepared to basement membrane of the same parietal yolk sac carcinoma grown in ascitic form[4]. The antibodies thus prepared react with epithelial basement membrane, vascular basement membrane and reticulin. These preparations have been previously used in the study of epithelial basement membrane[5]. The mounted coverslips in my experiments were randomized and coded, then examined independently by two observers who did not know the coding. The amount of fluorescence over cell masses producing epithelial basement membrane was scored on a scale of 0 to 4+ and the results are recorded in Table 2. A substantially greater fluorescence was recorded by both observers for the cultures grown in the presence of ascorbic acid.

Table 2. EFFECT OF ASCORBIC ACID ON FLUORESCENCE IN CULTURES OF PARIETAL YOLK SAC CARCINOMA EXPOSED TO ANTIBASEMENT MEMBRANE RABBIT GLOBULIN CONJUGATED WITH FLUORESCEIN ISOTHIOCYANATE

Without ascorbate		With ascorbate	
Observer No. 1	Observer No. 2	Observer No. 1	Observer No. 2
0	0	++	++
+	±	+	+
++	+	+++	++
+	±	++++	+++
++	+	+++	++
±	±	++++	+++

These observations suggest that the ability of cells to form a basement membrane is restricted when they are grown in medium devoid of vitamin C. Addition of the vitamin stimulates synthesis of hydroxyproline, a component of basement membrane protein, to an extent quantitatively similar to the stimulation of hydroxyproline synthesis by fibroblasts that form collagen. Antigenic components of epithelial basement membrane (presumably including basement membrane protein) produced in cell culture are also stimulated by the vitamin. Some of the pathological manifestations of vitamin C deficiency may well be explained by inability to synthesize epithelial and vascular basement membranes.

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Allosteric Interactions in $(Na^+ + K^+)$-ATPase

SODIUM and potassium ions are both required to activate the $(Na^+ + K^+)$-dependent-ATPase and each inhibits activation by the other[1]. The K^+ activates the turnover of enzyme hyperbolically at low Na^+ concentrations, but with increasing concentrations of Na^+ the activation curve becomes sigmoid[2]. Similarly at low concentrations of K^+, the Na^+ activation curve is hyperbolic but at high concentrations of K^+ it becomes sigmoid[1]. These data indicate the presence of at least two Na^+ and/or K^+ sites, respectively, but do not indicate the mechanism of interaction between these ions or sites[3]. Priestland and Whittam[3], and others, have suggested that direct competition between the cations for the activating sites can account for the sigmoidal activation curves in the presence of high concentrations of the other cation. Alternatively, Robinson[4] has argued that the sigmoidal activation curves, which are consistent with the heterotropic effects of an allosteric activator[5], are evidence for allosteric interactions in the $(Na^+ + K^+)$-ATPase. In support of this hypothesis he has presented evidence for a cooperativity in the K^+ activation of the *p*-nitrophenylphosphatase activity of the $(Na^+ + K^+)$-ATPase[4]. As Garrahan[6] has pointed out, however, these data are not conclusive.

In support of Robinson's interpretation we present more evidence for cooperative interactions between the Na^+ binding sites of this enzyme. In the presence of Mg^{2+} and P_i a readily reversible binding of ^{3}H-ouabain to this enzyme was observed[7,8]. The equilibrium level of this binding was lowered by the addition of Na^+, and zero in the presence of high Na^+. At low concentrations of ouabain ($2{\cdot}5\times10^{-7}$ M) the Na^+ inhibition curve was almost hyperbolic and the apparent K_m for Na^+ was 2·5 mM. A Hill plot of these data gave a slope of $n = 1{\cdot}11$. When the concentration of ouabain in the medium was increased, the apparent K_m for Na^+ increased and the inhibition curve became markedly sigmoid. At $2{\cdot}5\times10^{-5}$ M ouabain the apparent K_m for Na^+ was about 70 mM and n increased to 2·5. These observations suggest a cooperative interaction between the Na^+ sites and seem to represent a "standard example of a heterotropic allosteric interaction"[4,8].