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LIPID METABOLISM OF MAMMALIAN ERYTHROCYTES WITH SPECIAL REFERENCE TO CELLULAR AGING

A thesis presented in partial fulfillment
of the requirements for the degree
of PhD in Biochemistry
at Massey University

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ABSTRACT

Aspects of the lipid metabolism of mammalian erythrocytes, with special reference to changes in aging cells, have been investigated.

Bovine erythrocytes and leukocytes have been incubated with labelled palmitic acid, and the incorporation of the fatty acid into each cell type has been followed. A high level of incorporation was observed with leukocytes, mainly into the phospholipids and triglycerides. Incorporated palmitate took part in chain-lengthening processes and some $^{14}CO_2$ was produced during the incubations. Incorporation into the lipids of erythrocytes was very much lower than that observed for leukocytes and low leukocyte concentrations in red cell preparations accounted for a significant proportion of [1-^{14}C]palmitate uptake into the cell lipid. The importance of accounting for the metabolic activities of residual leukocytes has been stressed. After allowing for leukocyte contributions, a significant incorporation of palmitate into erythrocyte phospholipids, in particular phosphatidyl choline and phosphatidyl ethanolamine, was demonstrated. However, no significant uptake into the small quantities of triglyceride or cholesterol esters present in the erythrocytes could be detected.

Experiments have been carried out to examine variations in lipid content with cell age, in bovine erythrocytes fractionated by serial osmotic hemolysis. Only slight differences in cellular phospholipid or cholesterol content were found, and cholesterol:phospholipid ratios were constant in all fractions. No marked variation in cholesterol ester, triglyceride, or free fatty acid concentration with cell age could be detected.

Human red cells have been fractionated according to age by ultracentrifugation over discontinuous albumin density gradients. The efficiency of such age separation was examined by following the radioactivity distribution in the gradient when rat cells were fractionated at intervals after administration of reticulocytes labelled with [^{14}C]glycine. Considerable localisation of cells of particular ages in specific density bands was observed. Variations in lipid composition and in fatty

acid uptake into the cells have been investigated. A small decrease in lipid content with cell age was detected, the decrease being most marked between the youngest and all the other fractions. It is suggested that all the changes in lipid content which occur in aging red cells could take place during the transition from reticulocyte to erythrocyte.

Incorporations of labelled linoleate into intact red cells, and linoleate and palmitate into ghosts enriched with ATP and CoA have been examined. The major cell lipids which took up the acids were phosphatidyl choline, phosphatidyl ethanolamine, components tentatively identified as phosphatidic acid and diglyceride, and an unidentified non-polar lipid. A wide range of behaviour has been observed for different normal cell populations, both in total uptake, and in the distribution of the incorporated acid. In most cases uptake was predominantly into phosphatidyl choline, but in others uptakes into phosphatidyl choline and phosphatidyl ethanolamine were comparable. The observed range of behaviour can be explained as arising from differences in concentrations of the substrates required for fatty acid incorporation in the plasma in which the cells or ghosts were incubated.

Fatty acid uptakes into red cells and ghosts were also studied as a function of cell age. A wide diversity of behaviour for different blood samples was apparent. In some cases, uptake into all components was essentially independent of cell age, but in others, uptakes into specific components showed definite trends with age. Most noteworthy were a marked increase with age in uptake into phosphatidyl ethanolamine, with essentially constant uptake into phosphatidyl choline, and a decrease in uptake into phosphatidyl choline with uptake into phosphatidyl ethanolamine remaining constant. To account for the diversity of behaviour, it is suggested that changes in either enzyme availability or conformation, affecting cellular enzyme activity, occur as the cells age, and that only for certain plasma concentrations of the substrates required for fatty acid uptake, are these changes in enzyme activity evident.

In a single study, bovine erythrocytes have been labelled *in vivo* with [^{14}C]acetate, and levels of activity in the cell lipids followed. A decline in free cholesterol activity, arising from rapid equilibration with plasma cholesterol, has been demonstrated. A fall in activity that could be interpreted in terms of red cells being able to exchange some but not all of their phospholipids with plasma counterparts has also been found.

Roles for triglycerides and cholesterol esters in the erythrocyte, and properties of the erythrocyte phospholipid transacylation mechanism have been discussed. The possible importance of changes in lipid constitution and lipid metabolism in red cell aging has been considered.

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INTRODUCTION

Several very good reviews on aspects of red blood cell metabolism have recently been published[1-10], including "The Red Blood Cell" edited by Bishop and Surgenor, which brings together a number of comprehensive papers on this subject. As an introduction to the present study of erythrocyte metabolism, attention is confined to those aspects of cellular function which are either concerned with lipid metabolism, or which appear at this stage to be pertinent to the general problem of erythrocyte aging.

General Red Cell Metabolism

A red cell begins its life as an erythroblast in the bone marrow. It is considerably larger than a mature erythrocyte, contains a nucleus, mitochondria and microsomes, and has the ability to perform a wide range of metabolic reactions[11]. On shedding its nucleus, the cell becomes a reticulocyte, and either remains in the bone marrow or is released into the circulation. Between 1 and 2 per cent of circulating red cells are normally reticulocytes. Reticulocytes retain some mitochondria and posses a functional citric acid cycle with associated comparatively high Q_{O2} values[11]. These cells can synthesise purine nucleotides[12,13] and protein, in particular, hemoglobin,[14,15] and they appear to retain some ability to synthesise lipid from glycerol or acetate[16,17]. A number of the enzymes found in erythrocytes exhibit greater activity in reticulocytes[18-21]. In addition, reticulocytes also have higher nucleotide[22,23] and 2,3 diphosphoglycerate contents than mature cells[24]. They are larger and lighter than erythrocytes[25], and are reported to have higher lipid content[26] and relatively more phospholipid than cholesterol[27]. On maturation, reticulocytes lose all mitochondria and endoplasmic reticulum[11], and the ability to oxidise pyruvate[20], and synthesise de novo nucleotides[12,13], proteins[15,28] and lipids (from glycerol or acetate)[16,29,30]. Even with these losses in metabolic activity, erythrocytes still retain a

relatively wide array of enzymes[31].

The primary source of energy in erythrocytes is obtained from anaerobic glycolysis, which supplies about 90 per cent of the total energy requirement[11]. The hexokinase catalysed reaction does not appear to be saturated with respect to ATP and is rate limiting, and the other enzymes are present, at least in younger cells, in considerable excess[6,10]. The remaining 10 per cent of energy is derived from glucose oxidation via the phosphogluconate pathway, or "hexose monophosphate shunt"[10,11]. Apart from acting as a source of both ATP and ribose-5-phosphate, (for nucleotide synthesis), this oxidative pathway provides the cell with its only mechanism for generating reduced $NADP^+$ [32].

Energy Requirements of the Erythrocyte.

Oxygen transport by the erythrocyte proceeds as a result of concentration gradients[33] and hence causes no energy utilisation[11]. Inhibition of glycolysis, however, leads to K^+ and ATP loss, an increase in methemoglobin, and changes in cell morphology[34,35]. About 30 per cent of the energy produced is used to maintain sodium and potassium concentration gradients between cells and plasma[5]. There is good evidence that these are effected by a Na^+/K^+ dependent ATPase located in the cell membrane[36]. It has been observed that reduction in ATP levels causes changes in erythrocyte shape, producing sphering[37,38]. This evidence suggests that an energy input is required to maintain the normal biconcave disc form. Glutathione synthesis, which has an ATP requirement, can be performed by the erythrocyte[39]. In addition, ATP is required to form coenzyme A derivatives of fatty acids, which can be incorporated into phospholipids in the erythrocyte membrane[40,41].

Another energy requirement is for maintenance of hemoglobin, and some enzyme and structural proteins, in a reduced form. Hemoglobin, incubated _in vitro_, will convert to methemoglobin within a few days[42], and concurrently, oxidation of protein groups will gradually cause precipitation[15]. The cell has mechanisms for combating both types of reaction. Of the two known mechanisms for reducing

methemoglobin, one is NADH - dependent and the other NADPH - dependent. Under normal conditions the NADH - dependent reaction predominates[42]. In addition, erythrocytes contain the enzymes catalase and glutathione peroxidase[42,43], both capable of reducing cellular peroxides and thus preventing hemoglobin oxidation. Although glutathione peroxidase has been shown to combat oxidation by certain drugs, the importance of these mechanisms in preventing methemoglobin production in vivo has not been demonstrated[42]. Hexokinase and glyceraldehyde-3-phosphate dehydrogenase, as well as other enzymes, require reduced -SH groups to remain active[11]. Two -SH groups must also be preserved in hemoglobin. The cell requires a supply of reduced glutathione (GSH) to protect these groups against oxidation[22,44-49] In addition, Marks et al[50] have observed some protection of hemoglobin -SH groups against oxidation by NADPH, not mediated through GSH. Jacob and Jandl[51] have found that the cell membrane also contains protein -SH groups which are subject to oxidation, and that oxidation of these groups in vivo causes removal of the cell from circulation. Similar results have been obtained by Mills and Buell[52].

In order to combat oxidative damage, it is clear that the cell must have a supply of reduced pyridine nucleotides and glutathione. Regeneration of reduced glutathione after oxidation specifically requires NADPH as a cofactor, so basically the requirement is for the two pyridine nucleotides. Although NADH, required mainly for methemoglobin reduction, is regenerated during glycolysis, the only means of regenerating NADPH is by glucose metabolism via the phosphogluconate pathway. Supporting the proposition that the main purpose of this pathway is to regenerate NADPH for glutathione reduction is the evidence that the level of activity of this pathway is regulated primarily by GSH[53].

An interesting abnormality in red cell energy metabolism is glucose-6-phosphate dehydrogenase deficiency. This condition, found in humans, is genetically determined, and although it does not normally cause a shortened erythrocyte life-span, it is associated with a susceptibility to hemolytic anaemia induced by a

variety of oxidative drugs, and certain bacterial or fungal infections[32]. Examples of such agents are primaquine, quinine, fava bean, infectious hepatitis, and many others. As it is a key enzyme in glucose metabolism via phosphogluconate, deficiency in this enzyme results in a low rate of generation of NADPH, and hence GSH. Since a continuous supply of these cofactors is necessary to combat oxidative damage to proteins,[6] it appears that in glucose-6-phosphate dehydrogenase deficiency, under normal conditions, this requirement can be met, but in the presence of high concentrations of oxidising agents, regeneration of GSH is too slow to prevent oxidation of cell protein -SH groups, and the cells cannot survive. Low levels of reduced GSH are normally associated with G6PD deficiency[32]. Prins et al[54] also report an interesting case of hemolytic anaemia associated with erythrocytes in which the level of GSH was less than 10 per cent normal.

To summarise, the red cell metabolises glucose via two pathways, to produce ATP and a supply of reduced pyridine nucleotides. ATP is necessary for maintaining ionic concentration gradients, and its biconcave discoid shape, and for synthesising glutathione, some phospholipids and possibly other compounds. Reduced pyridine nucleotides are necessary to maintain certain cell constituents in a functional, reduced state.

Lipids of Mammalian Erythrocytes

The composition and metabolism of mammalian erythrocyte lipids has been reviewed very fully by de Gier and van Deenen[30], and other recent reviews are also available[7,55]. The great improvement in lipid separation and identification techniques over the past 10 years has led to a relative wealth of literature on the constitution of mammalian red blood cell lipids[56-72]. Since at least 95 per cent of the lipids are localised in the cell membrane[73], the red cell is a very useful system for investigating the role of lipids in membrane function. Lipid accounts for approximately 0.5 per cent of the total weight of the red cell, and 40 per cent of the membrane or ghost[30]. In all species, the lipid consists of

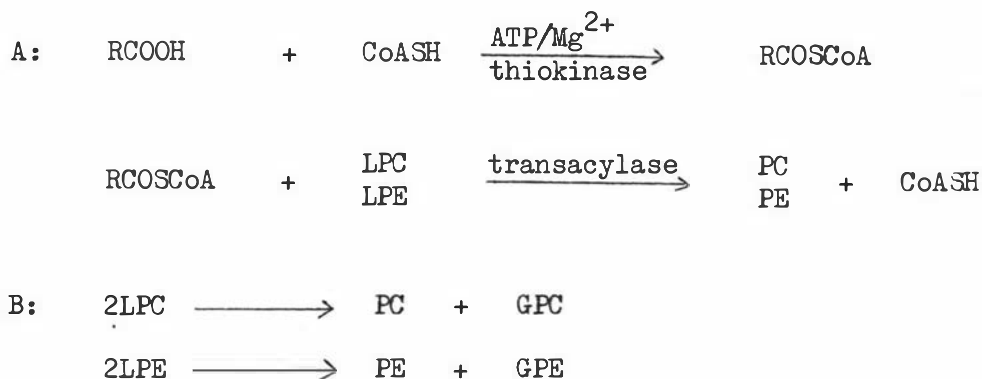
25-30 per cent cholesterol and 60-70 per cent phospholipid, the remainder including small quantities of glycolipids[74], and possibly triglycerides, cholesterol esters, diglycerides and free fatty acids. The major phospholipids are phosphatidyl choline (I), phosphatidyl ethanolamine (II), phosphatidyl serine (III) and sphingomyelin (IV). Species differences in phospholipid distribution exist, the most striking being observed with phosphatidyl choline, which is the major phosphatide in some species, but is present in very low concentrations, or may even be absent, in ruminant cells[57,58,61,64,68]. Low phosphatidyl choline levels are usually compensated by increases in sphingomyelin levels, and accordingly, there is little variation in choline-containing phospholipid. Fatty acid patterns also vary between species[63,75], but this has been shown to be primarily a dietary and not a genetic effect[76]. Changes in fatty acid pattern can affect erythrocyte permeability[63,75,77-79].

The lipid composition of abnormal erythrocytes has also been studied, and surprisingly few differences from normal have been detected. Acanthocytosis (abetalipoproteinemia), in which plasma phospholipid levels are very low, and sphingomyelin constitutes a relatively high proportion of the phospholipids, is associated with a decreased red cell phosphatidyl choline content, and a concomittant increase in sphingomyelin[80,81]. In patients with hepatic disease and associated anaemia, Neerhout[82] has observed varying degrees of elevation of red cell phosphatidyl choline and cholesterol concentrations. He has also examined red cell lipid levels in cases of plasma hyperlipemia and has been able to demonstrate no deviations from normality[83]. No other cases of abnormal lipid composition in hemolytic diseases, including spherocytosis and glucose-6-phosphate dehydrogenase deficiency, have been detected[30,84,85]. Normal lipid metabolism has also been observed in spherocytes[41]. Hence changes in erythrocyte shape are not necessarily associated with changes in either lipid composition or metabolism.

Initially it was considered that red cells were able to synthesise

lipid from acetate[86-88], but it is now known that although reticulocytes have this ability, it is absent in erythrocytes. The misconception arose from lack of correction for contributing white cells, which can carry out these biosynthetic reactions[89-92]. Pittman and Martin[93] have recently found evidence for an incomplete long chain fatty acid synthesising system, lacking only acetyl CoA carboxylase. In addition, mature erythrocytes are unable to use glycerol as a substrate for lipid synthesis[16,17]. Phospholipid synthesis from phosphate is very low, and restricted almost entirely to phosphatidic acid[94]. However, it has been shown by dietary experiments _in vivo_[75,76,95-101], and incorporation studies _in vitro_[40,41,75,94,102], that at least some phospholipid fatty acids can be renewed in mature red cells. Red cell ghosts, if supplied with ATP and coenzyme A, can also carry out these reactions[40,41]. Uptake into phosphatidyl choline, (PC) and, to a lesser extent, phosphatidyl ethanolamine (PE), has been observed, with the order of incorporation rate for different fatty acids being linoleate > oleate and palmitate > stearate > laurate[102,103]. These differences are more pronounced in whole cells than in ghosts[102]. The ability of intact cells and ghosts to esterify lysophosphatidyl choline (LPC) and lysophosphatidyl ethanolamine (LPE), whether present in the cells, or added to the medium, has also been demonstrated[41,104]. However, no phospholipase A activity can be detected in the erythrocyte[40,41,104]. Exchange of whole phospholipid molecules between plasma and erythrocytes has been observed[94,105-107]. Sakagami _et al_[105] have reported the following relative rates of exchange: lysophosphatidyl choline > phosphatidyl choline > sphingomyelin. Reed[106,107] has found that in humans and dogs, exchange rates for phosphatidyl choline and sphingomyelin are much greater than those for other phospholipids. From differences in the extent of influx and efflux he has proposed that the cells contain exchangeable and nonexchangeable pools of each lipid and that exchangeable pools turn over at rates of about 10 per cent per day.

Two mechanisms for fatty acid and lysophosphatide incorporation into erythrocyte lipids have been demonstrated[94,102,104].



(GPC = glycerophosphoryl choline; GPE = glycerophosphoryl ethanolamine). Mechanism A, initially identified by Lands and coworkers in rat liver microsomes[108,109,110], is ATP and coenzyme A dependent, and is favoured at low lysophosphatide concentrations[94]. Lands and his associates have shown that the mechanism exhibits positional selectivity, unsaturated acids generally preferring α-monoacyl, and saturated acids, β-monoacyl glycerophosphatides as substrates[109,110]. In this respect, differences in positional specificity were observed for acylation of LPC and LPE, which suggests the existence of more than one transacylase. Reaction rates depend more on the lysophosphatide isomer than on the acid already esterified[111,112].

Mechanism B was first postulated by Erbland and Marinetti[113]. It is favoured at high lysophosphatide concentrations and requires no ATP or coenzyme A[104]. An erythrocyte lysophospholipase has also been detected[104].

Although Mulder and van Deenen[102] report low incorporations of fatty acids into erythrocyte neutral lipids, Donabedian and Karmen[114] and Michaels _et al_[115] have described high uptakes into triglycerides. Fatty acid uptake into carnitine esters has also been observed[116,117].

Red cell cholesterol cannot be synthesised _in situ_[89,118], but can exchange freely with plasma cholesterol[118-121]. The exchange is independent of glucose

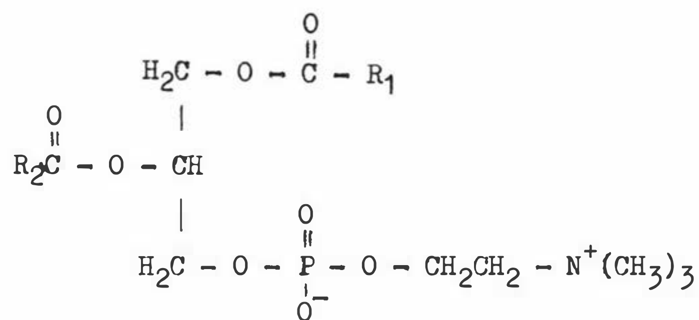
metabolism[68], and occurs at comparable rates with both fresh and stored cells[122]. It is pH dependent, relatively unaffected by temperature, but influenced by a wide variety of chemicals. The nature of these effects has led Brückdorfer and Green[122] to propose that hydrophobic bonding may be important in determining the rate of exchange. No net uptake by cholesterol depleted cells[121], or loss to cholesterol depleted plasma[123], is apparent, but incubation in plasma containing active cholesterol esterase results in a reduction in red cell free cholesterol, and an increase in cell fragility[124].

In order to obtain a fuller understanding of red cell lipid metabolism, a consideration of plasma lipid transformations is required. In addition to cholesterol esters, triglycerides, cholesterol and diacyl phospholipids, plasma also contains free fatty acids, lysophosphatidyl choline[125-127], and lysophosphatidyl ethanolamine[126,128]. Whereas the former all exist as lipoproteins, the fatty acids and lysophospholipids are transported almost entirely bound to albumin[129,130]. This form gives the red cells protection against lysis by the free lysophospholipids[131]. Plasma contains an active phospholipase A, which catalyses the deacylation of diacyl phospholipids[132,133]. Cholesterol esterase, which catalyses the reaction

Cholesterol + PC ⟶ Cholesterol ester + LPC

is also present[134,135].

In normal plasma, it appears that reactions proceed which decrease free cholesterol and diacyl phospholipid levels and increase lysophospholipid levels. Glomset[135] suggests that the cholesterol-lecithin acyl transfer mechanism could play a part in cholesterol exchange in cell membranes. If this interpretation is correct, the red cell transesterification mechanism could be important in removing lysophospholipids produced. As an alternative consideration, the plasma cholesterol esterase reaction could provide an essential supply of lysophospholipids for phospholipid metabolism in the membrane.



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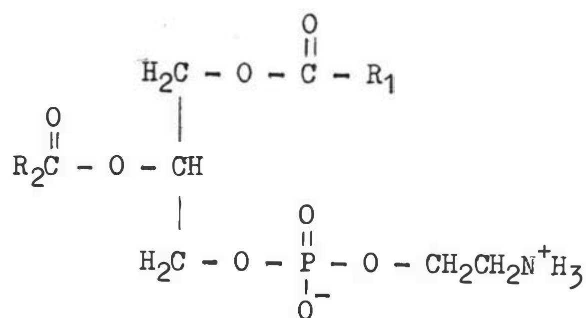

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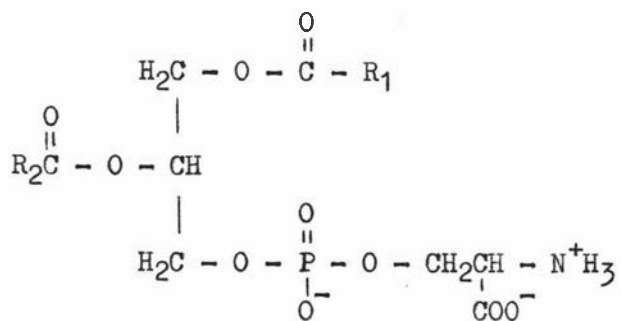
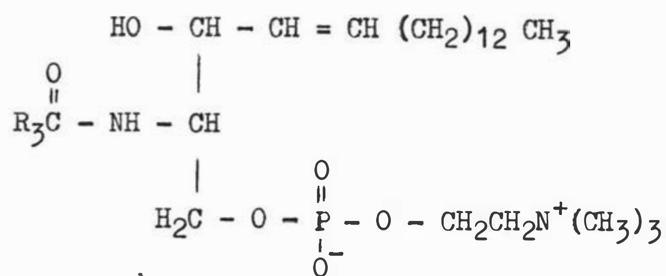


III

R_1 = long chain fatty acid, (predominantly saturated).

R_2 = long chain fatty acid, (predominantly unsaturated).



IV

R_3 = long chain fatty acid. (Sphingomyelins contain quite a high proportion of C-24 acids.)

The Erythrocyte Membrane

Erythrocyte lipids are an integral part of the cell membrane, and interpretations of their properties must be considered in relation to current theories on the structure of cell membranes.

Until recently, the most favoured model for membrane structure was the unit membrane hypothesis of Robertson[136,137], a modification of the Davson-Danielli paucimolecular membrane model[138]. It was deduced from interpretation of electron microscopic and x-ray diffraction studies, and explains many observed membrane properties, including electrical resistance and molecular birefringence. Many of the relevant deductions have come from the study of myelin. The unit membrane hypothesis requires that (1) there is one basic structure to which all membranes of all cells conform, and (2) this structure consists of a bimolecular leaflet of polar lipids, with polar groups orientated outward and electrostatically bonded to layers of protein, which may be visualised as extended polypeptide chains. Mucoprotein, mucopolysaccharide or enzymic protein may be attached on either side of the lipid bilayer.

However, recent information has led to questioning of these interpretations, and to suggestions that the model requires modification. These doubts have

been expressed by Korn[139], for example, who stressed the unlikelihood of a universal membrane, considering the diverse biochemical roles played by membranous structures including myelin, mitochondria, and plasma membranes, and the widely different chemical composition of different membranes, both in the nature of the polar lipid constituents and in lipid to protein ratios. Korn[139] has also pointed out that the electron microscopic and x-ray diffraction data do not necessarily require a unit membrane-type interpretation. More recent electron microscopic studies, using negative staining or freeze etching techniques, indicate the presence of repeating units[140-144]. Biochemical evidence now available also suggests that modifications to the unit membrane hypothesis are necessary. It has been shown that hydrophobic bonding between protein and lipid is important in membranes, and may even predominate over polar bonding[145-148]. The disassociation of membranes to yield lipoprotein subunits[149-151] favours an intimate association between lipid and protein rather than the presence of discrete phases. In no membrane system, including myelin, is there evidence for extended polypeptide chains. By contrast there is more to suggest random coiling with some α-helical configuration[152-157].

Green and his associates[158-160] have proposed an alternative membrane which is more compatible with available biochemical evidence. This model considers the membrane as a continuum made up of nesting or fused repeating units. The units are considered to be lipoprotein complexes, similar in size and shape in a particular membrane, but differing in chemical composition and function. Form, size and function of the repeating units can differ between membranes. The presence of lipids as micellular associates is postulated. Evidence for this type of structure has been obtained, mainly from chloroplast and inner mitochondrial membranes. As an example, it has been possible to disassociate inner mitochondrial membranes to give several different

lipoprotein electron transport complexes, which can reassociate into membranous structures. These complexes contain both catalytic and core proteins. The core proteins appear essential for alignment of the enzymes in the complex, and both types of protein are necessary for assembly of the units[160]. The presence of phospholipid is essential for membrane formation from inner mitochondrial subunits, and for enzymic activity, but the actual constitution of the phospholipids does not appear to be critical[160,161]. There is similarity in gross chemical and physical properties of the core proteins of the electron transport complexes, a mitochondrial structural protein which appears to be organisational in the oxidative phosphorylation mechanism[162], and structural proteins isolated from other membranes[163]. More detailed assessment of information and interpretations favouring a model of a membrane composed of repeating units has been well presented in reviews by Green and his associates[158-160]

Although most of the information supporting this model comes from studies on mitochondria and chloroplasts, there is some evidence available which suggests similarities with the erythrocyte membrane. Various proteins and lipoproteins have been separated from solubilised erythrocyte ghosts[164-169]. Although characterisation is far from complete, the properties of several of the proteins isolated are suggestive of aggregates of protein or lipoprotein subunits in the original membrane. Bakerman and Wasemiller[170] have isolated a protein which has comparable molecular weight and similarities in amino acid composition to the mitochondrial structural protein of Criddle <u>et al</u>[162]. This protein readily forms aggregates, and is capable of binding hemoglobin, whereas the mitochondrial protein binds myoglobin[171]. The infra-red absorption studies of Maddy and Malcolm[152], and optical rotatory dispersion and circular dichroism studies of Lenard and Singer[153], suggest that the proteins in the red cell membrane have partly a random coil, and partly α-helical conformation. Similar conformations have also been suggested for chloroplast, mitochondrial, and

bacterial membranes[154-157]. Infra-red analyses of the lipids in intact red cell membranes[172] indicate that very few of the hydrocarbon chains exist with a trans-planar configuration. This configuration is much more prevelant, however, in myelin and in synthetic lipid films. Lenard and Singer[173] have shown that the phosphorylated amines but not diglycerides, produced by the action of phospholipase C on erythrocyte ghosts, are released into solution. Even though 70 per cent of the lipid phosphorus is lost in this way, they could detect no change in membrane structure as revealed by circular dichroic spectrum. They have proposed a model for the erythrocyte membrane in which lipoproteins are stabilised by hydrophobic bonding, with little contribution by polar interactions, and in which most polar lipid groups are on the outer surface, in contact with the bulk aqueous phase.

Although our knowledge of the erythrocyte membrane is still far from complete, there is evidence supporting a structure similar to that proposed by Green and coworkers. This group has also presented convincing evidence for the localisation of all integrated metabolic sequences in membrane repeating units[174]. Any changes in the characteristics of the lipids, which are integral parts of the units, and which probably influence their conformation by hydrophobic interactions, could have profound effects on these metabolic activities[160]. Likewise, changes in lipid metabolism could reflect conformational changes in the membrane.

Separation of Erythrocytes according to Age

The phenomenon of erythrocyte aging has been studied by many investigators for many years, but the direct cause of the cellular changes which occur has yet to be established. A major problem which arises is the difficulty of separating cells of different ages from a normal circulating population. The first method of approach to the problem was to render an animal, highly anemic and induce reticulocytosis, either by repeated bleeding or administration of

acetylphenylhydrazine. This enables study of differences between reticulocytes and erythrocytes, and subsequently, when the reticulocytes have matured to young erythrocytes, study of the properties of a younger than normal population of cells. However, doubts have been expressed as to the normality of reticulocytes produced under such stress,[175,176] and although the method is useful for obtaining reticulocyte-enriched blood, it has distinct limitations in studying age changes in erythrocytes.

In 1950, Dreyfus et al[177] observed that young red cells tended to collect at the top of a column of centrifuged cells, and subsequently, other investigators have demonstrated an increase in density with erythrocyte age[25,178-180]. Other properties have been shown to differ in young and old cells, including susceptibility to osmotic hemolysis,[25,178,181,182] and surface charge, as demonstrated by electrophoretic mobility[183], agglutination by polylysine[183] and distribution between aqueous dextran and polyethylene glycol solutions[184,185]. Various methods of fractionation have been devised, based on these properties. The most popular of these has been centrifugation, either at normal speeds[177,180,186] or at high speeds,[25,178,186,187] at which most investigators have reported enhanced age separation. In contrast Prentice and Bishop[186] claim that the degree of age separation is independent of centrifugal force. An alternative procedure for separation of erythrocytes according to age is serial osmotic hemolysis in hypotonic NaCl solutions[181]. This technique has been used in one section of the present investigation. Results suggest that the age separations achieved by ultracentrifugation and serial osmotic hemolysis are comparable.

Another method which has been used[188], in particular by Löhr and Waller et al[34], is based on the Ashby differential agglutination technique originally used for measuring erythrocyte lifespan[189]. In this method, compatible but distinguishable donor cells are transfused into a recipient, and donor cells recovered at different times after transfusion, by differential agglutination of

recipient cells. The donor cells initially have a normal age distribution, but their mean age gradually increases, as no new cells are formed. Although improved age separations have been achieved by this method, it has only been applied to humans, for whom sufficient knowledge on blood groups, and a good supply of antibody, is available. In addition, the method is not particularly satisfactory, because it requires the transfusion of relatively large volumes of blood, and there is always the attendant risk, however slight, of incompatibility.

The methods discussed above are either not very practical, or produce enrichment of fractions with old or young cells, which is not very great. Modifications which improve separation of ages have been made. One limitation of the centrifugal separation method is cell trapping, which prevents alignment according to true density. Accordingly the system is not at equilibrium. Prankerd[179] reports improved separations by centrifuging cells over a 30 per cent albumin solution, which has a density almost as high as red cells and so provides more support for the cells. Danon and Marikovsky[190,191] have developed a method for centrifuging over mixtures of phthalate esters, with densities within the red cell range, which gives clear partition between two cell fractions.

Ultracentrifugation over albumin solutions appears to be the best method available for achieving good erythrocyte age fractionation. Bishop and Prentice[192], using a continuous albumin gradient with densities covering the red cell range have obtained an improved separation according to age, and have shown that in this system, equilibrium is achieved. Piomelli _et al_[193] have investigated ultracentrifugation over a discontinuous density gradient of isotonic albumin solutions, and obtained clearly separated erythrocyte fractions with widely different mean ages.

As ultracentrifugation over a discontinuous albumin gradient appears to give the best resolution of erythrocyte age, and, in addition, yields clearly defined and easily separated fractions, the procedure was modified slightly and

used in the present investigation, to study changes in the metabolism of red cell lipids with cell age.

The most common method of assessing the degree of age separation achieved by various techniques has been to examine the distribution of cells labelled with ^{59}Fe at various times after intravenous administration of the isotope[25,177-182,186,187,190-192]. Since only immature cells can synthesise hemoglobin[15], labelled cells are those formed during isotope administration, and ^{59}Fe distribution should reflect the distribution of these cells. However, if there is cell breakdown during the study, reutilisation of ^{59}Fe results in isotope distribution no longer being the same as the distribution of the originally labelled cells. An alternative method, used by Piomelli _et al_[193], examines the distribution of cells labelled by _in vitro_ incubation of whole blood with $[^{14}C]$ glycine. Reticulocytes are the only circulating red cells capable of synthesising heme or protein[14], although erythrocytes can take up $[^{14}C]$-glycine into their amino acid pool and use it to synthesise glutathione[194,195]. The cells can be returned to the circulation with only reticulocyte proteins permanently labelled. Reutilisation of any ^{14}C released from the cell amino acid pools and glutathione, or from broken down cells, is minimal, because of the dilution factors involved. Hence the distribution of radioactive red cell proteins is equivalent to the distribution of cells of a particular age. Piomelli _et al_[193] have compared both methods, and found, in the rabbit, that when ^{14}C-glycine shows the localisation of older cell groups in particular density fractions, ^{59}Fe activity, (presumably because of reutilisation from broken down cells), is spread throughout the cell population. Cells labelled with ^{14}C-glycine appear to be preferable for examining erythrocyte separation according to age, and in the present investigation, rat cells have been labelled in this manner to determine the extent to which age separation is achieved.

Metabolic and Physical Changes associated with Red Cell Aging

The red cell with its inability to divide, or when mature, to synthesise proteins, and with its relatively simple energetics, is a very good model for studying aspects of cell aging. The cell initially contains a full complement of enzymes and cofactors, but although there is essentially no protein hydrolysis, it is capable of existing only for a predetermined time. For mammalian erythrocytes, this time is between 1 and 4 months[196]. Attempts to resolve this problem have led to many studies on changes in erythrocyte structure, composition, and metabolic activity with age. Although various changes have been demonstrated, and some of these can be implicated as likely causes of erythrocyte malfunction, the direct cause of these changes, and of ultimate removal from circulation, has yet to be defined. There are several recent reviews on this subject[6,8,10,197].

Of the structural changes which have been observed in aging red cells, increases in density[25,177-180] and osmotic fragility[25,178,181,182,198] have already been discussed. A reduction in surface charge[183-185], which is due almost entirely to sialic acid residues[199], also occurs. It appears that as they age, the cells become smaller, and change shape slightly, decreasing in diameter and increasing in thickness[200,201]. However the size change that occurs on transformation from a reticulocyte into an erythrocyte is considerably greater than any changes occuring in the mature cell[25]. Danon and Perk[202] have examined electron microscopically, ghosts from young and old populations of erythrocytes, separated by centrifugation over phthalate esters. They have found a predominance of granular, folded, relatively thick ghosts in the younger fraction, and a predominance of smoother, thinner structures in the older fraction.

Differences in lipid content of young and old cells, separated by centrifugation, have been reported[179,200,201]. These are only significant when calculated on a per cell basis, and not if related to cell volume or surface area. Even then, the differences are small. In view of the much higher lipid content

of reticulocytes[26], it is possible that the differences between red cell fractions could be explained by variations in reticulocyte concentrations. No age differences in the relative amounts of cholesterol and phospholipid, or individual phospholipids, have been observed. Walker and Yurowski[203] have examined fatty acid patterns of lipids from rat red cells separated by ultracentrifugation, and have reported small but significant differences between fractions. Their re-examination of these distributions after feeding the animals on different diets showed slightly different rates of uptake of individual fatty acids in each fraction. The results were explained by postulating the presence of transacylases with different specificities, and age-dependent activities. Relatively large losses of both cholesterol and phospholipid, during in vitro storage under blood bank conditions have been reported[204].

There have been various reports, sometimes conflicting, on the levels of various constituents, and activities of various enzymes, in young and old erythrocytes. However reticulocytes have higher activities of many enzymes and higher levels of some cofactors than do erythrocytes, and in some studies the possible contribution by reticulocytes to age variation is not defined. Löhr and Waller and their coworkers[6,10,34,205-206] have observed decreases in enzyme activities and in concentrations of various constituents with age of cells separated by both differential hemolysis and differential agglutination following transfusion. It is unlikely that differences in transfused cells could be explained by levels of short-lived reticulocytes. Different enzymes became inactivated at different rates, the fastest rates being for glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphofructokinase and 6-phosphogluconic dehydrogenase. Very much slower rates of inactivation of hexokinase , lactic dehydrogenase and other glycolytic enzymes were observed[34]. In contrast, Brok et al[210] report marked decreases in hexokinase activity in the initial and final stages of erythrocyte aging. Marks and Johnson

and coworkers[19,211] obtained similar results to Löhr and Waller, by differential hemolysis, for glucose-6-phosphate, 6 phosphogluconic and lactic dehydrogenases. They also found little difference in purine nucleoside phosphorylase activity, and a phosphohexase isomerase activity difference which could be related to reticulocyte concentration. Waller[10] has calculated from these results that although hexokinase is relatively stable, the glycolytic reaction it catalyses remains rate limiting for almost the entire lifespan of the cell. Only towards the end does phosphofructokinase activity drop sufficiently for it to influence glycolytic rate. Similar conclusions have been reached by Rapoport[22] and Brewer and Powell[212].

Contrary results have been reported by Tada et al[18], who were able to relate the differences, between rabbit red cell samples, in activities of aldolase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase, and other factors to the different reticulocyte concentrations. Other enzymic activities which have been reported to decrease in aging cells are catalase[213-215], cholinesterase,[213-215] glyoxalase[213], and glutamic-oxalacetic transaminase[216]. In addition, slower rate of glutathione synthesis from ^{15}N-glycine has been described with older cells[206,217].

Sass et al[216], Löhr and Waller[206], and Rigas and Koler[218] have found a decrease in total, as well as reduced, glutathione, in aging cells, but Wagenknecht[219] could detect no change. A small difference in pyridine nucleotide level in top and bottom fractions of centrifuged cells was detected by Quarto Di Palo et al[220]. Allison[215] observed an exponential fall in NADH in transfused blood, but Löhr and Waller[6] detected no change until the cells were quite old and the effects of enzyme inactivation had become evident. They observed a similar pattern with ATP and ADP changes, no difference until about day 70 in humans, and then a gradual change in ATP:ADP, from about 5 to 1[206]. Bernstein[209] detected a difference in ATP and 2, 3-diphosphoglycerate concentration in cells separated by differential hemolysis, and Syllm-Rapoport et al[221] and Allison[214]

observed an ATP decrease in transfused cells. Brok *et al*[210] observed a decrease in ATP level with age roughly paralleling their decrease in hexokinase activity. However Shojania *et al*[24], although able to demonstrate higher ATP and 2,3-diphosphoglycerate levels in reticulocytes, could demonstrate no difference between cells at the top and bottom of a centrifuged column. There is some uncertainty concerning variation in methemoglobin concentration. Waller *et al*[206] and Brewer *et al*[224] observed an increase with age, but Beutler *et al*[225] and Betke *et al*[226] were unable to confirm this finding. Higher Na^+ and lower K^+ and Mg^{2+} concentrations have been measured in older cells[8,10].

Similar, but accelerated, changes have been observed in cells aged at constant pH in vivo[205,227].

Glucose-6-phosphate dehydrogenase deficient cells in general have normal or very slightly shortened lifespans[228], but Beutler *et al*[229] have shown that on exposure to an oxidising agent, it is the older cells which are selectively destroyed. Marks, Gross and coworkers[230,231] have related this to the higher levels of glucose-6-phosphate dehydrogenase found in the younger cells. Electron microscopic examination of a population of enzyme-deficient cells has revealed a predominance of granular membranes, in contrast to the predominance of smooth membranes in a normal population[232]. The cause of the difference is not known, but since they have found smooth membranes to be associated with older cells, they suggest that there may be some type of premature aging in glucose-6-phosphate dehydrogenase deficiency.

In interpreting these results, consideration must be given to the degree of separation of cell age achieved. It has been shown that in general osmotic hemolysis and centrifugation give fractions enriched with young or old cells, but the extent of separation depends on conditions. Differential agglutinati of transfused human blood yields cell samples with mean ages varying from about 60 to 120 days. It is quite possible that apparent similarities between old and

young cells could be a result of poor separation.

It is apparent that the largest differences so far detected between young and old erythrocytes are the activities of certain enzymes. However, as most of these enzymes are present in considerable excess, the effect of this inactivation may not be critical, and it is possible that other, smaller, changes contribute more to the senescence of the cell.

The mechanism by which an erythrocyte is removed from the circulation involves phagocytotis by cells of the reticuloendothelial system[9]. Whether the intact or hemolysed cell is phagocytised is not known. It appears that any badly damaged cells are removed by the liver, and slightly damaged ones by the spleen, but at least in the rabbit, the bone marrow is the most important destructive site. Any theory on red cell aging must explain how old cells either hemolyse, or become altered and recognisable to these sites of destruction.

Various suggestions of possible causes of red cell aging have been made. Marks, Johnson et al[19] suggest that a decrease in glucose-6-phosphate dehydrogenase, and hence a limited supply of NADPH could be important. Jacob and Jandl[51] discuss the importance of reduced glutathione as an antioxidant, especially in the protection of membrane -SH groups. Reduction in ATP content, and denaturation of membrane proteins have also been considered as causes of lipid loss, osmotic changes, and changes in enzyme activities[8,197]. More likely a series of changes, rather than any particular one, is ultimately responsible for red cell death. Waller and Löhr[6,10] have formulated a theory in which an overall rundown in energy metabolism is considered as the major cause. They suggest that the overall decrease in glycolytic rate, due to enzyme inactivation, and accelerated by reduced ATP production, which does not become obvious until the human cell is about 60 days old, causes a reduced supply of NADH and ATP. This results in increased methemoglobi formation, a gradual K^+ and Mg^{2+} loss, and a gradual change in the shape of the cell. A decrease in reduced glutathione concentration, caused by slower synthesis

as the ATP level falls, and slower regeneration as the NADPH level falls, renders the cell less able to combat oxidative changes to enzymes and membrane. They suggest that a combination of all these changes eventually bring about cell destruction. However the main problem remains unresolved, as to how the enzymes become inactivated in the first instance.

A theory in which glutathione plays a primary role in erythrocyte aging has been suggested[51]. Oxidation of membrane and enzyme proteins does occur if the cellular glutathione level is reduced, and once membrane -SH groups have been oxidised in vivo, the cell is removed by the spleen, before any change in glycolytic rate or ionic composition is evident. However the spleen is not normally the major site of red cell destruction. Jacob and Jandl[53] have observed that the protection given the cell by glutathione against an oxidising agent, such as acetylphenylhydrazine, is not quite complete and some hemoglobin and NADPH is oxidised. It is possible that a similar situation could exist in vivo, in which the built-in mechanism of the erythrocyte to combat oxidation is not 100 per cent efficient, and irreversible oxidative damage gradually accumulates in the aging cell.

Many questions are still unanswered, and to produce a satisfactory explanation of red cell aging, it is probably necessary to understand all the changes which occur. In this respect, the functioning of the cell membrane requires further investigation. The erythrocyte membrane contains about 60 per cent protein and 40 per cent lipid, and there are indications that the protein may be more important in determining its overall structure. As many of the cell enzymes, including all those involved in integrated metabolic sequences, are most likely localised in the membrane small alterations in membrane structure could have profound effects on erythrocyte metabolism. In this respect, Prankerd[197] notes that membrane bound enzymes appear to be more inactivated in older cells than soluble ones. At present insufficient information is available on the membrane proteins to undertake an investigation into their variation with age. However, the lipids, which are essential constituents of

the functioning membrane, and which appear to have a rather specialised metabolism, are sufficiently well documented for an investigation into variation in their composition and metabolism with cell age to be possible. Variations in both lipid composition and metabolism have been reported[200,201,203], and the present study was undertaken with a view to extending these results, and ascertaining whether alteration in lipid metabolism is a contributing factor to erythrocyte aging. Changes in lipid metabolism could also reflect structural alterations in the cell membrane.

MATERIALS AND EXPERIMENTAL METHODS

Materials

All solvents were distilled before use, and glassware was washed with chromic acid. The following chemicals were used: 2,5 diphenyloxazole (P.P.O.) and 1,4 di (diphenyloxazolyl) benzene (P.O.P.O.P.), (Nuclear Enterprises Scintillation Grade); bovine serum albumin, Cohn Fraction V, and coenzyme A, (Sigma Chemical Co); adenosine triphosphate and trimyristin (Koch-Light); cholesterol (BDH Laboratory Reagent, recrystallised); palmitic acid and oleic acid (BDH Laboratory Reagent); phosphatidyl choline and phosphatidyl ethanolamine (purified from egg); Merck silica gel G, (for thin-layer chromatography); Koch-Light silicic acid 100-120 mesh, (for column chromatography).

Beckman DU and Unicam SP500 Spectrophotometers were used.

A refrigerated centrifuge (MSE Mistral 2L) with swing-out head was used for separating blood plasma and cells. The same centrifuge, at room temperature, was used in the serial osmotic hemolysis procedure. A refrigerated Sorvall centrifuge, with angle head, was used for sedimenting red cells and ghosts after fractionation by means of albumin gradients.

Thin-layer plates were prepared with a Desaga spreader.

Radioactive Compounds

[2-^{14}C] Glycine, (specific activity 21.5 mC/mmole), [1-^{14}C] palmitic acid, (specific activity 36.6 mC/mmole), and [1-^{14}C] linoleic acid, (specific activity 53.5 mC/mmole) were obtained from the Radiochemical Centre, Amersham.

The glycine (purity 99 per cent quoted by the manufacturers), was stored in aqueous solution at 4°, and used within one month of arrival.

The fatty acids were stored in benzene at -15°. Purities were determined by TLC with the solvents used in later investigations and results are given in Table 1 and Fig. 1. At least 95 per cent of the radioactivity in the palmitate was located in the fatty acid spot, and there were no radioactive components

FIGURE 1: AUTORADIOGRAPH OF A TLC SEPARATION OF THE [1-^{14}C]LINOLEIC ACID AND [1-^{14}C]PALMITIC ACID PREPARATIONS

Solvent System HEA 70:30:1

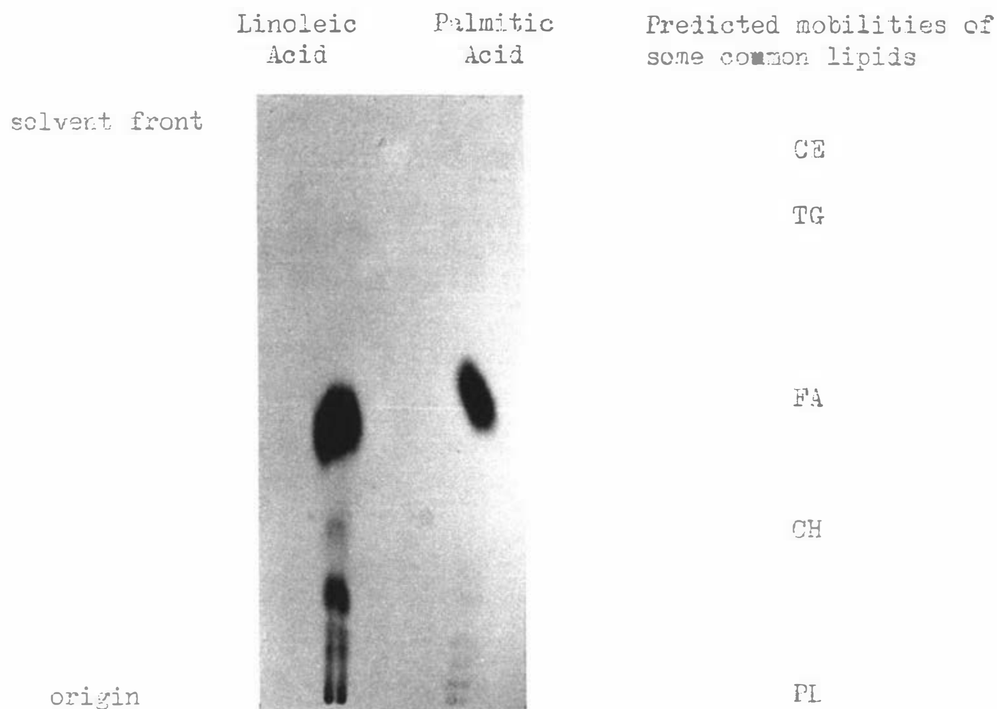


TABLE 1. PURITY OF THE [1-^{14}C]PALMITATE AND [1-^{14}C]LINOLEATE PREPARATIONS

Fraction		Per cent of total radioactivity [1-^{14}C] palmitate	[1-^{14}C] linoleate	Lipid with corresponding R_F
Solvent System HEA 70:30:1				
increasing R_F ↓	I	0.4	2	PL and other non-migrating components
	II	1.0	2.4	PL - Ch
	III	1.5	7.9	Ch
	IV	0.4	2.1	Ch - FFA
	V	95	86	FFA
	VI	1.5	0.3	TG and CE
Solvent System CMAW 65:25:8:4				
increasing R_F ↓	I	.01	.04	LPC and SP
	II	.02	.10	PC and PS
	III	.02	.12	PE
	IV	>99.95	99.7	NL

TLC separations of each preparation were performed, and the distributions of radioactivity on the gel performed. Abbreviations are tabulated in Appendix 2.

with Rf's similar to any phospholipids. Only 86 per cent of the linoleate activity was recovered in the free fatty acid spot, and although there were no components that could be mistaken for phospholipids, there were a series of radioactive components running between the fatty acid spot and the origin. It would appear that some autoradiolysis had occurred. Evidence that these extraneous compounds were not taken up by red cells on incubation in plasma comes from the findings that these bands were still present when the plasma lipid was separated after incubation, and that no equivalent bands were present in red cell lipid extracts.

Gas chromatographic analysis of the methylated $[1-^{14}C]$palmitate preparation showed that 95 per cent of the radioactivity was trapped in the column eluate corresponding to the palmitate peak.

Experimental Methods

Blood Collection

Bovine blood (250 - 500 ml) was collected from Jersey cows either into one seventh its volume of acid-citrate-dextrose (ACD) or into heparin solution.

Rat blood was collected into one quarter its volume of ACD. The animal was anaesthetised with ether and warmed under an infra-red lamp to stimulate blood flow through the tail. The tip of the tail was cut off and blood, (3-6 mls were obtainable), allowed to drip into anticoagulant. By moving further up the tail, a series of blood samples could be collected from the same animal.

Human blood was collected from healthy donors into heparinised tubes. Samples were taken within 1-2 hours of eating.

Separation of Cells

The blood was centrifuged in a swing-out head at 3000 revs/min for 30 min. Most of the upper plasma layer was removed and the remaining plasma plus a white cell-rich layer from the top of the cell column were collected

separately. For the larger volumes of bovine blood, a tube with a side opening was found to be more efficient in removing the white cell layer. For human and rat blood, the small amount of remaining plasma was gently stirred, to resuspend the very top layer of cells, and was aspirated with a Pasteur pipette. Red cells were washed twice with an equal volume of 0.9 per cent NaCl. At each centrifugation a white cell-rich layer was removed from the top of the cell column. Cell separation using this method was the best that could be achieved, but 1-5 per cent of the original white cells were still present in the red cell preparations.

Since even this low level of white cell contamination can contribute significantly to fatty acid uptake by red cell preparations, the possibility of using other methods of bovine blood cell separation was investigated. However, passage of the blood slowly through cotton wool columns removed only few white cells, and the addition of dextran to the blood[234] did not cause rapid sedimentation. The problems of blood cell separation, discussed in a review by Sparkes and Beutler[55], appear to be magnified when dealing with bovine blood. Bovine erythrocytes exhibit a slow sedimentation rate, due to lack of rouleau formation[273], which gives rise to greater leukocyte dispersal. This cell property could also be responsible for the absence of agglutination in the presence of dextran.

Ultracentrifugation using an angle head was also regarded as unsatisfactory because the mixing of upper cell layers on deceleration necessitated the removal of large quantities of erythrocytes with the leukocytes. Mulder and van Deenen[102], however, have employed this method, and report the elimination of all white cells by removing the top third of the cell column. This method is probably preferable in metabolic studies to making considerable corrections for residual leukocytes, but it requires the removal of a sizeable young population of cells, and is unsuitable in studies on variation in red cell metabolism with age. In the albumin density gradient method for fractionating red cells according to age, which is

described later, it is possible to trap the lighter white cells in a density band above the red cells. Although precipitation of rat hemoglobin prevented accurate counting of white cells, it appeared that for the rat, the method brought about efficient white cell separation. Human white cells, however, exhibited unusual differences in behaviour. Although during normal centrifugation they collected at the top of the cell column, in the albumin gradient procedure, they concentrated in one of the denser red cell bands. The albumin gradient method cannot therefore be used for complete separation of human red and white cells, and successive removal of cells from the top of a centrifuged column appears to be the best available method.

Enumeration of Cells

Red cell counts were made, after dilution in 0.9 per cent NaCl, with an Improved Neubauer hemocytometer and a Lietz microscope (magnification x 400). White cell counts were made at magnification x 100 after dilution in 1 per cent acetic acid. Packed cell volumes (PCV's) were determined by centrifuging in Wintrobe hematocrit tubes at 3000 rpm for 30 min. Difficulty in counting rat white cells was experienced, because of precipitation in acetic acid of hemoglobin released from lysed red cells.

For studies on human and rat blood, hemoglobin estimations rather than red cell counts were routinely performed. The relationship between cell volume and hemoglobin was determined in selected samples, and no variations between individuals were detected in blood from 3 (human) or 4 (rat) donors. Values of hemoglobin per cell were calculated from standard hematological data. This procedure was less time consuming and more accurate than direct cell measurement. (If 500 cells are counted, purely statistical grounds give a standard deviation of $\pm 4.5\%$)[235].

Hemoglobin (Hb) was estimated by the cyanmethemoglobin method[236]. Samples were diluted to 5 ml with Drabkin's diluent [KCN (.05g), $K_3Fe(CN)_6$(.20g) and

$NaHCO_3$(1.0g) to 1 l with water], and the absorbance of the solutions read at 540 mμ. Under these conditions,

	Hb absorbance/.01 ml packed cells	Packed cells/ml	Hb absorbance/10^8 cells
Human	.42 ± .01	1.15 x 10^{10} (Refs. 237,238)	.37 ± .02
Rat	.33 ± .01	1.65 x 10^{10} (Refs. 239,240)	.20 ± .01

Preparation of Red Cell Ghosts

Erthrocytes are impermeable to ATP and CoA[241]. During hemolysis the cells become permeable, but on resuspension in an isotonic medium, the ghosts become impermeable again. In order to prepare ghosts enriched with ATP and CoA it is necessary to add these cofactors at the time of lysis.

Ghosts were prepared for incubation by lysing erthrocytes with an equal volume of water containing ATP (4 μmoles/ml) and CoA (0.4 μmoles/ml). After standing 10 min, the same volume of double strength Krebs-Ringer phosphate (pH 7.4) solution (KRP)[242] was added to return the system to isotonicity. The final ATP and CoA concentrations, after addition of plasma, were 1 μmole/ml and 0.1 μmole/ml respectively.

Incubation of cells and ghosts with labelled fatty acids

All incubations were carried out at 37° in gently agitated conical flasks, plugged with cotton wool. In some bovine cell incubations, CO_2 was trapped by attaching outlets from the flasks to tubes containing NaOH, and passing a slow stream of CO_2-free air through the flasks.

Bovine blood cells were incubated for 5 hr with [1-^{14}C]palmitic acid in plasma, (total volumes about 30 ml and cell:plasma ratios 1:1). For one incubation Krebs-Ringer phosphate medium (pH 7.4)[242] was used. The [1-^{14}C]palmitic acid in benzene (0.1 - 0.2 ml) was added to a small volume of plasma or to the KRP solution, the benzene evaporated at 37°, an aliquot taken for counting, and a

known volume mixed with the cell suspension. At the end of each incubation, the cells were separated by centrifuging at 4°. When special efforts had been made to remove white cells prior to incubation, the red cells were washed twice with isotonic saline at 4°. When red and white cells were incubated together, plasma and white cells were collected separately as already described. Red cells were washed three times with cold saline, and the white cell fraction twice. Radioactivity in supernatants and washes was routinely measured.

Human cells or ghosts were incubated with either [1-^{14}C]palmitate or [1-^{14}C]linoleate in mixtures of plasma and KRP. Benzene solutions of the acids (1-2 µC in 0.1 - 0.2 ml) were evaporated to dryness, plasma immediately added, and the tubes shaken for at least 15 min, at 37° to dissolve the acids. Aliquots were taken for counting and addition to cell or ghost suspensions in KRP. Cell (or ghost) : KRP : plasma ratios were about 1:2:1 and total volumes were between 1 and 20 ml. Specific details for each study are given in the experimental section. Incubations were carried out for 3 hr. After incubations, cells were separated at 4°, by centrifuging at 4000 rpm for 10 min, and were washed twice with cold isotonic saline. Ghosts were centrifuged at 4°, at 20,000xg for 30 mins, and washed twice with cold saline. Supernatants were generally recentrifuged, to check on ghost recovery. Addition of albumin to the first wash aided removal of excess labelled free fatty acids from cells or ghosts.

Rat cells or ghosts were incubated with [1-^{14}C]palmitate. Cells from individual animals were incubated in their own plasma, but pooled cells or ghosts were incubated in KRP solution to avoid incompatibility reactions with plasma antibodies. Incubations were carried out for 3hr, total volumes were in the range 2-6 ml, and cell : medium ratios were between 1:2 and 1:5. Fatty acid addition and washing methods were the same as for human incubations.

Behaviour of Rat Red Cells during Incubation

If rat cells were first fractionated by density gradient centrifugation

in bovine serum albumin (BSA) solutions, and then incubated in KRP solution, considerable cell lysis and agglutination occurred and the cells became visibly deoxygenated. This deoxygenation could be overcome by shaking. In addition, if the cells were first incubated in plasma, and then fractionated in a BSA gradient, a high proportion either agglutinated or lysed. These phenomena were investigated further and the following information was obtained:

(1) Incubation of the cells for 3 hr at 37°, in their own plasma, resulted in no deoxygenation or agglutination and lysis.

(2) Incubation of the cells in KRP resulted in no deoxygenation or agglutination, but a small degree of lysis (1-2 per cent).

(3) Addition of BSA powder or solution to plasma caused the cells to become deoxygenated, but there was no lysis or agglutination in up to 8 per cent albumin solutions.

(4) Addition of BSA to KRP solutions caused both deoxygenation, and agglutination and lysis, of the cells. The amount of lysis depended on albumin concentration. (4 per cent lysis in 2 per cent albumin; 12 per cent lysis in 8 per cent albumin).

(5) Overcoming deoxygenation by shaking did not reduce lysis and agglutination.

(6) The cells could be either stood in contact with 10 per cent BSA solution for 15 min, or centrifuged over a BSA gradient, washed twice with KRP solution, and then incubated in plasma or KRP solution, with no deoxygenation or agglutination, and very little hemolysis (about 2 per cent) taking place.

These results suggest that:

(1) The presence of BSA or an impurity in the BSA preparation reduced the stability of rat erythrocytes, possibly by antibody-antigen reaction. The presence of rat plasma could eliminate this effect.

(2) The presence of BSA or an impurity in the BSA preparation caused hemoglobin in rat erythrocytes to become deoxygenated. The fate of the oxygen is not known,

but two possibilities can be suggested. Firstly, the presence of BSA could have caused an alteration in equilibrium constant for hemoglobin-oxygen binding. If this were the case, reversal of this shift on shaking would require explanation. Secondly, the oxygen could have been consumed. The identical effects of ultracentrifuged albumin solutions and fresh powdered albumin suggest that bacterial contamination is an unlikely cause of deoxygenation. However no explanation as to how the BSA preparation could stimulate oxygen uptake by the red cells can be given.

Lipid Extractions

Lipids were extracted from red cells, ghosts, and incubation media containing hemoglobin, with 20 vol of chloroform-isopropanol (7:11 v/v) by the method of Rose and Oklander[243]. This solvent is preferable to chloroform-methanol in that it extracts less pigment derived from hemoglobin, yet yields a good recovery of cell lipid. Plasma and white cell lipids were extracted with 20 vol of chloroform-methanol (2:1 v/v) by the method of Folch, Lees and Sloane-Stanley[244]. Some of the human red cell lipids were extracted under nitrogen to prevent autoxidation. Solvents were removed in a Büchi rotary evaporator below 45°C. Extracts were dissolved in chloroform-methanol (2:1 v/v), and shaken with 0.2 volumes KCl (0.05M) to remove water-soluble impurities[244].

Bovine lipids were evaporated to dryness, weighed, and stored in chloroform-methanol solution at 4°C. Some human and rat lipids were evaporated to dryness, immediately weighed, and dissolved in a known volume of chloroform-methanol for analysis. Others were immediately transferred to chloroform-methanol and stored under nitrogen, minimising the time during which the lipids were dry and more susceptible to autoxidation[71]. Extracts were stored in $CHCl_3$ - MeOH at -15°C.

Quantitative Analyses

Total Lipid and Neutral Lipid Determinations

Larger total lipid extracts (> 50 mg) from bovine cells, and neutral lipid fractions separated by column chromatography, were weighed directly. For smaller samples, a slight modification of the chromic acid oxidation method of Amenta[245] was used. Duplicate samples containing 20-200 µg lipid were evaporated to dryness, oxidised with a standard solution of potassium dichromate in concentrated sulphuric acid, and the colour change at 350 mµ determined spectrophotometrically. The amount of dichromate reduced, in oxidising the lipid to carbon dioxide and water, is a measure of the number of reducing equivalents per g lipid. This can be calculated*, and is found to vary between classes of lipid (Table 2). However the difference between phospholipid species is small, and the estimation is relatively insensitive to changes in chain length and degree of unsaturation of fatty acids.

A knowledge of the lipid constitution of a sample must be known before it can be accurately quantitated by this method. For red cell total lipid analyses, results were read from a curve corresponding to 70 per cent phospholipid, 25 per cent cholesterol and 5 per cent glyceride. Agreement between predicted and experimental slopes of standard curves was observed for cholesterol and trimyristin oxidation. No phospholipid standards were available, so the

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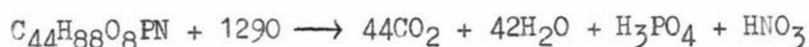
*For example, reducing equivalents per g phosphatidyl choline containing 2 stearate groups, (MW = 789):



$$C_{44}H_{88}O_8PN + 129O \longrightarrow 44CO_2 + 42H_2O + H_3PO_4 + HNO_3$$

$$\text{reducing equivalents per g} = \frac{\text{atoms oxygen required for complete oxidation}}{\text{MW}}$$

$$= \frac{129}{789}$$

$$= .163$$

Table 2: Reducing equivalents per g of common red cell lipids

Lipid Class	Reducing equivs/gm	Remarks
Cholesterol	.220	
Cholesterol ester	.210	No difference if acid is 12:0 or 18:0
Glyceride + fatty acid	.170 - .185 Mean .180	12:0 .170; 18:0 .184 13:3 .178; tri(18:0) .182
Phosphatidyl choline	.15 - .170	di(12:0) .150; di(18:0 .163
Lysophosphatidyl choline	.135 - .155	12:0 .135; 18:0 .155
Phosphatidyl ethanolamine	.15 - .170 Mean .16	di(18:0) .160 i.e. very little difference from PC
Sphingomyelin	.165 - .175	18:0 .167 i.e. very little difference from PC
Phosphatidyl serine	.14 - .16	di(18:0) .152
Glycolipid present in RBC	.13 - .17	

standard curve was inferred from those of cholesterol and triglyceride. Estimates of phospholipid in purified erythrocyte and plasma extracts, using this standard, were equivalent to those obtained by phosphorus analysis. Agreement between duplicates was regularly obtained, provided tubes were perfectly clean, and care was taken to remove all traces of organic solvent.

There are some indications that total lipid estimations by this method could be up to 10 per cent too low. Total lipid analyses for human red cells were equal to, or a little lower than, the sum of the measured phospholipid and cholesterol. These lipids normally constitute 90-95 per cent of the total lipid. In addition, values for total lipid per red cell obtained using this method were consistently a little lower than most literature values, whereas lipid phosphorus and cholesterol levels were more in agreement. However comparisons between total lipid estimates in different samples are not affected by a constant error.

Individual neutral lipids were analysed following elution from thin-layer chromatograms, and compared directly with standards.

Phosphorus determinations

Lipid phosphorus was determined by the method of Bartlett[246], which employs perchloric acid digestion and aminonaphtholsulphonic acid as reducing agent. Results were expressed in terms of either micrograms or microequivalents phosphorus. Approximate weights of phospholipid (µg) were calculated (µgP x 25) by assuming an average fatty acid chain length of just under C_{18}. Modifications were necessary when analyses were carried out on samples adsorbed to silica gel. Normally samples were digested over small gas flames, but when silica gel was present, a sand bath was used to give even heating, and to reduce the likelihood of bumping with consequent sample loss. The colour reaction is pH-dependent, and if the pH is too high the reagents alone yield an intense blue product[247]. If the usual quantity of acid was used, silica gel also caused this intense blue coloration, but if the quantity of acid was increased 1.5 times, the reaction proceeded normally. Silica gel blanks were low (absorbance: .05 - .07) and, provided no bumping occurred, good agreement between duplicates was obtained. Some variation in blanks reduced accuracy with small samples.

Cholesterol Determinations

Cholesterol in total lipid extracts was determined by the Liebermann-Burchard reaction using the method of Abel et al[248]. A disadvantage of this method is the relatively large amount of sample required (about 0.1 - 0.4 mg). High accuracy was not possible when the method was used at the lower limits and consequently cholesterol analyses on small samples were subject to relatively large errors.

Column Chromatography

Bovine erthrocyte lipids were separated on silicic acid columns, (Borgström[249]) into neutral and polar lipids. Neutral lipids were eluted with dry chloroform, and polar lipids consecutively with chloroform-methanol and

methanol.

Thin-Layer Chromatography (TLC)

All lipid samples were separated on 0.25 mm layers of silica gel G. Plates were activated at 110° for 1 hr and predeveloped in ether to remove impurities. For phospholipid separations, plates were reactivated and used within 15 min.

Solvent systems used for separating neutral lipids were[250]

hexane-ether-acetic acid (HEA)	70:30:1	(v/v/v)
hexane-ether-acetic acid	60:40:1	(v/v/v)
hexane-ether-acetic acid	30:70:1	(v/v/v)

and for phospholipid separations,

chloroform-methanol-acetic acid-water	65:25:8:4	(v/v/v/v)	(CMAW)[251]
chloroform-methanol-ammonia	14:6:1	(v/v/v)	(CMN)

Components on analytical plates were visualised by spraying with iodine, or with 20 per cent sulphuric acid and heating at 120°. Table 3 lists the components which have been identified by comparing their positions with those of standards. The incomplete resolution of cholesterol and diglyceride in these solvent systems prevented the unequivocal identification of possible diglyceride components in lipid mixtures. Some other components have been tentatively identified by comparison of their chromatographic behaviour with published values.

Preparative plates were sprayed with iodine, spots were marked, and appropriate areas of silica gel scraped off either for direct analysis or elution of the lipid components. The gel was transferred to narrow columns, and neutral lipids were eluted with 10 ml chloroform-methanol (2:1), phospholipids with 5 ml chloroform-methanol (1:2) followed by 5 ml methanol.

Table 3: Identification of lipid components separated by TLC.

Identified by direct comparison with standards	Identified from known Rf's and predicted presence in certain extracts
Phosphatidyl choline	Sphingomyelin
Phosphatidyl ethanolamine	Phosphatidyl serine
Cholesterol	Lysophosphatidyl choline
Triglyceride	Cholesterol ester
Free fatty acid	

Autoradiography

Autoradiographs of thin-layer chromatographic separations of lipid extracts were prepared by placing the plates in contact with Ilford X-ray film in light-proof boxes for 1-2 months. The lipid components were visualised with iodine and plates photographed for comparison with the X-rays.

Gas-Liquid Chromatography

Lipid samples were methylated by boron trifluoride-methanol, under nitrogen, in sealed tubes at 100°C, according to the method of Morrison and Smith[252]. Methyl esters were purified by TLC with HEA 90:10:1 as solvent. A Shandon Chromatograph, with an Apiezon (10% Apiezon L on Celite 545) stationary phase column (5' x 1/8") at 197°, argon carrier gas, and a ^{90}Sr detector was used for separation of radioactive components. Samples from the chromatograph were collected from the detector outlet into tubes containing toluene-moistened glass wool. For other analyses, an Aerograph Chromatograph, polyester column (stationary phase 12.5% DEGS on Gas Chrom P, dimensions 5' x 1/8") at 160°, helium carrier gas and hydrogen flame detector were used.

Quantitative Analysis of Lipids following Chromatographic Separation

Lipids extracted from bovine blood cells following incubation with

$[1-^{14}C]$palmitate were separated by column chromatography into neutral and phospholipids. These fractions were separated further by TLC using either HEA 70:30:1 or CMAW as solvent. When analysis for phosphorus or by the dichromate oxidation procedure, as well as radioactive assay, were performed, components were eluted and aliquots of eluate analysed. When only radioactive assay was required, components were counted directly on the silica gel. Generally duplicate TLC separations were performed, but if insufficient sample prevented this, analyses from one separation were carried out in duplicate.

Lipids extracted from ^{14}C-acetate labelled bovine erythrocytes were treated in a similar manner to above. Plasma lipids were separated directly by TLC. To determine radioactivity distributions within the molecules, cholesterol esters and phospholipids were hydrolysed in methanolic KOH, and radioactivity determined in ether extracts, before and after acidification, and in the residual aqueous phase.

After fractionation of bovine erythrocytes according to age by serial osmotic hemolysis, lipids were extracted from each lysate, and neutral and phospholipids separated by column chromatography. Large samples (about 10 mg) of neutral lipid were applied as bands to 20 x 20 cm plates and separated by TLC with HEA 70:30:1 as solvent. Lipid bands were eluted and duplicate dichromate analyses performed on each eluate. In one case, the phospholipids were separated in duplicate with CMN as solvent, and each band eluted and analysed for phosphorus.

Total lipid extracts from human and rat blood cells and plasma were fractionated directly by TLC. Neutral lipids were separated with HEA 60:40:1 as solvent, and appropriate areas of silica gel were scraped directly into counting vials for radioactive assay. Duplicate separations were performed on each sample. After separation of the phospholipids in the total lipid extracts, by TLC with CMAW as solvent, phosphorus and radioactivity determinations were performed directly on the gel. Duplicate separations were performed for each

type of analysis. Phosphorus recovery after TLC was assessed by measuring the radioactivity in the neutral lipid spot at the solvent front. Knowing the radioactivity distribution between neutral and phospholipid, and the total radioactivity and phosphorus in the samples, the amount of phosphorus applied was calculated and compared with the amount recovered. Although such a determination does not locate the source of any error found, it is a good check on the overall accuracy of all the analyses performed.

Radioactive Counting

A Packard Model 4000 scintillation counter was used for the studies on bovine cells, and a Packard Model 2000 was used for the remainder of the work. Non-aqueous samples were counted in a solution (10 ml) containing PPO (5 g) and POPOP (0.05 g) in toluene (1 l). Aqueous or silicic acid-containing samples were counted in a solution (10 ml) of PPO (5 g) and POPOP (0.2 g) in a mixture of toluene (600 ml) and ethanol (400 ml). Quenching was determined either with standard (^{14}C)-toluene or by the channels ratio method. These methods were equally good measures of solvent quenching, but there was no reliable relationship between channels ratio and the degree of colour quenching. Unquenched efficiences were about 75% in the toluene scintillator, and 60% in the toluene-ethanol scintillator, and background levels were about 20 and 40 counts/min respectively.

The presence of silica gel in the counting vials was found to have no effect on neutral lipid counts. The scintillator was probably sufficiently polar to elute all such lipid, and there was no increased quenching. For phospholipids, however, more quenching was observed when silica gel was present, and even after correction for this, counts were only 90-95 per cent of those in the absence of silica gel. This effect was probably due to some type of self-absorption, arising because the scintillation solution was insufficiently polar to completely elute the lipid from the gel. Samples of phospholipid were counted

both in the presence and absence of silica gel, and the following relative efficienies obtained:

phospholipid and any other components at the origin after TLC separation in HEA;	92 ± 4%
phosphatidyl ethanolamine (separated by TLC in CMAW);	95 ± 4%
phosphatidyl choline (separated by TLC in CMAW).	94 ± 4%

These values were used as correction factors when necessary.

$^{14}CO_2$ evolved during incubations of bovine blood cells was precipitated as $BaCO_3$.[253] The precipitate was collected on a small disc of filter paper and assayed for radioactivity in a liquid scintillator. Rough estimates of quenching were made from the dimensions of the filter paper disc[253], and results are interpreted on a semiquantitative basis only.

Red cell constituents labelled by incubation with ^{14}C-glycine, (see p.49), were analysed for radioactivity by a method based on that of Mahin and Loftberg[254]. Up to 0.1 ml of sample was digested with 72 per cent perchloric acid (0.1 ml) and 30% hydrogen peroxide (0.2 ml) for 1 hr at 70°C, in a tightly stoppered scintillation vial. After cooling, scintillation solution (15 ml) containing PPO (5 g) in a mixture of toluene (600 ml) and ethanol (400 ml) was added. The counting efficiency of the system was approximately 45%. Estimation of ^{14}C-glycine activity by this procedure and in toluene-ethanol-PPO-POPOP revealed that there was no detectable glycine loss during digestion. Digestion was necessary to decolorise the hemoglobin derivatives present and generally solubilise the tissue. No POPOP could be added to the scintillator as it reacts with hydrogen peroxide to give a green product.

Fractionation of Bovine Erythrocytes by Serial Osmotic Hemolysis

Red cell samples were subjected to a series of solutions of progressively decreasing NaCl concentration, using an adaptation of the method of Simon and

Topper[181]. Blood from 2 animals was examined. Red cells were separated from plasma and washed 3 times with isotonic saline. To the packed cells (100-300 ml) was added twice their volume of the least hypotonic NaCl solution (about 0.5 per cent). (Addition of NaCl solution to cells, rather than cells to NaCl solution, ensured that no cells were subjected to greater hypotonicity than any others. Release of contents from lysed cells causes a slight increase in salt concentration). After standing 10 min, the intact cells were sedimented by centrifuging in a swing-out head for 30 min at 2100 rpm (1000xg). The supernatant was separated, the cells washed with isotonic NaCl solution and recentrifuged. At a centrifugal force of 1000xg there was no appreciable sedimentation of ghosts. A NaCl solution of slightly lower concentration was then added to the cells, and the procedure repeated until 6 or 7 fractions had been obtained. Hemoglobin was estimated in each hemolysate and wash.

There was considerable cell swelling in hypotonic solutions, and the swollen cells became very dark and sticky. However on resuspension in an isotonic medium, their normal colour and texture returned. Hypotonic phosphate buffers (pH 7.4) caused the same changes.

Each hemolysate and wash were combined, (total volume 100-1000 ml) and evaporated under vacuum to a volume of 100-200 ml. Frothing of the protein solutions made this procedure very difficult, and temperatures of up to 65° were necessary to bring about evaporation within a reasonable time. Lipid was extracted from the concentrates with 10 volumes of chloroform-isopropanol 7:11 (v/v)

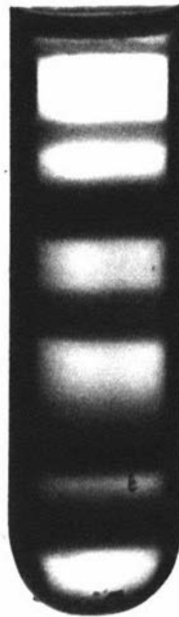
Fractionation of Human and Rat Erythrocytes by Ultracentrifugation over a Discontinuous Albumin Density Gradient

Fractionation Method

The method was basically that of Piomelli, Lurinsky and Wasserman[193], modified for use on a larger scale and with cells from different species. Red

Trapped white cells

Isotonic albumin solution of increasing density



Discrete bands of red cells

Densities of albumin solutions were such that the lightest was intermediate between those of red and white cells (1.075), and the heaviest was greater than that of red cells (about 1.110). Other densities were in the range 1.079 - 1.090, with differences between adjacent solutions being about .002. Washed cells were layered over the albumin gradient and the tube centrifuged in a swing-out head at 25,000 rpm at 4°C for 15 min.

FIGURE 2: A NORMAL RED CELL POPULATION AFTER ULTRACENTRIFUGING OVER A DISCONTINUOUS ALBUMIN GRADIENT

cells were collected, separated from plasma and most white cells, washed twice with and then mixed with isotonic saline to give about a 75 per cent suspension. Six isotonic bovine serum albumin (BSA) solutions were prepared, with densities ranging between 1.075 and 1.110. The lowest density was intermediate between those of red and white blood cells, the highest was greater than that of the heaviest red cells, and the other densities were within the red cell range. A discontinuous density gradient was prepared at 4°, by carefully layering these solutions in a 30 ml cellulose nitrate centrifuge tube. Interfaces between the albumin solutions were clearly visible. The cell suspension was carefully layered over the albumin, and the tube centrifuged at 4°, in a Spinco Model L Ultracentrifuge, for 30 min at 25,000 rpm in a swing-out head (SW25). Up to 3 ml packed cells could be applied per tube without overloading.

After centrifuging, provided the conditions were right, the tube contained 5 red cell bands between the albumin layers, no sedimented cells, and a film of white cells above the top albumin layer (Fig. 2). The different red cell bands were collected separately by piercing the bottom of the tube and forcing the contents to drip out. Albumin was separated from the cells by mixing with an equal volume of isotonic saline and centrifuging for 10 min at 4000 rpm. The cells, after washing twice with saline, were ready for further study.

Preparation of Isotonic Albumin Solutions

BSA powder was dissolved in water to give an approximately 40 per cent solution. The osmolality and density of the solution were measured, and the osmolality made up to 290 m Osm by adding solid NaCl*. The osmolality was

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*To calculate the amount of NaCl to add, it is necessary to know the amount of water in the solution, (since osmolalities are measured per kg solvent). This can be calculated from the solution density (d), volume (V) and the volume occupied per g albumin. This latter value was calculated (from the relationship between albumin concentration, determined from absorbance measurements at 280 mμ,

checked and if necessary slightly modified, and the density redetermined. Solutions of the required densities were prepared by diluting the bulk albumin with 0.92 per cent (290 m Osm) NaCl solution. The albumin concentration in these solutions was 30-40 per cent.

Densities were measured at 20°C, in a pyknometer standardised by weighing water. The density of 0.92 per cent NaCl was 1.005 g/ml and there was a linear relationship between albumin concentration and solution density.

Osmolalities were measured with a Fiske osmometer. Measurements could not be made on the concentrated albumin solutions, because of freezing problems. Values were obtained for serial dilutions with water, but when dilution factors were accounted for, it appeared that the higher dilution, the lower the apparent osmolality of the undiluted albumin. Such behaviour suggested that some interaction between albumin molecules was contributing to the measured osmolality value. An effect of this type would be more significant at higher concentrations.

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and solution density), to be 0.73 ml/g, which agrees with the value determined by Piomelli et al[193]. The general relationship is:

$$\text{Total volume } V = V_W + V_A$$

$$\text{Total weight } W = d \times V = W_W + W_A$$

where V_W = volume of water

W_W = weight of water = V_W

V_A = volume of albumin

W_A = weight of albumin

and $$V_A = 0.73\, W_A$$

$$\therefore \quad V_W = \frac{V(1 - .73d)}{.27}$$

Knowing the water volume, osmolality difference, and that a 0.54 mM NaCl solution corresponds to a 1 m Osm solution, the NaCl requirement can be calculated.

The nature of this effect has been investigated, and a means of correcting measurements to give true osmolalities proposed:

Consider $M = T + aC^2$

where M = measured apparent osmolality

T = true osmolality

C = albumin concentration

aC^2 = term involving interactions between albumin molecules

The possibility of such a relationship was examined for two albumin solutions, both of different osmolality but the same concentration (36% albumin, d = 1.100). Serial dilutions were made, and osmolalities measured (Table 4).

Since $T : T_1 : T_2 : T_3 : T_n = 1 : D_1 : D_2 : D_3 : D_n$

where the dilution is 1 : n

$$\text{then } M_n = \frac{1}{D_n} \times T + aC_n^2$$

$$\text{or } D_nM_n = T + aD_nC_n^2$$

Hence a plot of D_nM_n v. $D_nC_n^2$ should be linear with slope a and intercept T. Such plots have been made for these two examples (Fig. 3). This approximation appears to hold fairly well, since straight lines, with the same slope, can be drawn through both sets of points. Other albumin solutions have been examined and the same relationship holds for these also. In addition, when osmolalities calculated in this way are used to estimate the amount of NaCl required to bring a solution to a desired osmolality, good agreement between the amount of NaCl predicted and that actually required is obtained. When no correction is made, these values vary widely. It would appear, therefore, that some interaction between albumin molecules does affect osmolality measurement, and that by making an approximate correction for this effect, true osmolalities can be estimated. The necessary corrections at different albumin concentrations have been calculated from Fig. 3 and are shown in Table 5.

Albumin of the quality required for density gradient centrifugation

Table 4. Effect of albumin concentration on apparent osmolality

	Dilution	Water Dilution Factor D	Albumin Concentration C	Apparent Osmolality M
Solution A	0	-	36	not measurable
	1:1	$\frac{1.74}{.74}$	18	109 ± 1
	1:2	$\frac{2.74}{.74}$	12	65 ± 1
	1:3	$\frac{3.74}{.74}$	9	45 ± 1
Solution B	0	-	36	not measurable
	1:1	$\frac{1.74}{.74}$	18	115 ± 1
	1:2	$\frac{2.74}{.74}$	12	70 ± 1
	1:3	$\frac{3.74}{.74}$	9	49 ± 1
	1:4	$\frac{4.74}{.74}$	7.1	37 ± 1

Table 5. Differences between true and measured osmolalities in albumin solutions

Albumin concentration (per cent)	Osmolality difference (m Osm)
18	23
12	10
9	6
7	5

is comparatively expensive, but it was possible to recover it for reuse after each cell fractionation. Recovered solutions were largely freed from salts by dialysing for 1 day against at least 2 changes of water, and then freeze-dried. The albumin powder was redissolved, and solutions were stored at $-15^{o}C$. Reusing albumin had no obvious adverse effects on cell fractionation.

Piomelli et al[193] found that the use of Fraction V BSA powder, rather

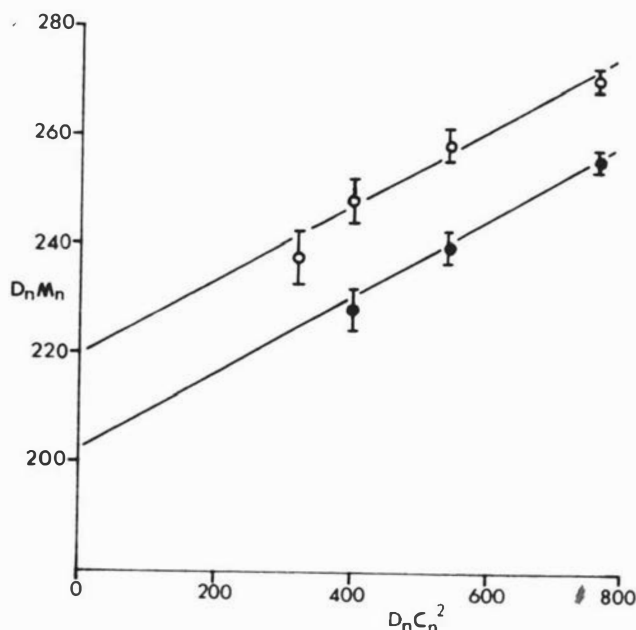

Figure 3: Variation in Apparent Osmolality with Albumin Concentration

than crystalline albumin, caused significant hemolysis of the oldest cells and less reproducibility. No such hemolysis could be demonstrated in the present study, perhaps because of differences in purity in the two commercial Fraction V albumin preparations.

Collection of Cell Fractions

After ultracentrifugation, each tube was stoppered and held firmly with its base resting on the top of a short, narrow polythene tube. An inlet through the stopper was connected to a variable pressure head, and the bottom of the tube was pierced with a syringe needle. The tube contents were allowed to drip slowly through the narrow polythene tube. Clearly defined bands were cleanly separated, provided the drop rate was slow enough to prevent streaming down the centre of the tube, and the temperature was kept constant to prevent

convective mixing. Cells did not stick to the walls of the centrifuge tube, and sedimented cells were not displaced.

Critical Factors in the Fractionation

(i) Osmolality. It is extremely important that solutions be strictly isotonic. Any deviations cause cells to shrink or swell, and hence change in density. The relationship between age and density for shrunken or swollen cells appears different from that for normal cells[257].

(ii) Temperature. The temperature dependence of cell density and albumin solution density is not the same, hence it is essential that the same constant temperature is maintained for all fractionations. Temperature changes are generally non-uniform throughout a solution, and cause convective mixing, so must be avoided from the time the gradient is prepared until the fractions are separated.

(iii) Density. The range of red cell densities in one population is very small. A density variation of .01 practically covers the whole range and four solutions within this range are required to produce five red cell bands. Small changes in albumin density therefore cause considerable variation in cell distribution. Density determinations are accurate to within ± .0005, but even this, combined with inevitable slight density changes during gradient preparation, and probable slight density variation between different cell populations, make it impossible to design a system yielding 5 equal red cell bands.

To reduce this problem, a preliminary run was always carried out on a small scale before a large scale fractionation; (3 ml tubes were centrifuged at 35,000 rpm in an SW39 head for 10 min). If an uneven cell distribution resulted, albumin solutions were modified, and the chances of getting 5 similarly sized bands of red cells greatly improved.

Distribution of Rat Red and White Cells in the Albumin Gradient

The densities of albumin solutions required for separation of rat cells

were in the range 1.090 - 1.075. White cells were concentrated above the d = 1.075 solution. Rough measurements indicated, in a case where most of the white cells had been separated prior to fractionation, that about 90 per cent of the remainder were in this layer, with the others in the top red cell layer. (i.e. Only 0.1 - 0.5 per cent of the original white cells were present in red cell fractions).

Reproducibility of red cell distribution is influenced both by slight variations in solution densities and differences between individuals. An example of reproducibility is given in Table 6. These results suggest that differences between individual rats are not very great.

Distribution of Human Red and White Cells in the Albumin Gradient

The albumin solutions necessary for human erythrocyte fractionation fell within the density range 1.090 - 1.078. Even allowing for some variation due to uncertainty of solution densities, it would appear that some difference does exist in erythrocyte density distribution between individuals (Table 7).

The behaviour of human white cells was unexpected. They tended not to collect in the $d < 1.075$ band, but rather in a band lying within the range of red cell densities. This phenomenon was independent of whether or not most of the white cells were removed prior to fractionation and consequently could not be attributed to a small atypical fraction of cells. Table 8 gives the results obtained for two cell samples. The following are possible reasons for this behaviour:

(1) white cells have a density within the red cell range;

(2) fractionated white cells are non-viable, and the density of non-viable cells is greater than that of viable ones;

(3) white cells either ingest or adsorb albumin molecules, causing their density to change.

The first appears unlikely because after normal centrifugation, the bulk

Table 6. Distribution of rat erythrocytes in albumin density gradients

Density	Per cent of cells in fraction		
	Rat A	Rat B	Rat C
1.095 - 1.085	24	23	38
1.085 - 1.083	38	35	36
1.083 - 1.080	27	29	16
1.080 - 1.075	10	10	6
< 1.075	1	2	3

Table 7. Distribution of human erythrocytes in albumin density gradients

Density	Per cent of cells in fraction						
	EJC	GGM	GL	VJC	CCW	MJW1	MJW2
> 1.090							17
1.090 - 1.088							20
1.088 - 1.086				9	16	22	30
1.086 - 1.084	4	14	6	11	20	21	15
1.084 - 1.082	11	16	19	17	20	29	22
1.082 - 1.080	31	23	25	22	28	17	
1.080 - 1.078	44	25	40	42	16	11	
1.078 - 1.075	10	22	5				

Table 8. Distribution of human white cells after albumin gradient fractionation

Albumin density	WRS per cent red cells	WRS total white cells x 10^{-5}	Albumin density	VJC per cent red cells	VJC total white cel x 10^{-6}
1.102 - 1.085	14	1.1	1.100 - 1.086	8	9
1.085 - 1.083	14	0.7	1.086 - 1.084	11	1.2
1.083 - 1.081	12	0.9	1.084 - 1.082	17	0.6
1.081 - 1.079	28	2.4	1.082 - 1.080	22	0.5
1.079 - 1.075	25	10	1.080 - 1.075	42	0.4
1.075	3	2.4			

of the white cells were clearly visible at the top of the cell column. Likewise the second, since freshly drawn blood behaved in this way, and also the fractionated cells were metabolically active as shown by their uptake of long-chain fatty acids. Considering the third possibility, it can be calculated that a white cell density change from 1.075 to 1.087, would be effected by an uptake of 2.2 μg albumin (d = 1.37) per 10^5 white cells (volume 500 μ^3), i.e. approximately 10^8 molecules per cell.

The difficulties presented by the comparable positioning of red and white cells in an albumin gradient were minimised by prior removal of the bulk of the white cells. In addition, if the highest albumin density just exceeded red cell densities, the heavier white cells sedimented completely. Under these conditions, white cell contamination of red cell preparations could be reduced to a very low level.

Examination of the Relationship between Density and Age in Rat Red Cells

A blood sample was incubated with [2-^{14}C]glycine to produce labelled reticulocyte proteins. The cells were reintroduced into the rat, and further blood samples were withdrawn at intervals following the administration. The red cells in each blood sample were fractionated by the albumin gradient procedure, and the total protein radioactivity in each fraction determined.

Rat blood (4 ml) was collected into heparin, and incubated with [2-^{14}C]glycine (10 μc) at 37° for 4 hr. The red cells were separated by centrifuging at 4° for 15 min at 3000 rpm, washed once with cold 0.9 per cent NaCl, and resuspended in saline (hematocrit about 75). Sterile equipment and solutions were used, and care was taken with manipulations to reduce the likelihood of contamination. Of the [2-^{14}C]glycine, 36 per cent was incorporated into the red cells, 38 per cent of this being associated with cellular protein. The red cell suspension, containing approximately 1.6 ml packed cells and 0.5 μC radioactivity in the cellular protein, was reinjected into a branch of the jugular vein of the same rat.

Blood samples (1-2 ml) were withdrawn from the rat at intervals after injection of the labelled reticulocytes. Washed red cells were fractionated by the albumin density gradient procedure, using 3 ml centrifuge tubes and spinning at 35,000 rpm for 15 min. Fractions were collected and the cells washed free of albumin. Hemoglobin, total radioactivity and protein radioactivity were estimated in the normal cell population and in each fraction.

Proteins were precipitated from cell lysates with 10 per cent trichloroacetic acid (TCA) and the precipitate was washed once with distilled water, digested, and assayed for radioactivity (see p.39).* Precipitation of protein and heme appeared complete, as no colour remained in the supernatant. The absence of radioactivity in the supernatants from cell samples in which only proteins should have been labelled, supports this interpretation.

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*Sephadex column chromatography and paper electrophoresis were initially examined as methods for protein separation, but a hemoglobin precipitate remained at the origin during electrophoresis, and formed an impenetrable layer at the top of the Sephadex column. Although the precipitate could be dissolved above pH 9, TCA precipitation was considered to be the most satisfactory method to adopt.

INCORPORATION OF $[1-^{14}C]$PALMITATE INTO THE LIPIDS OF BOVINE BLOOD CELLS*

This investigation was undertaken primarily to examine fatty acid uptake by erythrocyte neutral lipids, with a view to detecting a possible physiological role for these constituents. Mulder and van Deenen[102] and Oliviera and Vaughan[40], while investigating the uptake of fatty acids into red cell phospholipids, detected little incorporation into esterified neutral lipids, but the only detailed study has been that of Donabedian and Karmen[114], who reported high and variable incorporations into triglycerides in human cells.

Palmitate uptake by leukocyte populations was investigated basically because it was found to be necessary to account for the presence of leukocytes in erythrocyte preparations.

Preparations of bovine erythrocytes, containing varying numbers of leukocytes, were incubated either in plasma or Krebs-Ringer-Phosphate medium (KRP) with $[1-^{14}C]$palmitic acid. Lipids were subsequently extracted from washed red and white cells, separated and analysed. White cell contributions to uptakes by red cell preparations were estimated, and palmitate uptake into the individual components of each cell type assessed.

Incorporation into Leukocytes

The leukocytes showed a very high incorporation of $[1-^{14}C]$palmitate into their lipids (Table 9). In these results, account has been taken of the presence of red cells in white cell preparations from which lipids were extracted, (red cell: white cell ratios varied from 2:1 to 20:1). These levels of red cells made negligible contributions to radioactivity uptake, but corrections for red cell contributions to the extracted lipids have been made. It was possible to correct with reasonable accuracy, as white cells contain about 50 times the lipid present

*The results of this work have been published (ref. 255).

in red cells. Platelets were present but not enumerated.

Phospholipids and triglycerides were responsible for the majority of the uptake, the phospholipids attaining the highest total activity and the triglycerides the highest specific activity. Autoradiographs of the lipids separated by TLC (Figure 4) show these major spots, as well as faint cholesterol ester and diglyceride spots, but no radioactive cholesterol. Diglyceride is therefore primarily responsible for the radioactivity of samples containing both cholesterol and diglyceride, which could not be readily separated before analysis. The other, slow-running, radioactive components were not detectable gravimetrically and were not identified. One could be monoglyceride. Palmitate uptake by the phospholipids is summarised in Table 10, and an autoradiograph of a TLC separation of the phospholipids is shown in Figure 4. Of the phospholipids, phosphatidyl choline incorporated the most radioactivity, followed by sphingomyelin. It seems unlikely that the sphingomyelin radioactivity could be attributed to lysophosphatidyl ethanolamine (which could exhibit similar TLC mobilities in both solvent systems), in view of the very low incorporation into phosphatidyl ethanolamine, one of the major phospholipid components. The TLC behaviour of the remaining radioactive components suggests that one is lysophosphatidyl choline (running behind sphingomyelin in both solvents), and the other, phosphatidyl serine.

A further leukocyte sample, which was held for 8 hr at room temperature after collection, was incubated with palmitate. Incorporation was only 0.3 times that observed with fresh cells, and reduction of uptake into triglycerides was more pronounced than into phospholipids.

It is possible to estimate approximate times required to renew the palmitate in the different leukocyte lipids, by uptake of the free acid. Fatty acid distributions in the individual lipid components are not known, but by taking palmitate as comprising 25 per cent of the component fatty acids in each case, estimates can be made (Table 11). High turnover rates for some white cell lipids,

in particular triglyceride and phosphatidyl choline, are evident.

Only 65 per cent of the labelled fatty acids extracted from the cells was palmitic acid. The remainder were longer chain (mainly C_{18}) acids. Some radioactivity was also recovered as $^{14}CO_2$. A qualitative estimation showed that this amounted to roughly 20 per cent of the ^{14}C incorporated into the total leukocyte lipid.

After incubations were complete, plasma lipids were analysed for radioactivi Although reductions in the specific activities of the plasma free fatty acid were demonstrated, no decreases in fatty acid concentrations could be shown. The analyses may not have been sufficiently sensitive to detect small gravimetric changes. These results suggest that there is a mechanism for exchange of fatty acids between plasma and cellular lipids. This interpretation is supported by gas chromatographic analysis of the free fatty acids extracted from plasma after incubation with leukocytes. Only 70 per cent of the labelled fatty acid was palmitic, the remainder being longer chain (mainly C_{18}) acids. Therefore leukocytes can apparently synthesise labelled long chain acids from palmitic, and release these into the plasma. About 5 per cent of the plasma radioactivity was found in the phospholipids, which suggests that the white cell is able to release intact phospholipid molecules.

Discussion of Leukocyte Behaviour

No other studies on the uptake of plasma fatty acids by bovine blood leukocytes have been reported, but similar studies on leukocytes from some other animals and sources have been carried out[207,208,222,223]. When comparing the present results with these, it should be remembered that white cell populations are heterogeneous, and that their behaviour can depend on both the source of the cells and the treatment given them[58]. The distribution of radioactivity found in bovine blood leukocyte lipids is similar to that in human blood cells after incubation with labelled palmitate[208]. It is also similar to the distribution

Table 9: Radioactivity Distribution in the Lipids of Bovine White Cells after Incubation with [1-^{14}C]Palmitic Acid

	Per cent of total lipid weight	Per cent of total lipid radioactivity	dpm per 10^6 cells	Specific activity (dpm/mg x 10^{-3})
TL*			1300 ± 150	43 ± 8
FFA	7 ± 2	14 ± 1	180 ± 20	86 ± 5
PL	58 ± 3	49 ± 3	640 ± 80	37 ± 3
TG	8 ± 1	30 ± 3	390 ± 60	160 ± 30
CE	6 ± 1	1 ± 0.3	13 ± 4	7 ± 2.5
Ch+DG	21 ± 1	6 ± 2	80 ± 30	13 ± 4**

* 300 ± 50 mg/10^{10} cells.

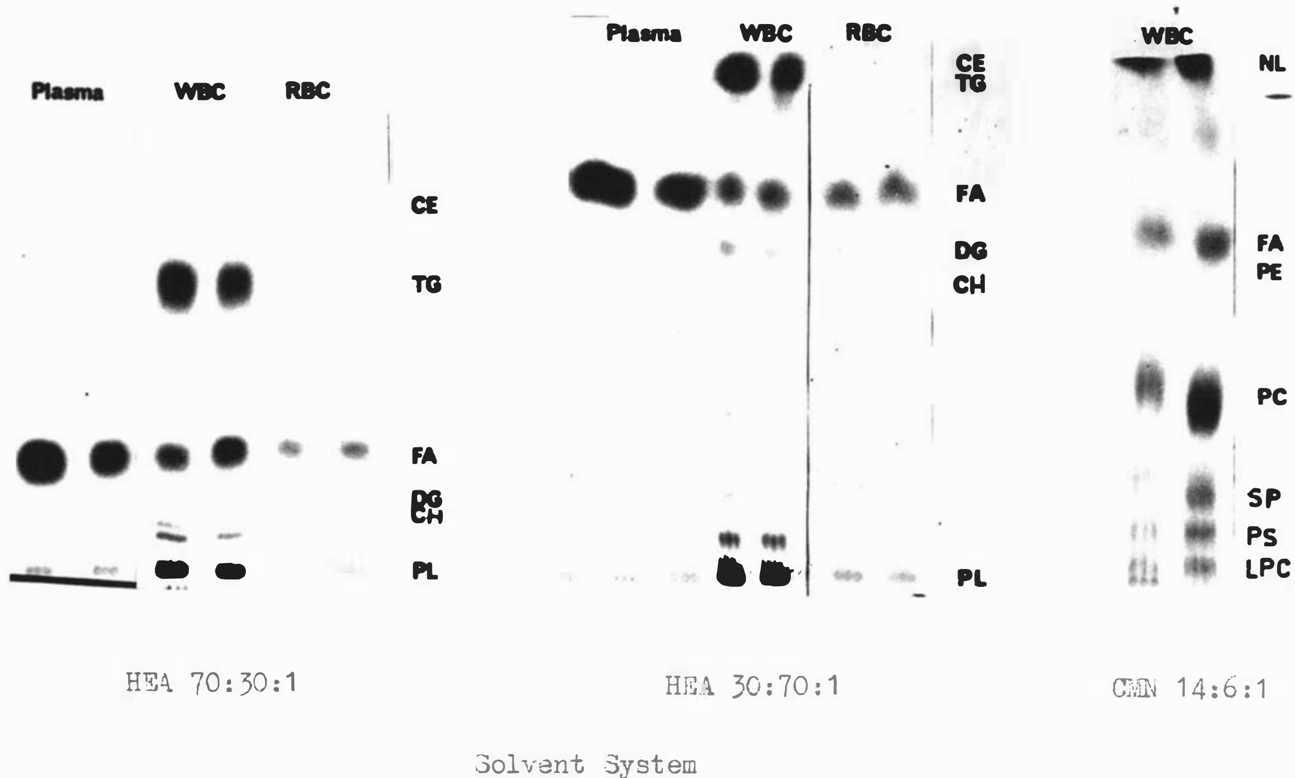
**The specific activity of pure DG would be considerably higher than this. Abbreviations are tabulated in Appendix 2. For incubation conditions see p. 28 . White cell concentrations varied between 5 x 10^6 and 50 x 10^6 cells per ml; higher concentrations resulted in slightly lower radioactivity incorporations per cell. From 9 to 63 per cent of the medium activity was incorporated into the cells. The total lipid was extracted, and separated in duplicate by TLC with HEA 70:30:1 as solvent. Results are the means (± 1 standard deviation) from 5 incubations of cells from 2 donors, and are related to a medium having an initial [1-^{14}C]palmitate activity of 10^5 dpm/ml, which corresponds to a palmitic acid specific activity of 8.0 x 10^5 dpm/µeq.

Table 10: Radioactivity distribution in the Phospholipids of White Cells after Incubation of the Cells with [1-^{14}C]palmitate

Phospholipid activity: 6400 dpm per 10^6 cells

	Per cent of Phosphorus	Per cent of activity
PE	18	4.2 ± 0.6
PS	12	6 ± 2
PC	29	60 ± 9
SP	32	20 ± 3
LPC and other components running behind SP	9	10 ± 3

Lipids were separated by TLC, solvent system CMAW 65:25:8:4. Results are the means (± 1 standard deviation) from 4 experiments.



The identification of PS and LPC is only tentative. Abbreviations are tabulated in Appendix 2.

FIGURE 4: AUTORADIOGRAPHS OF TLC SEPARATIONS OF TOTAL LIPIDS FROM BOVINE ERYTHROCYTES (RBC), LEUKOCYTES (WBC) AND PLASMA

by a chain-elongation of pre-existing fatty acids.

Incorporation into Erythrocytes

The lipid composition of bovine erythrocytes is given in Table 12. This is similar to that obtained by de Gier and van Deenen[64], and Hanahan, Watts and Pappajohn[59], although the latter report slightly higher triglyceride and cholesterol ester levels. Nelson[70] reports similar phospholipid and cholesterol levels, but his results suggest the absence of cholesterol esters and triglycerides from mammalian erythrocytes. Incorporation of radioactivity into the lipids of the red cell preparations is summarised in Table 13, and autoradiographs of TLC separations of the lipids are shown in Figure 4. Albumin, which binds almost all the plasma free fatty acids, appeared to have no effect on fatty acid uptake by the esterified lipids in these experiments. Although the greatest proportion of red cell radioactivity was present as unesterified fatty acid, comparatively high counts were recorded in the phospholipids. A significant amount of radioactivity was associated with the triglyceride fraction and only low activities were found in other fractions. These results show relatively high specific activities, and hence high incorporation rates, for minor components such as triglycerides.

However, 1-5 per cent of the original white cells were extracted along with the red cells, and, as shown in Table 14, these were responsible for a significant proportion of the radioactivity in the red cell extracts. When allowance was made for this contribution from the white cells (Tables 15 and 16), the incorporation of fatty acids into the phospholipids of red cells was reduced but was still significant. Corrected values showed no significant incorporation into triglycerides, and the amount of radioactivity in other neutral lipids (excluding free fatty acids) was so low as to be within the experimental error. Whether there was uptake into diglyceride, or diglyceride activity was an artifact arising from trailing free fatty acids, cannot be resolved, as radioactivity levels were too low to show on autoradiographs.

Contributions by white cells to individual phospholipid activities in red

cell extracts are shown in Table 17. Most components contained low activity and were subject to considerable correction for white cells, and accordingly the results are liable to comparatively large errors. The mean radioactivity distribution in the red cell phospholipids is given in Table 18, and this generally agrees with that of Mulder and van Deenen[102], who also found that most of the labelled fatty acids incorporated into bovine erythrocytes were present in the phosphatidyl choline and phosphatidyl ethanolamine fractions.

Approximate times requires for renewal of all the palmitate in the erythrocyte phosphatidyl choline and phosphatidyl ethanolamine, (the only components showing significant uptake), by uptake of the free acid from plasma can be calculated, (Table 19). Turnover of these components was about 20 times slower than for analagous white cell lipids. This suggests that under physiological conditions, the bovine erythrocyte could turn over the palmitate in its phosphatidyl choline several times during its lifespan (100 days). In the same time, palmitate in phosphatidyl ethanolamine could turn over less than once.

With higher concentrations of white cells in the incubation system, there was a decrease in the amount of radioactivity incorporated into the erythrocyte lipids (Table 20). Compensation for this decrease could be effected by relating uptakes to the amount of radioactivity added, after correcting for the amount taken up by white cells. The lower relative incorporations with higher white cell counts could have arisen from either (a) a reduction in substrate specific activity due to exchange of inactive white cell fatty acids for labelled palmitate, or (b) a reduction in substrate concentration due to net fatty acid uptake by the white cells. Whereas no gross reductions in plasma free fatty acids were measured, evidence was obtained for the exchange of white cell and plasma fatty acids, and accordingly the first alternative would appear more likely.

After one incubation, the total erythrocyte fatty acids were separated by gas chromatography and the radioactive components isolated. The fatty acids present

were mainly oleic (40%), palmitic (24%), and stearic (20%) acids. Of the radioactivity, 99% was still associated with palmitic acid. Hence in agreement with the results of Mulder and Van Deenen[102], on incorporation of linoleic acid into rabbit erythrocytes, no evidence for any active fatty acid chain altering processes in the erythrocyte could be demonstrated.

The autoradiographs in Figures 4a and 4b also show the radioactivity distribution in the plasma lipids in 2 experiments. About 1-4 per cent of the plasma activity was present in the phospholipids, and this varied only slightly with the number of white cells incubated. Most of the phospholipid activity was in each case associated with phosphatidyl choline. Plasma alone is unable to incorporate labelled fatty acid into phospholipid[102], and as only a small increase in labelled plasma phospholipid was associated with a large increase in white cell numbers, the ability to exchange intact phospholipid molecules appears to be a property of both cell types. This result supports previous evidence for exchange of some red cell and plasma lipids[94,105-107].

The present study has shown that the presence of comparatively small numbers of leukocytes in erythrocyte preparations can account for a high proportion of the observed incorporation of plasma fatty acids into the cell lipids. An apparently high rate of fatty acid incorporation into red cell triglycerides can be interpreted entirely in terms of contamination by only 2-5 per cent of the white cells normally present in blood. Such a result stresses the advisability of determining the extent of leukocyte presence in red blood cell samples before interpreting results of experiments in terms of red cell properties alone. A similar situation arose in the evaluation of erythrocyte synthesis from acetate, when it was shown that leukocyte contamination was responsible for the observed lipid synthesis[89-92].

Table 12: Lipid Composition of Bovine Erythrocytes

	Per cent of total weight	mg/10^{10} cells	mg/ml packed cells
TL		4.4 ± 0.2	4.0 ± 0.2
PL	70 ± 3	3.1 ± 0.2	2.8 ± 0.2
Ch	27 ± 1	1.20 ± 0.07	1.08 ± 0.07
CE	1.1 ± 0.7	0.048 ± 0.03	0.043 ± 0.03
TG	1.0 ± 0.7	0.044 ± 0.03	0.040 ± 0.03
FFA	1.2 ± 0.8	0.053 ± 0.03	0.048 ± 0.03

Lipids were extracted, and either separated by column chromatography into neutral and phospholipids and the neutral lipids separated further by TLC in HEA (70:30:1), or separated directly by TLC. 6 extracts were separated, analysed, each in duplicate, and results quoted are the means of these, ± 1 standard deviation.

Table 13: Radioactivity Distribution among the Lipids Extracted from Red Blood Cell Preparations after Incubation with [1-^{14}C]Palmitic Acid

	dpm per 10^{10} red cells									Range of specific activities (dpm per r
	A	B	C	D	E	F	G	H	J	
TL	7200	7700	5400	5000	5000	4800	11600	7900	7600	100–180
FFA	4500	4100	3100	3800	3800	3400	8700	6300	5900	3000–2000(
PL	2100	2700	1500	900	950	1150	920	930	1500	30–90
TG	410	580	230	150	130	110	320	340	35	140–1500
CE	70	54	47	25	25	24	66	90	24	20–250
Ch + DG	190	310	280	150	75	140	200	160	110	6–30
WBC extracted per 10^{10} RBC x 10^{-6}	1.33	1.2	0.55	0.29	0.26	0.25	Not determined			

For incubation conditions, see p. 28. The specific conditions for each experiment were as follows:

A : All but 5% of the white cells present in the original blood were separated prior to incubation.

B : Red cells were incubated with 5 times the number of white cells normally present in blood. All but 1% of these cells were separated after incubation.

C–F : Whole blood was incubated. 1–3% of the white cells were extracted.

G–J: White cells were separated before incubation. No cell counts were made but 1–5% of the original white cells were probably incubated and extracted.

J : Cells were incubated in a KRP medium.

Each experiment is shown separately, to show the dependence of uptake on the degree of white cell contamination. Lipids were separated in duplicate by TLC (solvent HEA 70:30:1). Uptakes are related to a medium having a /1-^{14}C/palmitate activity of 10^5 dpm/ml, (palmitate specific activity 8.0 x 10^5 dpm/μeq) at the end of the incubation. About 2% of the total radioactivity was recovered in the cell lipids.

Table 14: Radioactivity in the Red Cell Preparation Lipids Attributable to Extracts from White Cells

	dpm due to white cells per 10^{10} red cells					
	A	B	C	D	E	F
TL	1700 ± 140	3500 ± 230	1000 ± 70	450 ± 30	390 ± 30	370 ± 26
FFA	270 ± 50	290 ± 40	160 ± 25	80 ± 10	76 ± 10	35 ± 4
PL	880 ± 100	1900 ± 190	440 ± 40	240 ± 30	170 ± 20	180 ± 20
TG	490 ± 80	1000 ± 230	250 ± 30	110 ± 20	130 ± 50	115 ± 20
CE	20 ± 12	32 ± 27	17 ± 5	4 ± 3	6 ± 6	4 ± 3
Ch + DG	110 ± 30	160 ± 35	110 ± 20	30 ± 6	25 ± 14	20 ± 8

Values are calculated from the known number of white cells contaminating red cell preparations, and the radioactivity uptake by white cells from a medium having a /1-^{14}C/palmitate activity of 10^5 dpm/ml, and are quoted ± 1 standard deviation. Results given in this table and Table 13 are from the same experiments.

Table 15: Radioactivity Incorporated into Pure Erythrocyte Lipids, after Incubation of the Cells with /1-^{14}C/Palmitate

	dpm per 10^{10} red cells					
	A	B	C	D	E	F
TL	5500	4200	4400	4500	4600	4400
FFA	4200	3800	2900	3700	3700	3400
PL	1200	800	1100	650	800	950
TG	-80	-400	-20	40	-	-
CE	50	20	30	20	20	20
DG + Ch	80	150	170	120	50	120
Percentage of total activity due to WBC	24	45	19	9	8	8

Values are calculated from the data in Tables 13 and 14.

Table 16: Radioactivity Distribution in Pure Erythrocyte Lipids after Incubation of the Cells with [1-^{14}C]Palmitate

	Per cent of total lipid radio-activity	Per cent of esterified lipid radioactivity	dpm/10^{10} cells	Specific activity dpm/mg
TL			4700 ± 30	1000 ± 100
FFA	78 ± 5		3600 ± 200	43000 ± 15000
PL	20 ± 4	91 ± 5	900 ± 160	300 ± 70
TG	- ± 0.5	- ± 2	- ± 20	-
CE	0.5 ± 0.5	2 ± 2	20 ± 20	300 ± 300
CH+DG	2.5 ± 1.1	11 ± 5	120 ± 50	100 ± 60

Values are the means of the results from the 6 studies given in Table 15. They are related to an external [1-^{14}C]palmitate concentration of 10^5 dpm/ml, and are expressed ± 1 standard deviation.

Table 17: Distribution of [1-^{14}C]Palmitic Acid in the Phospholipids extracted from Red Cell Preparations, with Corrections for White Cell Contributions.

		Percentage distribution	Total dpm/ 10^{10}RBC	WBC contribution	RBC dpm/ 10^{10}RBC
Incubation A	PL		2100	900	1200
	PE	12 ± 7	250	30	220 ± 150
	PS	8 ± 5	170	60	110 ± 100
	PC	67 ± 3	1400	500	900 ± 150
	SP	10 ± 6	210	180	30 ± 50
	LPC + others	3 ± 3	60	110	- ± 60
Incubation D	PL		900	250	650
	PE	11 ± 6	100	10	90 ± 50
	PS	8 ± 4	70	10	60 ± 30
	PC	65 ± 4	580	130	450 ± 60
	SP	4 ± 3	36	50	- ± 40
	LPC + others	11 ± 6	100	40	60 ± 60
Incubation E	PL		950	150	800
	PE	22 ± 10	210	10	200 ± 100
	PS	5 ± 5	50	10	40 ± 40
	PC	51 ± 10	480	90	390 ± 80
	SP	16 ± 10	150	40	110 ± 60
	LPC + others	7 ± 7	70	20	50 ± 50
Incubation F	PL		1150	200	950
	PE	8 ± 5	90	10	80 ± 50
	PS	7 ± 4	80	20	60 ± 30
	PC	52 ± 5	600	100	500 ± 50
	SP	15 ± 6	170	40	130 ± 50
	LPC + others	18 ± 7	210	20	190 ± 70

Phospholipids were separated by TLC with CMAW 65:25:8:4 as solvent.

Table 18: Distribution of $[1-^{14}C]$Palmitic Acid Incorporated into Bovine Red Cell Phospholipids

	Per cent of Phosphorus	Per cent of activity
PE	23 ± 2	22 ± 12
PS	15 ± 2	6 ± 4
PC	4 ± 1	60 ± 9
SP	57 ± 3	6 ± 6
LPC + others	1 ± 1	7 ± 7

Values are the means of the results of the 4 incubations given in Table 17, and are quoted $\pm$ 1 standard deviation.
Phosphorus distribution values are the means of 8 analyses, performed during the course of the study.

Table 19: Estimation of Rates of Turnover of Palmitate in Bovine Erythrocyte Lipid Components.

Component	Activity taken up in 5 hr counts/min/10^{10} RBC	Palmitate taken up in 1 hr μeq/10^{10} RBC	Fatty acid in component μeq/10^{10} RBC	Palmitate in component μeq/10^{10} RBC*	Approximate renewal time (days)
PC	550	1.4×10^{-4}	0.3	0.1	30
PE	200	0.5×10^{-4}	1.8	0.2	170

*Fatty acid distributions taken from de Gier and Van Deenen[64].
Specific activity of the palmitate in the medium was approximately 8.0×10^5 dpm/μeq.

Table 20: Effect of Increasing White Cell Concentration in the Incubation System on Uptake of $[1-^{14}C]$Palmitate into Red Cell Phospholipids

	Incubation B	D	C	A	E
WBC incubated per 10^4 RBC	14	170	320	260	1500
Per cent total activity incorporated into WBC	-	9	15	17	63
RBC PL dpm/10^{10} cells, (i) related to total activity in system	1200	750	650	550	280
(ii) related to total less that taken up by WBC	1200	800	800	650	800

For descriptions of the conditions for each incubation, see footnote to Table 13.

THE LIPIDS OF BOVINE ERYTHROCYTES, SEPARATED ACCORDING TO AGE BY SERIAL OSMOTIC HEMOLYSIS

No role for the small quantities of cholesterol esters and triglycerides, apparently present in mammalian erythrocytes, has been established. In the previous chapter it was not possible to show any significant fatty acid uptake into these components, so it is unlikely that they are intermediates in the metabolism of other lipids. Another hypothesis is that triglycerides or cholesterol esters are present in the cell as a small but important energy reserve, and that breakdown of one of these components could be coupled with production of energy necessary for maintaining cell membrane functions. The requirements of such a hypothesis would be that cellular concentrations of the reserve components should decrease with cell age.

Two studies on variation in lipid content with age of erythrocytes separated by normal centrifugation[200] and ultracentrifugation[201] have been reported, but no analyses of minor neutral lipids have been made. The present study was therefore undertaken with the primary intention of examining variation in triglyceride and cholesterol ester levels in erythrocytes of different ages.

The Extent of Age Separation by Serial Osmotic Hemolysis

Correlation between osmotic fragility and erythrocyte age has been clearly demonstrated[25,178,179], and it is a common hematological procedure in the diagnosis of anemias to use osmotic fragility curves as indicators of abnormal red cell age distribution[256]. The normal procedure for measuring osmotic fragility involves the addition of cells to a range of hypotonic salt solutions and measurement of the relative proportions of intact to lysed cells in each solution. This procedure effectively shows fragility differences, but does not actually separate the different populations of cells.

Simon and Topper[181] have modified the procedure to allow cell separation by serial osmotic hemolysis. Cells lysed by the least hypotonic solution are

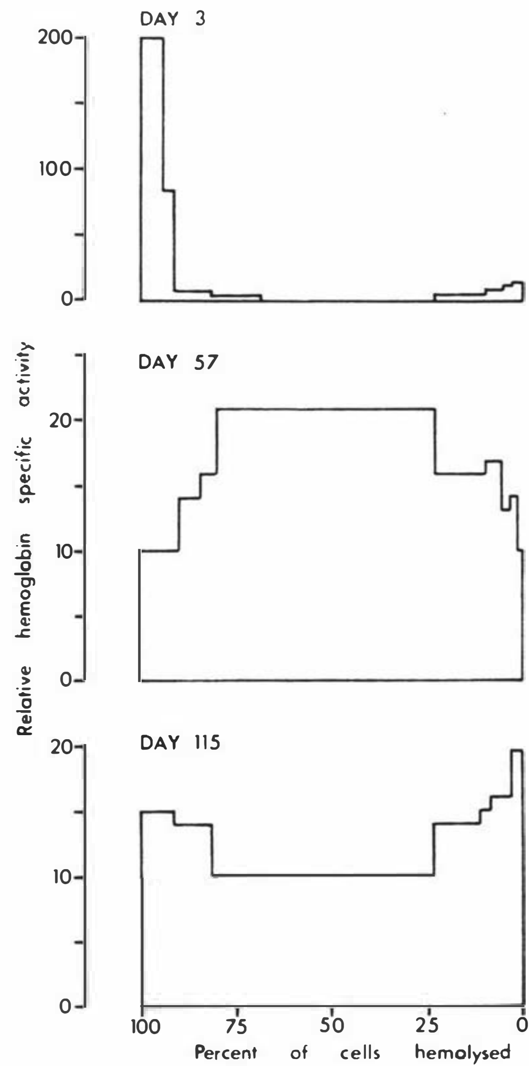


Figure 5: Serial Osmotic Hemolysis of Human Erythrocytes at Intervals after Administration of 59-Fe (Simon and Topper ref.181)

separated, the remaining cells subjected to the next dilute solution, and the process is continued until all the cells are lysed. In a study of the distribution of ^{59}Fe-labelled human red cells of particular ages between the various fractions, Simon and Topper[181] were able to demonstrate localisation of different aged cells (Figure 5), the mean age of each fraction increasing with increased fragility. Since any ^{59}Fe released from broken down cells is synthesised into new cells, distributions of ^{59}Fe generally underestimate the extent of localisation of cells of different ages[193]. Hence the age separation achieved by serial osmotic hemolysis should be greater than that suggested by Figure 5.

This separation appears to be comparable to that obtained by ultracentrifugation and an improvement on normal centrifugation. None of these procedures is as efficient as ultracentrifugation over discontinuous albumin density gradients, but the present investigation was undertaken before publication of the method of Piomelli _et al_[193]. Serial osmotic hemolysis should effectively separate very young cells, and yield other fractions enriched with cells of different ages. Analysis of hemolysates should clearly demonstrate any differences in very young cells, and any marked variations in constitution of mature erythrocytes.

Variation in Cellular Lipids with Osmotic Fragility

Studies on two cows were performed. The results in Table 21 show, for each animal, no significant difference in the amount of total lipid extracted from each fraction. Any variations are within the experimental error, which in some cases was relatively large because of difficulties encountered with hemoglobin estimations. The neutral and phospholipid distributions, also given in Table 21, were constant throughout all fractions.

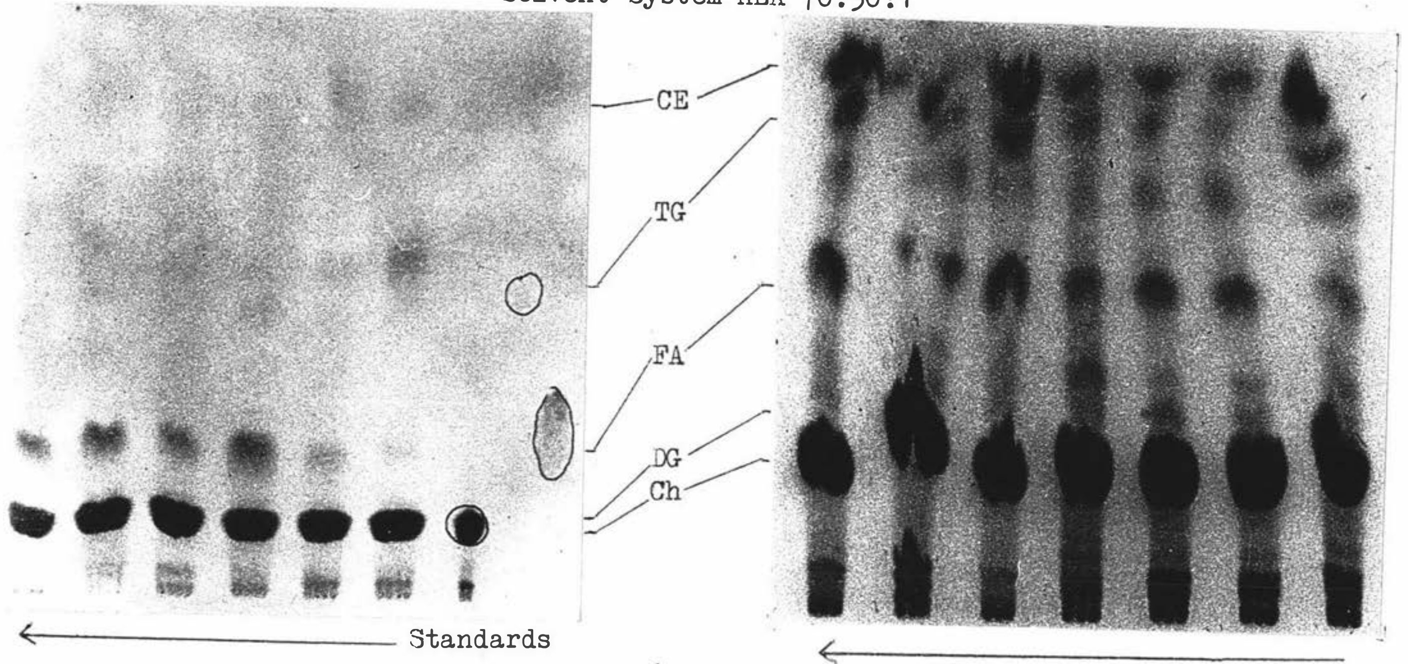
TLC separations are reproduced in Figure 6, and quantitative estimations of individual neutral lipids are given in Table 22. No obvious trends in cholesterol ester (CE), triglyceride (TG), or free fatty acid (FFA) content is obvious from the thin-layer plates, although these spots are faint and there is some random

intensity variation. The quantitative analyses, apart from some individual variations, also suggest constant levels throughout all fractions. The error estimates consider variation between duplicate analyses, and do not include extraction and separation procedures. Prevention of contamination and attainment of high accuracy was very difficult because each component was present at a very low level, and dichromate analysis is relatively non-specific. However, with a few exceptions, similar TG and CE levels were obtained for each fraction, and there were no variations which could be attributable to cell aging.

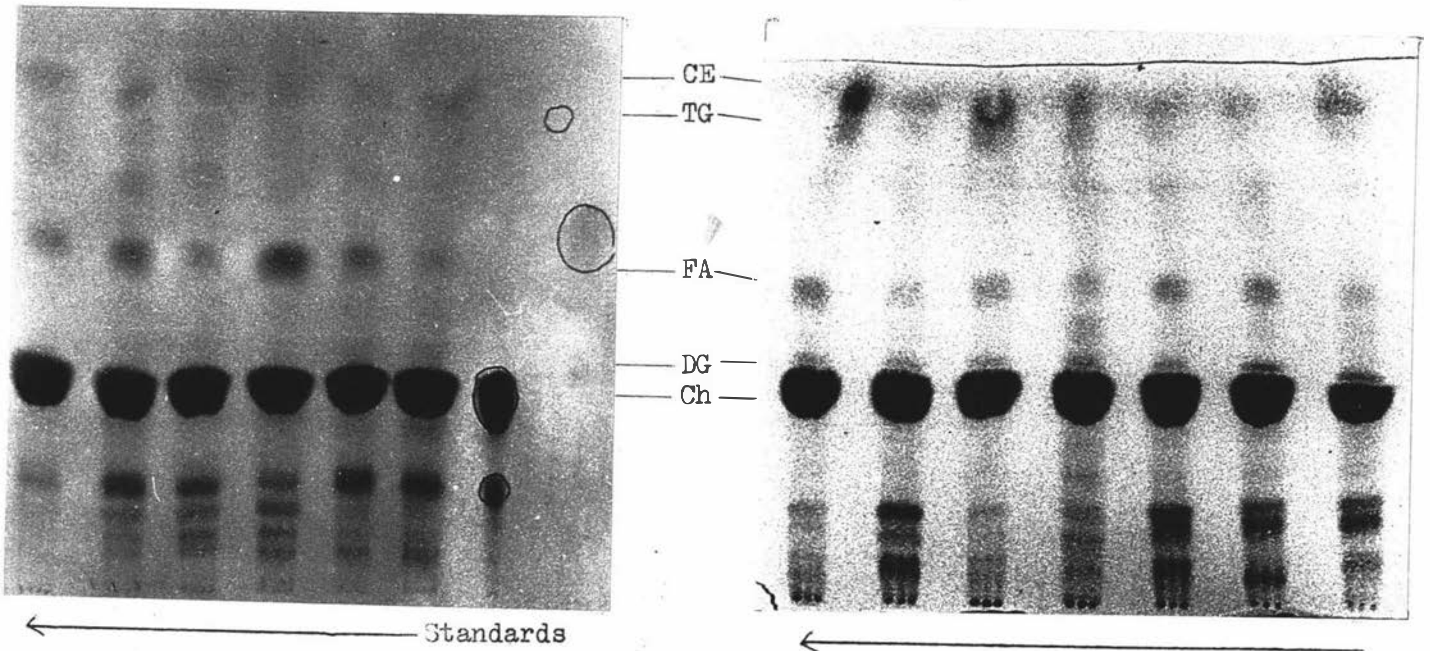
The relative amounts of the major phospholipids (Table 23), and the TLC phospholipid separations (Figure 7), do not show any differences between fractions, in excess of those covered by the experimental error. The greater variation in PE levels was probably caused by some oxidative destruction during evaporation of the hemolysates, (when it was necessary to maintain extracts at temperatures up to 65°).

Gas chromatographic separation of the phospholipid fatty acids revealed little variation between different age fractions (Table 24). Variations in most acids were small, and not significant. However a palmitate : oleate ratio of 1:1 in the youngest cell fraction, compared with a ratio of almost 1:2 in the others, could be significant, and could possibly arise from differences between bone marrow and plasma environments, from which the cells can take up fatty acids. The overall fatty acid patterns resemble others obtained for normal populations of bovine red cells[59,64]. Small autoxidative changes were probably responsible for variation in level of linoleic acid. The lower 24:0 level and higher levels of C-20 acids in the 0.42 per cent NaCl hemolysate were probably artifacts. Other studies on human and rat red cells, separated by ultracentrifugation, have suggested small differences between young and old cells in linoleate and arachidonate levels[201,203].

Westerman et al[200] have reported cellular total lipid differences of 0.7 mg, and van Gastel et al[201], differences of 1.3 mg between top and bottom fractions of ultracentrifuged human erythrocytes. The difference between middle and bottom



Solvent System HEA 30:70:1



Decreasing osmotic resistance and increasing cell age

Plates were sprayed with 20% sulphuric acid and charred. Major bands between cholesterol and the origin are degradation products of cholesterol, and correspond to a very small proportion of the total cholesterol. The band running between triglyceride and fatty acid has not been identified.

FIGURE 6: TLC SEPARATIONS OF NEUTRAL LIPIDS FROM BOVINE ERYTHROCYTES SEPARATED BY SERIAL OSMOTIC HEMOLYSIS

Solvent System

NL

Pigment

PE

PS

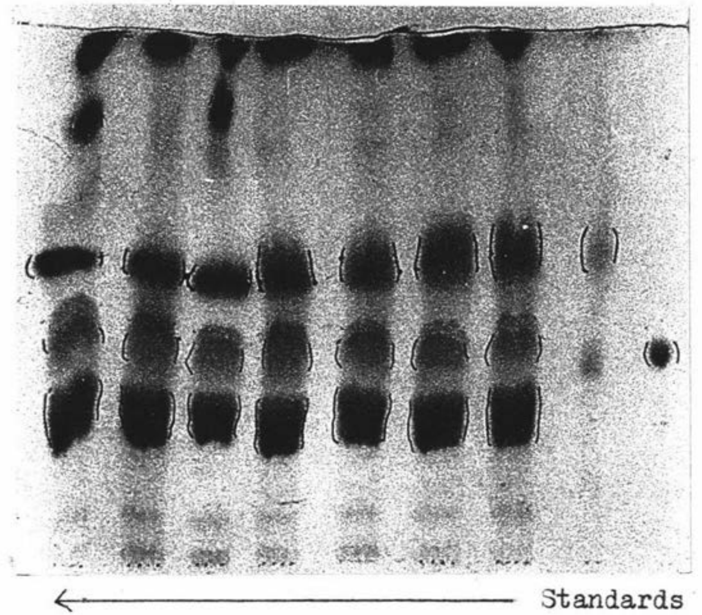
PC

SP

LPC

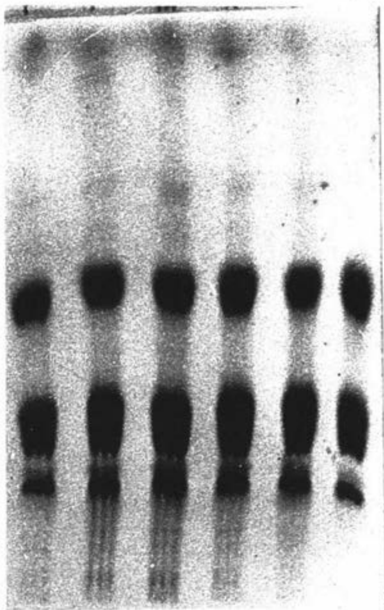
CMAW

65:25:8:4



CMN

14:6:1



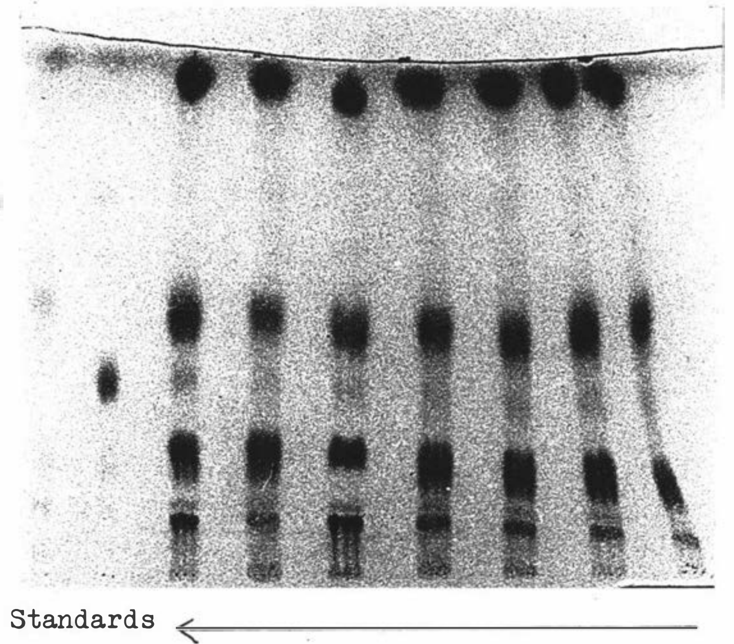
NL

PE

PC

SP

PS +
Pig-
ment



Decreasing osmotic resistance and increasing cell age

Plates were sprayed with 20% sulphuric acid and charred. Pigment contamination of some samples arose from hemoglobin breakdown prior to extraction.

FIGURE 7: TLC SEPARATIONS OF PHOSPHOLIPIDS FROM BOVINE ERYTHROCYTES SEPARATED BY SERIAL OSMOTIC HEMOLYSIS

Table 21: Lipid Content of Bovine Erythrocyte Fractions Separated by Serial Osmotic Hemolysis

	Hemolysing NaCl concentration (per cent)	Per cent in supernatant	No. cells in supernatant $\times 10^{-10}$	TL extracted mg	TL per 10^{10} cells	Per cent NL	Per cent PL
			Cow A				
increasing cell age ↑	0.51	8 ± 1	33	120 ± 6	3.7 ± 0.3	31 ± 2	69 ± 2
	0.48	22 ± 1	90	360 ± 18	3.9 ± 0.2	31 ± 2	69 ± 2
	0.45	17 ± 1	70	285 ± 15	4.1 ± 0.2	29 ± 2	71 ± 2
	0.42	20 ± 1	82	315 ± 15	3.8 ± 0.2	32 ± 2	68 ± 2
	0.39	9 ± 1	37	158 ± 8	4.3 ± 0.3	28 ± 2	72 ± 2
	0	24 ± 1	98	370 ± 18	3.8 ± 0.2	30 ± 2	70 ± 2
			Cow B				
increasing cell age ↑	0.48	7 ± 1	8.3	38.5 ± 3	4.6 ± 0.4	30 ± 2	70 ± 2
	0.45	12 ± 1	14	59.2 ± 3	4.3 ± 0.3	31 ± 2	69 ± 2
	0.42	7 ± 1	8.2	44 ± 4	5.4 ± 0.5	31 ± 2	69 ± 2
	0.39	30 ± 1	35.5	158 ± 8	4.4 ± 0.2	29 ± 2	71 ± 2
	0.36	20 ± 1	23.5	89 ± 4	3.8 ± 0.2	29 ± 2	71 ± 2
	0.33	21 ± 1	24.5	102 ± 5	4.1 ± 0.2	31 ± 2	69 ± 2
	0	2.6 ± 0.3	3.1	15 ± 1	4.8 ± 0.4	30 ± 2	70 ± 2

Volume of packed cells fractionated: Cow A 325 ml (4.10×10^{12} cells)
Cow B 100 ml (1.20×10^{12} cells)

Neutral and phospholipids were separated on silicic acid columns with 95 - 105 per cent recoveries. Errors in cell fractionation and lipid analysis have been estimated. Abbreviations are tabulated in Appendix 2.

Table 22: Neutral Lipids in Bovine Erthrocytes Separated by Serial Osmotic Hemolysis

Hemolysing NaCl concentration (per cent)	Per cent of Total Neutral Lipid Ch	FFA	TG	CE
	Cow A			
0.51	80 ± 3	8 ± 2	5.3 ± 0.2	6.8 ± 0.2
0.48	77 ± 3	11.5 ± 1	5.8 ± 0.5	6.0 ± 2
0.45	86 ± 1	5.5 ± 0.2	4.3 ± 0.2	5.2 ± 0.2
0.42	80 ± 2	11 ± 1	4.5 ± 0.2	4.1 ± 0.5
0.39	85 ± 1	6.8 ± 0.2	4.4 ± 0.2	4.1 ± 0.5
0	86 ± 2	4.0 ± 0.5	5.0 ± 0.5	5.0 ± 0.2
(↑ increasing cell age)				
	Cow B			
0.48	80 ± 1	7.7 ± 0.5	6.0 ± 0.2	6.5 ± 0.5
0.45	91 ± 2	3.8 ± 0.5	2.7 ± 0.2	2.5 ± 1
0.42	85 ± 2	4.6 ± 0.2	3.8 ± 0.5	6.5 ± 0.5
0.39	93 ± 1	3.0 ± 0.5	1.9 ± 0.2	1.7 ± 0.2
0.36	92 ± 1	4.8 ± 0.5	1.7 ± 0.2	1.7 ± 0.2
0.33	91 ± 2	3.7 ± 0.2	2.6 ± 0.5	2.9 ± 0.5
0	75 ± 1	7.8 ± 0.2	5.2 ± 0.2	11.8 ± 0.5
(↑ increasing cell age)				

Each sample was separated by TLC with HEA 70:30:1 as solvent, and duplicate dichromate analyses performed on each eluted band. Errors quoted are a measure of the variation between duplicate analyses.

Table 23: Major Phospholipids in Bovine Erythrocytes Separated by Serial Osmotic Hemolysis (Cow B).

Hemolysing NaCl concentration (per cent)	Per cent of Recovered Phosphorus SP	PE	PS	PC
0.48	54 ± 3	26 ± 3	15 ± 3	5 ± 2
0.45	62 ± 3	18 ± 2	17 ± 3	4 ± 1
0.42	54 ± 3	29 ± 3	14 ± 2	3 ± 1
0.39	55 ± 3	22 ± 1	19 ± 3	3.5 ± 0.5
0.36	58 ± 3	22 ± 1	16 ± 2	4 ± 1
0.33	57 ± 3	23 ± 1	14 ± 2	5 ± 2
0	60 ± 2	24 ± 1	13 ± 1	2 ± 0.5

Each sample was separated in duplicate by TLC in CMN. Bands were eluted and analysed. Estimates of errors include both separation and analysis.

Table 24: Comparison of the Major Fatty Acids Present in the Phospholipids of Differen Populations of Bovine Erythrocytes. (Cow B)

Fatty acid designation	Fatty Acid Components (per cent by weight)						
	Hemolysing NaCl concentration						
	0.48%	0.45%	0.42%	0.39%	0.36%	0.33%	0%
16:0	14	15	14	15	15	15	18
16:1	2.2	1.6	1.6	1.1	1.7	2.5	2.5
17:0	1.5	1.0	1.3	0.7	1.1	1.3	1.8
18:0	13	14	13	14	14	15	16.5
18:1	27	26	24	27	26	26	18
18:2	7.8	3.3	8.0	2.7	✓	2.0	2.5
18:3&20:0	1.7	1.0	4.7	0.5	0.3	✓	0.9
20:1*	-	0.5	4.4	✓	0.3	-	-
21:0*	1.5	-	2.3	1.4	1.1	1.3	1.0
22:0	4.9	4.9	6.5	5.5	5.4	5.2	5.4
23:0*	✓	2.0	-	1.4	2.1	2.8	5.0
24:0 + 20.4	21	26	15	23	26	24	23
24:1*	3.4	3.9	3.0	5.8	5.2	4.0	3.6

*tentative designation

✓present but not measurable

Other minor components each amounted to less than 1% of the total. The reproducibility of estimates from duplicate chromatographic separations was approximately $\pm$ 5%.

PROPERTIES OF THE LIPIDS FROM RED CELL POPULATIONS, FRACTIONATED ACCORDING TO AGE BY DISCONTINUOUS ALBUMIN GRADIENT ULTRACENTRIFUGATION

Variations in lipid metabolism with red cell age have been examined, in order to investigate the possibility of a relationship between membrane function and cell age. Variations in lipid level and composition have also been studied. Investigations of the lipid constitution of cells of different ages have been reported[200,201], but the improved age separation which could be achieved in the present study should allow more accurate assessment of the observed variations.

Cells were separated according to age by ultracentrifugation over a discontinuous albumin density gradient, (a slight modification of the method of Piomelli et al[193], see p.40). The degree of separation achieved was assessed by fractionating rat red cells at intervals after administration of reticulocytes labelled with $[2\text{-}^{14}C]$glycine.

Uptakes of $[1\text{-}^{14}C]$linoleate and $[1\text{-}^{14}C]$palmitate into ghosts from fractionated cells were studied. Ghosts were incubated in plasma/Krebs-Ringer-phosphate solution, in the presence of ATP and CoA. Linoleate uptake into intact, fractionated cells, from a similar medium, lacking ATP and CoA, was also measured. The purpose of the comparison was to examine whether variations in levels of these cofactors with age could cause variation in the rate of fatty acid uptake.

Initial studies were performed on rat red cells, but in view of the problems which arose when dealing with rat blood (p.29), it was decided to centre the experimental work on human red cell populations. With human cells, no problems arose from hemoglobin precipitation, or cell lysis and deoxygenation in the presence of albumin. In addition, sufficient blood for each study could be obtained from one human donor.

The problem of misinterpreting results, because of white cell contamination, made it necessary to measure uptakes of palmitate and linoleate by white cells. Corrections, (which were generally small), could then be applied to incorporations into red cell preparations.

As a basis for comparison of fatty acid uptake into different aged fractions, normal populations of erythrocyte ghosts were also incubated with both labelled acids.

Variation in Age of Populations of Rat Red Cells Separated by Albumin Gradient Ultracentrifugation

Reticulocytes labelled with [2-^{14}C]glycine were administered to a rat. Red cell samples were withdrawn at intervals, fractionated by centrifugation over discontinuous albumin gradients, and the radioactivity in each fraction analysed (Table 25 and Figure 8). The radioactivity was initially concentrated in the lightest red cell fraction, but gradually became concentrated in fractions of increasing density as the cells aged. Although relatively sharp fractionation of radioactivity was achieved for the samples taken 0, 39 and 55 days after administration, fractions of the day 5 sample, and more particularly the day 22 sample, were not as well-defined. This was probably a result of osmotic variation. During this study, the separation technique was still being developed, and it was subsequently found that the albumin solutions used for the day 5 and day 22 separations had osmolalities of 310 and 325 mOsm respectively. Hence some cell shrinkage occurred during these separations, and since density changes caused by cell shrinkage are probably not related to age[257], the true distributions according to age were partly obscured. However the distribution patterns seen in the other samples show that rat red cells are efficiently separated into fractions of different mean age by ultracentrifuging over a discontinuous albumin gradient.

Each blood sample taken (1-2 ml) removed a significant amount of the total blood of the rat (20-25 ml). This would have stimulated the production of young, unlabelled cells, and hence caused a bias towards the lighter fractions in the red cell distributions in the density gradients. However the cells were removed every 15 days, and the cell life span is 60 days, so the changes in distribution should have been slight.

The results obtained by Piomelli _et al_[193] for rabbit erythrocyte fractionation

using this procedure are shown in Figure 9. The rat cell fractionations, apart from those affected by osmolality variation, are comparable to these. Piomelli et al[193] have proposed a model which fits their results, and from this model they have calculated the mean ages of the cells in the different fractions. The model for rabbit cells, (lifespan 60-70 days), considered 7 cell age groups distributed in 10 hypothetical layers, 40 per cent of the cells in each group concentrated in 2 consecutive layers, and the rest distributed normally on either side. The model also took into account random destruction of the cells, i.e. that there are normally more younger cells in circulation than older ones. Table 26 gives the mean ages of 5 equal fractions of rabbit erythrocytes calculated by Piomelli et al[193] from this model, and also calculated from their experimental results. Approximations of the mean ages of similar fractions of rat erythrocytes calculated from the results of the present study, (assuming the same degree of random destruction of rat and rabbit cells), and are given in Table 26. The latter are approximate, as distributions of cells of only a few ages were measured. They also underestimate the separation of different ages, since data from separations in hypertonic media have been included in the calculations. Both the model and experimental data give very good separation of mean cell age for the rabbit. Considering the approximations and data available for calculation, the separation achieved for rat cells is probably equally good.

It is likely that human cells would be separated to a similar degree. After centrifugation of human, rat or rabbit red cells, there is an excess of young cells at the top, and old cells at the bottom, of the cell column. The albumin gradient technique would be expected to improve this age separation for human cells, as it does for rabbit or rat cells. On this assumption, the range of mean ages of 5 equal human red cell fractions have been calculated (Table 26).

With the achievement of such a variation, it should be possible to detect any variability of cell properties with age. Likewise any parameter which shows little or no variation between fractions would be unlikely to vary with cell age. On this

surmise, the lipid composition of, and fatty acid uptake by, different density fractions of red cells have been examined.

Table 25: Distribution of Radioactivity Among Rat Blood Cells of Different Density, at Intervals After [2-^{14}C]glycine Labelled Reticulocyte Administration

Days after Adminis-tration	Density	Per cent of cells	Relative Hemoglobin content	Total Radio-activity*	Protein Radio-activity*	Total activity Per unit Hb	Protein activity Per unit Hb
0	1.075						
	1.0805	7	0.75	28500	18800	38000	25000
	1.083	15	1.75	27000	9700	15400	5540
	1.085	38	4.40	46500	14700	10600	3340
	1.0865	23	2.65	20500	5050	7800	1900
	1.110	17	2.00	8800	2080	4400	1040
		Total cells				11400	3000
5	1.075						
	1.0845	12	1.65	8300	8200	5100	5000
	1.086	16	2.35	15600	15500	6600	6550
	1.088	39	5.50	17800	17500	3200	3150
	1.0895	7	1.05	2500	2180	2450	2150
	1.110	26	3.65	3650	3600	1000	950
		Total cells				3050	2650
22	1.075						
	1.091	7	0.35		230		650
	1.0965	28	1.43		2000		1400
	1.0985	16	0.84		1800		2150
	1.110	49	2.50		5000		2000
		Total cells				1750	1650
39	1.075						
	1.081	14	0.63		210		340
	1.083	39	1.82		620		340
	1.085	19	0.87		730		830
	1.087	18	0.84		950		1140
	1.108	10	0.46		750		1650
		Total cells					910
55	1.075						
	1.081	3	0.40		125		310
	1.083	26	3.35		420		120
	1.085	29	3.80		700		180
	1.087	26	3.25		870		270
	1.108	15	2.00		1360		680
		Total cells					410

*After day 5, total and protein radioactivities were identical.

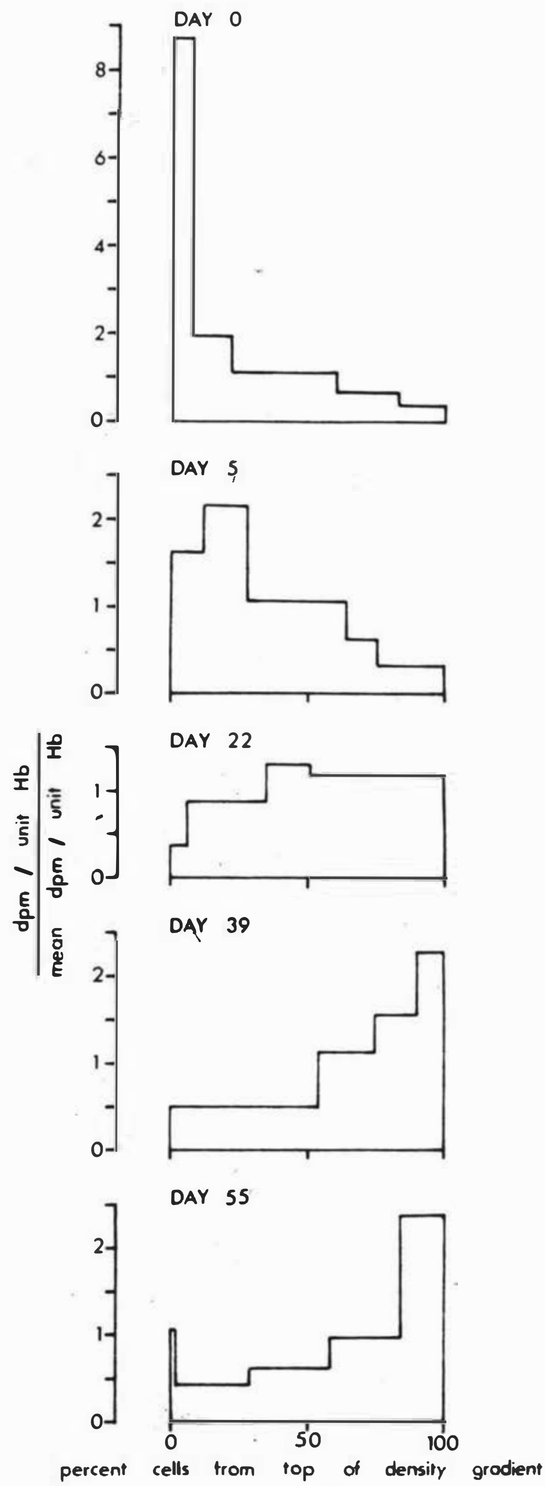


Figure 8: Discontinuous Albumin Gradient Ultracentrifugation of Rat Red Cells at Intervals after Administration of Reticulocytes Labelled with 14C-Glycine

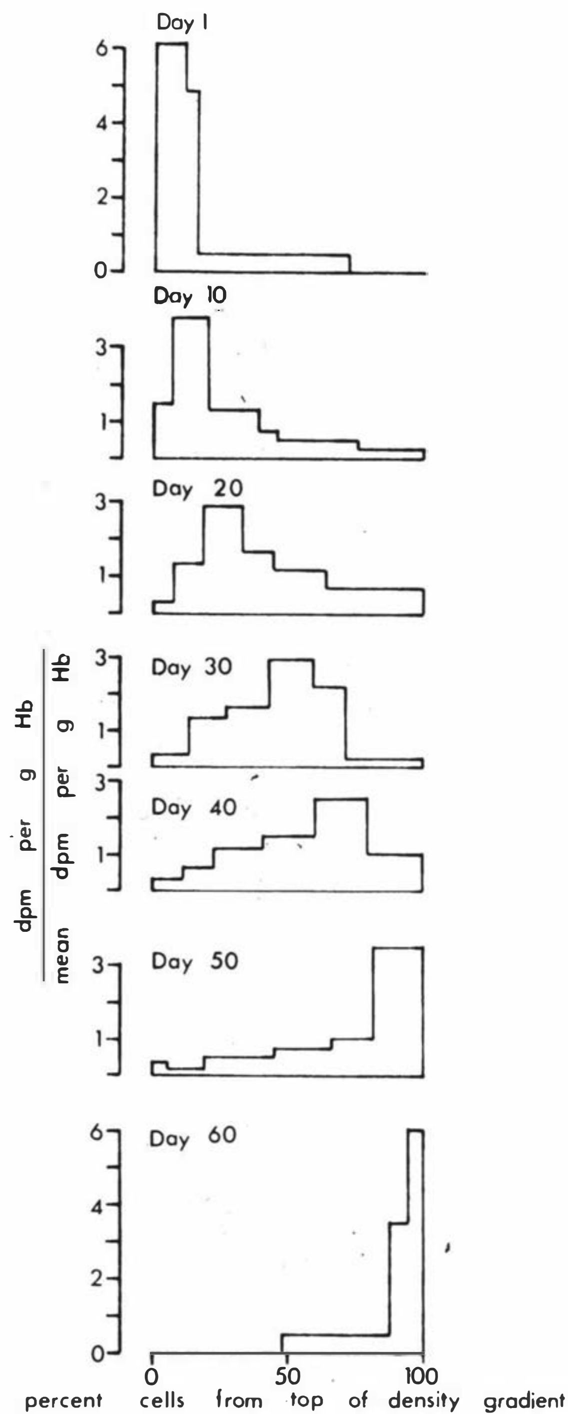


Figure 9: Discontinuous Albumin Gradient Ultracentrifugation of Rabbit Red Cells at Intervals after Administration of Reticulocytes Labelled with 14C-Glycine (Piomelli et al. ref.193)

Table 26: Comparison of the Mean Ages of Fractions of Rabbit, Rat, and Human Red Cells, Separated by Density Gradient Centrifugation

Cummulative per cent of cells from top of density gradient	Mean Age (days)			
	Rabbit[193] Lifespan 60-70 days		Rat Lifespan 60 days	Human Lifespan 120 days
	From model	From experiment	From experiment	Interpolate from rabbit data
0 - 20	9	7	14	15
20 - 40	18.5	20	20	40
40 - 60	26	30	26	60
60 - 80	39	35	30	75
80 - 100	51.5	50	38	100

Table 27: A Summary of the Basic Conditions for Each Study

WRS Sex M — Whole population of red cell ghosts incubated with linoleate
ghosts:plasma:KRP 1:0.87:2 (v/v/v)

plasma fatty acid activity:	1.20×10^6 dpm/ml
RBC incubated:	5.0×10^{10}
WBC per 10^{10} RBC:	6×10^5
radioactivity recovered in ghosts:	4.4%
total recovery:	95%

CD Sex F — Whole population of red cell ghosts incubated with linoleate
ghosts:plasma:KRP 1:1:2 (v/v/v)

plasma fatty acid activity:	1.46×10^6 dpm/ml
RBC incubated:	3.25×10^{10}
WBC per 10^{10} RBC:	1.5×10^5
radioactivity recovered in ghosts:	6.4%
total recovery:	91%

WRS Sex M — Whole population of red cell ghosts incubated with palmitate
ghosts:plasma:KRP 1:0.87:2 (v/v/v)

plasma fatty acid activity	1.64×10^6 dpm/ml
RBC incubated:	5.25×10^{10}
WBC per 10^{10} RBC:	6×10^5
radioactivity recovered in ghosts:	4.1%
total recovery:	94%

WRS Sex M — White cells incubated with linoleate + CoA/ATP
cells:plasma:KRP 0.2:1.1:1.4 (v/v/v)

plasma fatty acid activity:	0.72×10^6 dpm/ml
WBC incubated:	49×10^6
RBC incubated:	0.18×10^{10}
radioactivity recovered in cells:	19%
total recovery:	94%

EJC Sex F — White cells incubated with palmitate, ATP and CoA
cells:plasma:KRP 0.2:1:5 (v/v/v)

plasma fatty acid activity:	2.10×10^6 dpm/ml
WBC incubated:	12.6×10^6
RBC incubated:	0.16×10^{10}
radioactivity recovered in cells:	4.2%
total recovery:	100%

MJW(I)* Sex M — Red cells incubated with linoleate
cells:plasma:KRP 1:1:2 (v/v/v)

plasma fatty acid activity: 2.82×10^6 dpm/ml

density:	1.075		1.080		1.082		1.084		1.086		1.110
per cent of RBC:		11		17		29		21.5		21.5	
RBC incubated x 10^{-10}		0.33		0.92		1.75		1.30		1.35	
WBC per 10^5 RBC		1		2		5		3		8	
radioactivity recovered in cells (%)		0.90		0.62		0.61		0.52		0.65	
total recovery (%)		92		89		92		89		91	

MJW(II)* Sex M Red cells incubated with linoleate
cells:plasma:KRP 1:0.5:2 (v/v/v)
plasma fatty acid activity: 4.60×10^6 dpm/ml

density:	1.075	1.0815	1.0835	1.085	1.0865	1.090
per cent of RBC:	5.5	16.5	12.5	18	18	29
RBC incubated x 10^{-10}:	0.43	1.25	0.95	1.40	1.40	2.25
WBC per 10^5 RBC:	3	3	3	3	3	7
Radioactivity recovered in cells (%):	3.6	2.3	2.9	2.6	3.5	8.5
total recovery (%):	95	96	95	98	98	69

GL Sex M Red cells incubated with linoleate
cells:plasma:KRP 1:1:2 (v/v/v)
plasma fatty acid activity: 4.75×10^6 dpm/ml

density:	1.075	1.079	1.0805	1.0815	1.0835	1.0865
Per cent of RBC:	10	34.5	30	18.5	7	
RBC incubated x 10^{-10}:	0.63	2.12	1.85	1.15	0.45	
WBC per 10^5 RBC	2	2	2	1	1	
Radioactivity recovered in cells (%):	1.6	1.9	2.1	2.4	1.1	
total recovery (%):	99	104	98	96	98	

MJW(I)* Sex M Red cell ghosts incubated with linoleate, ATP and CoA
ghosts:plasma:KRP 1:1:2.2 (v/v/v)
plasma fatty acid activity: 2.82×10^6 dpm/ml

density:	1.075	1.080	1.082	1.084	1.086	1.110
per cent of RBC:	11	17	29	21.5	21.5	
RBC incubated x 10^{-10}:	0.39	1.08	1.70	1.22	1.19	
WBC per 10^5 RBC:	1	2	3	3	5	
Radioactivity recovered in ghosts (%):	3.3	3.1	3.0	2.9	2.7	
Total recovery (%)	70	78	85	83	90	

MJW(II)* Sex M Red cell ghosts incubated with linoleate, ATP and CoA
ghosts:plasma:KRP 1:0.5:2 (v/v/v)
plasma fatty acid activity: 4.3×10^6 dpm/ml

density:	1.075	1.0835	1.0855	1.088	1.0935	1.102
Per cent of RBC:	18.5	15	29.5	24	13	
RBC incubated x 10^{-10}:	0.68	0.55	1.08	0.90	0.47	
WBC per 10^5 RBC:	5	4	2	3	5	
Radioactivity recovered in ghosts (%):	3.9	4.5	7.0	5.7	6.0	
Total recovery (%):	92	95	86	77	86	

CCW Sex F Red cell ghosts incubated with linoleate, ATP and CoA
ghosts:plasma:KRP 1:1.5:3.3 (v/v/v)
plasma fatty acid activity: 1.97×10^6 dpm/ml

density:	1.075	1.080	1.082	1.084	1.086	1.105
per cent of RBC:	16	28	20	20.5	16	
RBC incubated x 10^{-10}:	1.05	1.85	1.34	1.35	1.07	
WBC per 10^5 RBC:	2	2	1	2	2	
Radioactivity recovered in ghosts (%):	5.0	4.6	4.8	4.3	5.6	
Total recovery:	91	95	99	95	97	

VJC Sex M Red cell ghosts incubated with palmitate, ATP and CoA
ghosts:plasma:KRP 1:1:2.25 (v/v/v)
plasma fatty acid activity: 3.62×10^6 dpm/ml

density:	1.075	1.080	1.082	1.084	1.086	1.110
per cent of RBC:	42	22	17	11	8.5	
RBC incubated x 10^{-10}:	3.15	1.65	1.32	0.84	0.62	
WBC per 10^5 RBC:	2	5	7	20	210	
Radioactivity recovered in ghosts (%):	2.4	2.7	2.7	2.9	6.5	
total recovery (%):	89	88	86	86	87	

GGM Sex M Red cell ghosts incubated with palmitate, ATP and CoA
ghosts:plasma:KRP 1:1:3.6 (v/v/v)
plasma fatty acid activity: 3.26×10^6 dpm/ml

density:	1.075	1.078	1.080	1.082	1.0845	1.102
per cent of RBC:	22	25	23	16.5	14	
RBC incubated x 10^{-10}:	2.50	2.80	2.60	1.87	1.60	
WBC per 10^5 RBC:	8.5	8.0	7.5	7.5	5.5	
Radioactivity recovered in ghosts (%):	7.5	4.0	3.7	3.4	4.5	
total recovery:	108	107	101	96	99	

EJC Sex F Red cell ghosts incubated with palmitate, ATP and CoA
ghosts:plasma:KRP 1:1:4.4 (v/v/v)
plasma fatty acid activity: 2.10×10^6 dpm/ml

density:	1.075	1.078	1.080	1.082	1.084	1.086
per cent of RBC:	10.5	44	31	10.5	3.6	
RBC incubated x 10^{-10}:	0.54	2.30	1.62	0.55	0.19	
WBC per 10^5 RBC:	0.5	0.5	0.5	0.5	1	
radioactivity recovered in ghosts (%):	4.9	4.9	3.3	4.2	2.6	
total recovery:	106	96	98	98	101	

* Denotes separate blood samples

Abbreviations are tabulated in Appendix 2

Lipid Content of Human Red Cell Populations with Different Mean Ages

Nine red cell samples were fractionated according to age by albumin gradient ultracentrifugation, and the lipids extracted from the cells, or ghosts of the cells, in each fraction. Details concerning fractionations are summarised in Table 27. The levels of total lipid, phospholipid and cholesterol per cell are given in Tables 28, 29 and 30, and also presented graphically in Figures 10,11 and 12. The percentages of cholesterol and phospholipid in each extract are given in Table 31. It was not possible to analyse for cholesterol in some of the smaller samples.

Variation between Donors

On inspection, it is apparent that there were some differences between cell and ghost lipid extracts. In general, ghost preparations yielded more total lipid than did cells, on a per cell basis, but there was closer agreement between cells and ghosts for cholesterol and phospholipid. In addition, the amounts of lipids extracted from different ghost preparations are less reproducible. This can be seen more clearly in Figure 13 in which lipid levels are compared with one another, and with a selection of literature values. The additional components in ghost extracts, (shown by TLC), appeared to be triglycerides and cholesterol esters. However, if these lipids were true ghost constituents, they should also be present in cell extracts, and hence it is likely that they arose from another source, probably plasma. It is possible that ghosts could bind plasma lipids or lipoproteins strongly enough to withstand normal washing procedures. It is well known that the method of ghost preparation can affect lipid recovery[73]. In this study it was necessary to use a method yielding ghosts enriched with ATP and CoA, which may not have given complete lipid recovery. These factors led to greater variations in ghost as compared with cell lipid parameters, and prevented the use of data on ghosts as more than a guide for assessing any age variation. Phospholipid levels, however, were more reliable than total lipid levels, and hence justified more detailed consideration.

The literature values for the lipid content of human erythrocytes show a

good deal of variation, and much of this could be ascribed to differences in the relationship between cell numbers and volume. In general there is closer agreement when lipid levels are related to cell volume. In the present investigation, hemoglobin concentrations were measured in each cell sample and in a normal population of packed cells. By assuming a standard packed cell volume[237,238], cell numbers in each fraction were calculated. Since cell size, but not hemoglobin content, is reputed to change with age[25,200,201], hemoglobin concentrations could not be related directly to cell volumes in different fractions. Reported phospholipid and cholesterol values generally agree with the present results (for cell extracts), but reported total lipid values are a little higher. Possible reasons for this discrepancy have been discussed, (see p.33). It is clear that total lipid levels should all be lower by a constant factor, and hence comparisons between different age fractions should not be affected.

Variations with Age

No large variations between age fractions in cellular levels of total lipid, phospholipid or cholesterol (Tables 28, 29, 30 and Figures 10, 11, 12), and no differences in phospholipid or cholesterol percentages are apparent (Table 31). The overall trend is a slight decrease in the amount of all lipid classes with cell age, but the biggest difference lies between the youngest and all the other fractions. Any variation in these fractions is almost covered by experimental error. Although there is much more fluctuation in ghost lipids, in most cases a similar overall pattern is evident.

Distributions of major phospholipids were determined in different age fractions and are given in Table 32. The CMAW solvent did not give complete phosphatidyl choline (PC) and phosphatidyl serine (PS) separation, and if this was required it was necessary to separate using a CMN solvent as well. Since differences rather than absolute amounts, were of prime interest, a second separation was not always performed. Lipid extracts from MJW(I), GGM, VJC and CCW red cells contained

less phosphatidyl ethanolamine (PE) than did others, and in fact showed phospholipid distributions similar to those obtained by Dodge and Phillips[71] for human red cell lipids which had undergone varying degrees of autoxidation*. Phospholipid distributions for other donors agreed closely with literature values[66,67,72]. For the donors for which no autoxidation is evident (GL, MJW(II) cells and ghosts, EJC), no variation in phospholipid constitution between fractions of cells of different ages is evident. This is true for both cell and ghost extracts. This result agrees with those of Westerman et al[200] and van Gastel et al[201], who were not able to detect phospholipid differences in fractions of centrifuged cells.

The fractionation by serial osmotic hemolysis of bovine red cells (pp.63-69) also revealed little difference in either lipid level or constitution. Westerman et al[200] obtained differences of 0.7 mg, 0.4 mg and 0.18 mg per 10^{10} cells for total lipid, phospholipid and cholesterol, between the top and bottom 10 per cent of a column of centrifuged red cells. Van Gastel et al[201] took the top, middle and bottom 15 per cent of a sample of ultracentrifuged red cells. Their differences between top and bottom were 1.3 mg total lipid, 0.5 mg phospholipid and 0.2 mg cholesterol per 10^{10} cells, and between middle and bottom were 0.3 mg, 0.3 mg and 0.1 mg. Both groups measured small differences in cell dimensions between old and young cells, and when lipid values were related to cell volume or surface area, differences mostly became insignificant, or at least very low. Van Gastel et al[201] regard their total

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* Autoxidation of red cell lipids causes decreases in PE and phosphatidyl serine (PS) concentrations, and apparent increases in sphingomyelin (SP) and lysophosphatidyl choline (LPC) concentrations. This is due to PE and PS breakdown products exhibiting similar TLC mobilities to SP and LPC[71]. After it was realised that autoxidation was occuring, it was eliminated by extracting and storing lipids under nitrogen, and always keeping them in solution. The conditions responsible for autoxidation were either drying for 1-2 hr under vacuum, prior to weighing, or storage in chloroform at 4°C or -15°C for varying periods.

lipid differences as least significant, because of inherent inaccuracies in the determinations. Variations measured in the present study are of a similar order to those of Westerman _et al_[200] and van Gastel _et al_[201]. In fact the enhanced age separation has revealed no greater differences between young and old cells, and has shown the biggest differences in lipid content to be between the very youngest and the remaining cells. As reticulocytes are considerably larger than erythrocytes[25], and contain more lipid[26], it would appear that the transition between reticulocyte and erythrocyte could account for most of the observed lipid difference between young and old red cells.

Table 28: Total Lipid Extracted from Human Red Cells of Different Ages

Donor	Est. Error	Fraction I	II	III	IV	V	VI
		increase in density and age →					
				cells			
MJW(I)	± 0.20	4.40	3.55	3.50	3.60	3.50	
GL	± 0.15	3.80	3.45	3.40		3.35	
MJW(II)	± 0.15	4.35	3.85	3.90	3.70	3.70	3.35
				ghosts			
CCW	± 0.15	4.60	4.10	3.95	3.80	4.00	
MJW(II)	± 0.20	6.30	7.30	5.10	4.50	4.80	
MJW(I)	± 0.20	4.10	4.25	3.85	4.20	4.35	
GGM	± 0.15	4.00	3.40	3.75	4.00	4.45	
VJC	± 0.20	3.70	3.65	3.20	3.25	3.20	
EJC	± 0.15		3.90		4.20	4.60	

Values are quoted in mg per 10^{10} cells. Errors arising from extraction and analysis are estimated. For fraction sizes and densities, see Table 1. These results are presented graphically in Figure 10.

Table 29: Phospholipid Extracted from Human Red Cells of Different Ages

Donor	Est. Error	Fraction I	II	III	IV	V	VI
				increase in density and age →			
				cells			
MJW(I)	± 0.20	3.50	3.30	3.25	3.35	3.40	
GL	± 0.15	3.65	3.40	3.40		3.25	
MJW(II)	± 0.15	4.30	3.90	3.95	3.70	3.70	3.30
				ghosts			
CCW	± 0.15	4.55	3.85	3.80	3.70	3.90	
MJW(I)	± 0.15	4.20	4.50	4.10	3.10	3.50	
MJW(I)	± 0.20	4.00	3.65	3.45	3.55	3.80	
GGM	± 0.15	3.90	3.20	3.55	3.65	3.90	
VJC	± 0.20	3.65	3.55	3.10	3.00	2.90	
EJC	± 0.15		3.65		3.65	3.70	

Values are quoted in µeq per 10^{10} cells. Errors arising from extraction and analysis are estimated. For fraction sizes and densities, see Table 1. These results are presented graphically in Figure 11.

Table 30: Cholesterol Extracted from Human Red Cells of Different Ages

Donor	Est. Error	Fraction I	II	III	IV	V	VI
				increase in density and age →			
				cells			
MJW(I)	± 0.05		0.97	1.02	0.93	0.90	
GL	± 0.05	1.05	1.00	1.00		0.90	
MJW(II)	± 0.05		1.02	0.97	1.00	0.94	0.94
				ghosts			
CCW	± 0.05	1.15	1.00	0.95	0.95	1.00	
MJW(I)	± 0.05		1.05	0.92	1.00	1.05	
GGM	± 0.05	0.90	0.80	0.92	0.95	1.00	
VJC	± 0.05	0.85	0.80	0.75	0.70	0.70	

Values are quoted in mg per 10^{10} cells. Errors arising from extraction and analysis are estimated. For fraction sizes and densities see Table 1. These results are presented graphically in Figure 12.

Table 31: Percentages of cholesterol and phospholipid in the lipid extracted from human red cells of different ages

Donor	Phospholipid I	II	III	IV	V	VI	Cholesterol I	II	III	IV	V	VI
	increase in density and age →						increase in density and age →					
	cells											
MJW(I)	61	72	71	72	75			25	29	26	25	
GL	74	76	76	75	74		27	29	29	30	26	
MJW(II)	76	78	78	77	76	76		26.5	25	27	25	28
	ghosts											
CCW	76	73	74	75	75		25	24	24	24	24	
MJW(II)	52	48	62	55	55							
MJW(I)	72	66	70	65	68			25	24	23	24	
GGM	74	73	73	72	68		22	24	25	24	23	
VJC	76	76	76	72	70		24	23	24	21	21	
EJC	64	71	69	67	62							

For fraction sizes and densities, see Table 1.

Table 32: Percentage Distribution of the Major Phospholipids in Human Red Cells of Different Ages

Donor	Phospholipid	Fraction I	II	III	IV	V	VI
		increase in density and age →					
		Per cent of Lipid Phosphorus					
GL	PE	30 ± .5	30 ± .5	32 ± 1	29 ± 1	30 ± 2	
	PS+PC	43 ± 2	42 ± 1	42 ± 1	41 ± 2	42 ± 3	
	SP	26 ± 2	26 ± 1	25 ± 1	27 ± 2	27 ± 1	
	LPC	0.5 ± 0.2	1.5 ±0.5	1.5 ± 1	3 ± 2	1 ± 1	
MJW(II) cells	PE	30 ± 1	27 ± 1	29 ± 0.5	29 ± 0.5	29.5 ± 1	34 ± 1
	PS+PC	48 ± 1	49.5 ± 1	44 ± 0.5	45 ± 1	46.5 ± 0.5	43 ± 1
	SP	22 ± 2	23 ± 1	27 ± 0.5	26 ± 1	24 ± 0.5	23 ± 0.5
MJW(II) ghosts	PE	28 ± 1	29 ± 2	29 ± 2	30 ± 1	30 ± 1	
	PS+PC	48 ± 2	48 ± 2	49 ± 2	47 ± 2	47 ± 2	
	SP	24 ± 1	23 ± 1	22 ± 1	23 ± 1	23 ± 1	
EJC	PE	30 ± 1	31 ± 1	30 ± 1	31 ± 2	30 ± 2	
	PS+PC	46 ± 2	41 ± 2	41 ± 2	41 ± 2	44 ± 2	
	SP	22 ± 2	25.5 ± 1	27 ± 1	25 ± 2	}26 ± 2 (SP + LPC)	
	LPC	2 ± 1	2 ± 1	2 ± 1	3 ± 1		

Table 32, cont.

	Phospholipid	I	II	III	IV	V	VI
CCW	PE	24.5 ± 0.5	22.5 ± 0.5	18.5 ± 1	20.5 ± 0.5	20 ± 0.5	
	PS+PC	38 ± 1	41 ± 1	43 ± 1	43 ± 1	38 ± 2	
	SP	29 ± 1	30 ± 0.5	30 ± 1	30 ± 0.5	32 ± 2	
	LPC	8.5 ± 0.5	6 ± 0.5	8.5 ± 0.5	6 ± 1	10 ± 0.5	
VJC	PE	21 ± 1	17 ± 1	15.5 ± 1	16 ± 1	17.5 ± 1	
	PS	8 ± 2	7 ± 2	8 ± 2	9 ± 2	6 ± 2	
	PC	34 ± 2	34 ± 2	34 ± 2	35 ± 2	33 ± 2	
	SP	26 ± 2	28 ± 2	28 ± 2	28 ± 2	27 ± 2	
	LPC	11 ± 2	13 ± 2	14 ± 2	12 ± 2	15 ± 2	
MJW(I) cells	PE	16 ± 2	20 ± 1	18 ± 1	13 ± 1	17 ± 1	
	PS+PC	36 ± 3	32 ± 2	34 ± 2	34 ± 2	32 ± 2	
	SP	32 ± 2	31 ± 1	30 ± 1	29 ± 2	30 ± 2	
	LPC	16 ± 2	17 ± 2	17 ± 2	23 ± 3	21 ± 2	
MJW(I) ghosts	PE	15 ± 1	20 ± 1	18 ± 1	22 ± 1	16 ± 1	
	PS+PC	33 ± 1	37 ± 2	34 ± 2	36 ± 2	37 ± 2	
	SP	28 ± 1	35 ± 2	36 ± 1	30 ± 1	36 ± 1	
	LPC	24 ± 1	9 ± 1	13 ± 2	12 ± 1	11 ± 0.5	
GGM	PE	26 ± 2	19 ± 0.5	16 ± 1	16.5 ± 0.5	21.5 ± 0.5	
	PS+PC	36 ± 2	43 ± 1	40 ± 1	39 ± 1	39 ± 1	
	SP	28 ± 1	31 ± 1	33 ± 1	31.5 ± 1	29 ± 1	
	LPC	9 ± 2	6 ± 1	10 ± 2	13 ± 1	10.5 ± 1	

Phospholipids were separated by TLC in CMAW 65:25:8:4. Some distributions were verified by TLC separations in CMN 14:6:1. Phosphorus analyses were carried out directly on the silica gel. Each separation was performed in duplicate, and errors between separations are estimated.

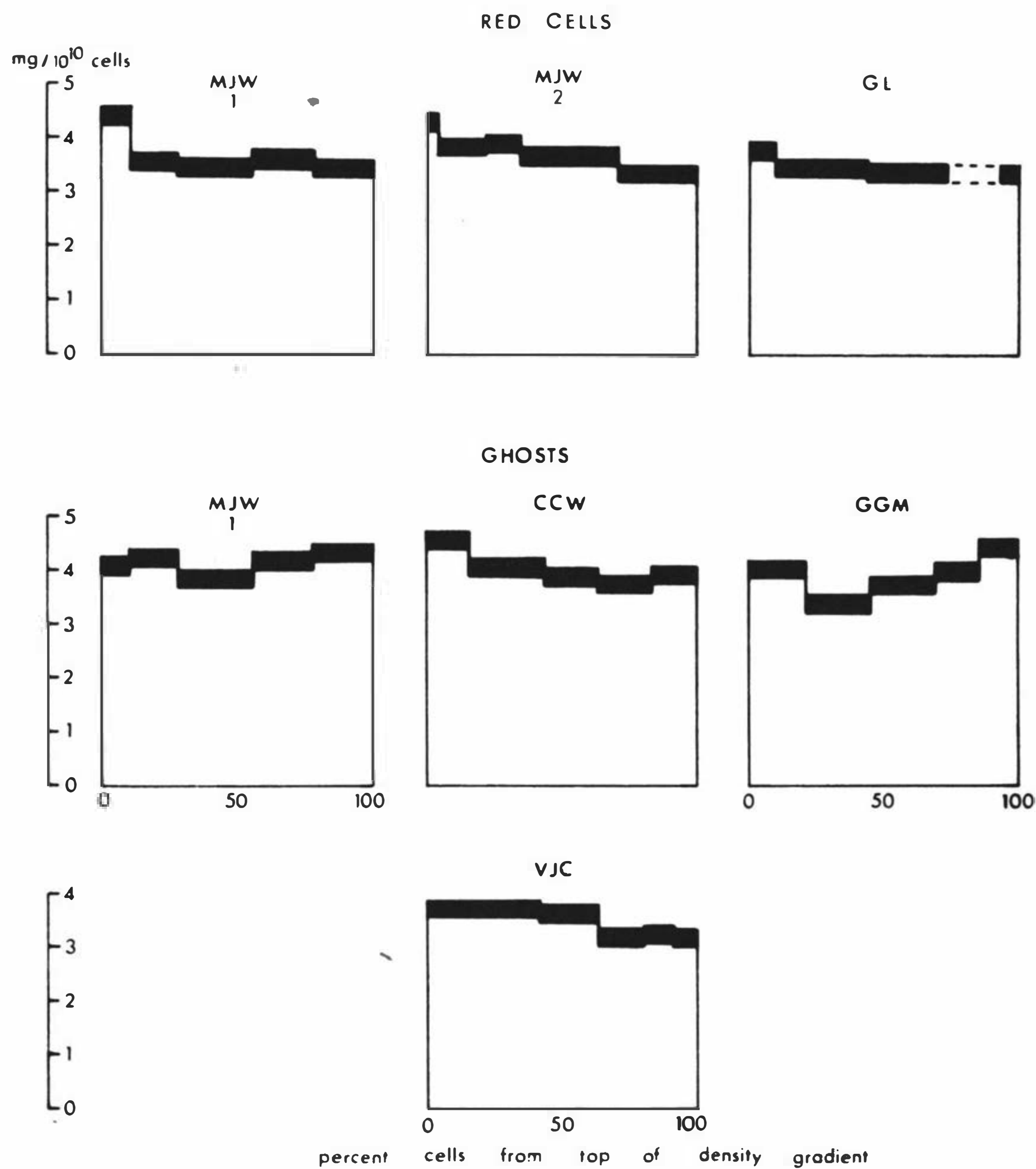

Figure 10: Variation in Human Red Cell Total Lipid Content with Cell Age

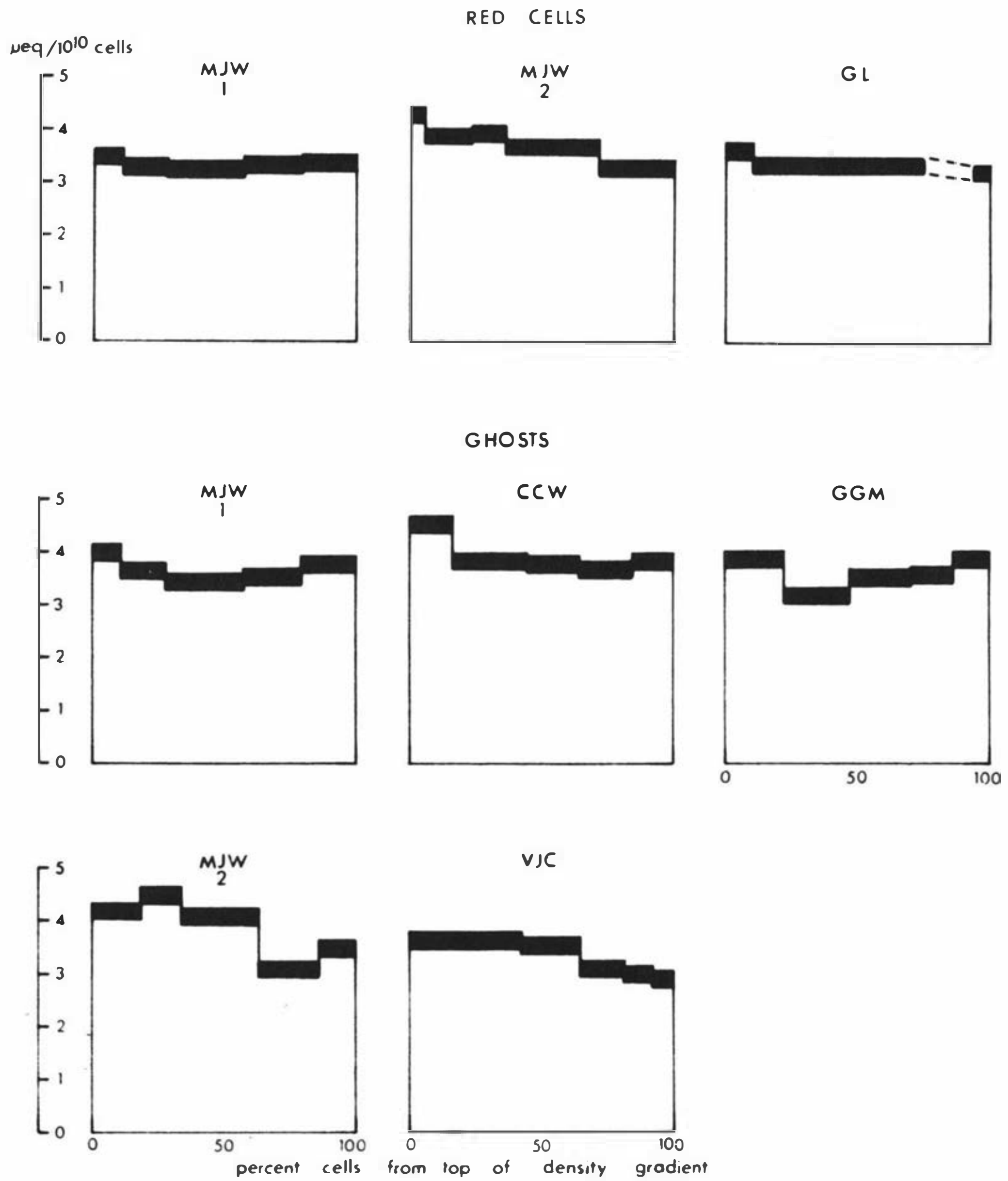

Figure 11: Variation in Human Red Cell Phospholipid Content with Cell Age

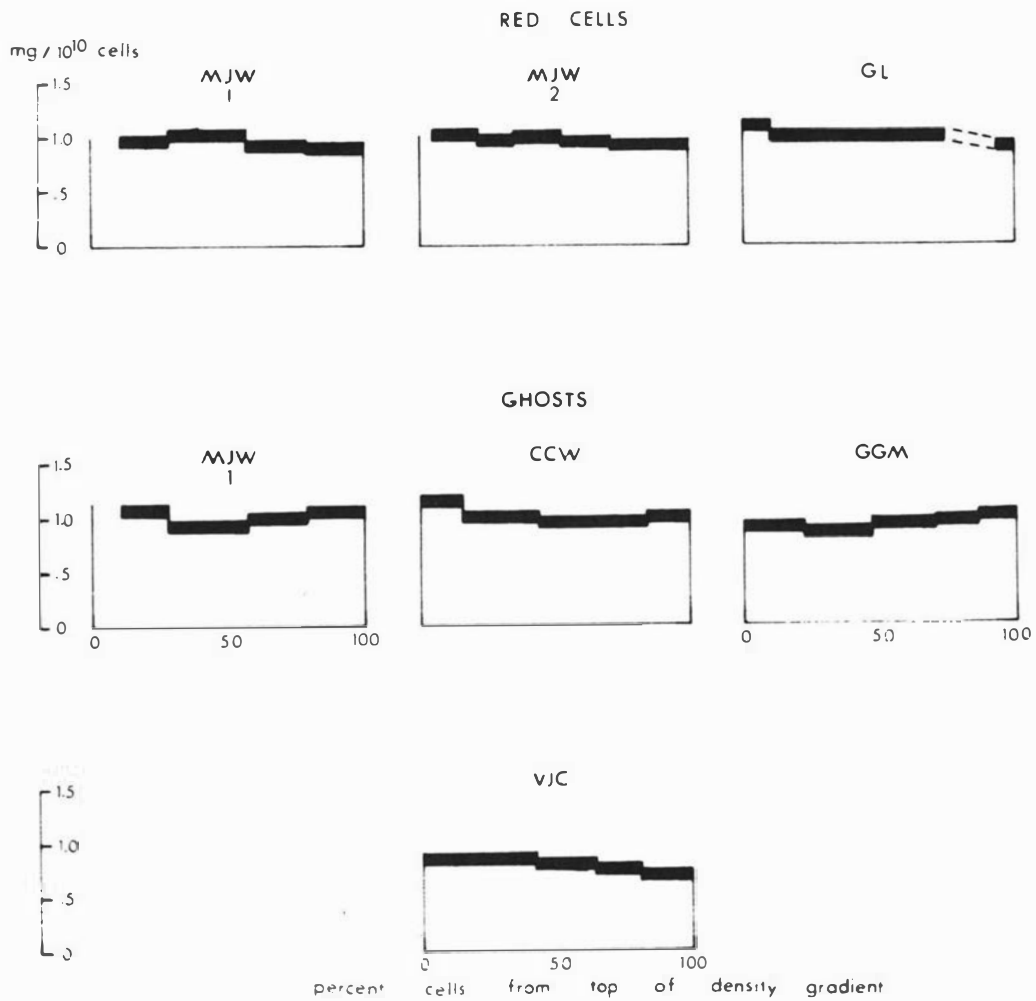

Figure 12: Variation in Human Red Cell Cholesterol Content with Cell Age

References

a Harris, Prankerd and Westerman[263]

b Phillips and Roome[264]

c Williams, Kuchmak and Witter[67]

d Dodge and Phillips[71]

e de Gier and van Deenen, Verloop and van Gastel[265]

f Kates, Allison and James[266]

g Munn and Crosby[267]

h Erickson, Williams, Bernstein, Arvin, Jones and Macy[268]

i Hill, Kuskis and Beveridge[48]

j Ways and Hanahan[66]

k Dodge, Mitchell and Hanahan[73]

l Farquhar[65]

m Reed, Swisher, Marinetti, and Eden[60]

n Parpart and Dzieman[240]

o Westerman, Pierce and Jensen[200]

p van Gastel, van den Berg, de Gier and van Deenen[201]

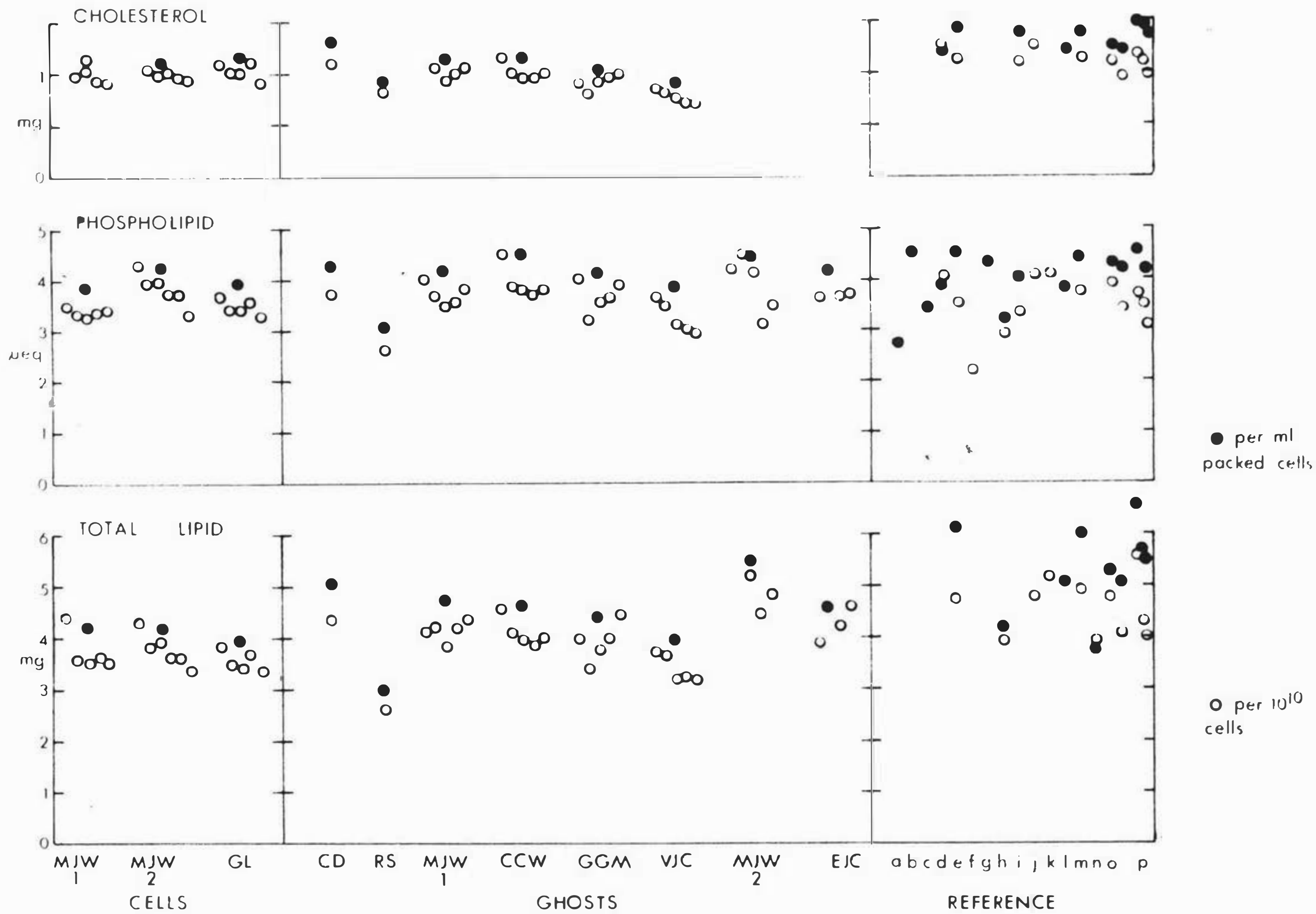

Figure 13: Comparison of Amounts of Lipids Extracted from Red Cell Samples with Literature Values

Variation in Fatty Acid Uptake with Human Red Cell Age

Red cells were fractionated by albumin gradient ultracentrifugation, and incubated either as intact cells or ghosts, with [1-^{14}C]linoleate or [1-^{14}C]palmitate. Table 27 summarises separation and incubation conditions for each study. Lipids were extracted from washed cells or ghosts, and radioactive components were identified and estimated quantitatively. In two studies, lipids were also extracted from incubation media.

Expression of Results

1. As can be seen in Table 27, medium radioactivity concentrations varied between experiments. In order to relate fatty acid uptakes in different experiments it has been assumed that uptake was proportional to concentration. (This seems reasonable, providing the enzyme catalysing fatty acyl-CoA formation was not saturated. Calculations on p.128 suggest that the assumption is justified.) In this case, rate of radioactivity uptake $\propto$ [RCOOH] x specific activity

$$\propto [^{14}\text{C-RCOOH}].$$

Most of the free fatty acids in plasma are albumin bound[129]. It is the concentratio of those which are not, (only about 0.01 per cent of the total), which is the critical determinant of reaction rate. Dilution of the plasma will cause some dissociation of the albumin-fatty acid complexes, and concentrations of non-albumin bound fatty acids should remain essentially constant. Therefore the rate of radio- activity uptake should be independent of plasma dilution. Accordingly all uptakes have been related to a plasma radioactivity concentration of 10^6 dpm/ml.

Since the [1-^{14}C]linoleate preparation used was only 85 per cent pure (see p2 an appropriate correction has also been made for this.

2. For comparing properties of different cell fractions, radioactivity incorporations can be expressed in a variety of ways. Of these, results expressed either in terms of specific activities or per μeq phospholipid are the most

desirable, because of the variations found in ghost lipid recoveries. Uptakes on a per cell basis are affected by lipid loss, and on a total lipid basis, by the variable amounts of excess neutral lipid recovered.

3. In some incubations, enough white cells were present to contribute to radioactivity uptakes. Accordingly white cell contributions were estimated and appropriate corrections made.

4. Autoxidative changes occurred in some lipid samples, affecting primarily PE and PS levels. No incorporation of fatty acids into PS has been detected, so in this context, loss of PS can be disregarded. PE radioactivity would have been reduced, and apparent SP and LPC levels increased. In order to correct for PE autoxidation, random breakdown has been assumed, and observed and predicted PE levels used to obtain a correction factor for radioactivities. This is only an approximation, as polyunsaturated acids are more likely to be oxidised[71]. However, as arachidonate is the major polyunsaturated acid present, and phospholipid molecules tend to contain one saturated and one unsaturated fatty acid chain, the assumption is probably reasonable.* A similar type of assumption is involved in the measurement of specific activities. Both corrected and uncorrected PE radioactivities have been quoted.

Results are expressed in terms of specific activity or per μeq phospholipid, after allowing for white cell contributions, and normalising to a plasma activity of 10^6 dpm/ml. Data to which none of these corrections has been applied are tabulated in Appendix 1.

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* An attempt was made to examine autoxidative changes in PE labelled with palmitate and linoleate. However prolonged standing in air at room temperature or at 15° failed in this case to bring about any changes, and no correction factors could be obtained.

Identification of Radioactive Lipid Components

The identification of lipid components after TLC has been discussed (p. 35). Radioactive components were identified both by comparing autoradiographs with TL plates from which they were prepared, and comparing the positions of radioactive spots with standards.

Radioactive lipids identified in red cell extracts were:

1. Positive identification: free fatty acid (FFA), triglyceride (TG), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and lysophosphatidyl choline (LPC)*, (Figures 14-19).

2. Tentative identification: diglyceride (DG); two faint bands, one almost ahead of and one almost behind cholesterol, were sometimes present, (Figures 16,17). These were tentatively identified as diglyceride isomers. Phosphatidic acid (PA); in most extracts there was evidence for a component running with or just behind the solvent front in CMAW, and not migrating in HEA (see Figures 16,19). It was first postulated by comparing total phospholipid radioactivities measured after separation using both solvent systems, and later demonstrated by eluting the origin after HEA separation, separating the components in CMAW, and measuring the solvent front activity. Autoradiography of a 2 dimensional TLC separation using both these solven also showed presence of this component. It was not separated from the bulk phospholipid by TLC in CMA 185:15:1, but autoradiographs of CMN separations (Figures 17,19) suggest that in this solvent it could run just behind SP. The component was tentatively identified as phosphatidic acid, which exhibits similar TLC behaviour to that observed.

3. Not identified: One or two bands, migrating between cholesterol and the origin in HEA, and between the solvent front and free fatty acid in CMN (Figures 16,17) have not been identified and are designated "components X".

---

* Abbreviations are tabulated in Appendix 2.

Some faint streaking in the LPC,SP region can be seen in autoradiographs of autoxidised samples.

Since the [1-^{14}C]linoleate was not completely pure (see p. 24), the question arises as to whether the trace impurities could have been taken up by the red cells. This appears to be unlikely because (a) after incubation, the impurities were still present, with no apparent reduction in concentration, in the medium (Figure 14), and (b) no impurity bands were detectable in the autoradiographs of the cell lipid (Figure 14).

Table 33: Incorporation of ^{14}C-Fatty Acids into Human White Blood Cell Lipids

Approximate white cell lipid levels: 500 mg TL/10^{10} cells
500 μeq PL/10^{10} cells

Component	[1-^{14}C]linoleate uptake (Donor WRS) Per cent of esterified activity	Activity per 10^6 cells (dpm)	[1-^{14}C]palmitate uptake (Donor EJC) Per cent of esterified activity	Activity per 10^6 cells (dpm)
TL		4200		2800
TG	47 ± 0.5	2000	57 ± 2	1600
DG	} 5.5 ± 0.2	} 230	2.2 ± 0.3	60
X			2.2 ± 0.3	60
PA	4.0 ± 1.0	170	2.0 ± 1.0	55
PE	2.3 ± 0.3	100	1.6 ± 0.5	45
PS	0.9 ± 0.5	40	0.9 ± 0.5	25
PC	39 ± 0.5	1600	29 ± 1	800
SP	0.6 ± 0.1	25	6.0 ± 0.5	170
LPC	0.9 ± 0.1	40	1.1 ± 0.3	30

Duplicate TLC separations with both HEA 60:40:1 and CMAW as solvents were performed. Estimates of errors in separation and analysis have been made. Errors in white cell counts are of the order 10-20 per cent. Experimental details are given in Chapter 2 and Table 27, and data uncorrected for plasma activity concentrations and red cell contributions in Appendix 1, Table 33a.

Table 34: Incorporation of $[1-^{14}C]$linoleate into Normal Populations of Human Red Cel

Component	Donor CD			Donor WRS		
	Per cent of esterified activity	Activity per µeq PL (dpm)	Specific activity dpm/µeq	Per cent of esterified activity	Activity per µeq PL (dpm)	Specific activity dpm/µeq
TL		17300			12200	
TG	2.4 ± 1	400		2.9 ± 1	350	
DG, X	5.2 ± 0.2	900		7.6 ± 0.3	930	
PA	- ± 1	-		4.5 ± 1	550	
PE	10.5 ± 0.3	1800	6000	6.3 ± 0.2 (7.0)	770 (860)	3300
PC	80 ± 0.5	14000	38000	70 ± 0.5	8500	26500

Duplicate TLC separations with both HEA 60:40:1 and CMAW as solvents were carried out. PA activities were estimated from differences in "phospholipid" activity between the two separations. Values for PE activities in parentheses have been corrected for autoxidation. Estimates of errors associated with separation, analysis and correction for white cell contributions have been made. Experimental details are given in Chapter 2 and Table 27, and data uncorrected for plasma activity concentration and white cell contributions in Appendix 1. All abbreviations are tabulated in Appendix 2.

Table 35: Incorporation of $[1-^{14}C]$palmitate into a Normal Population of Human Red Cell Ghosts, (Donor WRS)

Component	Per cent of esterified activity	Activity per µeq PL (dpm)	Specific activity dpm/µeq
TL		11000	
TG	1.4 ± 1	150	
DG, X	13.5 ± 0.5	1500	
PA	9.0 ± 1	1000	
PE	8.7 ± 0.3(10.0)	950(1100)	4200
PC	58 ± 0.5	6400	20000

For the explanation of results, see the footnote to Table 34.

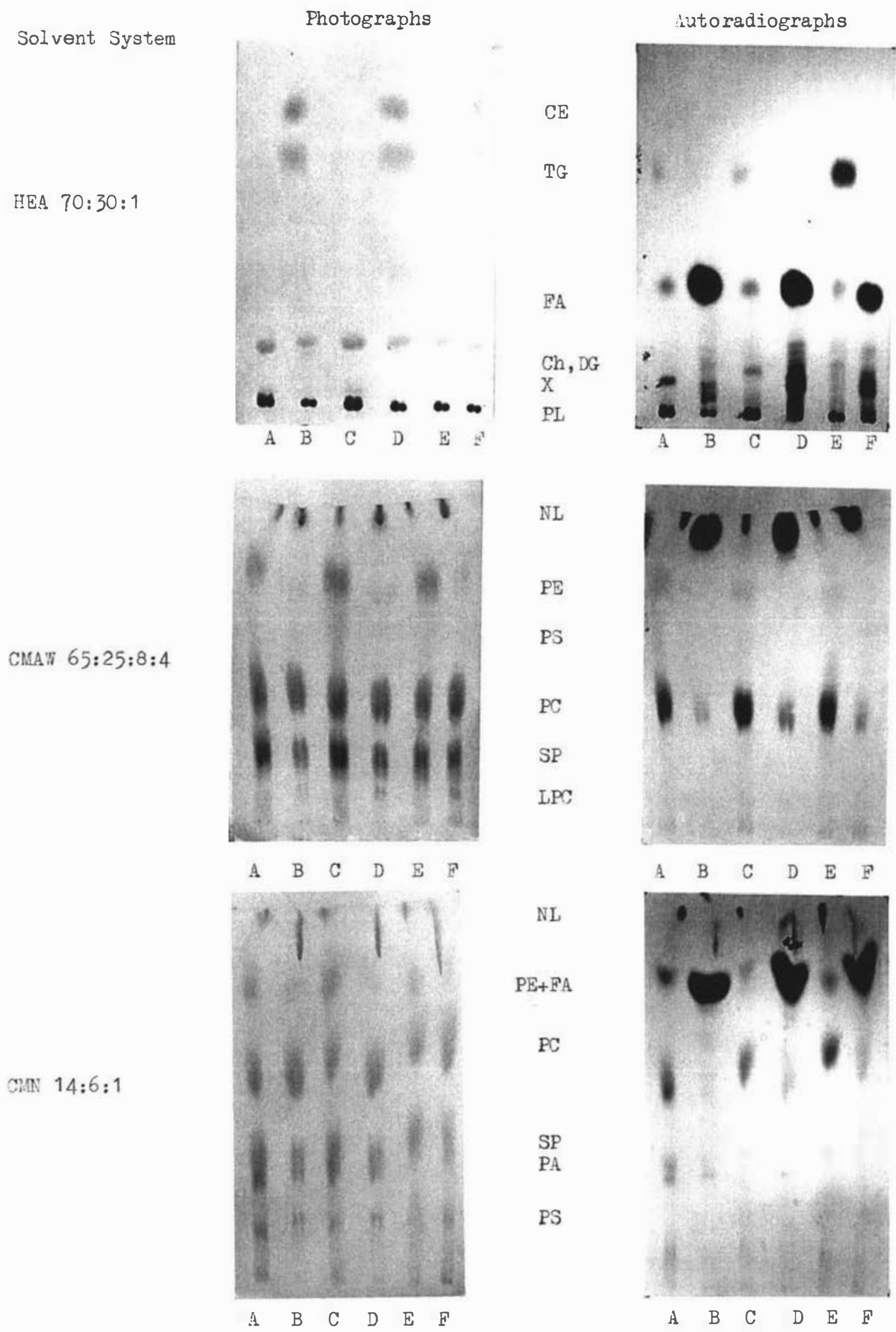


FIGURE 14: TLC SEPARATIONS OF LIPIDS FROM HUMAN RED CELL GHOSTS (A) AND PLASMA (B) AFTER INCUBATION WITH [1-^{14}C]PALMITATE; RED CELL GHOSTS (C) AND PLASMA (D) AFTER INCUBATION WITH [1-^{14}C]LINOLEATE; AND WHITE CELLS (E) AND PLASMA (F) AFTER INCUBATION WITH [1-^{14}C]LINOLEATE (DONOR WRS)

Linoleate and Palmitate Uptake by Human Red Cells

Cells from different blood samples showed considerable variation in fatty acid uptake. This variation could not be accounted for by differences between linoleate and palmitate uptake, or differences in the behaviour of cells and ghosts. For this reason, results for each set of incubations are presented separately.

Data on the uptake of both acids into white cell lipids are given in Table 33. As white cell levels in red cell preparations were very low, only approximate counts could be made, and the order of the fatty acid incorporation due to white cells estimated. However since it has already been shown that fatty acid uptake into bovine red cell TG is not significant, and comparison of observed TG activities and estimates of white cell contributions suggest a similar situation for human cells, white cell contributions to activities in other components were calculated from TG activities and the radioactivity distributions in Table 33.

Tables 34 and 35 summarise the incorporation of linoleate and palmitate into normal populations of red cell ghosts. Photographs and autoradiographs of TLC separations of lipid extracts from WRS cells are shown in Figure 14. Both fatty aci were predominantly taken up into PC, with slower uptake into PE, PA, DG, and probably two other unidentified components. Overall linoleate uptake appeared to be slightly faster than palmitate uptake. Other investigators have observed similar patterns of uptake into PC and PE[40,41,102]. Incorporation of fatty acids into red cell diglyceride and phosphatidic acid has not been reported.

Incorporations into cells and ghosts, after fractionation according to age, are given in Tables 36-38 (cells and linoleate), Tables 39-41 (ghosts and linoleate) and Tables 42-44 (ghosts and palmitate). As well as the components listed in the tables, free fatty acid fractions were also radioactive. Levels of free fatty acid activity varied quite widely, depending on the efficiency of washing after incubati Very small amounts of TG activity were measured, but were accountable for in terms of white cell contamination. Apart from a trace of palmitate-labelled PS (see GGM,

Figure 19) no incorporation into other components could be detected. Although low activity (less than 2 per cent of the total) was often measured in other components, it was never seen as discrete bands, and was most likely due to slight trailing of other labelled components.

Photographs and autoradiographs of TLC separations of lipids from WRS, MJW(II) cells and ghosts, CCW, VJC and GGM are shown in Figures 15,16,17,18,19 respectively. Graphical representation of variations in fatty acid incorporation into total esterified lipid (Figure 20), phosphatidyl choline (Figure 21), phosphatidyl ethanolamine (Figure 22), phosphatidic acid (Figure 23), and diglyceride and components X (Figure 24) are given. Figure 25 compares radioactivity distributions in the esterified lipids, and Figure 26, PE : PC specific activity ratios.

From rates of uptake into the different cell lipids it is possible to calculate times required for the renewal of all the palmitate or linoleate in each component. These calculations have been made for uptakes into both normal population and age fractions of cells and ghosts (Table 45), and show considerable variation between individuals in renewal time for each component. In particular the difference between 5 and 250 days for cell PE linoleate is noteworthy. Normal cells, incubated *in vitro* under near physiological conditions appear to be able to turn over their entire PC linoleate only once, or even less, in a lifetime (120 days). Their PE linoleate turnover rate appears to vary from only about one third right up to 20 times per lifetime. Ghost turnover rates are considerably faster.

The main features of each set of incubations of fractionated cells or ghosts are briefly as follows:

MJW(I) cells and linoleate: Total incorporation fell slightly with cell age. This is attributable to a fall in incorporation into PC, the major labelled component. Uptake into PE was relatively constant, but autoxidation effects tend to obscure the true picture. Radioactivities in other labelled lipids increased very slightly with age. On a percentage basis, a decrease in PC was accompanied by an increase in

other activities. The ratio of PE to PC specific activities increased with cell age.

<u>MJW(II) cells and linoleate</u>: Total incorporations were higher than normally observed with intact red cells. PE was responsible for most of the additional uptake, and there was a marked increase in PE activity with cell age, (see Figure 15) It appears that PC activity decreased slightly with cell age, DG activity increased, and there were no definite trends in PA or X activities. Incomplete separation of DG and X in the oldest fraction could account for its observed distribution. The most striking feature is the marked increase in the per cent of activity associated with PE, with corresponding decrease in PC, and as a result, the much higher ratio of PE to PC specific activity for older cells.

<u>GL cells and linoleate</u>: Apart from low activity in the small, oldest cell fraction, there was an overall increase in fatty acid uptake with cell age. Activities in all components of the oldest fraction were uniformly lower than would b expected from a comparison with other fractions, and it is possible that this could be due to an error in a fundamental measurement. Uptakes into both PC, the major labelled component, and PE increased with cell age, but DG and X were relatively constant, and PA low and variable. Variation in PA could in part be attributable to errors in estimation. Percentage distributions were relatively uniform, but PE to PC specific activity ratios were slightly higher in older fractions.

<u>MJW(I) ghosts and linoleate</u>: Linoleate incorporation was predominantly into PC, and this showed a slight decrease with cell age. Uptake into PE could have decreased slightly, but again autoxidation effects tend to obscure the true picture. PE to PC specific activity ratios were essentially constant, and no obvious variation in uptake into minor components is evident.

<u>MJW(II) ghosts and linoleate</u>: The same overall pattern as that for uptake in MJW(II) cells is evident. There was a marked increase in uptake into PE with age, and virtually no change in uptake into PC (see Figure 16). Uptakes into minor components varied a little, with that into PA increasing slightly with cell age.

CCW(I) ghosts and linoleate: Uptake into all esterified lipids was essentiall constant, whether expressed in terms of absolute uptake or percentage distribution. Combined DG and X activity was higher than usual. Autoradiography shows this activity to be evenly divided between two components (Figure 17).

VJC ghosts and palmitate: As a relatively large number of white cells accumulated in the densest red cell fraction, and made a large contribution to fatty acid uptake, accuracy of red cell data for this fraction is limited. Nevertheless a generally constant pattern of total incorporation is evident. Uptake into PE was fairly constant and that into PC decreased very slightly with cell age. Uptakes into PA, DG and X were higher in older cells. PE:PC specific activity ratios were essentially constant. High TG activity due to white cell contamination can be seen the autoradiographs (Figure 18).

GGM ghosts and palmitate: There was a slight increase in total fatty acid uptake with age, mainly attributable to uptake into PE. PC activities were constant and consequently there was a marked increase in PE:PC specific activity ratio with cell age. Activities of all components in the second oldest sample were uniformly lower than expected from comparison with other fractions, possibly because of an error in a fundamental measurement. DG and X activities apparently decreased, and PA activity increased with cell age. Autoradiography shows a low uptake into PS in all fractions. PE activities were higher than average, and the overall picture resembles that for MJW(II).

EJC ghosts and palmitate: Again total activities were essentially constant, and there were no marked variations with age in PC or PE activities, either in terms of absolute or relative uptake. DG and X activities decreased slightly with cell age. Fluctuations in PA activity were due, at least in part, to inaccuracy of lipid separation.

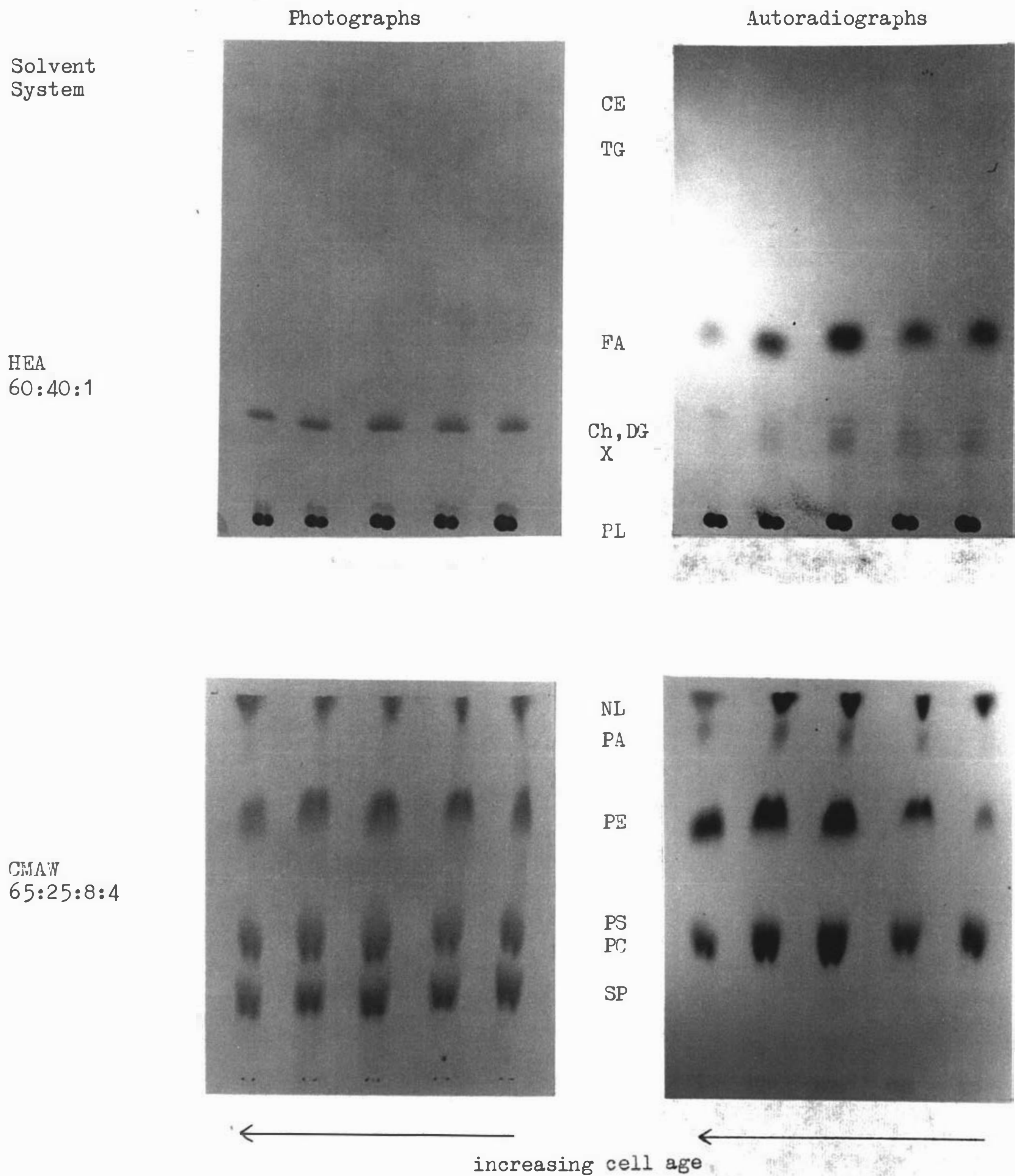


FIGURE 16: TLC SEPARATIONS OF LIPIDS FROM GHOSTS OF HUMAN RED CELLS, FRACTIONATED ACCORDING TO AGE, AFTER INCUBATION WITH [1-^{14}C]LINOLEATE (DONOR MJW(II))

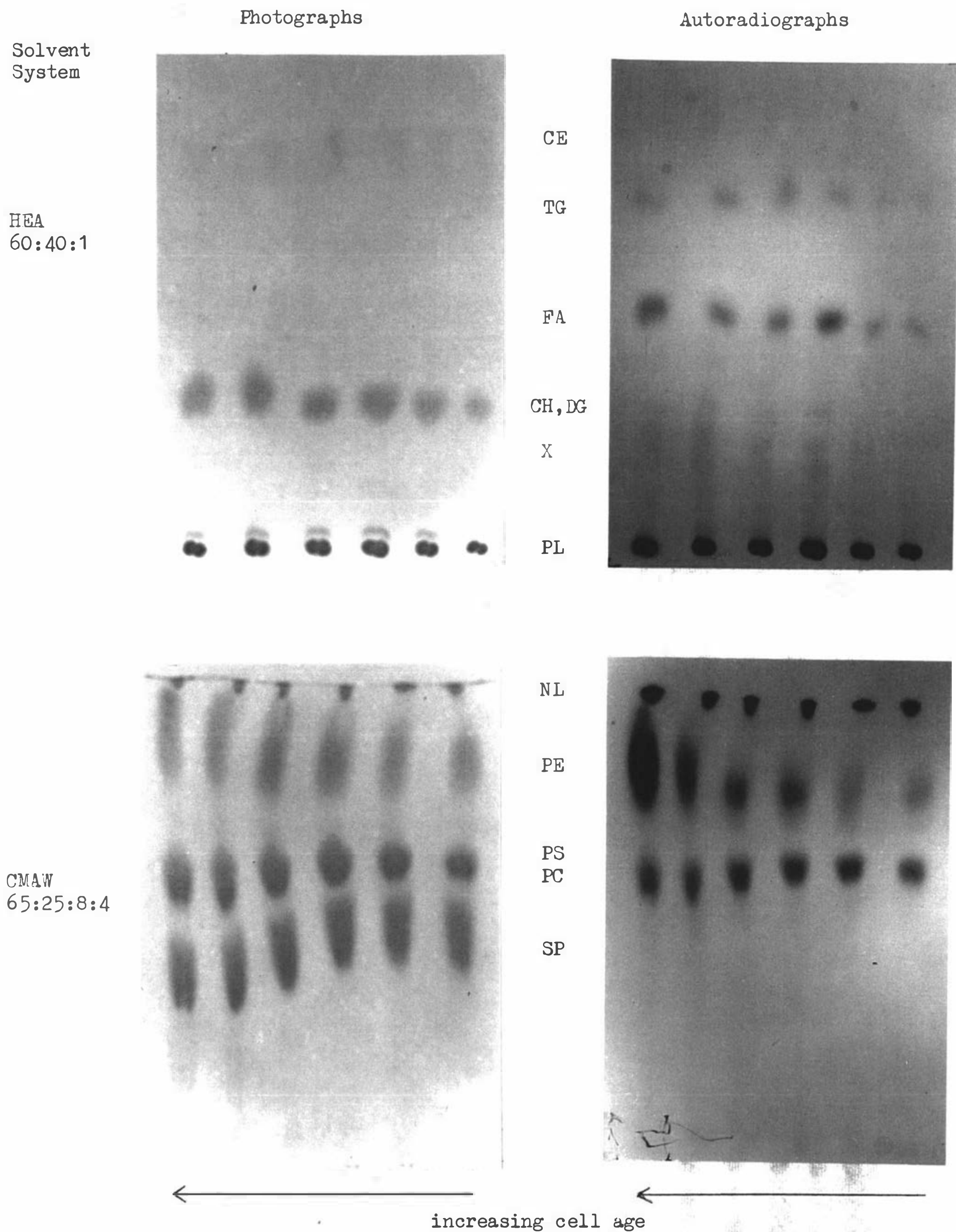
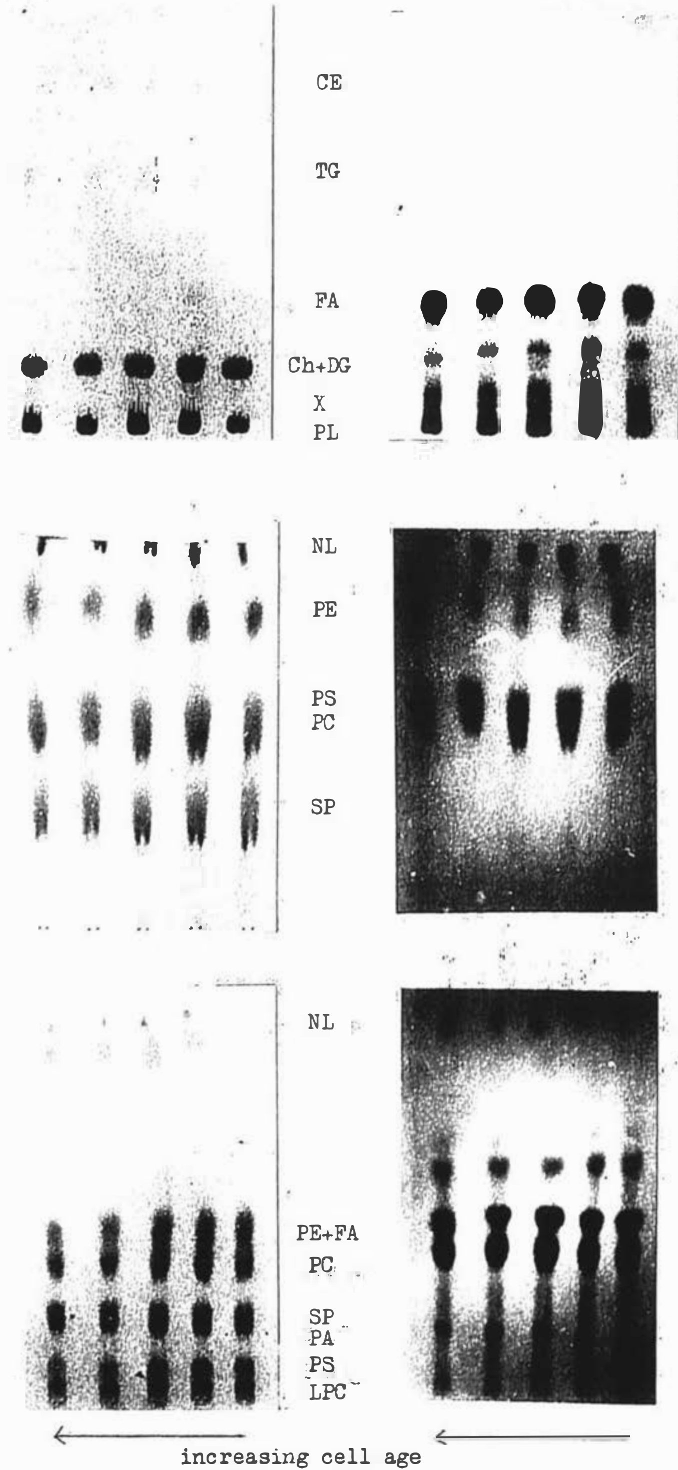


FIGURE 15: TLC SEPARATIONS OF LIPIDS FROM HUMAN RED CELLS FRACTIONATED ACCORDING TO AGE, AFTER INCUBATION WITH [1-^{14}C]LINOLEATE (DONOR MJW(II))



E 17: TLC SEPARATIONS OF LIPIDS FROM GHOSTS OF HUMAN RED CELLS, FRACTIONATED ACCORDING TO AGE, AFTER INCUBATION WITH [1-^{14}C]LINOLEATE (DONOR CCW)

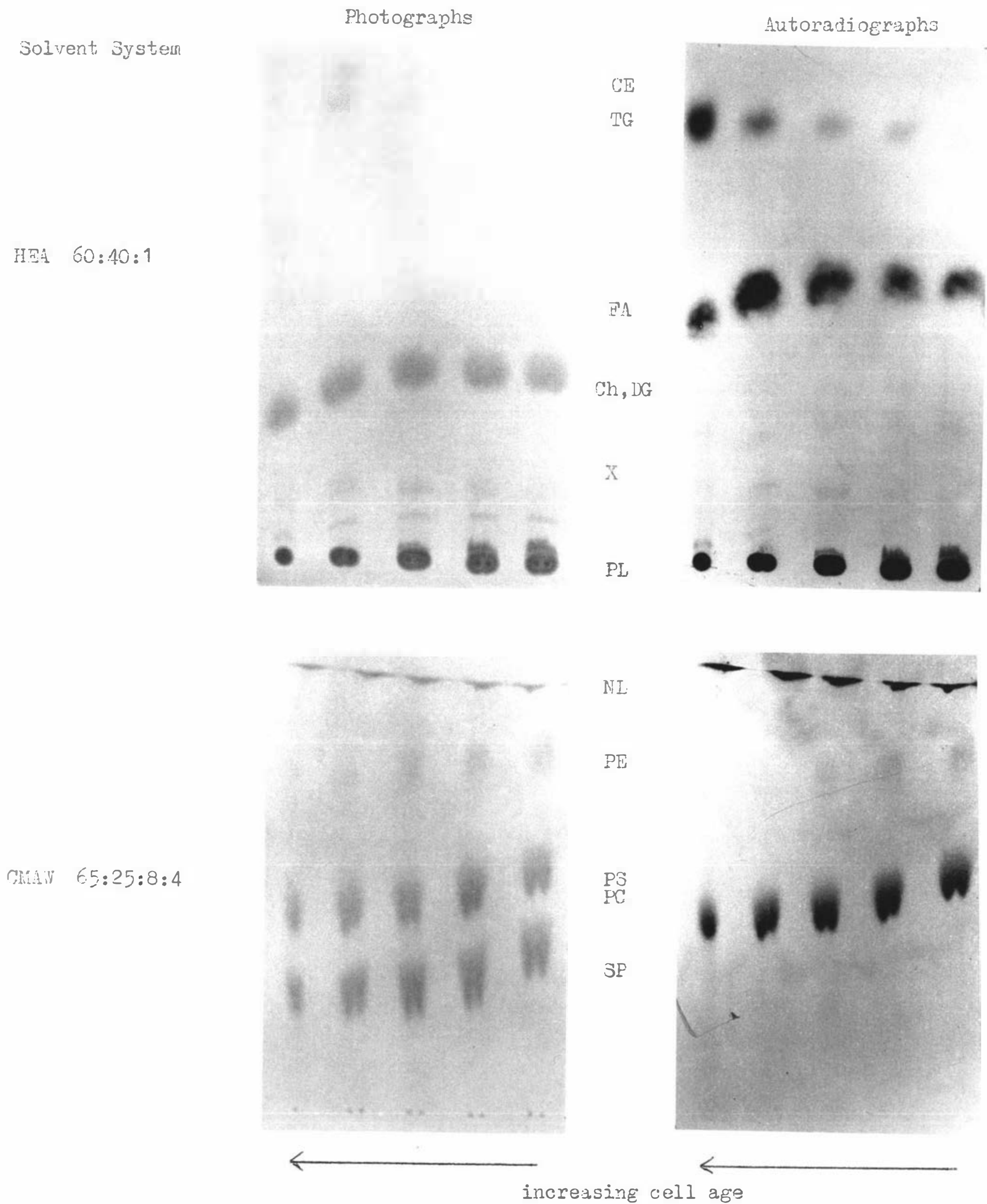


FIGURE 18: TLC SEPARATIONS OF LIPIDS FROM GHOSTS OF HUMAN RED CELLS, FRACTIONATED ACCORDING TO AGE, AFTER INCUBATION WITH [1-^{14}C]PALMITATE (DONOR VJC)

Photographs

Autoradiographs

Solvent System

CMAW 65:25:8:4

NL
PA
PE
PS
PC
SP
LPC

CMN 14:6:1

NL
FA
PE
PC
SP
PA
PS
LPC

increasing cell age

FIGURE 19: TLC SEPARATIONS OF LIPIDS FROM GHOSTS OF HUMAN RED CELLS, FRACTIONATED ACCORDING TO AGE, AFTER INCUBATION WITH [1-^{14}C]PALMITATE (DONOR GGM)

Table 36: Incorporation of $[1-^{14}C]$linoleate into Human Red Cells Fractionated According to Age. (donor MJW(I))

	density range of cells				
	1.075 – 1.080	1.080 – 1.082	1.082 – 1.084	1.084 – 1.086	1.086 – 1
Percentage distribution of the radioactivity in the esterified lipids					
DG + X	6 ± .5	9.5 ± 1	13.5 ± .5	16 ± 1	21 ± 2
PA	17 ± 2	19 ± 2.5	14 ± 3	29 ± 3	38 ± 4
PE	5(8) ± 1	9.0(12) ± 2.5	8.5(13) ± 1.5	10.5(21) ± .5	10(16) ± 2
PC	59 ± 1.5	55 ± 1.5	55 ± 1.5	30 ± 1	18 ± 1
SP + LPC	15 ± 1	11 ± 1	10 ± 1	14 ± 1	12.5 ± 1
Activity per µeq phospholipid (dpm)					
TL (esterified)	2100	1550	1450	1250	1350
DG + X	125	150	200	200	280
PA	350	300	200	360	520
PE	105(165)	145(185)	125(185)	130(250)	135(230)
PC	1230	850	800	370	250
SP + LPC	310	175	150	170	170
Specific activity (dpm/µeq)					
PE	650	700	700	1000	800
PC	3500	2800	2300	1050	780

These results are presented graphically in Figures 20 - 26. For an explanation of results, see the footnote to Table 34.

Table 37: Incorporation of $[1-^{14}C]$linoleate into Human Red Cells Fractionated According to Age, (Donor MJW(II))

	density range of cells					
	1.075	1.0815	1.0835	1.085	1.0865	1.090
Percentage distribution of the radioactivity in the esterified lipid						
DG	3.2 $\pm$ 0.2	4.3 $\pm$ 0.2	10.5 $\pm$ 0.2	10.0 $\pm$ 0.2	8.8 $\pm$ 0.2	1.1 $\pm$ 0.2
X	4.1 $\pm$ 0.2	5.4 $\pm$ 0.2	5.5 $\pm$ 0.2	4.8 $\pm$ 0.2	3.7 $\pm$ 0.2	2.2 $\pm$ 0.2
PA	9 $\pm$ 1.5	11 $\pm$ 1.0	10 $\pm$ 1.0	14 $\pm$ 1.0	10 $\pm$ 1.0	6.5 $\pm$ 1.0
PE	30 $\pm$ 0.5	31 $\pm$ 1.5	45.5 $\pm$ 0.5	40 $\pm$ 1.0	55 $\pm$ 0.5	80 $\pm$ 1.0
PC	54 $\pm$ 1.0	49 $\pm$ 1.5	28.5 $\pm$ 0.5	32 $\pm$ 1.0	22 $\pm$ 0.5	9.6 $\pm$ 0.5
Activity per µeq phospholipid (dpm)						
TL(esterified)	3500	2550	2900	3000	4150	11700
DG	110	110	300	300	360	130
X	140	140	160	145	150	260
PA	310	280	290	420	400	760
PE	1050	800	1320	1200	2300	9400
PC	1900	1250	820	960	910	1120
Specific activity (dpm/µeq)						
PE	3500	3000	4500	4100	8000	27500
PC	5400	3500	2700	3000	2650	3800

These results are presented graphically in Figures 20 - 26. For an explanation of results, see the footnote to Table 34.

Table 38: Incorporation of $[1-{}^{14}C]$linoleate into Human Red Cells Fractionated According to Age, (Donor GL)

	density range of cells				
	1.075 – 1.079	1.079 – 1.0805	1.0805 – 1.0815	1.0815 – 1.0835	1.0835 – 1.0865
Percentage distribution of the radioactivity in the esterified lipids					
DG	5.7 ± 0.3	7.0 ± 0.3	7.5 ± 0.3	5.2 ± 0.3	8.0 ± 0.5
X	11.0 ± 0.3	10.5 ± 0.3	12.8 ± 0.3	9.2 ± 0.3	17 ± 0.5
PA	3.5 ± 3.0	6.5 ± 1.0	2.5 ± 1.0	8.0 ± 2.0	4.5 ± 2
PE	7.5 ± 0.5	8.5 ± 0.3	8.5 ± 0.5	10.0 ± 1.5	10.0 ± 0.5
PC	72 ± 1.0	68 ± 1.0	68 ± 0.5	66 ± 0.5	62 ± 0.5
Activity per μeq phospholipid (dpm)					
TL(esterified)	1480	1950	2100	2400	1080
DG	85	135	160	125	85
X	160	210	270	220	185
PA	50	125	50	190	50
PE	110	165	180	240	110
PC	1070	1350	1450	1600	670
Specific activity (dpm/μeq)					
PE	360	540	560	820	370
PC	3400	4300	4800	5200	2100

These results are presented graphically in Figures 20 - 26. For an explanation of results, see the footnote to Table 34.

Table 39: Incorporation of [1-^{14}C]linoleate into Ghosts of Human Red Cells Fractionated According to Age (Donor MJW(I))

	density range of cells				
	1.075 – 1.080	1.080 – 1.082	1.082 – 1.084	1.084 – 1.086	1.086 – 1.110
Percentage distribution of the radioactivity in the esterified lipids					
DG + X	1.8 ± 0.2	2.2 ± 0.3	4.2 ± 0.3	3.8 ± 03	5.6 ± 0.3
PA	4.0 ± 1.0	4.5 ± 1.0	5.5 ± 1.0	2.0 ± 1.0	7.5 ± 1.0
PE	5.2(9.5) ± 0.5	5.1(7.0) ± 0.3	3.8(5.5) ± 0.3	4.4(5.5) ± 0.5	4.1(7.0) ± 0.3
PC	77 ± 1.0	85 ± 1.0	85 ± 1.0	85 ± 1.0	75 ± 1.0
SP + LPC	13.2 ± 0.3	3.3 ± 0.3	3.3 ± 0.3	4.2 ± 0.3	7.4 ± 0.3
Activity per μeq phospholipid (dpm)					
TL(esterified)	7500	6700	6600	6400	5600
DG + X	135	150	280	245	310
PA	300	300	360	125	410
PE	390(720)	350(470)	255(360)	285(350)	240(390)
PC	5800	5700	5600	5400	4200
SP + LPC	970	220	220	270	410
Specific activity (dpm/μeq)					
PE	2600	1750	1450	1300	1500
PC	18500	17500	17500	17200	13000

These results are presented graphically in Figures 20 - 26. For an explanation of results, see the footnote to Table 34.

Table 40: Incorporation of [1-^{14}C]linoleate into Ghosts of Human Red Cells Fractionated According to Age (Donor MJW(II))

	density range of cells				
	1.075 – 1.0835	1.0835 – 1.0855	1.0855 – 1.088	1.088 – 1.0935	1.0935 – 1.102
Percentage distribution of the radioactivity in the esterified lipids					
DG	3.8 ± 0.5	3.6 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	1.1 ± 0.2
X	3.1 ± 0.2	3.2 ± 0.2	1.3 ± 0.2	2.2 ± 0.2	1.1 ± 0.2
PA	11 ± 2.0	8 ± 1.5	8 ± 1.5	8 ± 2.0	10 ± 1.0
PE	16 ± 0.5	38 ± 0.5	43 ± 1.0	49 ± 1	56.5 ± 1.0
PC	63 ± 2.0	46 ± 0.5	45 ± 1.0	35 ± 1	30 ± 1.0
Activity per μeq phospholipid (dpm)					
TL(esterified)	3500	3900	6600	7500	7500
DG	130	140	150	160	80
X	105	125	85	160	80
PA	380	320	540	600	760
PE	540	1500	2850	3800	4200
PC	2150	1800	2950	2600	2250
Specific activity (dpm/μeq)					
PE	1900	5000	9800	12200	14000
PC	6200	5200	8200	7600	6600

These results are presented graphically in Figures 20 - 26. For an explanation of results, see the footnote to Table 34.

Table 41: Incorporation of [1-^{14}C]linoleate into Ghosts of Human Red Cells Fractionated According to Age (Donor CCW)

	density range of cells				
	1.075 – 1.080	1.080 – 1.082	1.082 – 1.084	1.084 – 1.086	1.086 – 1.105
Percentage distribution of the radioactivity in the esterified lipids					
DG + X	20 ± 0.5	23.5 ± 0.5	21.5 ± 0.4	24 ± 0.4	26 ± 0.4
PA	9.5 ± 1.0	8.0 ± 1.5	10 ± 1.5	8.5 ± 2.0	9.0 ± 2.0
PE	8.1 ± 0.3	5.5 ± 0.3	5.7 ± 0.3	5.4 ± 0.3	5.9 ± 0.3
	(8.7)	(6.6)	(8.3)	(7.0)	(8.0)
PC	58 ± 0.5	60 ± 0.5	57 ± 0.5	58 ± 0.5	58 ± 1.0
Activity per μeq phospholipid (dpm)					
TL(esterified)	12000	12500	12800	14000	13200
DG + X	2400	2950	2750	3300	3500
PA	1100	1000	1250	1250	1250
PE	970	700	720	750	800
	(1050)	(850)	(1050)	(1000)	(1100)
PC	6900	7500	7200	8000	7700
Specific activity (dpm/μeq)					
PE	3900	3150	3900	3600	3900
PC	20000	22000	21000	23000	22000

These results are presented graphically in Figures 20 - 26. For an explanation of results, see footnote to Table 34.

Table 42: Incorporation of [1-^{14}C]palmitate into Ghosts of Human Red Cells Fractionated According to age (Donor VJC)

	density range of cells				
	1.075 – 1.080	1.080 – 1.082	1.082 – 1.084	1.084 – 1.086	1.086 – 1.110
Percentage distribution of the radioactivity in the esterified lipids					
DG	3.0 ± 0.5	3.5 ± 0.5	3.8 ± 1.0	11 ± 1.0	11 ± 2.0
X	5.5 ± 0.5	6.5 ± 0.5	10 ± 1.0	13 ± 1.0	22 ± 2.0
PA	10 ± 2.0	11 ± 3.0	8.5 ± 2.0	18.5 ± 3.0	18 ± 4.0
PE	10 ± 0.5	9.8 ± 0.5	7.2 ± 0.5	5 ± 1.0	7 ± 2.0
	(12.5)	(15.5)	(12.5)	(9)	(11)
PC	67 ± 2.0	66 ± 1.0	60 ± 1.0	45 ± 2.0	41 ± 4.0
LPC + SP	3.0 ± 0.5	4.5 ± 0.5	12 ± 1.0	4.0 ± 1.0	1.0 ± 1.0
Activity per µeq phospholipid (dpm)					
TL(esterified)	3800	3900	3600	4700	4800
DG	115	140	130	520	520
X	210	250	340	620	1000
PA	370	430	280	850	800
PE	370	380	250	230	300
	(460)	(600)	(430)	(430)	(500)
PC	2500	2600	2000	2050	1900
LPC + SP	115	180	400	180	50
Specific activity (dpm/µeq)					
PE	1750	2200	1550	1450	1800
PC	7400	7700	5800	5800	5800

These results are presented graphically in Figures 20 - 26. For an explanation of results, see the footnote to Table 34.

Table 43: Incorporation of [1-^{14}C]palmitate into Ghosts of Human Red Cells Fractionated According to Age (Donor GGM)

	density range of cells				
	1.075 – 1.078	1.078 – 1.080	1.080 – 1.082	1.082 – 1.0845	1.0845 – 1.102
Percentage distribution of the radioactivity in the esterified lipids					
DG + X	10.5 ± 0.5	8.8 ± 0.5	6.8 ± 0.5	6.7 ± 0.5	5.6 ± 0.5
PA	5.5 ± 1.0	7.0 ± 1.0	9.5 ± 1.0	11.5 ± 1.0	8.5 ± 1.0
PE	13.5 ± 0.5	13 ± 0.5	13.5 ± 0.5	24 ± 0.5	30.5 ± 0.5
		(18)	(23)	(35)	(37)
PC	61 ± 1.0	62 ± 1.0	54 ± 1.0	48 ± 1.0	49 ± 1.0
SP + LPC	3.8 ± 0.5	4.5 ± 0.5	9.7 ± 0.5	7.5 ± 0.5	5.5 ± 0.5
Activity per μeq phospholipid (dpm)					
TL(esterified)	5400	6200	6600	4300	7200
DG + X	570	550	460	290	400
PA	300	450	600	520	600
PE	720	830	920	1050	2200
		(1100)	(1500)	(1500)	(2600)
PC	3300	3800	3500	2050	3500
SP + LPC	200	280	630	320	390
Specific activity (dpm/μeq)					
PE	2800	4400	5600	5700	10200
PC	10200	10400	10400	6300	10000

These results are presented graphically in Figures 20 - 26. For an explanation of results, see footnote to Table 34.

Table 44: Incorporation of [1-^{14}C]palmitate into Ghosts of Human red Cells Fractionated According to age (Donor EJC)

	density range of cells				
	1.075 – 1.078	1.078 – 1.080	1.080 – 1.082	1.082 – 1.084	1.084 – 1.086
Percentage distribution of the radioactivity in the esterified lipids					
DG	8.7 ± 0.5	6.5 ± 0.3	6.1 ± 0.3	5.5 ± 0.5	4.5 ± 1.0
X	5.2 ± 2.0	2.0 ± 0.5	1.2 ± 0.3	2.0 ± 0.2	- ± 0.5
PA	6.0 ± 2.0	- ± 1.5	1.0 ± 1.0	7.0 ± 1.5	1.5 ± 1.5
PE	11.7 ± 3.0	12.8 ± 0.5	14 ± 0.5	12.2 ± 0.5	19 ± 1.5
PC	56 ± 2.0	71 ± 1.0	72 ± 1.0	65 ± 1.0	68 ± 2.0
Activity per µeq phospholipid (dpm)					
TL(esterified)	7200	8900	8400	7400	7600
DG	630	570	520	410	330
X	370	180	100	150	-
PA	450	-	80	550	110
PE	830	1150	1200	910	1450
PC	4100	6300	6100	4800	5200
Specific activity (dpm/µeq)					
PE	2700	3600	3900	3000	4000
PC	14000	21000	21000	17500	17500

These results are presented graphically in Figures 20 - 26. For an explanation of results, see the footnote to Table 34.

Table 45: Estimation of Approximate Rates of Linoleate and Palmitate Turnover in Human Erythrocyte or Ghost Lipid Components, as a Result of Uptake of the Free Fatty Acid from Plasma

Component	Activity taken up in 3 hr from plasma, 10^6 dpm per ml (dpm per μeq PL)	Plasma FFA taken up per hr (μeq per μeq cell PL) x 10^5	Linoleate (or palmitate) in component μeq/μeq PL	Fraction replaced per hr (Per cent)	Renewal time (days)
		Cells and linoleate			
PC	500-1500	2-6	0.15	.013-.04	100-300
PE (normal)	200	0.8	0.045	.018	250
PE (high uptake)	1000-9000	4-36	0.045	.09-.8	5-45
		Ghosts and linoleate			
PC	3000-8000	12-50	0.15	.15-.35	12-30
PE (normal)	500-1000	2-4	0.045	.07-.13	30-60
PE (high uptake)	500-4000	3-25	0.045	.07-.55	7-60
		Ghosts and palmitate			
PC	2500-5000	15-30	0.15	.07-.15	30-60
PE (normal & high uptake)	500-2500	3-15	0.045	.04-.2	20-100

Data used in calculations:

Plasma FFA concentration[257-259] 0.8 μeq per ml*
Linoleate[260,261] 15% 0.12 μeq per ml
Palmitate[260,261] 24% 0.19 μeq per ml
A plasma radioactivity concentration of 10^6 dpm per ml is equivalent to: Linoleate specific activity 8×10^6 dpm per μeq; Palmitate specific activity 5×10^6 dpm per μeq.

Red cell phospholipids

PC	33% total	23% linoleate[67,72]	0.15 μeq linoleate per μeq PL
		32% palmitate	0.21 μeq palmitate per μeq PL
PE	30% total	7.5% linoleate	0.045 μeq linoleate per μeq PL
		12.5% palmitate	0.075 μeq palmitate per μeq PL

*Plasma FFA concentrations can vary over a wide range, (normally about 0.3 - 1 ueq/ml). As uptakes appear proportional to fatty acid concentration, they would vary accordingly.

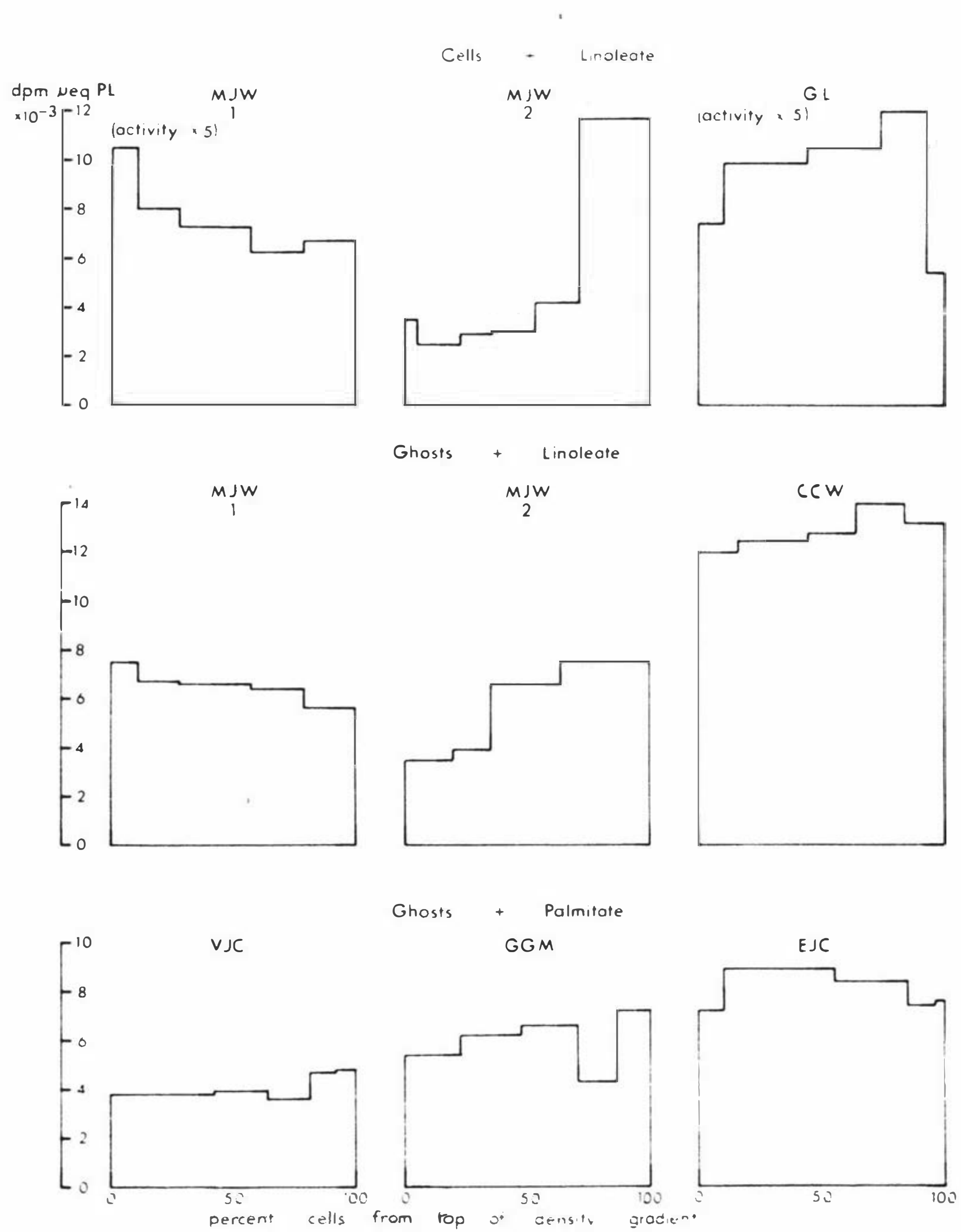


Figure 20: Incorporation of Labelled Fatty Acids into the Total Esterified Lipids in Human Red Cells of Different Ages

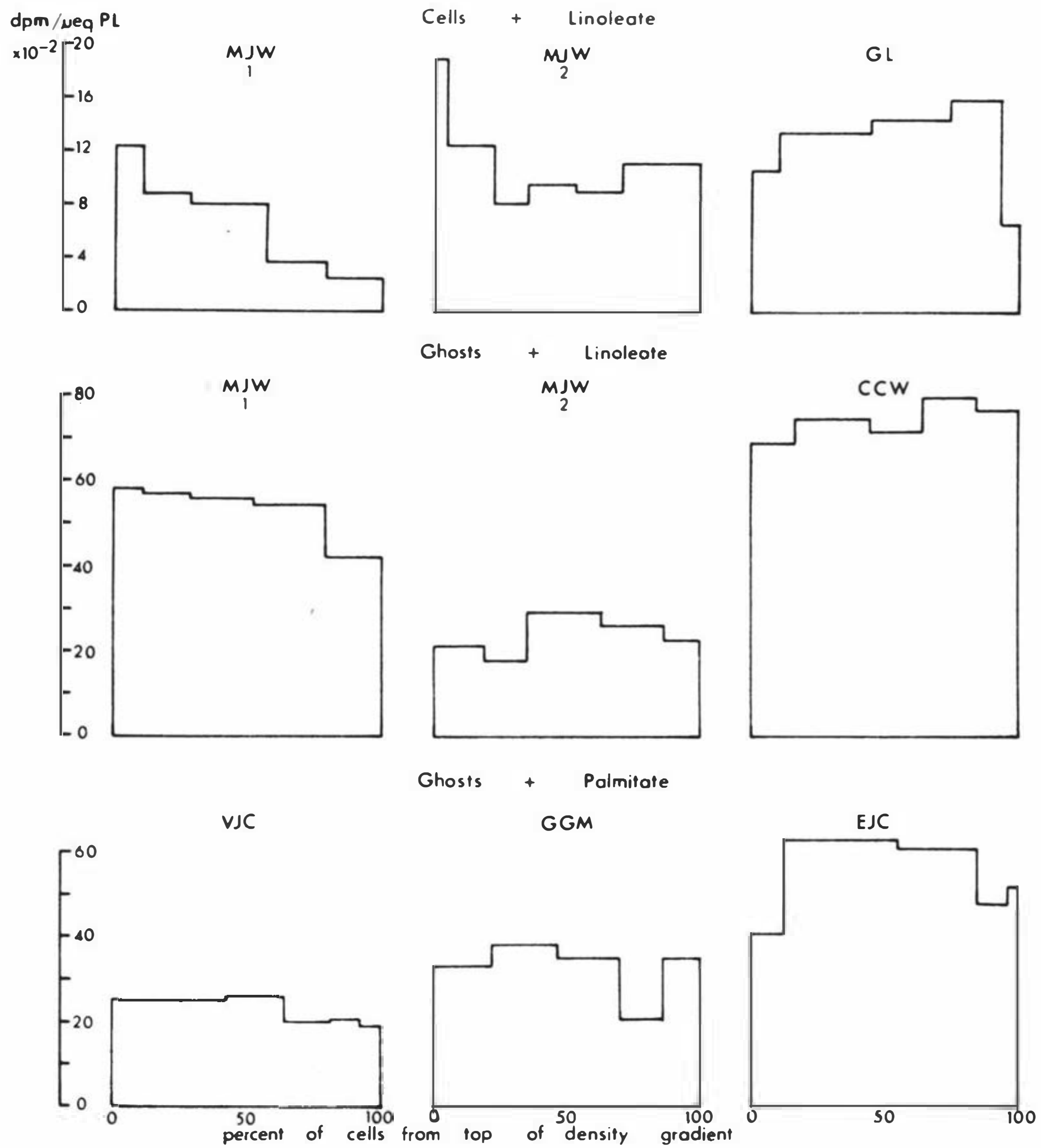


Figure 21: Incorporation of Labelled Fatty Acids into Phosphatidyl Choline in Human Red Cells of Different Ages

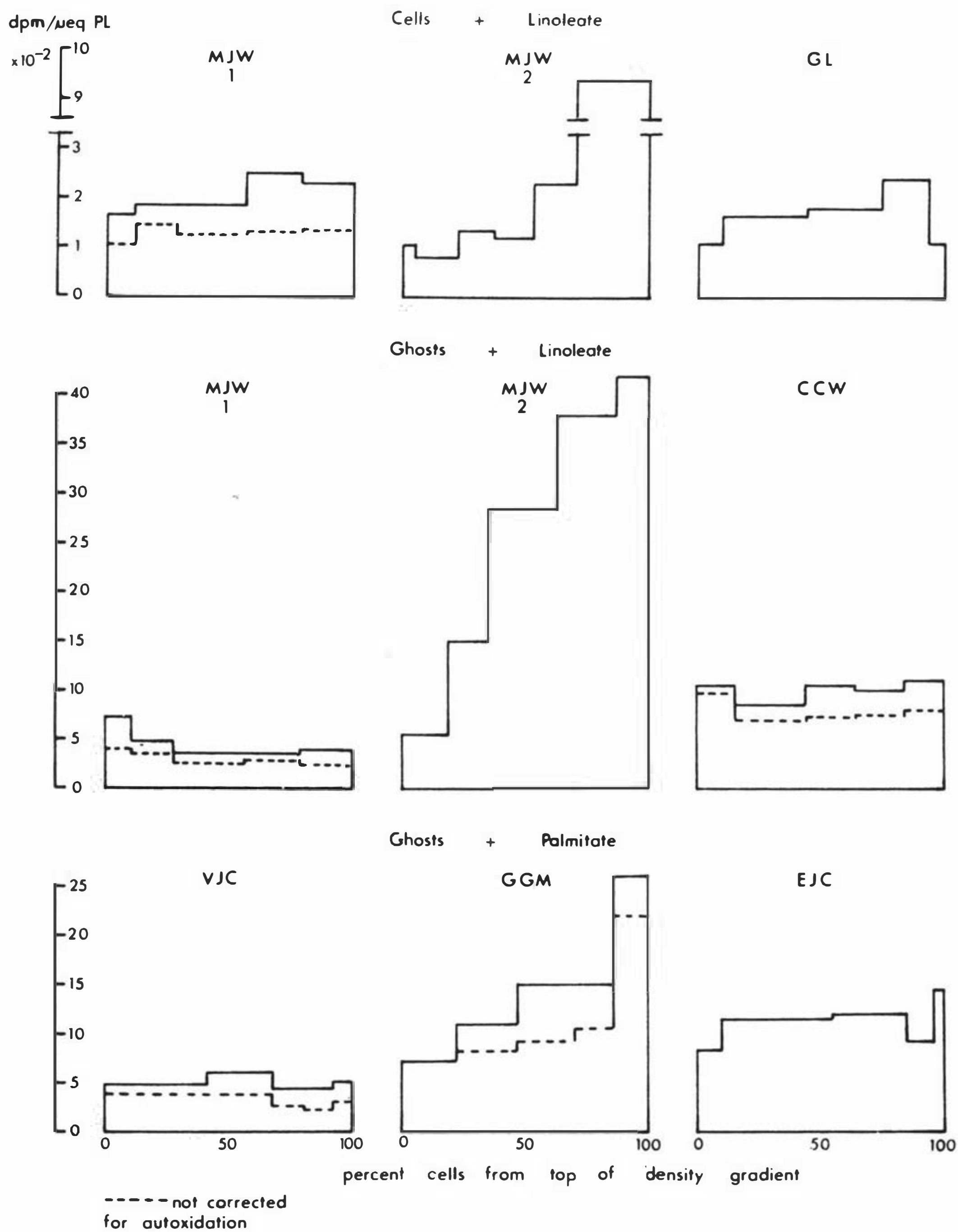


Figure 22: Incorporation of Labelled Fatty Acids into Phosphatidyl Ethanolamine in Human Red Cells of Different Ages

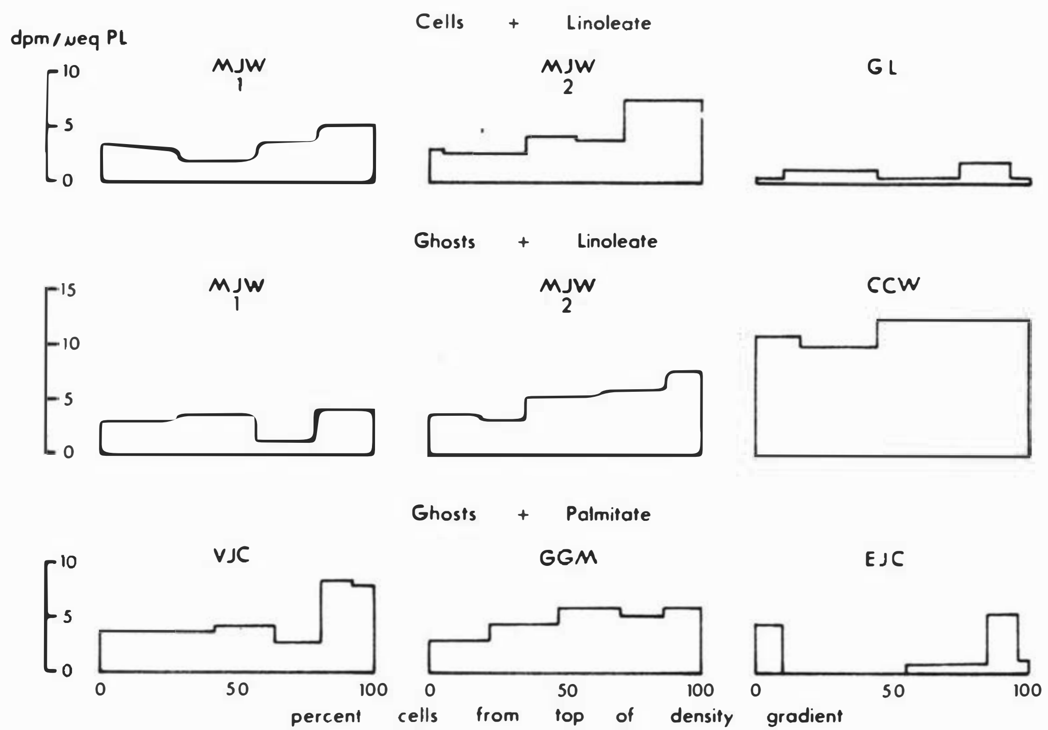


Figure 23: Incorporation of Labelled Fatty Acids into Phosphatidic Acid in Human Red Cells of Different Ages

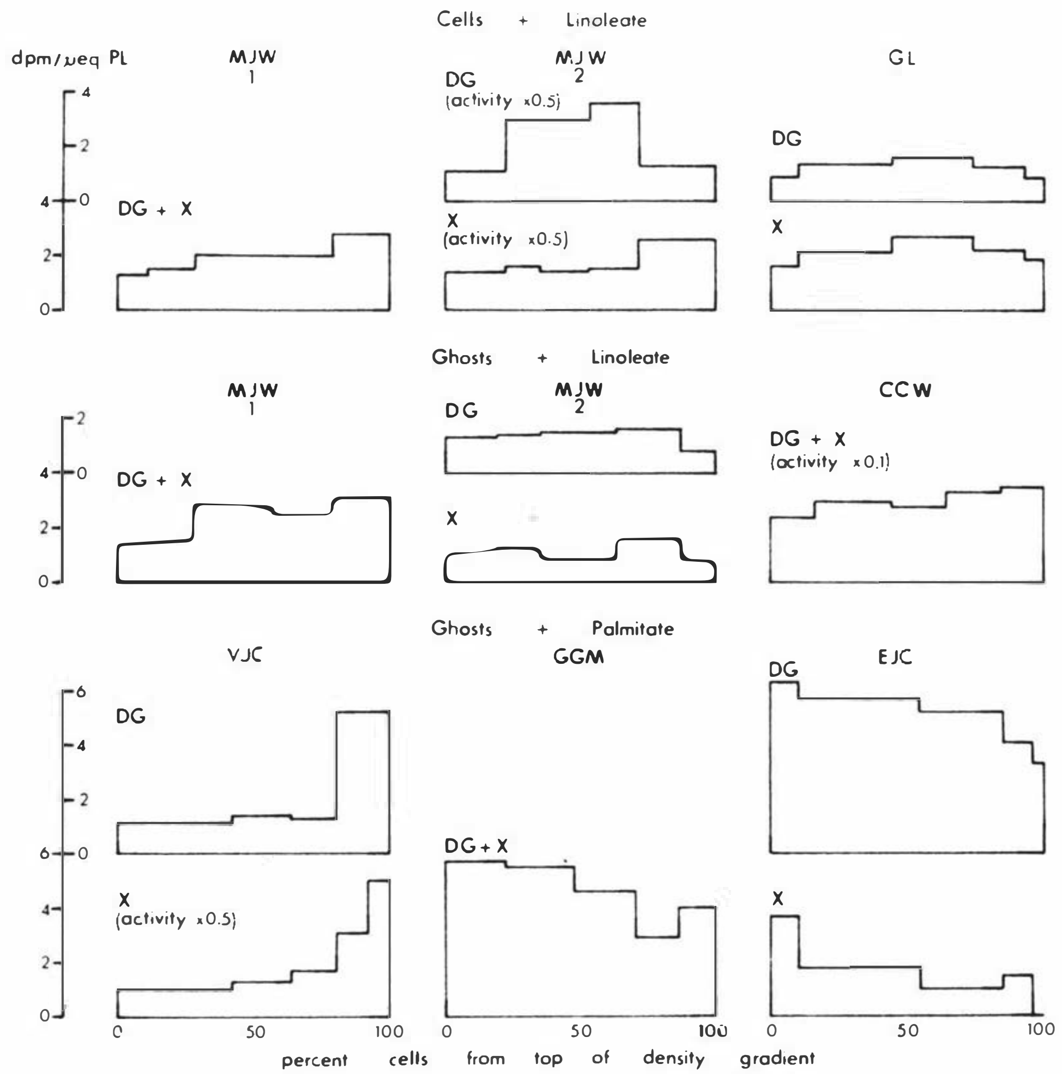


Figure 24: **Incorporation of Labelled Fatty Acids into Diglycerides and Components X in Human Red Cells of Different Ages**

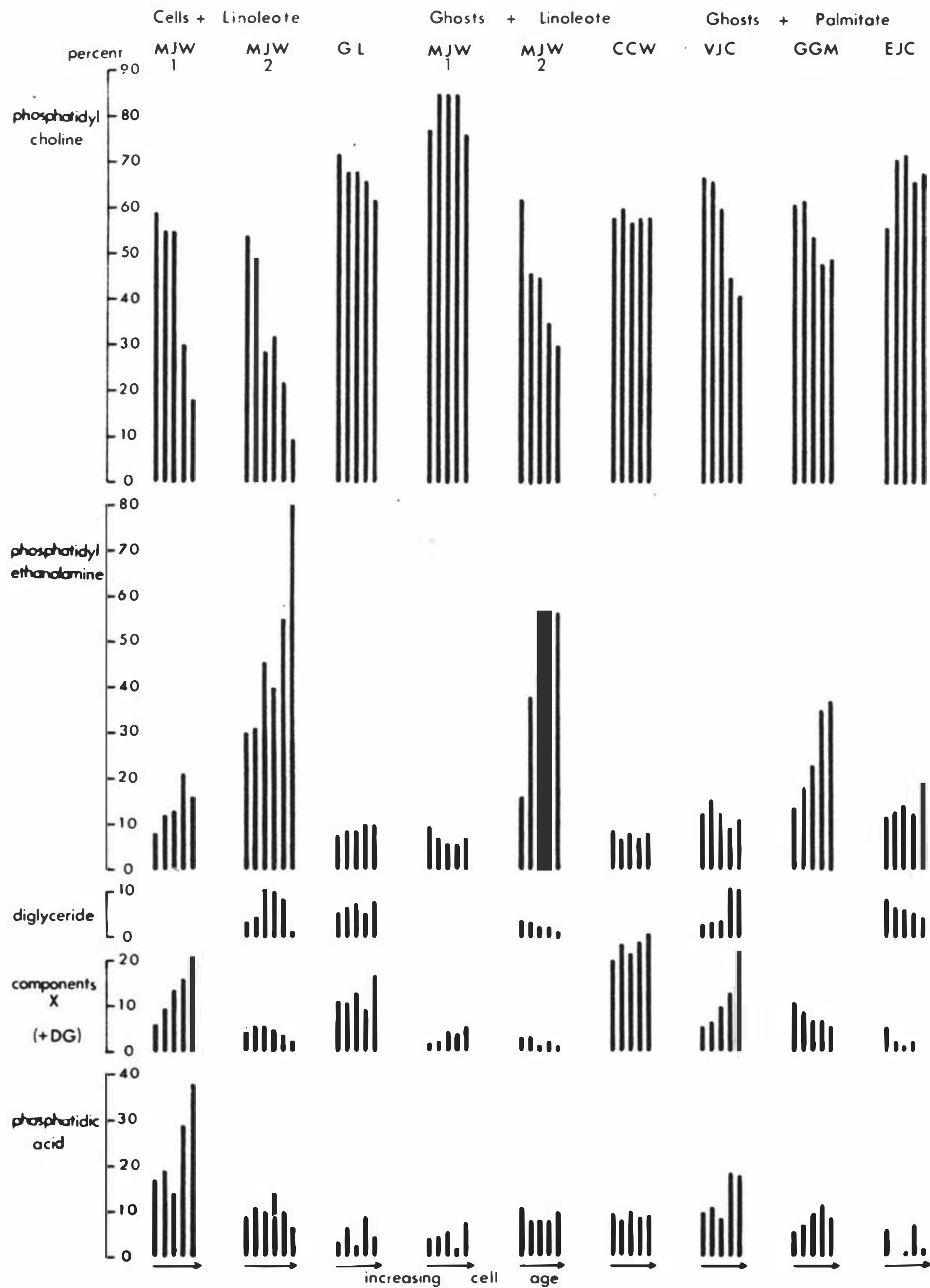


Figure 25: Percentage Distribution of Fatty Acids Esterified by Human Red Cells of Different Ages

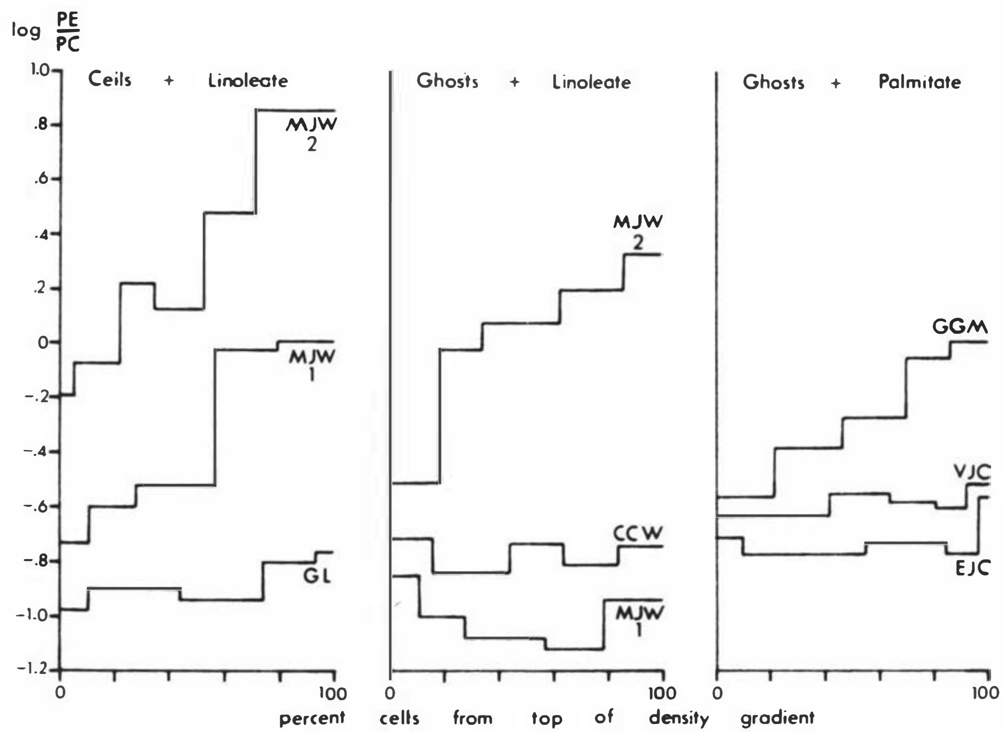


Figure 26: Phosphatidyl Ethanolamine to Phosphatidyl Choline Specific Activity Ratios in Human Red Cells of Different Ages

Comparison between Different Blood Samples

On comparing the different sets of incubations of red cells or ghosts with plasma fatty acids, it is obvious that a wide range of behaviour is possible. For an ATP and CoA dependent process, it would be expected that uptake into cells to which these cofactors were not added would be lower than that into enriched ghosts. Lower uptakes were observed for two of the cell samples, but for the other, the extent of fatty acid incorporation was comparable to that for ghosts.

On the average, linoleate uptake into ghosts was slightly higher than palmitate uptake. However variation between individuals was too great to assess the difference.

Uptake of a particular acid would appear to depend on cell concentration. There is some correlation between uptake into total esterified lipid (Figure 20) and cell dilution during incubation:

	cell dilution
cells + linoleate	MJW(I) 1:4 = GL 1:4 > MJW(II) 1:3.5
ghosts + linoleate	CCW 1:5.8 > MJW(I) 1:4.2 > MJW(II) 1:3.5
ghosts + palmitate	EJC 1:6.4 > GGM 1:5.6 > VJC 1:4.25

Variation between sets of incubations is particularly evident for the distribution of the fatty acid taken up, between the different cell lipids. However this cannot be explained in terms of differences between palmitate and linoleate uptake, or between uptake into cells and ghosts.

Blood samples were taken from all donors shortly after breakfast, hence variations in plasma composition would be expected. As the esterification reactions under study make use of external substrates, namely free fatty acids and lysophospholipids[41,102,103], variations in concentrations of these would considerably influence both total incorporation rate and the distribution of the incorporated fatty acid. Variation in plasma constitution could have two effects. Firstly, different sets of incubations have been compared by assuming that uptake of a fatty acid is proportional to its concentration. If this were not so, the lower plasma specific activities would be associated with higher incorporations. However no such

correlation is evident. Secondly, concentrations of plasma lysophospholipids, and precursors of diglycerides and the unidentified nonpolar lipids (X) if present, could vary independently. This would affect both the overall fatty acid uptake, and the distribution of the acid taken up.

Variation in Fatty Acid Uptake with Cell Age

The relationship between fatty acid uptake and red cell age also varied considerably between different blood samples. For incorporation into PC and PE, the following relationships have been observed:

(a) both remain constant with age,

(b) both increase slightly with age,

(c) an increase with age in uptake into PE with a constant uptake into PC,

(d) a constant uptake into PE with a decreasing uptake into PC.

On this basis there are two fairly distinct types of behaviour; with the first, the ratio of PE:PC specific activities increases markedly with cell age (c and d), but with the second, this ratio remains virtually unchanged (a and b). Uptakes into PA, DG and X were mostly constant or slightly increasing with age, although in two cases uptakes into DG and X decreased slightly. No correlation between the behaviour of these components and either PC or PE is evident.

Although plasma variation could account for variation between different sets of incubations, it alone cannot account for variation between different fractions of the same cell population, as all fractions were incubated in identical media. Behaviour does not appear to be necessarily characteristic of a particular donor. Two blood samples were removed at different times from the same donor (MJW), and both cells and ghosts from each incubated. Although cells and ghosts from the same blood sample behaved similarly, there was a marked difference in behaviour between the two samples. In addition, behaviour is apparently independent of the fractionation procedure, as both MJW(II) fractionations were carried out independently.

Before suggesting possible interpretations of these results, it should be

ascertained that the observed differences in red cell age dependence of fatty acid uptake are genuine. Firstly the accuracy of results in any incubation should be considered. Estimates of errors arising out of TLC separation, quantitative analysis, and correction for white cells have been made and are shown in the tables. Measurements of total activity per unit phospholipid should be reproducible to within ± 5 per cent. These errors are insufficient to explain the observed variations between cell fractions. Because of the method of estimation, PA radioactivities are the least reliable. Radioactivity uptakes are related to the amount of phospholipid extracted, and hence no error would have been introduced by uniform lipid loss during extraction. There is no evidence for gain of extraneous phospholipid (Figures 11,13) causing erroneously low uptakes.

A possible source of error is in the constitution of the incubation systems. These systems were prepared by adding proportionate amounts of KRP and plasma to packed cells, and errors in measurement of these volumes would have been greatest for the smallest cell samples. Dilution errors should not have affected concentrations of fatty acid available for uptake into the cells, (as dissociation of the albumin-fatty acid complexes accompanies dilution). However as it appears that cellular acyl transferases were not saturated , fatty acid uptakes would have been dependent on cell concentration (see p.128), and hence affected by errors in dilution. It is very unlikely that such errors would be large and able to account fo the ranges of incorporation observed. In addition, percentage distributions of esterified activities should be unaffected by any errors in cell dilution.

In the studies in which different fractions show a wide range of behaviour, variation does appear to occur progressively with age rather than randomly, hence there appears to be no cause to doubt that the major variations in fatty acid uptake with age are real. Small variations, especially in minor components such as PA, DG and X, could be attributable to experimental error.

Another possibility that should be discussed is whether the lack of variation in uptake with cell age observed in some experiments could be due to lack of

significant age fractionation. The same density gradient centrifugation technique was employed in all experiments, solutions were always isotonic, and albumin densities were modified to suit the particular cells. Definition of cell bands was clearer in some separations than in others but these bands were always discernable. Although small variation in the efficiency of age separation could thus have arisen, there is no reason why any marked differences, or lack of separation, should have been achieved.

It seems reasonable, therefore, to assume that genuine differences in behaviour of red cells of different ages have been observed. As these differences cannot be explained solely in terms of substrate concentrations in the incubation medium, they must be due to different capabilities of young and old cells.

Incorporation of Labelled Fatty Acid into the Lipids in Human Plasma

When plasma was incubated alone with [1-^{14}C]palmitate or [1-^{14}C]linoleate, radioactive components in lipid extracts were similar to those present before incubation, (see p. 24), and no radioactivity corresponding to any phospholipid could be detected. However, after incubation with either acid in the presence of red cell ghosts, some activity was present in plasma PC and LPC (Figure 14).

The relationship between radioactivity released into plasma and age of red cell ghosts was investigated and the results are presented in Table 46. Percentages of activity in the phospholipids were very low, and hence very sensitive to very small amounts of trailing fatty acid. PC activities were significantly higher than the control, but PE and LPC activities were less conclusive. No correlation with age of the incubated cells could be deduced from these results.

Table 46: Radioactivity Distributions in Plasma Lipids after Incubating with Human Red Cell Ghosts of Different Ages and [1-^{14}C]Palmitate (Donor GGM)

	Percentage of Radioactivity			
	solvent front	PE	PC	LPC
Plasma incubated alone	>99.9	.02	.02	.02
Plasma incubated with cells				
Fraction I	99.7	.078	.18	.035
II	99.7	.085	.15	.025
increasing III	99.7	.085	.16	.026
IV	99.8	.082	.10	.034
cell age V	99.5	.12	.25	.12

Plasma lipids were extracted and separated by TLC with CMAW 65:25:8:4

Lipid Content of Rat Red Cell Populations with Different Mean Ages

General information on incubations and fractionations is summarised in Table 47. Of the three fractionations only one was suitable for studying variation in lipid levels. Data from Rats C are presented in Figure 27 and Table 48, in which a comparison is also made with data from normal cell populations and with literature values. Lipid levels in the cells of different ages varied in the ranges observed for normal populations, and agreement with literature values is good. It appears that there was a slight decrease in cell phospholipid and total lipid with age, and that this decrease occurred mainly between the two youngest fractions. Distributions of both phospholipids and neutral lipids, although fluctuating somewhat, showed no definite trends with age. Results are therefore in agreement both with those obtained for albumin gradient fractionation of human red cells, and serial osmotic hemolysis of bovine cells.

Table 48: Variation in Lipid Content of Rat Red Cells, Fractionated According to Age

	Rats C increasing age of cells →					Normal cell populations (a)	Literature Values (b)	(c)
Total lipid mg/10^{10} cells	3.10	2.65	2.95	2.55	2.90	2.85 ± 0.1	3.15	
Phospholipid µeq/10^{10} cells	2.40	2.10	2.20	2.05	2.15	2.45 ± .05	2.75	61
Cholesterol mg/10^{10} cells	0.75	0.70	0.85	0.65	0.80	0.68 ± .05	0.79	28
Per cent Phospholipid	61 ± 1	64 ± 1	60 ± 2	62 ± 2	58 ± 2	65 ± 2	67	61
Cholesterol	24 ± 1	27 ± 1	29 ± 2	24 ± 1	28 ± 2	24 ± 3	25	28
FFA	6 ± 1	3 ± 1	4 ± 1	4 ± 1	5 ± 2		-	
TG	7 ± 1	3 ± 1	5 ± 2	5 ± 2	5 ± 2		-	
CE	7 ± 1	3 ± 1	5 ± 2	4 ± 1	6 ± 2		-	
Percentage distribution of major phospholipids								
PE	29 ± 2	21 ± 2	30 ± 2	26 ± 2	23 ± 3	24 ± 2	22	17-23
PS						16 ± 2	14	
PC	56 ± 2	63 ± 2	59 ± 1	60 ± 2	65 ± 3	48 ± 2	48	58-63
SP	16 ± 2	16 ± 1	11 ± 2	14 ± 1	12 ± 2	12 ± 1	13	18-22

Neutral lipids were separated in duplicate by TLC in HEA 70:30:1, phospholipids in CMAW 65:25:8:4. Estimates of errors arising from chromatographic separation and analysis have been made.

(a) Mean of 3 cell samples
(b) Nelson[68,70]
64,262

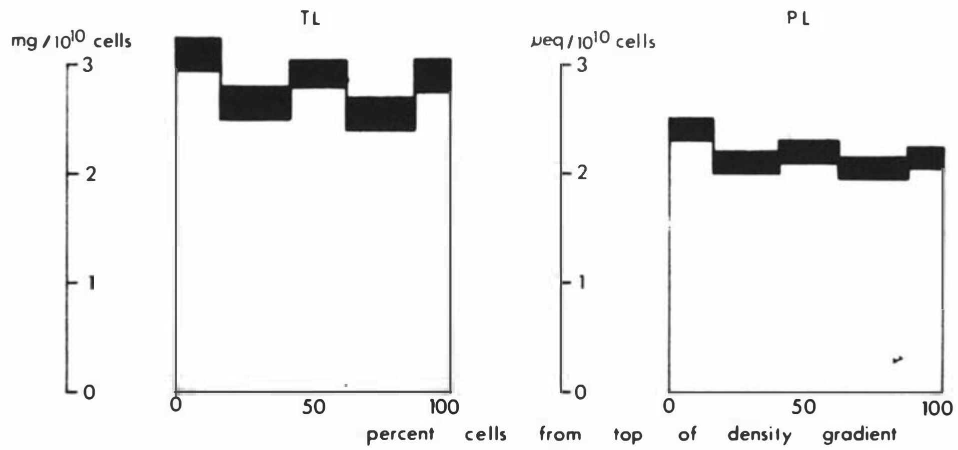


Figure 27: Variation in Rat Red Cell Total Lipid and Phospholipid Contents with Cell Age (Rats C)

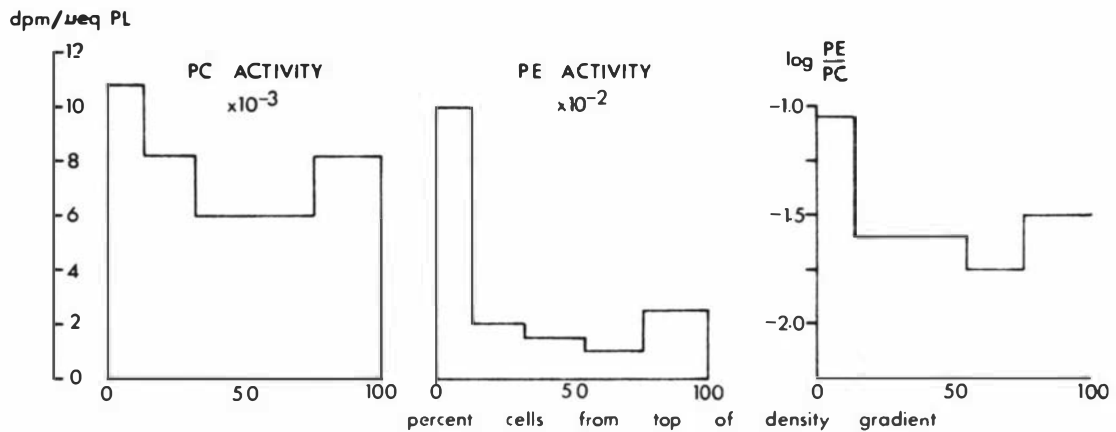


Figure 28: Incorporation of Palmitic Acid into the Lipids of Rat Red Cells of Different Ages (Rats D)

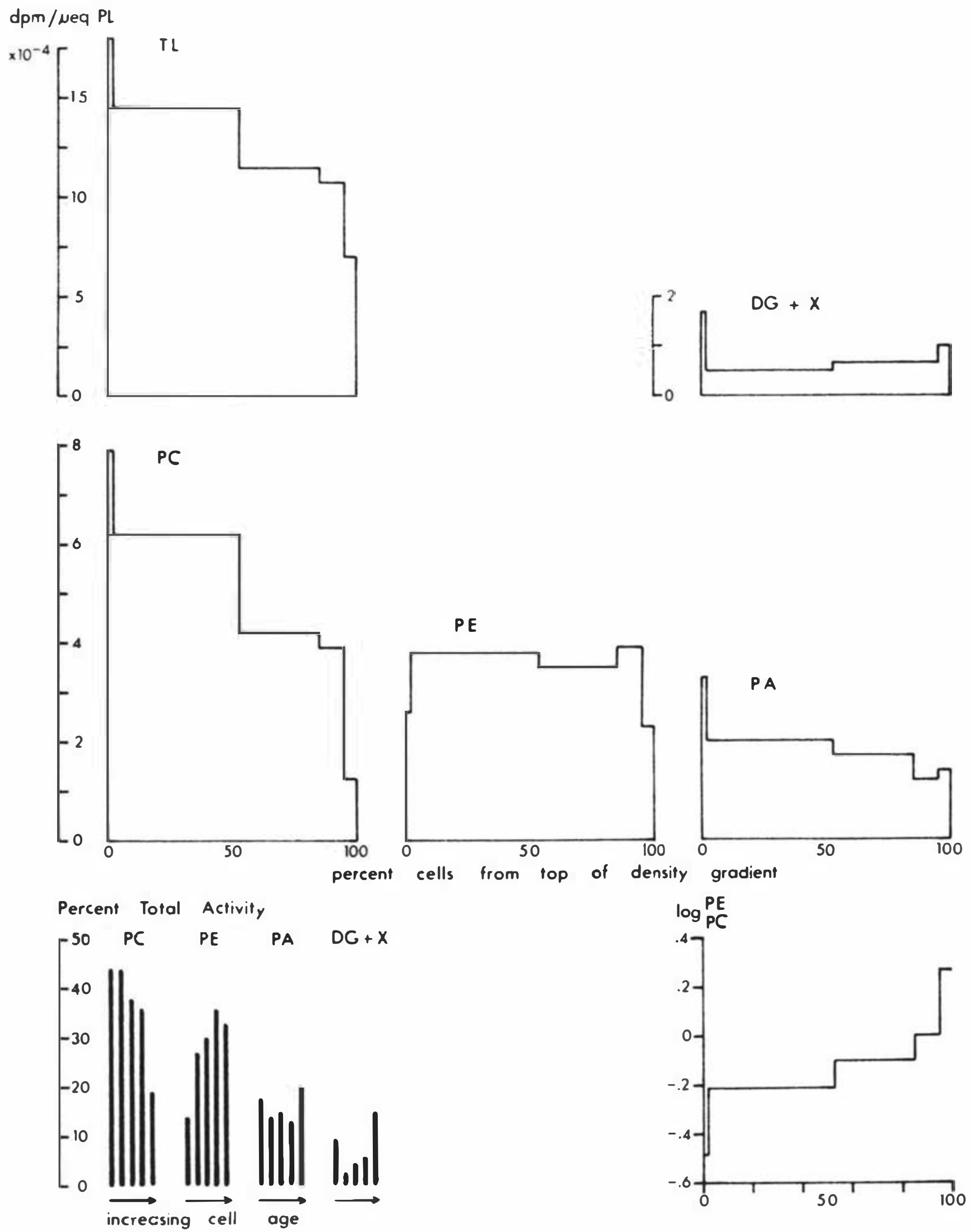


Figure 29: Incorporation of Palmitic Acid into the Lipids of Rat Red Cell Ghosts of Different Ages (Rats E)

Variation in Uptake of Palmitic Acid with Age of Rat Red Cells

Data are presented in the same way as for experiments with human cells. Incorporation and fractionation conditions are summarised in Table 47.

Normal Cell Populations

Uptakes into white cell lipids are shown in Table 49. Total uptake was of a similar order to that observed for human white cells, but TG activity was considerably lower. Incorporations into red cells, and ghosts to which ATP and CoA were added after preparation, are shown in Table 50. Although white cell contamination reduced quantitative accuracy in the cell incubation, both cells and ghosts incorporated the acid predominantly into PC, and to a much lesser extent into PE, PA, and probably DG. Traces of LPC activity were also detectable. After incubations, supernatant lipids were extracted and analysed. Some PC and LPC activity was evident, with PC activity amounting to about 0.2 per cent of the total.

Cell Populations of Different Ages

Radioactivity incorporation into fractions of red cells, separated according to age after incubation with palmitate, is shown in Table 51 and Figure 28. Lipids were extracted from cells which had not been washed free of albumin following density gradient fractionation. Hence any lipids present in the albumin were also extracted, and for this reason, radioactivity incorporations are related to cell numbers. Almost all uptake was into PC, and it appears that there were roughly equal decreases in PE and PC activities with cell age. Considerable cell agglutination occurred during incubation and fractionation. The relationship between cell age and agglutination is not known, and the densest, agglutinated fraction probably does not contain only the oldest cells. It is also possible that some alterations to other cells occurred, which could affect age-density relationships. As a result of the difficulties encountered in this experiment, cells were separated before incubation in subsequent studies.

Table 47: Summary of Conditions for Separation and Incubation of Rat Red Cells.

Rat A: Incubation of Red Cells and White Cells with /1-^{14}C/palmitate.

Red and White cells were incubated together in plasma, with activity 1.27×10^{6} dpm/ml
cells:plasma = 1:1.42 v/v
Red cell extract 3.0×10^{10} RBC
1.2×10^{6} WBC per 10^{10} RBC
White cell extract 17×10^{6} WBC
1.1×10^{8} RBC per 10^{6} WBC
Radioactivity recovered in RBC 3.0%
Radioactivity recovered in WBC 10.8%
overall recovery 98%

Rat B: Incubation of Red Cell Ghosts with /1-^{14}C/palmitate.

Ghosts were prepared by hemolysis in hypotonic phosphate buffer (Dodge et al[73]). They were separated but not washed and ATP and CoA were added to give final concentrations of about 4µM and 0.3µM respectively.

Ghosts were incubated in plasma, with activity 1.08×10^{6} dpm/ml
ghosts:plasma 1:2
ghosts extracted 3.0×10^{10}
WBC per 10^{10} RBC 0.4×10^{6}
Radioactivity recovered in RBC 5%
Overall recovery 95%

Rats C: Analysis of lipids from the pooled red cells from 4 rats after fractionation of the cells according to age

density	1.075		1.081		1.083		1.085		1.087		1.108
per cent of RBC		16		25		21		25		12	
RBC extracted $\times 10^{-10}$		2.45		3.75		3.2		3.9		1.8	

Rats D: Cells from 4 rats were incubated separately in plasma with /1-^{14}C/palmitate.

Each sample was fractionated according to age and the pooled fractions analysed. Incubation prior to separation resulted in some cell agglutination and sedimentation during ultracentrifugation. Sedimented cells were separated and extracted. Cells were not washed free of albumin before extraction.

Average plasma activity: 1.22×10^{6} dpm/ml (0.91 - 1.56)

density	1.075		1.081		1.083		1.085		1.087		1.108	
per cent of RBC		13		19		21		20		25		
RBC extracted $\times 10^{-10}$		0.81		1.20		1.30		1.20		1.55		5.05*

No white cell counts could be made.
*Agglutinated cells.

Rats E: Cells from 4 rats were fractionated according to age.

ATP and CoA enriched ghosts from pooled fractions were incubated with /1-^{14}C/palmitate in KRP pH 7.4, containing 0.5 per cent albumin.

Medium activity: 1.09×10^{6} dpm/ml
ghosts: KRP = 1:4

density	1.075		1.079		1.081		1.083		1.085		1.103
Per cent of RBC		2		51		32		9		5	
RBC incubated $\times 10^{-10}$		0.21		5.3		3.4		0.98		0.55	

No white cell counts could be made.

Ghost Populations of Different Ages

Table 52 and Figure 29 show the incorporation of palmitate into age fractions of red cell ghosts. Uptakes were higher than that into the normal ghost population, probably because ATP and CoA, added at the time of lysis, were able to penetrate into the ghosts. Some loss of lipid and gain of impurity occurred in the smaller samples, but as there appeared to be little gain in phospholipid, by relating uptakes to PL levels, errors from this source should be minimised. It appears that there was a decrease in uptake into PC with age, and relatively constant uptake into PE. The ratio of PE : PC activity increased markedly with age, as in some human studies. Incubations were carried out in KRP with added albumin. Lysophospholipids and free fatty acids, which occur in plasma as albumin complexes[129,130], would be present in the albumin, and therefore available as substrates.

Both these studies, therefore, conform to the general pattern observed for human cells and ghosts, although experimental inadequacies do not allow as critical an assessment.

Table 49: Incorporation of /1-^{14}C/palmitate into rat white blood cell lipids (Rat A)

Component	Per cent of Activity in Esterified Lipids	Activity per 10^6 cells (dpm)
TL		6900
CE	1 ± 0.5	70
TG	8.7 ± 0.5	600
DG+X	5.5 ± 0.2	400
PA	11 ± 1	750
PE	6.0 ± 0.2	400
PS	1.0 ± 0.1	70
PC	64 ± 1	4400
SP	1.4 ± 0.1	100
LPC	0.5 ± 0.1	30

Contributions by red cells were negligible.
Errors in white cell counts could be ± 20%. Data uncorrected for plasma activity concentration are given in Appendix 1, Table 49a.

Table 50: Incorporation of /1-^{14}C/palmitate into Normal Populations of Rat Red Cells and Red Cell Ghosts.

Component	Red Cells Rat A			Red Cell Ghosts Rat B		
	Per cent of activity in esterified lipids	Activity per μeq PL (dpm)	Specific activity dpm/μeq	Per cent of activity in esterified lipids	Activity per μeq PL (dpm)	Speci acti dpm/μ
TL		1700 ± 300			11000 ± 500	
TG		-		-	-	
DG+X	13 ± 3	240		3.2 ± 0.2	350	
PA	- ± 4	-		9 ± 2	1000	
PE	7 ± 3	130	550	1.0 ± 0.2	100	450
PS	4 ± 2	60		-		
PC	67 ± 4	1200	2500	81 ± 2	9000	19000
SP	2 ± 2	30				

Uncorrected data is given in Appendix 1, Table 50a.

Table 51: Incorporation of [1-^{14}C]palmitate into Rat Red Cells Fractionated According to Age (Rats D)

	density range of cells					
	1.075	1.081	1.083	1.085	1.087	1.108
Percentage distribution of the radioactivity in the esterified lipids						
PE	8.0 ± 0.5	2.3 ± 0.5	2.4 ± 0.5	1.5 ± 0.5	2.7 ± 0.5	3.0 ± 0.5
PC	89 ± 0.5	95 ± 0.5	95 ± 0.5	96 ± 0.5	95 ± 0.5	94 ± 0.5
Activity per 10^{10} cells (dpm; accuracy in TL ± 10%)						
TL(esterified)	12200	8700	6400	6200	8400	14200*
PE	1000	200	150	100	250	450
PC	10800	8200	6000	6000	8000	13500

*Sedimented cells

These results are presented graphically in Figure 28. Uncorrected data is given in Appendix 1.

Table 52: Incorporation of [1-^{14}C]palmitate into Ghosts of Rat Red Cells, Fractionated According to Age (Rats E)

density range of cells: 1.075, 1.079, 1.081, 1.083, 1.085, 1.103

Percentage distribution of the radioactivity in the esterified lipids					
DG or X	9.3 ± 0.5	2.5 ± 0.2	4.6 ± 0.4	6.0 ± 0.5	15 ± 0.5
PA	18 ± 2.0	14 ± 2.0	15 ± 2.0	13 ± 2.0	20 ± 2.0
PE	14 ± 0.5	27 ± 0.5	30 ± 0.5	36 ± 0.5	33 ± 0.5
PC	44 ± 0.5	44 ± 0.5	38 ± 0.5	36 ± 0.5	19 ± 0.5
Activity per µeq phospholipid (dpm)					
TL(esterified)	180000	145000	116000	108000	70000
DG or X	17000	5200	6200	6500	10000
PA	33000	20000	17000	12000	14000
PE	26000	38000	35000	39000	23000
PC	79000	62000	44000	39000	12500

These results are presented graphically in Figure 29. Uncorrected data is given in Appendix 1.

METABOLISM *IN VIVO* OF BOVINE ERYTHROCYTE LIPIDS LABELLED WITH ^{14}C-ACETATE*

This investigation was instigated when a cow was given an intravenous infusion of ^{14}C-acetate, primarily to study milk lipid production. Since erythrocyte and plasma lipids would also become labelled, a study was made of the fate of the radioactivity incorporated into the erythrocyte lipids. Only one animal was available for an entire study, so caution must be observed in interpreting the result directly and the value of the study is more as a guide for interpreting related studies on red cell lipid metabolism. However it was possible to perform a preliminary investigation, involving two red cell samples, on a similar animal, to compare this with the main study.

Bovine erythrocytes have a lifespan of about 107 days[196]. Mature red cells cannot synthesise lipids from acetate or phospholipid precursors, but can exchange a variety of lipid components for plasma counterparts[7,30,94]. In an animal administere $[^{14}C]$acetate, radioactivity could enter the erythrocyte lipids either by direct synthesis from acetate prior to maturation, or by replacement of lipids of mature cells by radioactive plasma lipids, synthesised at other sites in the body. Incorporated radioactivity could be lost by exchange with plasma constituents, metabolic breakdown, or removal with the cell at the end of its lifespan. $[^{14}C]$Acetate administered to a cow is rapidly metabolised, and most of the radioactivity is quickly lost by oxidation or milk production[270], so persisting radioactiv lipids would be predominantly those synthesised at the time of acetate administration

$[^{14}C]$Acetate (10mC in isotonic saline) was infused into the jugular vein of a Jersey cow, over a 24 hr period. Blood samples were collected at intervals after infusion, and the red cell and plasma lipids extracted and the radioactivity in individual components measured.

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*The results of this investigation have already been published, ref. 269.

Results and Discussion

Table 53 shows the lipid constitution of the blood samples analysed. The reproducibility obtained in these analyses indicates that variation in absolute amounts of cell or plasma lipid could not be responsible for observed differences in radioactivity differences.

The disappearance of radioactivity from the erythrocyte total lipid, cholesterol, and phospholipid fractions during the study is shown in Figure 30. As the cow was not available until a fortnight after the infusion, no earlier samples could be taken. The total activities, and specific activities, of the cholesterol ester, triglyceride and free fatty acid fractions were lower than those of cholesterol or phospholipid. On day 15, the triglyceride and free fatty acid fractions each contained 1 count/min/10^{10} cells or 15 counts/min/mg; in later samples activities were less than half this. No cholesterol ester activity was detectable in any sample. The [^{14}C]acetate dose was not high enough to accurately assess turnover of these components. In the preliminary study, red cells withdrawn 31 days after acetate infusion had a phospholipid activity of 37 $\pm$ 2 and a cholesterol activity of 19 $\pm$ 2 counts/min/10^{10} cells, and after 72 days the phospholipid activity was 33 $\pm$ 2 and the cholesterol activity 11.5 $\pm$ 2 counts/min/10^{10} cells. The rates of disappearance of both components compare well with those observed in the main study.

A comparison of red cell and plasma phospholipid and cholesterol specific activities is given in Table 54. No activity was detected in any other plasma lipid. As in the dog and human[118-121], the plasma free and esterified cholesterol, and erythrocyte free cholesterol appear to be in equilibrium with each other, but not with the erythrocyte esterified cholesterol.

The fall in radioactivity of the erythrocyte cholesterol with time produced a similar curve to that typically obtained for plasma cholesterol[271]: an initial changing slope due to slow and variable rates of equilibrium between the different body cholesterol pools, followed by exponential decay representing true metabolic

Table 53: Lipid Content of Blood Samples Collected from ^{14}C-Acetate Labelled Cows

Time after acetate adminis- tration(days)	Erythrocytes Total lipid per 10^{10} cells (mg)	Erythrocytes Per cent phospho- lipid	Plasma Cholesterol ester	Plasma Cholesterol	Plasma Phospholipi
			Per cent		
15	4.95	61			
34	4.9	64			
61	4.95	62	54	27	10
100	5.0	65	53	29	9
114	5.0	64	51	29	10
135	4.95	64	54	28	8
Cow B (preliminary study)					
31	4.3	71			
72	5.0	69			

Table 54: Specific Activities of Erythrocyte and Plasma Cholesterol and Phospholipid

	Days after [^{14}C]acetate administration					
	15	34	61	100	114	135
Erythrocyte esterified cholesterol	0 ± 5	0 ± 5				
Erythrocyte free cholesterol	80 ± 3	27 ± 2	15 ± 1	10 ± 1	7.5 ± .5	4.5 ± .5
Plasma esterified cholesterol*			15 ± 1	10 ± 1	7.0 ± 1	5 ± .5
Plasma free cholesterol			14 ± 1	7.5 ± 1	11 ± 4	4.5 ± .5
Erythrocyte phospholipid	17 ± 1	17 ± 1	16 ± 1	15 ± 1	10.5 ± 1	9.5 ± .5
Plasma phospholipid			6 ± 1	3.3 ± .5	3.0 ± .5	2.0 ± .5

*Calculated from total cholesterol ester activity less fatty acyl contributions, and corresponding to at least 97% of the total activity.

Components were separated by TLC in hexane-ether-acetic acid 70:30:1

Each separation was carried out in duplicate, and results are quoted ± 1 standard deviation as counts/min/mg.

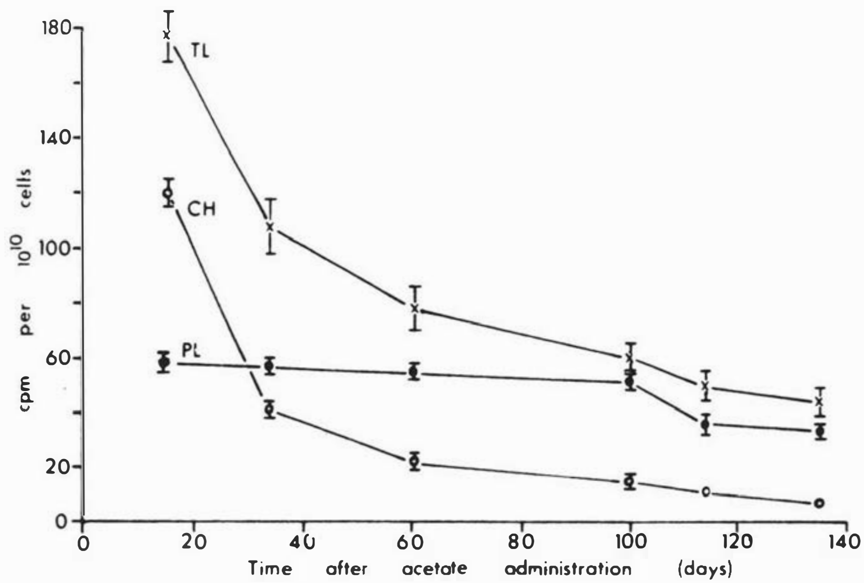


Figure 30: Disappearance of Radioactivity from Bovine Erythrocyte Lipids in vivo, following Administration of 14C-Acetate

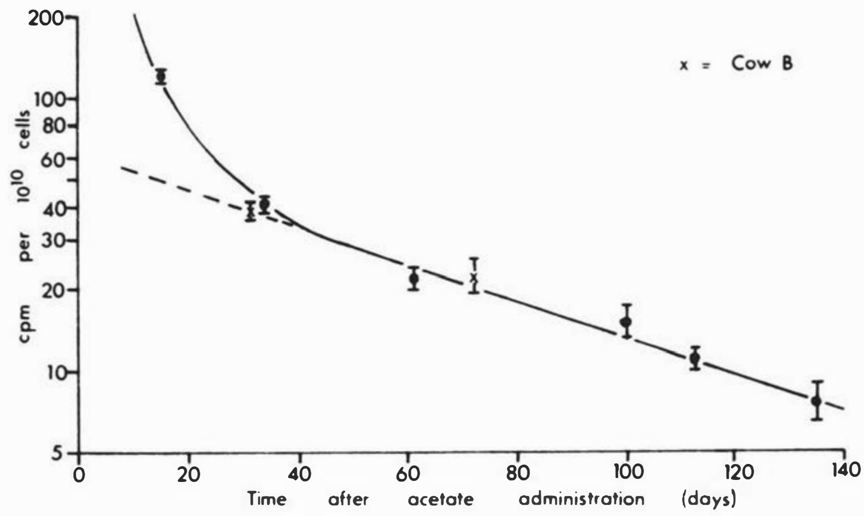


Figure 31: Log Plot of the Disappearance of Radioactivity from Erythrocyte Cholesterol

turnover. This is shown in the log plot in Figure 31. The slope of the graph suggests that equilibrium was achieved after about 40 days, and a cholesterol half life of about 45 days. No other data on bovine blood cholesterol turnover have been reported, but for comparison, human plasma cholesterol takes about 60 days to equilibrate, and then exhibits a half-life of 50-100 days[271,272].

From Figure 30, it is evident that although plasma phospholipid activity fell very gradually during the experiment, red cell phospholipid activity fell as if it were influenced by two processes. Firstly there appeared to be a gradual fall paralleling that in plasma, and secondly a sharper drop between 100 and 114 days after acetate administration. At this time, cells produced during isotope administration were reaching the end of their lifespan. If this is a true picture of the situation, it could be explained by the existence of some erythrocyte lipid constituents capable of replacement by plasma counterparts, and others which remain intact throughout the life of the cell. (Although, the two rates of fall of phospholipid activity are clearly present in the animal studied, confirmation in another animal would be desirable before considering detailed interpretation).

The distribution of isotope between fatty acyl and non-fatty acyl moieties of the erythrocyte phospholipid is shown in Table 55. If the phospholipids were uniformly labelled, about 65 per cent of the activity would be in the fatty acids. The lower levels found could result from some phospholipids being removed from the cells less readily than their fatty acid constituents.

Table 55: Location of Radioactivity in Erythrocyte Phospholipids

Days after [^{14}C]acetate administration	Per cent activity in the fatty acids
15	46 ± 5
34	51 ± 5
100	51 ± 5

Phospholipids were hydrolysed in methanolic KOH and the fatty acids extracted. Each result is quoted ± the estimated error.

The cell phospholipids, examined in two samples, showed very similar distributions of isotope (Table 56), and no localisation of isotope in any one fraction is evident. The main difference is the lower phosphatidyl choline activity in the later sample. Low counts prevent any detailed interpretation of these results.

That some erythrocyte phospholipids can be replaced in part or whole has already been demonstrated by in vitro incorporation studies[40,41,102,104,105–107] and in vivo dietary studies[75,95–101]. The results of this investigation are in agreement with these observations. They suggest that some erythrocyte phospholipid constituents cannot be replaced during the lifespan of the cell, and that the fatty acid constituents can exchange slightly more readily than the whole phospholipid molecules.

Low radioactivity levels prevent more detailed interpretation of this study, but to achieve sufficiently high activity in cattle, the radioactivity dose would not be practicable. However a similar experiment in a smaller animal given a relatively higher dose of [^{14}C]acetate could prove valuable.

Table 56: Distribution of Radioactivity Throughout the Erythrocyte Phospholipids

	Percentage of Phospholipid Weight	Percentage of Phospholipid Radioactivity	
		After 15 days	After 34 days
PE	23 ± 2	17 ± 2	16 ± 2
PC	4 ± 1	8 ± 1	3 ± 1
SP	57 ± 3	62 ± 2	80 ± 3
PS	15 ± 2	11 ± 2	
Others	1 ± 1	2 ± 1	1 ± 1

Phospholipids were separated by TLC with CMW as solvent. Each result is quoted ± the estimated error.

GENERAL DISCUSSION

The initial aim of the present investigation was to examine the role of the metabolism of red cell membrane lipids in the process of erythrocyte aging. In order to pursue this aim, rates of incorporation of exogenous free fatty acids into the individual cell lipids were investigated. Cells were fractionated according to age in early studies by serial osmotic hemolysis and in subsequent experiments by ultra-centrifugation over discontinuous albumin density gradients. From examination of the separated fractions, information was obtained on the dependence of both lipid constitution and fatty acid uptake on erythrocyte age. A study of the turnover of erythrocyte lipids *in vivo* was also made. The purpose of this general discussion, is to group together relevant experimental results and to consider specific aspects of red cell lipid metabolism.

Metabolism of Esterified Neutral Lipids in the Erythrocyte

Palmitate or linoleate incorporated into erythrocytes is present almost entirely in the phospholipids. After correction for white cell contributions, no uptake into triglycerides, in bovine, rat, or human red cells, could be demonstrated, and incorporations into cholesterol esters were very low or absent. Although there was enhanced fatty acid incorporation into phospholipids of red cell ghosts incubated with ATP and CoA, there was still no significant incorporation into triglycerides or cholesterol esters.

These results extend the work of Mulder and van Deenen[102], who incubated rabbit erythrocyte ghosts with labelled fatty acids and by scanning a thin-layer chromatogram, observed little activity corresponding to any esterified neutral lipid. In contrast, Donabedian and Karmen[114], and Michaels *et al*[115] have reported considerab incorporation of labelled fatty acids into the triglycerides of human erythrocytes. These incorporations were very sensitive to incubation conditions and subsequent treatment of the cells. The differences between their results and those obtained in the present study could be due to the extent to which allowance has been made

for the presence of white cells in the incubation system. It has been shown that standing for 8 hr before incubation reduces uptake into white cells to about 30 per cent, but has little effect on red cell incorporation. White cell levels in the human erythrocyte preparations used by Donabedian and Karmen[114] were of the same order as those in the bovine red cell preparations used in the present study, and such levels were found to contribute significantly to fatty acid uptake.

It appears, therefore, that renewal of erythrocyte triglyceride and probably cholesterol ester fatty acids by uptake from plasma is either not possible, or of an extremely low order and hence not detectable by the present methods. After incubation with [1-^{14}C]palmitate, the specific activity of bovine erythrocyte triglycerides was much lower, and that of cholesterol esters no higher, than the total phospholipid specific activity, and both were considerably lower than those of phosphatidyl choline and phosphatidyl ethanolamine, the only phospholipids showing significant uptake. It has been postulated that possible roles for minor neutral lipids in the erythrocyte could be as intermediates in the transfer of fatty acids to other major erythrocyte lipid constituents[30]. However the observed specific activities do not support their involvement in transfer of plasma fatty acids to red cell phospholipids. In addition, the very low cholesterol ester activity would suggest the absence of cholesterol-lecithin acyltransferase, an enzyme present in plasma, and in some other tissues[135].

There is some evidence from the studies with bovine red cells, and more substantial evidence from studies with human cells and ghosts, for fatty acid uptake by one or two components with similar thin-layer chromatographic mobilities to diglycerides. Such incorporations cannot be interpreted in terms of white cell contamination. Since labelled fatty acid incorporation into a component which is probably phosphatidic acid has been demonstrated, it is likely that labelled diglyceride is formed by the action of phosphatidic acid phosphatase, which has been shown by Hokin and Hokin[282] to be present in red cell ghosts. In contrast to other systems diglycerides are not required by the erythrocyte for synthesis of triglycerides

or major phospholipids[94]. However Hokin and Hokin[282,283] have also demonstrated the presence of diglyceride kinase in the red cell membrane. This, combined with the phosphatase, constitutes the phosphatidic acid cycle, the net reaction of which is the hydrolysis of ATP.

The other human red cell lipids which took up some labelled fatty acid could not be identified. These components were not detectable on thin-layer plates sprayed with iodine or sulphuric acid, and exhibited different mobilities from monoglyceride.

If cholesterol esters or triglycerides were present in the erythrocyte membrane as essential energy reserves, they would be expected to undergo relatively rapid turnover, or, if they were critical determinants of erythrocyte lifespan, their concentrations should gradually fall to zero as the cell ages. No detectable turnover of either constituent could be demonstrated. Variation with age in the levels of these components has been investigated, both in bovine erythrocytes separated by serial osmotic hemolysis and rat erythrocytes separated by albumin gradient centrifugation. Although measurements fluctuated a little because of difficulties in accurately analysing such minor components, no significant decrease with age in either triglyceride or cholesterol ester could be detected. Age separations should have been sufficient to show if either component were substantially reduced in the older cells. It is unlikely, therefore, that either of these components functions as an energy reserve in the red cell.

According to Nelson[70], erythrocytes contain no triglycerides or cholesterol esters. He suggests that these components are present only in extracts contaminated with white cells or plasma. Information obtained in the present study would not support this contention. Firstly, extracts of cells fractionated by serial osmotic hemolysis contained as much of these components after 15 saline washes as they did after 3 washes. Triglyceride and cholesterol esters were also present in white cell-free hemolysates of well washed erythrocytes. Secondly, it was shown that after

administration of ^{14}C-acetate to a cow, cholesterol ester specific activity was significantly lower in red cell lipid extracts than in plasma extracts. Thirdly, although levels of white cells in red cell preparations were sufficient to account for all fatty acid uptake into triglycerides, they were not high enough to account for all the triglyceride extracted. Against these arguments it could be proposed that some plasma components could be so strongly bound to the erythrocytes so as not to be removed by saline washing, and in the case of cholesterol ester, not in equilibrium with plasma counterparts. However, if such close association exists between erythrocytes and these triglycerides and cholesterol esters, they are more realistically cellular constituents. Variation in levels observed in different investigations could be due to analytical difficulties, rather than the variable presence of plasma or white cell contaminants.

It would appear that these classes of lipid are true red cell constituents. Although it has been shown that they are unlikely sources of energy, or intermediates in phospholipid or cholesterol metabolism, it has not been possible to demonstrate alternative cellular functions. Perhaps this is simply because of the existence of specific triglyceride and cholesterol ester binding sites in the cell membrane, and apart from filling these sites, they fulfil no physiological role. However in this capacity they could be important contributors to membrane conformation.

Incorporation of Fatty Acids into Erythrocyte Phospholipids

[1-^{14}C]palmitate, taken up from plasma into bovine erythrocytes, was found to be almost exclusively localised in the phosphatidyl choline (60 per cent) and phosphatidyl ethanolamine (20 per cent) fractions. Approximate times required for the cells to renew all the palmitate in these components, by uptake from plasma, were 30 and 170 days respectively. The ratio of phosphatidyl ethanolamine to phosphatidyl choline activity is higher than generally found for fatty acid uptake into most mammalian erythrocytes or ghosts[40,41,102]. However bovine erythrocytes are very low in phosphatidyl choline, and for this reason, a proportionately lower

incorporation into this fraction, in bovine cells, as also found by Mulder and van Deenen[102], would be expected.

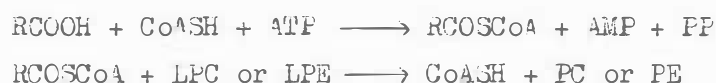
The existence of phosphatidyl choline in ruminant erythrocytes has been doubted by some authors[57,58,68], but others[59,64,61] report the presence of low concentrations, as found in this study. In addition, significant uptake of labelled palmitate into bovine phosphatidyl choline has been observed. Although Olivera and Vaughan[40] were unable to detect any fatty acid uptake into phosphatidyl choline in sheep ghosts, Robertson and Lands[41] observed, when lysophosphatidyl choline was added to the system , a fatty acid uptake of a similar order as that for cells of other species. Hence under physiological conditions, i.e. in the presence of plasma containing fatty acids and lysophosphatidyl choline, phosphatidyl choline synthesis in erythrocytes of both species could occur. This implies the presence of phosphatidyl choline in ruminant erythrocytes, but it is possible that there is relatively rapid release into plasma of the synthesised molecules, and as a consequence the cellular concentrations remains very low. Such conditions would correspond to high turnover rate.

In human and rat erythrocytes, or ghosts enriched with ATP and CoA, most of the uptake of plasma palmitate or linoleate was into phosphatidyl choline and phosphatidyl ethanolamine. Uptake into another component, with thin-layer chromatographic mobilities resembling phosphatidic acid, and tentatively identified as such, was also observed. Uptakes into ghosts were higher than into cells, but no differences between the two in radioactivity distribution were apparent. There was, however, considerable variation between individuals both in total incorporation and particularly in the distribution of the acid taken up. The most striking differences were in the ratios of uptake into phosphatidyl choline and phosphatidyl ethanolamine. In some cases these were comparable, in others uptake into phosphatidyl choline was ten times the greater. The nature of the uptake appeared to depend on incubation conditions rather than specific donor. Although incorporation of linoleate was slightly higher than palmitate, no dependence of radioactivity distribution on

the nature of the acid was apparent. However dependence of total uptake per cell on fatty acid concentration and on cell concentration was evident. Such variation has not been reported for uptake into ghosts or cells from synthetic media rather than from plasma[40,41,103], but Mulder and van Deenen[102] also observed wider ranges of uptake from plasma media.

Mulder and van Deenen[102] also observed the same order of difference as in the present study for uptake of plasma linoleate and palmitate into the phosphatidyl choline of rabbit red cells or ghosts. However Waku and Lands[103] observed that acylation of 1-acyl glycero-3-phosphoryl choline (1-acylGPC), the predominant plasma lysophosphatidyl choline isomer, with linoleoylCoA was about 7 times faster than with palmitylCoA, in buffered suspensions of human ghosts. It is possible that variation between media is responsible for these ratio differences.

Fatty acid uptake into erythrocyte phosphatidyl choline and phosphatidyl ethanolamine proceeds via the following reactions[94,103]:



$$\text{RCOOH} + \text{CoASH} + \text{ATP} \longrightarrow \text{RCOSCoA} + \text{AMP} + \text{PP}$$
$$\text{RCOSCoA} + \text{LPC or LPE} \longrightarrow \text{CoASH} + \text{PC or PE}$$

Lands and Hart[274] have observed phosphatidic acid formation in liver microsomes via a similar transacylation mechanism:

$$\text{RCOSCoA} + \text{LPA} \longrightarrow \text{PA} + \text{CoASH}$$

Although this mechanism has not been demonstrated, presumably it could also operate in the erythrocyte.

Specificity with regard to lysophospholipid isomer has been observed, and this does not appear to be identical in microsomal and erythrocyte systems. For liver microsomes[108-112,274], specificities are different for lysophosphatidyl choline, lysophosphatidyl ethanolamine, and lysophosphatidic acid isomers, which suggests the involvement of more than one transacylase. Waku and Lands[103] report that in human ghost suspensions, 1-acylGPC is preferentially acylated by unsaturated rather than saturated acids, but very little acylation of 2-acylGPC occurs. In contrast, Mulder and van Deenen[102] report that the bulk of the labelled palmitate in-

corporated from plasma into rabbit red cells or ghosts via this mechanism is localised in the 1-position. No information is available on the specificities of acylation of lysophosphatidyl ethanolamine and lysophosphatidic acid by erythrocytes.

It is suggested that the variation in fatty acid uptake by red cells observed in the present study can be explained by:

1. Linoleate uptake by human erythrocyte ghosts being slightly faster than palmitate uptake.
2. Independent variations in plasma concentrations of free fatty acid and lysophospholipid substrates giving rise to variations in both fatty acid incorporation and distribution of the acid incorporated.
3. Cell enzymes not being saturated, and hence any increase in cell concentration (i.e. enzyme concentration) not resulting in an equivalent increase in total fatty acid uptake.

For this explanation to hold, the following conditions must be satisfied:

1. Plasma substrates must be available for esterification by the cells.
2. Plasma lysophospholipid and free fatty acid levels must vary, both between individuals and in one individual at different times.
3. Substrate concentrations must vary within a range for which cell enzymes are not saturated.

It is obvious that fatty acids added to plasma can be incorporated into red cells or ghosts, and increases in fatty acid uptake cells or ghosts have been observed on addition of exogenous lysophosphatidyl choline or lysophosphatidyl ethanolamine[41,102]. Plasma substrates are therefore available for esterification by erythrocytes.

Mammalian plasma contains free fatty acids (about 5 per cent of the total lipid)[275], lysophosphatidyl choline (20 per cent of the phospholipid in rats, 8 per cent in humans), and lysophosphatidyl ethanolamine[125-128]. The findings of Mulder and

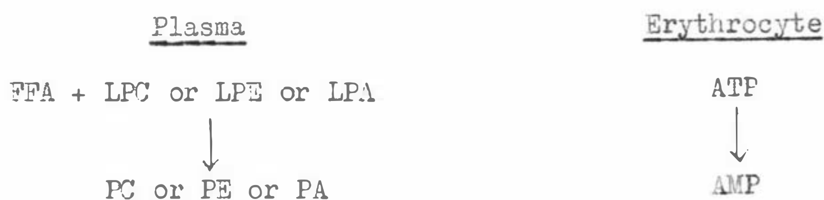
van Deenen[102] would suggest the presence of 2-acylGPC, which has been found in rat tissue[276] and in plasma under some conditions[281], as well as 1-acylGPC, in plasma. No information is available on the plasma lysophosphatidyl ethanolamine isomers, or on the occurrence in plasma of lysophosphatidic acid. Plasma free fatty acid levels vary inversely with glucose level and are thus lowered during glucose absorption[260,275]. Phospholipid levels vary between individuals[276-278] and also appear to increase during lipid absorption[233], although the extent to which lysophospholipid levels are affected is not known. It is possible that variation in substrate levels could arise in the cells themselves. They are such minor constituents that a demonstration of this would be very difficult, but constancy of other cell lipid constituents as against variability in plasma lipids would suggest that variations in plasma lysophospholipid concentrations are more likely.

With regard to the saturation of cell enzymes in the present investigation, Waku and Lands[103] have observed for human ghosts at similar concentrations to those in the present study, proportionality between esterification rate and 1-acylGPC concentration up to about 60 μM and linoleoylCoA concentration up to about 10 μM. Although total concentrations of plasma palmitate and linoleate are about 100 μM, albumin binding reduces the concentrations of the free species to about 0.01 per cent of this[129]. The total concentration of lysophosphatidyl choline is about 600 μM, and that of lysophosphatidyl ethanolamine considerably lower than this, but albumin binding again results in much lower concentrations of the free species[130]. Assuming that the K_m values of Waku and Lands[103] are similar for different lysophospholipids and fatty acids, it can be seen that concentrations of free lysophospholipids should have been well below 60 μM and in the rate influencing range, and even in the presence of exces CoA and ATP, free fatty acid concentrations would have been too low for the cell enzymes to be saturated with acylCoA. Hence the cell enzymes should not have been saturated with respect to fatty acid or lysophospholipid substrates, providing only the uncomplexed species are able to react. Under physiological conditions this situation would be accentuated even more, as cell concentrations would be 2 to 3

times higher. Available information therefore supports the postulated explanation for individual variations in fatty acid uptake into erythrocytes, and suggests that variations in plasma lysophospholipid levels occur, both between individuals, and at different times in one individual.

It is often considered that erythrocyte metabolic activity is closely controlled by the relative constancy of the plasma environment. However for lysophospholipid esterification within the cell membrane, this does not appear to be so. Activity is markedly influenced by fluctuations in plasma free fatty acid or lysophospholipid concentrations. Times required for turnover for all the linoleate in human red cell phosphatidyl choline have been found to vary from 100 to 300 days, and in phosphatidyl ethanolamine, 10 to 250 days. Times for ghosts are shorter but show similar variations. Palmitate turnover rates in bovine erythrocyte phosphatidyl choline are slightly faster than in humans, but because of the much lower cellular concentration, they correspond to similar rates of fatty acid uptake. Since the mechanism involves the hydrolysis of ATP, the cell may be required to supply considerably more energy under some plasma conditions. If substrate concentrations were particularly high, this demand could conceivably place the cell under considerabl stress.

Release of phospholipids, specifically phosphatidyl and lysophosphatidyl choli from bovine, human and rat red cells into plasma has been observed. Such results are in accord with previous reports of phospholipid exchange[105-107]. Erythrocytes lack phospholipase A[40,41,102]. Hence for erythrocyte lipid levels to remain constant every acylation involving the uptake of plasma substrates must be accompanied by the equivalent loss of diacyl lipids, presumably by release into plasma. Thus the mechanism constitutes a cycle which can be represented:



Speculative Roles for the Erythrocyte Lysophospholipid Acylation Mechanism

The ability to esterify lysophospholipids is a property of the erythrocyte membrane. It is possible, although not proven, that this could be a general property of all cell membranes. Possible roles for, and consequences of, this mechanism can be suggested.

Lysophospholipids can destroy red cell membrane function and cause homolysis[231]. Although in plasma albumin binding counteracts this toxicity[131], this protection may not be sufficient at increased concentrations. Mulder and van Deenen[104] have suggested that both the acylation mechanism and the ability to transfer an acid from one lysophospholipid molecule to another could be means by which the red cell protects itself against damage by lysophospholipids.

The net result of the mechanism can be considered as the conversion of plasma fatty acids and monoacyl phospholipids into plasma diacyl phospholipids. There are at least two mechanisms for the reverse reaction, firstly by phospholipase A action[132,133] and secondly by the action of cholesterol-lecithin acyltransferase[135], which catalyses acid transfer from the 2-position of phosphatidyl choline to cholesterol, and which results in the build up of 1-acylGPC. The red cell could be responsible for the reacylation of 1-acylGPC. The overall reaction would then be the synthesis, in plasma, of cholesterol esters from fatty acids and cholesterol, at the expense of red cell ATP. Glomset[135] has suggested the possible involvement of the cholesterol esterification mechanism in membrane cholesterol removal: cholesterol could be picked up from membranes, by lipoproteins with free sites produced by esterification of other cholesterol molecules, and thus be transported to the liver for catabolism. Membrane cholesterol could be replaced by synthesis within the cell. As red cells cannot synthesise cholesterol[89,118], and no depletion in cell cholesterol level is observed <u>in vivo</u>, removal of red cell membrane cholesterol must be accompanied by cholesterol transfer from plasma. However lysophospholipid esterification by red cells need not be concerned with reactions of their own cholesterol,

but could be the means by which plasma lysophospholipids, formed during uptake of cholesterol from other tissues, are reacylated.

Alternatively, deacylation of phosphatidyl choline rather than cholesterol esterification could be the primary function of plasma cholesterol-lecithin acyl transfer. If phospholipid turnover, requiring uptake of exogenous fatty acids and lysophospholipids, were necessary for membrane function, this plasma reaction could be required to supply lysophospholipid substrates. It is noteworthy that patients with familial cholesterol ester deficiency (normal or elevated plasma free cholesterol levels, but less than 10 per cent normal cholesterol ester levels), who have no demonstrable plasma lecithin-cholesterol acyltransferase, are also anemic[279,280].

As transacylation appears to be a cyclic process between cells and plasma, there is the interesting possibility that it could be involved in a transport process. Any substances selectively bound to monoacyl but not diacyl phospholipids could be transported into the cells, released during esterification of the carrier, and retained when the diacyl derivative is released. However such a condition is highly speculative as no substance capable of active or facilitated transport into the red cell is known to have such binding affinities.

It has been shown that erythrocyte transacylation rates are dependent on plasma substrate concentrations. A feature of this is the apparent lack of control by the cell over the reaction rates. Hence it would seem more likely that the mechanism is involved in maintenance of stable plasma concentrations, rather than primarily in cell metabolism. On these grounds hypotheses requiring the removal of lysophospholipids via this mechanism, either because of their toxicity to the cells or their production during cholesterol esterification, are favoured.

Variation in Lipid Composition with Erythrocyte Age

It has been shown that the total lipid content of human red cell samples decreased slightly with mean cell age. The pattern of this decrease is such that it could be interpreted as the only time when changes in red cell lipid content

occur being the transition from reticulocyte to erythrocyte. Results of other investigations could also be explained by this hypothesis[26,200,201]. Reticulocytes are larger than erythrocytes[25], and contain internal membranous structures. On maturation, when these structures disappear, a drop in cell lipid content would be expected to occur.

No variation in the relative amounts of lipid components with cell age was apparent. Experimental accuracy was not sufficient to eliminate the possibility of minor changes in lipid distribution or total cell lipid. However, considerable age separation was achieved, and only very small changes could have avoided detection.

It has been suggested that lipid loss could be responsible for the increased density of old erythrocytes[179]. However an erythrocyte with density about 1.08 conta only 0.5 per cent lipid with average density about 1.00. It is obvious that loss of the entire cell lipid would cause a negligible change in cell density. A far more likely cause of the observed increase in density with cell age is the decrease in cell size but not hemoglobin content[25,200,201,179], and hence increase in cellular hemoglobin concentration. Since hemoglobin has a relatively high density, cell density should also increase.

It seems unlikely that changes in lipid composition in aging red cells cause major modifications to cell structure and function. The difference in appearance of young and old cell membranes, observed by Danon and Perk[202], more likely arises from conformational changes involving reorganisation of interacting lipids and proteins, rather than differences in lipid composition.

<u>Fatty Acid Uptake into Red Cells as a Function of Age</u>

Human erythrocytes have been fractionated according to age and either cells or ghosts incubated with labelled palmitate or linoleate. The fatty acids were taker up, predominantly into phosphatidyl choline and phosphatidyl ethanolamine, but also into phosphatidic acid,diglyceride and an unidentified nonpolar lipid. No constant pattern of variation in uptake with age has emerged, and the different patterns are

Table 57: Summary of the Types of Relationship between Fatty Acid Uptake and Erythrocyte Age

Donor	Cell Type	Substrate	PC	PE	PA	DG	X
CCW	ghosts	linoleate	—	—	—		—
EJC	ghosts	palmitate	—	—	—	↓	↓
VJC	ghosts	palmitate	—	—	↑	⇑	⇑
GL	cells	linoleate	↑	↑	↑	—	—
MJW(I)	ghosts	linoleate	↓	—	—	↑	↑
MJW(I)	cells	linoleate	⇓	—	—	↑	↑
MJW(II)	ghosts	linoleate	—	⇑	↑	—	—
MJW(II)	cells	linoleate	—	⇑	↑	⇑	—
GGM	ghosts	palmitate	—	⇑	↑		↓

Uptake per ueq cell or ghost phospholipid

⇑ increasing markelly with age

↑ increasing slightly with age

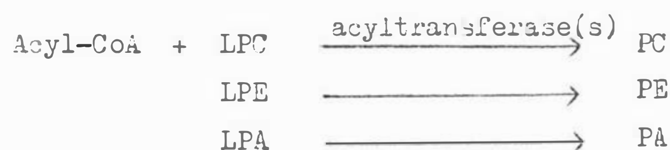
— no change with age

↓ decreasing slightly with age

⇓ decreasing markedly with age

Table 58: Mechanism for Fatty Acid Uptake into Erythrocyte Phospholipids

$$FA + CoA + ATP \xrightarrow{\text{thiokinase}} Acyl\text{-}CoA + AMP$$



$$Acyl\text{-}CoA + LPC \xrightarrow{\text{acyltransferase(s)}} PC$$
$$LPE \longrightarrow PE$$
$$LPA \longrightarrow PA$$

Possible variable factors affecting rates of fatty acid uptake:

Constant under all conditions studied	Possible variation between different populations	Possible variation within a population
ATP, CoA, ghost incubations	Plasma FA Plasma LPC, LPE, LPA	ATP, CoA, Cell incubation Cell FA Cell LPC, LPE, LPA thiokinase activity acyl-CoA acyltransferase activities

summarised in Table 57. Two broad classes of behaviour are evident; one with incorporation into phosphatidyl ethanolamine about 10 per cent or less of the total, and the ratio of phosphatidyl ethanolamine to phosphatidyl choline activity essentially unaffected by cell age; the other with higher phosphatidyl ethanolamine incorporations, and ratios showing a marked increase with cell age. Generally total fatty acid uptakes were unchanged or increased to varying extents with cell age. However in two studies on cells from the same blood sample there was a decrease, so all three behaviour patterns must be possible. Similar results were obtained in studies on rat blood.

Differences in the pattern of age dependence of fatty acid uptake do not correlate with whether intact cells or CoA and ATP enriched ghosts were incubated, or whether the acid was palmitic or linoleic.

An explanation of this complex behaviour pattern is not immediately apparent. However a consideration of the mechanism of fatty acid uptake shows potential sites of variability between cells of different ages. This information is examined in Table 58 for uptake into phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidic acid. Mechanisms for uptake into other components have not been elucidated.

Although there could be cellular ATP and CoA concentration variations with age the lack of distinctive behaviour differences between cells and ghosts enriched with these cofactors suggests that variation in fatty acid uptake with age is not a result of changes in ATP or CoA levels.

Exogenous substrates are available for the esterification reaction, and as cells in the absence of added lysophospholipids can still incorporate fatty acids, endogenous substrates must also be available[40,41,102]. As cell and plasma fatty acids undergo rapid exchange, and fatty acid uptake seems to be proportional to concentration, any variation in cellular level should not affect the amount of radioactive acid taken up from the plasma. Concentrations of ^{14}C-acylCoA should therefore be directly related to thiokinase activity.

Rates of fatty acid uptake into different erythrocyte phospholipids are

affected by concentrations of their lyso derivatives. Depending on relative availability, either cellular or plasma concentrations could dominate in rate determination. Although in humans, lysophosphatidyl choline is about 5 times more concentrated in plasma than in cells[67,72,126,127], plasma need not be necessarily the prime lysophospholipid source. However it would appear likely that plasma is the main source, because there is no known mechanism for lysophospholipid production within the red cell. The extremely low fatty acid uptake into the phosphatidyl ethanolamine of erythrocyte ghosts from a buffered saline medium[40], compared with uptake from plasma, also favours plasma being the major source of lysophosphatidyl ethanolamine. From this evidence, and the observation that there are no detectable variations in major red cell phospholipid levels with age, it is unlikely that variations in cellular lysophospholipid concentrations are responsible for variations in fatty acid uptake with age. Lysophospholipids provided primarily from plasma can cause only interpopulation variation in fatty acid uptake. Variation with age must then be a result of changes in levels of enzymic activity.

Both these factors, namely variation in enzymic activity with cell age coupled with variation in substrate concentration between plasmas, could account for the observed range of dependence of fatty acid uptake on red cell age.

It has been shown that normal plasma lysophospholipid levels are sufficiently low that cellular acyltransferases should not be saturated, (see p.128). This means that, assuming plasma is the main source of these substrates, an increase in enzyme concentration would not bring about an equivalent increase in reaction rate. Under these conditions, variation in enzyme levels between cells of different ages could exist without causing a difference in rate of fatty acid uptake into the cells. If, however, lysophospholipid concentrations were higher, enzymes could approach saturation, and increased uptake into those cells with higher enzyme activities would result. If plasma lysophospholipid, particularly lysophosphatidyl ethanolamin concentrations were elevated under some conditions, increases in erythrocyte

phosphatidyl ethanolamine acyltransferase and possibly phosphatidic acid acyltransferase activities, and a decrease in phosphatidyl choline acyltransferase activity, with cell age, could account for the major differences between studies.

Such a hypothesis is supported by increases in uptake into phosphatidyl ethanolamine with age being associated with higher overall uptake into this lipid. It requires that MJW(II) and GGM plasma lysophosphatidyl ethanolamine and MJW(I) plasma lysophosphatidyl choline concentrations were high. Until it has been demonstrated that alterations in lysophospholipid levels are capable of causing changes in the relationship between fatty acid uptake and erythrocyte age, this explanation must be put forward as speculative. However Walker and Yurowski[203] have also proposed the existence of erythrocyte acytransferases with different specificities and age-dependent activities, from studies of rates at which dietary changes influence fatty acid composition of cells of different ages.

An increase on aging in the ability of the red cell to acylate lysophosphatidyl ethanolamine and a decrease in ability to acylate lysophosphatidyl choline is suggested. An increase in enzymic activity with age is an unusual occurrence in a cell which is incapable of protein synthesis. However such an increase could be brought about, not by an increase in the number of enzyme molecules, but by either an increase in availability of existing molecules, or an alteration in conformation and improvement in catalytic ability of existing molecules. As these enzymes are membrane-localised, it is conceivable that changes in conformation of the membrane itself could bring about either of these changes. Since in some studies an increase in total esterification rate with cell age was observed, it would seem more likely that there are changes in activity of more than one enzyme, rather than a change in specificity of a single enzyme.

An increase in acyltransferase activity, accompanying erythrocyte aging, shoul therefore be indicative of changes occurring in the conformation of the cell membrane

In considering whether the ability to carry out lipid turnover is a determinan

of erythrocyte lifespan, it has been shown that:

(1) The rate of turnover is very susceptible to changes in environmental conditions.

(2) The relationship between turnover and cell age also appears to be influenced by environmental conditions.

(3) No changes in cell lipid composition with cell age, as a result of this mechanism are apparent.

On these grounds, it is unlikely that an alteration in ability to esterify fatty acids is in any way responsible for erythrocyte aging.

With regard to the primary cause of erythrocyte aging, the importance of maintenance of membrane function has been considered[8,197]. It has been suggested[6,10] that a gradual decrease in glycolytic activity occurs on aging, and that this, coupled with the effects it produces, could ultimately be responsible for cell death. The importance of maintaining cellular constituents, and in particular membrane proteins, in a reduced state has also been demonstrated[51,53]. There is convincing evidence that both the glycolytic and pentose phosphate pathways are membrane localised in the erythrocyte[174]. Changes in membrane conformation could markedly alter activities of these and any other membrane bound enzymes, and could initiate the gradual rundown in energy metabolism proposed by Löhr and Waller[6,10]. Perhaps the incomplete oxidative protection given to the cell by glutathione observed by Jacob and Jandl[53] under certain conditions _in vitro_, is paralleled _in vivo_, and oxidative alteration of membrane conformation initiates the aging process.

In the present investigation evidence has been obtained which suggests that conformational changes to the erythrocyte membrane occur on aging.

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APPENDIX I: BASIC DATA FOR FATTY ACID INCORPORATION INTO HUMAN AND RAT ERYTHROCYTES

Table 33a: Incorporation of Labelled Fatty Acids into Human White Cell Lipids

WRS

[1-^{14}C]linoleate uptake into total esterified lipid: 3000 dpm/10^6 cells.
RBC contribution is negligible.

EJC

[1-^{14}C]palmitate uptake into total esterified lipid: 6600 dpm/10^6 cells.
Significant RBC contributions, estimated from uptake into EJC ghost lipid (dpm per 10^6 WBC per 10^6 dpm/ml plasma)

	TL	TG	DG	X	PA	PE	PC
WBC + RBC	3200	1600	90	70	65	95	1050
RBC	380	10	25	10	12	50	260

Table 34a: Incorporation of [1-^{14}C]linoleate into Normal Populations of Human Red Cell Ghosts

CCW(II)

Uptake into total esterified lipid: 25,200 dpm/ueq PL
Significant WBC contributions, estimated from data in Tables 27 and 33: (dpm per ueq PL per 10^6 dpm/ml plasma)

TG (only significant contribution)		
	RBC + WBC	490
	WBC	80

WRS

Uptake into total esterified lipid: 15,600 dpm/μeq PL.
Significant WBC contributions, estimated from data in Tables 27 and 33: (dpm per μeq PL per 10^6 dpm/ml plasma)

	TL	TG	DG+X	PA	PC
RBC + WBC	34000	2100	2550	1500	23000
WBC	2500	1200	140	90	950

Table 35a: Incorporation of [1-^{14}C]palmitate into a normal population of Red Cell Ghosts

WRS

Uptake into total esterified lipid: 18,700 dpm/μeq PL
Significant WBC contributions, estimated from data in Tables 27 and 33; (dpm per 10^{10} RBC per 10^6 dpm/ml plasma).

	TL	TG	DG+X	PC	SP
RBC + WBC	30000	1350	3800	16500	970
WBC	1600	920	80	480	100

Table 36a: Incorporation of [1-^{14}C]linoleate into Human Red Cells Fractionated According to Age (Donor MJW(I))

	density range of cells				
	1.075–1.080	1.080–1.082	1.082–1.084	1.084–1.086	1.086–1.110
Uptake into total esterified lipid dpm/µeq PL	6000	4500	4500	3700	4400
RBC and WBC activity per 10^{10} RBC per 10^{6} dpm/ml plasma					
TL	7500	5500	5200	4400	5300
TG	40	150	190	110	300
DG + X	450	500	670	670	1000
PA	1230	1000	670	1200	1800
PE	350	470	420	430	470
PC	4350	2850	2800	1330	1050
SP	480	280	200	210	580
LPC	620	300	280	370	
Significant WBC contributions, estimated from TG activities* and the linoleate distribution in Table 33:					
TL	85	340	420	260	680
TG	40	160	200	120	320
DG + X	-	20	25	15	35
PA	-	-	15	10	25
PE	-	-	10	-	15
PC	35	140	160	100	260

*This assumes uptakes to be a fifth that observed for WRS white cells. Lower uptakes were probably due to the absence of ATP and CoA in MJW samples.

Table 37a: Incorporation of [1-^{14}C]linoleate into Human Red Cells Fractionated According to Age (Donor MJW (II))

	density range of cells					
	1.075–1.0815	1.0815–1.0835	1.0835–1.085	1.085–1.0865	1.0865–1.090	1.090–
Uptake into total esterified lipid dpm/µeq PL	17600	12400	14500	14500	18400	56000
RBC and WBC activity per 10^{10} RBC per 10^{6} dpm/ml plasma						
TL	16400	10500	12400	11600	15600	40000
TG	540	280	460	210	120	680
DG	520	440	1240	1120	1340	480
X	660	540	660	540	560	900
PA	1400	1100	1200	1600	1600	2600
PE	4600	3100	5200	4500	8500	31000
PC	8600	5100	3600	3700	3500	4300
Significant WBC contributions estimated from TG activities* and the linoleate distribution in Table 33:						
TL	1200	650	950	450	200	1500
TG	550	300	450	200	100	700
DG + X	60	40	50	20	-	80
PA	50	30	40	-	-	60
PC	450	250	350	170	80	600

*This assumes uptakes to be a fifth to a half that observed for WRS white cells

Table 38a: Incorporation of $[1-^{14}C]$linoleate into Human Red Cells Fractionated According to Age (Donor GL)

	density range of cells 1.075 – 1.079	1.079 – 1.0805	1.0805 – 1.0815	1.0815 – 1.0835	1.0835 – 1.0865
Uptake into total esterified lipid dpm/μeq PL	7900	10300	11100	12100	5700
RBC and WBC activity per 10^{10} RBC per 10^{6} dpm/ml plasma					
TL	6200	7300	8000	9000	3950
TG	450	480	380	320	150
DG	330	480	570	450	280
X	620	710	950	800	600
PA	230	470	200	700	165
PE	430	570	630	870	370
PC	4200	4700	5200	5800	2350
Significant WBC contributions estimated from linoleate uptake into WRS cells (Table 33).					
TL	850	850	850	440	440
TG	400	400	400	200	200
DG + X	40	40	40	20	20
PA	35	35	35	20	20
PE	20	20	20	10	10
PC	320	320	320	160	160

Table 39a: Incorporation of $[1-^{14}C]$linoleate into the Ghosts of Human Red Cell Fractionated According to Age (Donor MJW(I))

	density range of cells 1.075 – 1.080	1.080 – 1.082	1.082 – 1.084	1.084 – 1.086	1.086 – 1.110
Uptake into total esterified lipid dpm/μeq PL	21000	18800	18500	17900	15700
RBC and WBC activity per 10^{10} RBC per 10^{6} dpm/ml plasma					
TL	30500	26000	23200	23000	21500
TG	80	65	50	60	100
DG + X	540	540	950	900	1250
PA	1200	1100	1250	440	1750
PE	1550	1350	850	1000	900
PC	23000	22000	19500	19500	16200
SP + LPC	4000	850	760	950	1600
Significant WBC contributions estimated from uptake into WRS cells (Table 33)*					
TL	400	800	1300	1300	2100
TG	200	400	600	600	1000

*These estimates of TG activities are about 10 times the observed activities. As the MJW cells were stored overnight before incubation, such a decrease in WBC incorporation would be expected (see p. 52). Since TG activities were so low, WBC contributions to uptakes into other components would be negligible, an corrections are required.

Table 40a: Incorporation of $[1-^{14}C]$linoleate into Ghosts of Human Red Cells Fractionated According to Age (Donor MJW(II))

	density range of cells				
	1.075 – 1.0835	1.0835 – 1.0855	1.0855 – 1.088	1.088 – 1.0935	1.0935 – 1.102
Uptake into total esterified lipid dpm/µeq PL					
	15000	16800	28500	32500	32500
RBC and WBC activity per 10^{10} RBC per 10^6 dpm/ml plasma					
TL	14500	17500	27000	23500	26000
TG	100	100	100	300	< 280
DG	580	620	640	520	280
X	460	540	380	520	280
PA	1800	1400	2200	2000	2600
PE	2400	6400	11800	11200	14800
PC	9400	7800	12200	8200	7800
Significant WBC contributions estimated from uptake into WRS cells (Table 33)*					
TL	2100	1700	800	1300	2100
TG	1000	800	400	600	1000

*Estimates of TG activities are much lower than those observed. The same footnote as that to Table 39a applies.

Table 41a: Incorporation of $[1-^{14}C]$linoleate into Ghosts of Human Red Cells Fractionated According to Age (Donor CCW)

	density range of cells				
	1.075 – 1.080	1.080 – 1.082	1.082 – 1.084	1.084 – 1.086	1.086 – 1.105
Uptake into total esterified lipid dpm/µeq PL					
	23500	24500	25000	27000	26000
RBC and WBC activity per 10^{10} RBC per 10^6 dpm/ml plasma					
TL	53500	48500	49000	51000	52000
TG	340	410	280	380	410
DG + X	11000	11500	10500	12200	12500
PA	5050	4100	5050	4400	4400
PE	4400	2650	2850	2750	2900
PC	31500	29000	28000	30000	29000
Significant WBC contributions estimated from uptake into WRS cells (Table 33)					
TL	800	800	400	800	800
TG	400	400	200	400	400

No other WBC contributions are significant.

Table 42a: Incorporation of $\lceil 1-{}^{14}C \rceil$palmitate into Ghosts of Human Red Cells Fractionated According to Age (Donor VJC)

density range of cells	1.075 – 1.080	1.080 – 1.082	1.082 – 1.084	1.084 – 1.086	1.086 – 1.110
Uptake into total esterified lipid dpm/µeq PL	14400	15900	14300	21500	57500
RBC and WBC activity per 10^{10} RBC per 10^6 dpm/ml plasma					
TL	14500	15500	12500	17500	43000
TG	500	900	900	2100	16500
DG	430	510	450	1550	2200
X	800	950	1100	1850	3600
PA	1400	1500	900	2800	3100
PE	1400	1400	800	770	1300
PC	9500	9500	6800	7200	14000
LPC + SP	430	650	1300	830	2100
WBC contributions estimated from TG activities* and the palmitate distribution in Table 33					
TL	900	1600	1600	3700	29000
TG	500	900	900	2100	16500
DG	20	30	30	80	650
X	20	30	30	80	650
PA	-	30	30	70	600
PE	-	25	25	60	450
PC	250	450	450	1000	8500
LPC + SP	60	120	120	250	2000

*This assumes to be between half and one times that observed for EJC white cells

Table 43a: Incorporation of $\lceil 1-{}^{14}C \rceil$palmitate into Ghosts of Human Red Cells Fractionated According to Age (Donor GGM)

density range of cells	1.075 – 1.078	1.078 – 1.080	1.080 – 1.082	1.082 – 1.0845	1.0845 – 1.102
Uptake into total esterified lipid dpm/µeq PL	20200	22800	23500	16500	25000
RBC and WBC activity per 10^{10} RBC per 10^6 dpm/ml plasma					
TL	24000	22500	25500	18500	30000
TG	2600	2200	2400	1350	1350
DG + X	2300	1880	1680	1200	1650
PA	1200	1400	2200	1950	2400
PE	2850	2600	3150	4000	8600
PC	13500	13200	13200	8600	14500
SP + LPC	980	1100	2400	1400	1700
Significant WBC contributions estimated from uptake into EJC cells (Table 33)					
TL	2400	2200	2100	2100	1500
TG	1400	1300	1200	1200	900
DG + X	110	100	100	100	70
PA	50	45	40	40	20
PE	40	35	30	30	20
PC	700	650	550	550	400
SP + LPC	170	160	140	140	90

Table 44a: Incorporation of $[1-^{14}C]$palmitate into Ghosts of Human Red Cells Fractionated According to Age (Donor EJC)

density range of cells

1.075 1.078 1.080 1.082 1.084 1.086

Uptake into total esterified lipid dpm/μeq PL	15300	18700	17800	15500	16000
RBC and WBC activity per 10^{10} RBC per 10^{6} dpm/ml plasma					
TL	19500	32000	24000	27000	28500
TG	650	650	370	830	1500
DG	1650	2050	1450	1480	1300
X	1000	610	280	550	-
PA	1100	-	280	1850	370
PE	2300	4100	3400	3200	5400
PC	10700	22500	17000	17500	19500
Significant WBC contributions estimated from uptake into EJC cells (Table 33)					
TL	140	140	140	140	280
TG	80	80	80	80	160

No other contributions are significant

Table 49a: Incorporation of $[1-^{14}C]$palmitate into Rat White Blood Cell lipids (Rat A)

Uptake into total esterified lipid: 9000 dpm/10^{10} WBC

Table 50a: Incorporation of $[1-^{14}C]$palmitate into Normal Populations of Rat Red Cells and Red Cell Ghosts

Rat A (cells)

Uptake into total esterified lipid: 6000 dpm/μeq PL

White cell contributions (dpm per 10^{10} RBC)

	TL	TG	DG+X	PA	PE	PS	PC	SP
RBC + WBC	15000	850	1350	300	1000	300	10500	250
WBC	10500	950	600	1200	600	100	6800	150

Rat B (ghosts)

Uptake into total esterified lipid: 16500 dpm/μeq PL

White cell contributions (dpm per 10^{10} RBC per 10^{6} dpm/ml plasma)

	TL	TG	DG+X	PA	PE	PC
RBC + WBC	23000	250	800	2000	350	18000
WBC	2800	250	160	300	160	1800

Table 51a: Incorporation of [1-^{14}C]palmitate into Rat Red Cells Fractionated According to Age (Rats D)

	density range of cells					
	1.075 – 1.081	1.081 – 1.083	1.083 – 1.085	1.085 – 1.087	1.087 – 1.108	1.108 –
TL dpm/10^{10} cells	14000	10000	7300	7500	10500	17000
TG	140	70	40	10	40	80

No WBC counts could be made, but very low TG activities indicate that negligible uptake was due to WBC contamination.

Table 52a: Incorporation of [1-^{14}C]palmitate into Ghosts of Rat Red Cells Fractionated according to age (Rats E)

	density range of cells				
	1.075 – 1.079	1.079 – 1.081	1.081 – 1.083	1.083 – 1.085	1.085 – 1.103
TL dpm/μeq PL	200000	158000	127000	118000	76000
TG	8000	1600	1300	2800	2300

No WBC counts could be made, but TG counts suggest that WBC contamination was low. More contamination of the lightest fraction is likely.

APPENDIX 2: ABBREVIATIONS

AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CE	cholesterol ester
Ch	cholesterol
CMAW	chloroform-methanol-acetic acid-water
CMN	chloroform-methanol-ammonia
CoA(CoASH)	coenzyme A
DG	diglyceride
dpm	disintegrations per minute
FFA	free fatty acid
GPC	glycero-3-phosphoryl choline
GPE	glycero-3-phosphoryl ethanolamine
GSH	reduced glutathione
GSSG	oxidised glutathione
Hb	Hemoglobin
HEA	hexane-ether-acetic acid
KRP	Krebs-Ringer-phosphate
LPA	lysophosphatidic acid
LPC	lysophosphatidyl choline
LPE	lysophosphatidyl ethanolamine
LPL	lysophospholipids
MeOH	methanol
NAD^+(NADH)	nicotine adenine dinucleotide
$NADP^+$(NADPH)	nicotine adenine dinucleotide phosphate
NL	neutral lipid
PA	phosphatidic acid
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PL	phospholipid
PS	phosphatidyl serine
RBC	red blood cell
SF	solvent front
SP	sphingomyelin
TCA	trichloroacetic acid
TLC	thin-layer chromatography
WBC	white blood cell
X	unidentified radioactive lipids

16:0, 18:0, 18:1 etc. fatty acid with 16(18,18) carbons and 0 (1 double bonds.

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INCORPORATION OF [1-^{14}C]PALMITATE INTO THE LIPIDS OF BOVINE BLOOD CELLS

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SUMMARY

1. Bovine erythrocytes and leukocytes have been incubated with [1-^{14}C]palmitic acid, and the incorporation of the fatty acid into each cell type has been followed.

2. A high level of incorporation was observed with leukocytes, mainly into the phospholipids and triglycerides. The processes appeared to involve an exchange rather than a net uptake mechanism. Incorporated palmitate took part in chain-lengthening processes and some $^{14}CO_2$ was produced during the incubations.

3. Incorporation into the lipids of erythrocytes was very much lower than that observed for leukocytes. Low leukocyte concentrations in red cell preparations accounted for a significant proportion of [1-^{14}C]palmitate uptake into the cell lipid. The importance of accounting for these leukocytes has been stressed.

4. After allowing for leukocyte contributions, a significant incorporation of palmitate into the erythrocyte phospholipids, in particular phosphatidyl choline and phosphatidyl ethanolamine, was demonstrated. However, no significant uptake into the small quantities of triglycerides or cholesterol esters present in the erythrocytes could be detected.

INTRODUCTION

Phospholipids and free cholesterol are the major lipids present in mammalian erythrocytes. It has been established that the cholesterol and much of the phospholipid can exchange with similar components of the blood plasma, but that they cannot be synthesised by the erythrocyte[1,2]. In addition to these lipids, erythrocytes contain small amounts of glycolipids, triglycerides, cholesterol esters and unesterified fatty acids. A role for the minor neutral lipids has not been defined, although it has been suggested that they may act as intermediates in the turnover of the major erythrocyte lipids[1]. The turnover of either triglycerides or cholesterol esters has not been studied in detail. MULDER AND VAN DEENEN[3] and OLIVEIRA AND VAUGHAN[4], while investigating the uptake of radioactive long chain fatty acids into red cell phospholipids, detected little incorporation into esterified neutral lipids. Since the

uptake of a small amount of labelled substrate into a minor cellular constituent may represent a high turnover rate, a detailed examination of [1-^{14}C]palmitate incorporation into erythrocyte neutral lipids was undertaken, with a view to detecting a possible physiological role for these constituents.

It is generally accepted that the energy requirements for erythrocytes are primarily met by anaerobic glycolysis, and that the cellular lipid does not make a significant contribution[5]. However, at least 95% of the lipid is localised in the cell membrane[6], and it is possible that a lipid component could be a source of maintenance energy for the membrane itself. This possibility has been considered in interpreting the results of the present study.

As the presence of leukocytes in erythrocyte preparations affected the level of palmitate incorporation, it was necessary to determine the nature of [1-^{14}C]palmitate uptake by the leukocytes, and this information is also reported.

MATERIALS AND METHODS

[1-^{14}C]Palmitic acid, with a specific activity of 36.6 mC/mmole was obtained from the Radiochemical Centre, Amersham, and stored in benzene at $-4°$. Using the thin-layer chromatographic technique employed in this study, at least 95% of the palmitate radioactivity was recovered in the free fatty acid spot. Autoradiography showed no radioactivity elsewhere on the plate. Using gas chromatography, 95% of the radioactivity was trapped in the column eluate corresponding to the palmitate peak.

All solvents were distilled before use. Koch-Light silicic acid, 100–120 mesh, activated for 12 h at 110°, was used for column chromatography, and Merck silica gel G was used for thin-layer chromatography. Layers were predeveloped with ether to remove impurities and activated at 110° for 1 h before use. For phospholipid separations, plates were used within 15 min of activation.

Blood was collected from Jersey cows into one-seventh of its volume of acid citrate–dextrose. If red and white cells were to be incubated separately, or together in abnormal proportions, the blood was spun in an MSE swing-out head centrifuge for 30 min. at 3000 rev./min. Most of the upper plasma layer was separated, and the remaining plasma plus a white cell-rich layer from the top of the cell column were collected separately. A tube with a side opening was used to aspirate horizontal layers. The red cells were washed 3 times with an equal volume of 0.9% NaCl. At each centrifuging, a white cell-rich layer was removed from the top of the cell column. Using this method, 1–5% of the original white cells were present in the red cell preparations*.

Red cell and white cell preparations of known composition were incubated in plasma (total volumes about 30 ml) with [1-^{14}C]palmitic acid. For one incubation a Krebs–Ringer phosphate medium (pH 7.4) was used. Cell to plasma ratios were

* Because the degree of radioactivity incorporation was affected by the presence of white cells, the possibility of using other methods of cell separation was investigated. Passage of the blood slowly through cotton wool columns removed only few white cells, and the addition of dextran to the blood did not cause rapid red cell sedimentation[7]. Ultracentrifugation using an angle head was also regarded as unsatisfactory because the mixing of upper cell layers on deceleration necessitated the removal of large quantities of erythrocytes with the leukocytes.

approx. 1:2. The [1-^{14}C]palmitic acid (1–2 μC) in benzene (0.5–1 ml) was added to a small volume of plasma or to the Krebs–Ringer phosphate solution, the benzene evaporated at 37°, an aliquot taken for counting, and a known volume mixed with the cell suspension. Incubations were carried out for 5 h at 37°, in gently agitated conical flasks, either plugged with cotton wool or with outlets to tubes containing NaOH, in which CO_2 was trapped by passing a slow stream of CO_2-free air through the flask. At the end of each incubation, the cells were separated by centrifuging at 4°. When special efforts were made to remove white cells prior to incubation, the red cells were washed twice with 0.9% NaCl at 4°. When red and white cells were incubated together, plasma and white cells were collected separately as described. Red cells were washed with cold saline 3 times and the white cell fraction twice. Cell counts were made on each sample using an Improved Neubauer counting chamber. White cells were stained with methylene blue in 1% acetic acid.

Red cell lipids were extracted with 20 vol. of chloroform–isopropanol (7:11, v/v) by the method of ROSE AND OKLANDER[8]. Plasma and white cell lipids were extracted with 20 vol. of chloroform–methanol (2:1, v/v) using the method of FOLCH, LEES AND SLOANE-STANLEY[9]. Solvents were removed in a Büchi rotary evaporator below 45° and the lipids were washed[9], evaporated to dryness, and weighed.

The neutral and polar lipids of the erythrocytes were separated on silicic acid columns using the method of BORGSTRÖM[10]. Neutral lipids, which had been eluted with dry chloroform, were separated further by thin-layer chromatography using either hexane–ether–acetic acid (70:30:1, v/v/v) or (30:70:1, v/v/v) as the developing solvent[11]. Phospholipids, which had been eluted from the silicic acid columns with chloroform–methanol and methanol, were separated by thin-layer chromatography using either chloroform–methanol–conc. ammonia (14:6:1, v/v/v) or chloroform–methanol–acetic acid–water (65:25:8:4, v/v/v/v)[12]. Plasma and white cell lipids were separated directly by thin-layer chromatography. Analytical plates were sprayed with iodine, or 20% sulphuric acid and charred, and the spots were identified by comparing their positions with those of standards. Autoradiographs were prepared by placing the plates in contact with Ilford X-ray film for 6 weeks. Preparative plates were sprayed with iodine, and the silicic acid containing each lipid band scraped off either into a vial for scintillation counting or into a narrow glass column for elution. Neutral lipids were eluted completely with 10 ml of chloroform–methanol (2:1, v/v) and phospholipids with 5 ml chloroform–methanol (1:2, v/v) followed by 5 ml methanol. Equivalent results were obtained by counting a lipid sample either adsorbed to silicic acid or after elution from it.

A Packard model 4000 scintillation counter was used. Non-aqueous samples were counted in a solution (15 ml) containing PPO (5 g) and POPOP (0.05 g) (Nuclear Enterprises) in toluene (1 l). Aqueous or silicic acid-containing samples were counted in a solution (15 ml) of PPO (5 g) and POPOP (0.2 g) in a mixture of toluene (600 ml) and ethanol (400 ml). Quenching was determined either with standard [^{14}C]toluene or by external standardisation. The normal background activity was approx. 25 counts/min.

Total amounts of lipid in samples were determined using a modification of the method of AMENTA[13]. Duplicate fractions of eluates containing 20–200 μg lipid were evaporated to dryness, oxidised with a standard chromic acid solution, and the colour change determined spectrophotometrically. Standard graphs of weight *versus* absor-

bance change were determined for each class of lipid. Phosphorus was assayed by the method of BARTLETT[14], and $^{14}CO_2$ was estimated by precipitation as $BaCO_3$ (ref. 15).

Lipid samples for gas–liquid chromatography were methylated by the method of MORRISON AND SMITH[16]. A Shandon Chromatograph, with an apiezon stationary phase column at 197°, argon carrier gas, and a ^{90}Sr detector was used. Samples from the chromatograph for radioactive assay were collected from the detector outlet into tubes containing toluene-moistened glass wool.

RESULTS

Leukocyte incorporation of [1-^{14}C]palmitic acid

The leukocytes showed a very high incorporation of [1-^{14}C]palmitate into their lipids (Table I). No other studies have been reported of fatty acid uptake into bovine

TABLE I

RADIOACTIVITY DISTRIBUTION AMONG WHITE BLOOD CELL LIPIDS AFTER INCUBATION OF THE CELLS WITH [1-^{14}C]PALMITIC ACID

Cells were incubated in plasma as described. White cell concentrations varied between $5 \cdot 10^6$ and $50 \cdot 10^6$ cells per ml. When the concentration was high, the radioactivity incorporated per cell was slightly lower. From 9 to 63% of the medium activity was incorporated into the cells. The total lipid was extracted and separated by thin-layer chromatography with hexane–ether–acetic acid (70:30:1, v/v/v). Results are the means from 5 incubations, each quoted ± 1 standard deviation, and expressed as incorporation per 10^{10} cells from a medium having an initial [1-^{14}C]palmitate activity of 10^5 counts/min per ml, which corresponds to a palmitic acid specific activity of $8.0 \cdot 10^5$ counts/min per mg.

	Percent of total lipid weight	*Percent of total lipid radio-activity*	*Counts/min per 10^{10} cells $\times 10^{-4}$*	*Specific activity (counts/min per mg $\times 10^{-3}$)*
Total lipid*			1300 ± 150	43 ± 8
Free fatty acid	7 ± 2	14 ± 1	180 ± 20	86 ± 5
Phospholipid	58 ± 3	49 ± 3	640 ± 80	37 ± 3.3
Triglyceride	8 ± 1	30 ± 3	390 ± 60	160 ± 30
Cholesterol ester	6 ± 1	1 ± 0.3	13 ± 4	7 ± 2.5
Cholesterol and diglyceride	21 ± 1	6 ± 1	80 ± 20	13 ± 2.6

* 300 ± 50 mg/10^{10} cells.

leukocytes with which these results may be compared. The phospholipids and triglycerides were responsible for the majority of the uptake, the phospholipids attaining the highest total activity and the triglycerides the highest specific activity. Autoradiographs of the lipids separated by thin-layer chromatography (Figs. 1a and 1b) show these major spots as well as faint cholesterol ester and diglyceride spots, but no radioactive cholesterol. Diglyceride is therefore primarily responsible for the radioactivity of samples containing both cholesterol and diglyceride, which could not be readily separated before analysis. The other, slow-running, radioactive components were not detectable gravimetrically and were not identified. One could be monoglyceride. Palmitate uptake by the phospholipids is summarised in Table VI, and an autoradiograph of a thin-layer chromatographic separation of the phospholipids is shown in Fig. 1c. Of the phospholipids, phosphatidyl choline incorporated the most radioactivity, followed by sphingomyelin. It seems unlikely that the sphingomyelin radioactivity could be attributed to lysophosphatidyl ethanolamine (which could

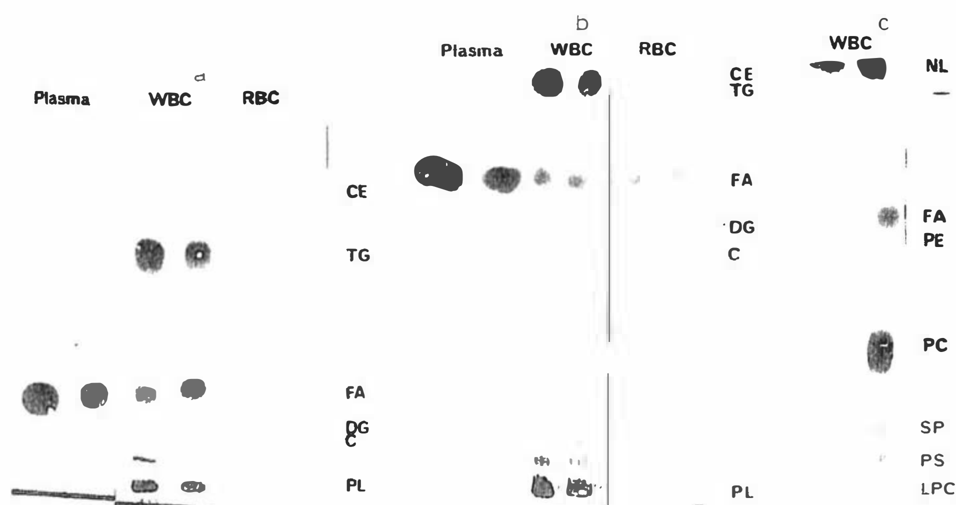

Fig. 1. Autoradiographs of thin-layer chromatographic separations of total lipid extracts from bovine erythrocytes (RBC), leukocytes (WBC) and plasma, after incubation with [1-^{14}C]palmitic acid. The identification of phosphatidyl serine and lysophosphatidyl choline is only tentative. a. Solvent system hexane–ether–acetic acid (70 30 1, v/v/v). b. Solvent system hexane–ether–acetic acid (30 70 1, v/v/v). c. Solvent system chloroform–methanol–ammonia (14 6 1, v/v/v). Abbreviations: CE, cholesterol ester; TG, triglyceride; FA, free fatty acid; DG, diglyceride; C, cholesterol; NL, neutral lipid; PL, phospholipid; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PS, phosphatidyl serine; SP, sphingomyelin; LPC, lysophosphatidyl choline.

exhibit similar thin-layer chromatographic mobilities in both solvent systems), in view of the very low incorporation into phosphatidyl ethanolamine, one of the major phospholipid components. The thin-layer chromatographic behaviour of the remaining radioactive components suggests that one be lysophosphatidyl choline (running behind sphingomyelin in both solvents), and the other, phosphatidyl serine.

Only 65% of the labelled fatty acids extracted from the cells was palmitic acid. The remainder were longer chain (mainly C_{18}) acids. Some radioactivity was also recovered as $^{14}CO_2$. A qualitative estimation showed that this amounted to roughly 20% of the ^{14}C incorporated into the total leukocyte lipid.

After incubations were complete, the plasma lipids were analysed for radioactivity. Although reductions in the specific activities of the plasma fatty acid were demonstrated, no decreases in fatty acid concentrations could be shown. The analyses may not have been sufficiently sensitive to detect small gravimetric changes. These results suggest that an exchange occurs between the fatty acids of the plasma and cellular lipids. This interpretation is supported by gas chromatographic analysis of the fatty acids extracted from plasma after incubation with leukocytes. Only 70% of the labelled fatty acid was palmitic, the remainder being longer chain (mainly C_{18}) acids. Therefore leukocytes can apparently synthesise labelled long chain acids from palmitic, and exchange these for plasma fatty acids. About 5% of the plasma radioactivity was found in the phospholipids, which suggests that the white cell is able to release intact phospholipid molecules.

Although white cell samples for lipid extraction normally contained red cells (ratio of red to white cell counts 2 to 20), the contribution of radioactivity by red cell components to that of any leukocyte component was very slight. A red cell

contains about one-fiftieth of the amount of lipid present in a white cell, and accordingly it was possible to obtain fairly accurate lipid weights for white cells by an appropriate correction. Platelets, although present in the white cell fractions, were not counted.

Erythrocyte incorporation of [1-^{14}C]palmitic acid

The lipid composition of bovine erythrocytes is given in Table II. This is

TABLE II

LIPID COMPOSITION OF BOVINE ERYTHROCYTES

Lipids were extracted, and either separated by column chromatography into neutral and phospholipids and the neutral lipids separated further by thin-layer chromatography in hexane–ether–acetic acid (70:30:1, v/v/v), or separated directly by thin-layer chromatography. 6 extracts were separated, analysed, each in duplicate, and results quoted are the means of these, ± 1 standard deviation.

	Percent of total weight	*mg/10^{10} cells*
Total lipid		4.4 ± 0.2
Phospholipid	70 ± 3	3.1 ± 0.2
Cholesterol	27 ± 1	1.20 ± 0.07
Cholesterol ester	1.1 ± 0.7	0.048 ± 0.03
Triglyceride	1.0 ± 0.7	0.044 ± 0.03
Free fatty acid	1.2 ± 0.8	0.053 ± 0.03

similar to that obtained by DE GIER AND VAN DEENEN[17], and HANAHAN, WATTS AND PAPPAJOHN[18], although the latter report slightly higher triglyceride and cholesterol ester levels. Incorporation of radioactivity into the lipids of the red cell preparations is summarised in Table III, and autoradiographs of thin-layer chromatographic separations of the lipids are shown in Figs. 1a and 1b. Albumin, which has been shown to bind fatty acids in plasma, appeared to have no effect on fatty acid uptake by the esterified lipids in these experiments. Although the greatest proportion of red cell radioactivity was present as unesterified fatty acid, comparatively high counts were recorded in the phospholipids. A significant amount of radioactivity was associated with the triglyceride fraction and only low activities were found in other fractions. These results give relatively high specific activities, and hence high incorporation rates, for minor components such as triglycerides.

However, 1–5% of the original white cells were extracted along with the red cells, and, as shown in Table IV, these were responsible for a significant proportion of the radioactivity in the red cell extracts. When allowance was made for this contribution from the white cells (Table V), the incorporation of fatty acids into the phospholipids of red cells was reduced but was still significant. Corrected values showed no significant incorporation into triglycerides, and the amount of radioactivity in other neutral lipids (excluding free fatty acids) was so low as to be within the experimental error. Some of the counts isolated with cholesterol probably arose from contamination by trailing fatty acids on the thin-layer plates.

The distribution of radioactivity among the red cell phospholipids is shown in Table VI. Most components contained low counts and accordingly the results are liable to comparatively large errors. However, the results, in general, agree with those of MULDER AND VAN DEENEN[3], who also found that most of the labelled fatty

TABLE III

RADIOACTIVITY DISTRIBUTION AMONG THE LIPIDS EXTRACTED FROM RED BLOOD CELL PREPARATIONS AF INCUBATION WITH [1-^{14}C]PALMITIC ACID

Incubations were carried out as described. The specific conditions for each experiment were as follows: (i) but 5% of the white cells present in the original blood were separated prior to incubation. (ii) Red cells v incubated with 5 times the number of white cells normally present in blood. All but 1% of these cells v separated after incubation. (iii)–(vi) Whole blood was incubated. All but 1% of the white cells were separa after incubation. (vii)–(ix) White cells were separated before incubation. No cell counts were made but 1- of the original white cells were probably incubated and extracted. (vii) The cells were incubated in a Kr Ringer phosphate medium. About 2% of the total radioactivity was recovered in the red cell lipids. White c which were present during the incubations incorporated from 8–63% of the total [1-^{14}C]palmitate, and ca a corresponding decrease in the amount available to the red cells. By relating red cell uptakes to initial ra activity concentrations less the amounts taken up by the white cells, *i.e.*, the concentrations at the end of experiments, different incubations are comparable. Each experiment is shown separately, to show the depend of uptake on the degree of white cell contamination. Each lipid extract was separated in duplicate by thin-la chromatography. Results are expressed in terms of radioactivity per 10^{10} cells taken up from a medium ha a [1-^{14}C]palmitate activity of 10^5 counts/min per ml, or a palmitate specific activity of $8.0 \cdot 10^5$ counts/min mg, at the end of the incubation.

	Counts/min per 10^{10} red cells									*Range o specific activitie (counts/ per mg)*
	Expt. i	*Expt. ii*	*Expt. iii*	*Expt. iv*	*Expt. v*	*Expt. vi*	*Expt. vii*	*Expt. viii*	*Expt. ix*	
Total lipid	7200	7700	5400	5000	5000	4800	11600	7900	7600	100–18
Fatty acid	4500	4100	3100	3800	3800	3400	8700	6300	5900	3000–20
Phospholipid	2100	2700	1500	900	950	1150	920	930	1500	30–90
Triglyceride	410	580	230	150	130	110	320	340	35	140–15
Cholesterol ester	70	54	47	25	25	24	66	90	24	20–25
Cholesterol and diglyceride	190	310	280	150	75	140	200	160	110	6–30
White cells extracted per 10^{10} red cells	$1.33 \cdot 10^6$	$1.2 \cdot 10^6$	$0.55 \cdot 10^6$	$0.29 \cdot 10^6$	$0.26 \cdot 10^6$	$0.25 \cdot 10^6$	Not determined			

TABLE IV

RADIOACTIVITY IN THE RED CELL PREPARATION LIPIDS ATTRIBUTABLE TO EXTRACTS FROM WHITE CELLS

Values are calculated from the known number of white cells contaminating the red cell preparations and the radioactivity uptake per 10^{10} white cells from a medium having a [1-^{14}C]palmitate activity of 10^5 counts/min per ml, and are quoted ± 1 standard deviation. Results given in this table and Table III are from the same experiments. In Expt. i, the white cells were incubated separately with [1-^{14}C]palmitic acid.

	Counts/min due to white cells per 10^{10} red cells					
	Expt. i	*Expt. ii*	*Expt. iii*	*Expt. iv*	*Expt. v*	*Expt. vi*
Total lipid	1700 ± 140	3500 ± 230	1000 ± 70	450 ± 30	390 ± 30	370 ± 26
Fatty acid	270 ± 50	290 ± 40	160 ± 25	80 ± 10	76 ± 10	35 ± 4
Phospholipid	880 ± 100	1900 ± 190	440 ± 40	240 ± 30	170 ± 20	180 ± 20
Triglyceride	490 ± 80	1000 ± 230	250 ± 30	110 ± 20	130 ± 50	115 ± 20
Cholesterol ester	20 ± 12	32 ± 27	17 ± 5	4 ± 3	6 ± 6	4 ± 3
Cholesterol and diglyceride	110 ± 30	160 ± 35	110 ± 20	30 ± 6	25 ± 14	20 ± 8

acids incorporated into bovine erythrocytes were present in the phosphatidyl choline and phosphatidyl ethanolamine fractions.

With higher concentrations of white cells in the incubation system, there was an apparent decrease in the amount of radioactivity incorporated into the erythrocyte lipids. A probable explanation is that the comparatively rapid exchange of the plasma

TABLE V

RADIOACTIVITY DISTRIBUTION AMONG PURE ERYTHROCYTE LIPIDS AFTER INCUBATION OF THE CELLS WITH [1-^{14}C]PALMITIC ACID

Values are calculated from the results shown in Tables III and IV. They are the means of the 6 incubations, are related to an external [1-^{14}C]palmitate concentration of 10^5 counts/min per ml which corresponds to a palmitic acid specific activity of $8.0 \cdot 10^5$ counts/min per mg, and are quoted $\pm$ 1 standard deviation.

	Percent of total lipid radioactivity	*Counts/min per per 10^{10} cells*	*Specific activity (counts/min per mg)*
Total lipid		4700 ± 30	1000 ± 100
Free fatty acid	78 ± 5	3600 ± 200	43000 ± 15000
Phospholipid	20 ± 4	900 ± 180	300 ± 70
Triglyceride	— ± 0.5	— ± 20	—
Cholesterol ester	0.5 ± 0.5	20 ± 20	300 ± 300
Cholesterol and diglyceride	2.5 ± 1.1	120 ± 50	100 ± 60

TABLE VI

RED AND WHITE BLOOD CELL PHOSPHOLIPID ACTIVITY DISTRIBUTION AFTER INCUBATION OF THE CELLS WITH [1-^{14}C]PALMITIC ACID

Cells were incubated as described. Phospholipids were separated by thin-layer chromatography with chloroform–methanol–acetic acid–water (65:25:8:4, v/v/v/v) as developing solvent. Synthetic phosphatidyl choline and phosphatidyl ethanolamine (L. Light and Co.) and sphingomyelin extracted from sheep brain were used in identification; phosphatidyl serine was tentatively identified from its R_F. White cell contamination has been accounted for in calculating the red cell radioactivity distribution. The mean results from 5 (red cell) and 4 (white cell) experiments are quoted $\pm$ 1 standard deviation.

	Percent of phosphorus		*Percent of activity*	
	Red cells	*White cells*	*Red cells**	*White cells***
Phosphatidyl ethanolamine	21	18	22 ± 12	4.2 ± 0.6
Phosphatidyl serine	17	12	6 ± 4	6 ± 2
Phosphatidyl choline	6	29	60 ± 9	60 ± 9
Sphingomyelin	50	32	6 ± 6	20 ± 3
Components running behind sphingomyelin	6	9	7 ± 7	10 ± 3

* 900 phospholipid counts/min per 10^{10} cells.
** $6.4 \cdot 10^6$ phospholipid counts/min per 10^{10} cells.

fatty acids with the leukocyte lipid components leads to a reduction in the specific activity of the fatty acids being incorporated into the erythrocytes.

After one incubation, the total erythrocyte fatty acids were separated by gas chromatography and the radioactive components isolated. The fatty acids present were mainly oleic (40%), palmitic (24%), and stearic (20%) acids. Of the radioactivity, 99% was still found associated with palmitic acid. Hence in agreement with the results of MULDER AND VAN DEENEN[3], on incorporation of linoleic acid into rabbit erythrocytes, no evidence for any active fatty acid chain altering processes in the erythrocyte could be demonstrated.

The autoradiographs in Figs. 1a and 1b also show the plasma radioactivity distribution in 2 experiments. About 1–4% of the plasma activity was present in the phospholipids, and this varied only slightly with the number of white cells incubated. Most of the phospholipid activity was in each case associated with phosphatidyl choline. Plasma alone is unable to incorporate labelled fatty acid into phospholipid[5],

and as only a small increase in labelled plasma phospholipid was associated with a large increase in white cell numbers, the ability to exchange intact phospholipid molecules appears to be a property of both types of cell.

DISCUSSION

It has been shown that the presence of comparatively small numbers of leukocytes in erythrocyte preparations can account for a high proportion of the observed incorporation of plasma fatty acids into the cell lipids. An apparently high rate of fatty acid incorporation into red cell triglycerides can be interpreted entirely in terms of contamination by only 2–5% of the white cells normally present in blood. This result stresses the advisability of determining the extent of leukocyte presence in red blood cell samples before interpreting results of experiments in terms of red cell properties alone. A similar situation arose in the evaluation of erythrocyte lipid synthesis from acetate, when BUCHANAN[19] and others showed that leukocyte contamination was responsible for the observed lipid synthesis. The problems of blood cell separation are discussed in a review by SPARKES AND BEUTLER[20]. Difficulties are magnified when dealing with bovine blood, which exhibits a slow erythrocyte sedimentation rate, giving rise to greater leukocyte dispersal, and no erythrocyte aggregation in the presence of dextran[7]. The lack of rouleau formation by bovine erythrocytes causes their slow sedimentation[21], and it could be the same property of the cell surface which prevents their aggregation in the presence of dextran.

The method employed, of removing the upper layer of cells after successive centrifugations, still left a final 2–5% of leukocytes in erythrocyte samples. MULDER AND VAN DEENEN[3] report the removal of all white cells from blood of different species, including cattle, by a method involving ultracentrifugation of the cell suspension at $100\,000 \times g$ and removal of the upper third of the cell column. Although this results in the removal of a sizeable young population of red cells[22], it is probably preferable in metabolic studies to making considerable corrections for residual leukocytes.

Bovine erythrocytes were found to take up [1-^{14}C]palmitic acid from the suspending medium. Apart from highly radioactive unesterified fatty acids associated with the cells, the remaining activity was located in the phospholipids. Phosphatidyl choline (60%) and to a lesser extent phosphatidyl ethanolamine (20%) contained most of the incorporated radioactivity. Similar experiments by other workers, who studied rabbit or human erythrocytes or ghosts[3,4,23], have resulted in higher incorporations into phosphatidyl choline and lower into phosphatidyl ethanolamine. However, these erythrocytes contain much more phosphatidyl choline than do bovine cells, and for this reason, a proportionately lower incorporation into bovine phosphatidyl choline, as found by us and also by MULDER AND VAN DEENEN[3], might be expected.

In this study, at least some of the red cell phospholipid molecules, particularly lecithins, are apparently released into the surrounding plasma. Results obtained for erythrocytes from other species are in agreement with this finding[24–26].

No isotope incorporation into the erythrocyte triglycerides and only very low incorporation into the cholesterol esters could be demonstrated. These results are supported by the work of MULDER AND VAN DEENEN[3], who incubated rabbit erythrocyte ghosts with labelled fatty acid, and qualitatively found little activity corre-

sponding to any esterified neutral lipid by scanning a thin-layer chromatograph. Our results suggest, therefore, that the renewal of erythrocyte triglyceride and probably cholesterol ester fatty acids by uptake from plasma is either not possible or of such a low order that it was not detected by our methods. The specific activity of the red cell triglycerides was found to be much lower, and that of the cholesterol esters no higher, than the total phospholipid specific activity, and both were considerably lower than those of the individual phospholipids exhibiting the highest incorporations. Hence with regard to the role of these minor neutral lipids in overall cell metabolism, these results do not support a postulate that they act as intermediates in the transfer of plasma fatty acids to erythrocyte phospholipids.

High incorporation of plasma palmitic acid into bovine leukocyte lipids has been found. The phospholipids, especially phosphatidyl choline, and the triglycerides were largely responsible for this uptake, but other components were involved to a lesser extent. Results suggest that exchange rather than net uptake took place. The [1-^{14}C]palmitate incorporated appeared to be quite actively metabolised, both to longer chain fatty acids and to $^{14}CO_2$. No other studies on the uptake of plasma fatty acids by bovine leukocytes have been reported, but similar studies on leukocytes from some other animals and sources have been carried out[27–30]. When comparing our results with these, the non-homogeneity of white cell samples must be considered, and differences in leukocyte behaviour have been found, depending on both the source of the cells and the treatment given them[20]. The distribution of radioactivity found in bovine blood leukocyte lipids is similar to that in human blood cells after incubation with [^{14}C]palmitate[30]. It is also similar to the distribution in rabbit polymorphonuclear leukocytes from peritoneal exudate, after incubation with labelled fatty acids, reported by ELSBACH[27,28]. The present findings seem to be representative of general leukocyte behaviour.

In his experiments, ELSBACH was unable to detect significant release of labelled phospholipid into the incubation medium, or a release of radioactivity from the cells on resuspension in an inactive medium. However EVANS AND MUELLER[29], although able to measure net uptake of fatty acids by guinea pig peritoneal exudate leukocytes, found no significant uptake into the blood leukocytes from the same animal. The differences between our finding that bovine blood leukocytes can release radioactive fatty acids to their plasma surroundings and the results of ELSBACH would appear due to a difference in behaviour of leukocytes from different sources. ELSBACH also demonstrated production of $^{14}CO_2$ and radioactive water soluble products, but there have been no other reports of chain lengthening of labelled fatty acids. The existence of such a process is substantiated, however, by the finding of MIRAS, MANTZOS AND LEVIS[31] that most of the [1-^{14}C]acetate incorporated into human leukocyte lipid is effected by a chain-lengthening process.

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A recent paper* reports a considerable incorporation of labelled fatty acids into the triglycerides of human erythrocytes. The extent of the incorporation was very sensitive to incubation conditions and subsequent treatment of the cells.

It is suggested that differences between the results reported in this communication and in the paper by DONABEDIAN AND KARMEN could be due to the extent to which allowance has been made for the presence of white cells in the incubation systems. Our results with bovine red cells showed that standing for 8h before incubation reduced the uptake of fatty acids into white cells to about 30%, but this procedure had little effect on red cell incorporation. The white cell levels in the human red cell preparations of DONABEDIAN AND KARMEN are of the same order as those in our bovine red cell preparations, and such levels were found to contribute significantly to fatty acid uptake by the cells.

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Metabolism *in vivo* of bovine erythrocyte lipids

A rather unique opportunity arose for us to study a Jersey cow which had been given a 24-h intravenous infusion of 10 mC of [^{14}C]acetate. Only one animal was available, but results are reported, not necessarily for direct interpretation, but more as a possible guide for interpreting related studies on red cell lipid metabolism. Blood samples were collected, and the fate of the radioactivity incorporated into the red cell lipids investigated.

Bovine erythrocytes have a lifespan of about 107 days. Of their total weight, 0.5% is lipid, largely phospholipid and cholesterol, and small amounts of tri- and diglyceride, cholesterol ester, free fatty acid and glycolipid[2,3]. Mature red cells cannot synthesise lipid from acetate, but exchange of some lipid molecules, or their fatty acyl moieties, between cells and plasma can occur[3-6]. In an animal administered [^{14}]acetate, radioactivity could enter the erythrocyte lipids either by direct synthesis from acetate prior to maturation, or by exchange of cell lipids for radioactive plasma lipids, which had been synthesised from acetate at other sites in the body. Incorporated radioactivity could be lost by exchange with plasma constituents, metabolic breakdown, or removal with the cell at the end of its lifespan. [^{14}C]Acetate administered to a cow is rapidly metabolised, and most of the radioactivity is quickly lost by oxidation or milk production[7], so persisting radioactive lipids would be predominantly those synthesised at the time of acetate administration.

Blood (250 ml) was withdrawn into ACD. Erythrocytes were separated from leukocytes and plasma and washed three times in 0.9% saline. Methods of lipid extraction, separation and analysis were similar to those reported in a previous paper[3]. Neutral lipids were separated by thin-layer chromatography on silica gel G

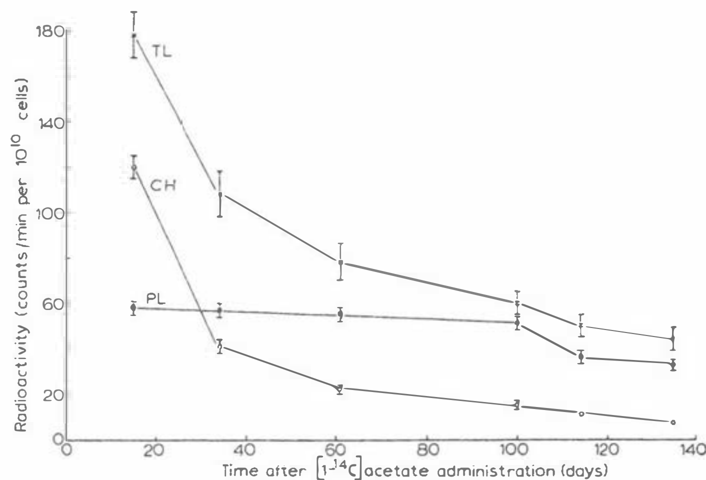

Fig. 1. Disappearance of radioactivity from bovine erythrocyte lipids *in vivo*, following administration of [1-^{14}C]acetate. TL, ×——×, total lipid radioactivity, CH, o——o, cholesterol; PL, ●——●, phospholipid. The standard error in each determination, carried out in duplicate, is shown.

developed with hexane–ether–acetic acid (70:30:1, v/v/v); phospholipids were developed with chloroform–methanol–conc. ammonia (14:6:1, v/v/v). The proportion of radioactivity in the component fatty acids of the phospholipids or cholesterol esters was determined following hydrolysis in methanolic KOH.

The disappearance of radioactivity from the erythrocyte total lipid, cholesterol, and phospholipid fractions during the study is shown in Fig. 1. As the cow was not available until a fortnight after the infusion, no earlier samples could be taken. The total activities and specific activities of the cholesterol ester, triglyceride and free fatty acid fractions were lower than those of cholesterol or phospholipid. On the day 15, the triglyceride and free fatty acid fractions each contained 1 count/min per 10^{10} cells or 15 counts/min per mg; in later samples activities were less than half this. No cholesterol ester activity was detactable in any sample. The [^{14}C]acetate dose was not high enough to accurately assess turnover of these components.

TABLE I

SPECIFIC ACTIVITIES OF ERYTHROCYTE AND PLASMA CHOLESTEROL AND PHOSPHOLIPID

Each determination was carried out in duplicate, and results are quoted ± 1 standard deviation as counts/min/mg.

	Days after [^{14}C]acetate administration					
	15	*34*	*61*	*100*	*114*	*135*
Erythrocyte esterified cholesterol	0 ± 5	0 ± 5				
Erythrocyte free cholesterol	80 ± 3	27 ± 2	15 ± 1	10 ± 1	7.5 ± 0.5	4.5 ± 0 5
Plasma esterified cholesterol*			15 ± 1	10 ± 1	7.0 ± 1	5 ± 0.5
Plasma free cholesterol			14 ± 1	7.5 ± 1	11 ± 4	4.5 ± 0.5
Erythrocyte phospholipid	17 ± 1	17 ± 1	16 ± 1	15 ± 1	10.5 ± 1	9.5 ± 0.5
Plasma phospholipid			6 ± 1	3.3 ± 0.5	3.0 ± 0.5	2.0 ± 0.5

* Calculated from total cholesterol ester activity less fatty acyl contribution.

A comparison of red cell and plasma phospholipid and cholesterol specific activities is given in Table I. No activity was detected in any other plasma lipid. As in the dog and human[4,6], the plasma free and esterified cholesterol and erythrocyte free cholesterol appear to be in equilibrium with each other, but not with the erythrocyte esterified cholesterol. The fall in erythrocyte cholesterol activity with time produced a similar curve to that typically obtained for plasma cholesterol[8], an initial changing slope due to slow and variable rates of equilibration between the different body cholesterol pools, followed by exponential decay representing true metabolic turnover Equilibrium was achieved after about 40 days, and a cholesterol half-life of about 45 days is suggested. No other data on bovine blood cholesterol turnover have been reported, but for comparison, human plasma cholesterol takes about 60 days to equilibrate, and then exhibits a half-life of 50–100 days[8].

Although the plasma phospholipid activity fell very gradually during the experiment, the red cell phospholipid activity fell as if it were influenced by two processes. Firstly there appeared to be a gradual fall paralleling that in the plasma, and secondly a sharper drop between 100 and 114 days after acetate administration. At this time, cells produced during isotope administration were reaching the end of their lifespan. If this is a true picture of the situation, it could be explained by the existence of some erythrocyte constituents capable of exchange with plasma counterparts, and others which remain intact throughout the life of the cell.

The distribution of isotope between the fatty acyl and non-fatty acyl moieties of the erythrocyte phospholipid was determined for samples taken on days 15, 34 and 100. In each case between 46% and 51% (mean 49 ± 5%) was associated with the fatty acids. If the phospholipids were uniformly labelled, this figure would be about 65%. The lower levels found would result from some phospholipid molecules being removed from the cells less readily than their fatty acid constituents.

The cell phosphatides, examined on days 15 and 34, showed very similar distributions of isotope, and no localisation of isotope in any phosphatide was evident. Low counts prevent any detailed interpretation of these results.

In a single study, bovine erythrocytes have been labelled *in vivo* with [^{14}C]-acetate. A decline in free cholesterol activity, arising from rapid equilibration with plasma cholesterol, has been demonstrated. A fall in activity that could be interpreted in terms of the erythrocyte being able to exchange some but not all of its phospholipid with plasma counterparts has also been found. The availability of only one animal and insufficient detail concerning individual phosphatides, prevents a full explanation of this behaviour at present.

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