We have examined labelling of the large protein in Na^+- or K^+-containing media. The enzyme adopts specific Na^+- or K^+-dependent conformations[8,12–14] and so it was interesting to ask whether these different states are accompanied by a change in accessibility of the protein to INA in the bilayer. In six separate experiments with different concentration ratios of label to protein we observed 10–25% greater incorporation into the large chain in the presence of KCl than with NaCl. This may mean that the conformational change following binding of K^+ leads to an altered relationship of protein to lipid.

In attempts to localise the 12 K fragment we exploited the fact that major fragments of the large chain can be produced by controlled tryptic cleavage in the presence of NaCl or KCl because the conformational response of the protein to cation binding is reflected in exposure of different bonds to tryptic attack[8,9]. Thus, fragments with MW 58 K and 46 K were formed by primary cleavage of the large chain in presence of KCl. A fragment of 78 K was seen after digestion in the presence of NaCl. The distribution of INA in protein fragments generated by controlled trypsinolysis of enzyme labelled at low INA concentrations is shown in Figs 2 and 3. These results show clearly that the binding of INA does not prevent the conformational response of the large chain to Na^+ and K^+. In the presence of KCl (Fig. 2) most of the radioactivity of the large chain was transferred to the 46 K fragment, but some labelling of the 58 K fragment was also seen. Comparison of radioactivity in the peaks with the content of protein obtained from absorbance scans of the gels showed that about 80% of the label was transferred to the 46 K fragment and only 20% to the 58 K fragment. Subsequently a 36 K fragment and the terminal 12 K peptide appear. The 46 K and the 36 K fragments therefore include segments which are embedded in the bilayer. The low concentration of label in the 58 K fragment may be due either to labelling of different parts of the large chain or to variation of the position of the tryptic split within the large chains in the preparation.

In the presence of NaCl (Fig. 3), the distribution of label was much more distinct. The 78 K fragment was completely devoid of radioactivity and the terminal 12 K fragment seems to arise almost by primary cleavage of the large chain. A minor fraction of the label, at most 2%, was found in a 37 K fragment. This implies that the radioactive 12 K segment or segments are localised near one end of the large chains which give rise to the 78 K fragment in the presence of NaCl.

Our results support previous evidence that iodonaphthylazide acts as a hydrophobic probe which labels membrane proteins from within the bilayer. The selectivity of the label for a small segment of the large peptide does not imply that other regions of the protein are not embedded in the membrane, for the labelled segment may hinder access of the label to other areas of the large peptide or indeed to the glycoprotein component. Preferential binding to this segment, or kinetic factors could explain the discrimination against unlabelled regions of protein and lipid side chains. Our work illustrates that combining specific labelling from within the lipid core with proteolytic digestion at the membrane surface may be an important tool for identifying segments of membrane proteins in intimate contact with the lipids.

We thank Dr T. Bercovici for ^{125}I-INA and Mrs Rivka Goldshlegger and Annie Sloth for excellent technical assistance. P.L.J. was a recipient of a short-term fellowship from EMBO.

STEVEN J. D. KARLISH
Biochemistry Department, Weizmann Institute of Science, Rehovot, Israel
PETER LETH JORGENSEN
Institute of Physiology, University of Aarhus, Aarhus, Denmark
CARLOS GITLER
Membrane Department, Weizmann Institute of Science, Rehovot, Israel

Received 7 July; accepted 12 August 1977.

1. Jorgensen, P. L. *Biochim. biophys. Acta* **356**, 36–52 (1974).
2. Kyte, J. *J. Biol. Chem.* **247**, 7642 (1972).
3. Jorgensen, P. L. *Biochim. biophys. Acta* **356**, 53–67 (1974).
4. Klip, A. & Gitler, C. *Biochem. biophys. Res. Commun.* **60**, 1155–1162 (1974).
5. Gitler, C. and Klip, A. in *Perspectives in Membrane Biology* (eds Estrado, O. S. & Gitler, C.) 149 (Academic, New York, 1974).
6. Sigrist-Nelson, K., Sigrist, H., Bercovici, T. & Gitler, C. *Biochim. biophys. Acta* **468**, 163–176 (1977).
7. Bercovici, T. & Gitler, C. *Biochemistry* (submitted for publication).
8. Jorgensen, P. L. *Biochim. biophys. Acta* **401**, 399–415 (1975).
9. Jorgensen, P. L. *Biochim. biophys. Acta* **446**, 97–108 (1977).
10. Jorgensen, P. L. *Meth. Enzym.* **32B**, 277–290 (1974).
11. Karlish, S. J. D., Yates, D. W. & Glynn, I. M. *Nature* **263**, 251–253 (1976).
12. Jensen, J. & Ottolenghi, P. *Biochem. J.* **159**, 815–817 (1976).
13. Hegevary, C. & Post, R. L. *J. biol. Chem.* **246**, 5234–5240 (1971).
14. Norby, J. G. & Jensen, J. *Biochim. biophys. Acta* **233**, 104–116 (1971).

Synthesis of haemoglobin Wayne in erythroid cells

HETEROZYGOTES for haemoglobin (Hb) Wayne possess two minor haemoglobin components that migrate more rapidly than HbA on electrophoresis at *p*H8.6. Each of the minor haemoglobin components contains an abnormal α chain in which the carboxyl-terminal tripeptide sequence, Lys-Tyr-Arg, has been replaced by an octapeptide[1]. The slower of the two components, henceforth designated Wayne-Asn, has the following octapeptide sequence: Asn-Thr-Val-Lys-Leu-Glu-Pro-Arg, whereas the faster one (Wayne-Asp) has exactly the same sequence except that asparagine at position 139 is replaced by aspartic acid. (These components, formerly designated[1] Wayne-1 and Wayne-2, respectively, have been renamed for clarity.) This is the first variant described in which deamidation of the gene product is believed to occur. The purpose of this study was to demonstrate the proposed deamidation and to explain another interesting feature of the Hb Wayne phenotype in heterozygotes; namely, the presence of the variant haemoglobins in markedly reduced quantities relative to HbA (3% and 4%, respectively, for Hb Wayne-Asn and Hb Wayne-Asp).

Peripheral blood red cells from two heterozygotes with Hb Wayne and bone marrow cells from one of them were incubated for 60 min in the presence of ^{3}H-labelled leucine. A fraction of the labelled haemolysates was converted into globin immediately by precipitation in acid–acetone. The α/β ratios obtained, based on total counts and the specific activities, indicate that the synthesis of α chains is equal to that of the β chains in the peripheral blood and bone marrow (balanced synthesis). Moreover, a free α chain peak could not be demonstrated after chromatography of the haemolysates on Sephadex G-100 gel filtration columns.

In order to compare more precisely the specific activities of the various α chains (Table 1), it was necessary to first purify the haemoglobins in the labelled haemolysates by column chromatography of the haemolysates on carboxylmethyl cellulose[2]. This was followed by preparative isoelectric focusing of the peaks containing the Hb Wayne fractions using LKB columns and finally globin chain separation[3]. The Wayne chain fractions obtained were shown to be free of contamination by peptide chromatography of tryptic digests.

In the peripheral blood incubations the amount of radioactivity corresponding to the α^{Wayne} chains was less than 11% of the total α chain radioactivity (Table 1). The corresponding value in the bone marrow was 11.4%. These figures were considerably lower than the 25% expected on the basis of the two loci believed to be present for α chains. The $\alpha^{Wayne\text{-}Asn}/\alpha^{A}$ specific activity ratio was 2.40 to 2.46 in the peripheral blood and 3.1 in the bone marrow, suggesting instability of the $\alpha^{Wayne\text{-}Asn}$ chains. In both peripheral blood and bone marrow, the specific activity of the $\alpha^{Wayne\text{-}Asp}$ chains was lower than that of the $\alpha^{Wayne\text{-}Asn}$ chains (Table 1). Time course experiments using peripheral blood showed no change in the $\alpha^{Wayne\text{-}Asn}/\alpha^{Wayne\text{-}Asp}$ ratio from 4 min to 3.5 h (not shown).

In order to evaluate the difference in labelling of the two types of variant α chains a pulse-chase experiment was performed. Peripheral blood red cells from a heterozygote were incubated in the presence of ^{3}H-leucine for 15 min (pulse) after which they were washed and a portion was immediately frozen. The remaining cells

Table 1 Incorporation of ^{3}H-leucine into α and β chains following incubation of red cells for 60 min

	α/β ratios of globin from crude haemolysates		Radioactivity of purified α chains: Specific activity (c.p.m. per mg)			% Of total α chain radioactivity		
	Total counts	Specific activity	$\alpha^{Wayne\text{-}Asn}$	$\alpha^{Wayne\text{-}Asp}$	α^{A}	$\alpha^{Wayne\text{-}Asn}$	$\alpha^{Wayne\text{-}Asp}$	α^{A}
Peripheral blood (WS)	0.97	0.94	644	115	262	8.36	1.80	89.84
Peripheral blood (GS)	1.04	0.96	968	202	403	6.96	1.95	91.09
Bone marrow	1.12	0.91	40,039	837	12,880	11.16	0.27	88.57

A fraction of the crude haemolysates was converted to globin immediately by precipitation in acid acetone. The globin obtained was chromatographed on columns of Sephadex G-100 in 20% HCOOH[15]. After chromatography globin fractions were recovered by freeze drying. Another fraction of the haemolysate was used for purification of the various haemoglobin components for accurate determination of radioactivity in the α^{Wayne} chains. Globin chain separation was carried out according to Clegg *et al.*[3] Aliquots of 1 ml of each fraction were added to 10 ml Instagel (Packard) and counted by scintillation spectroscopy. The total radioactivity under each peak was summated. The various chain components were then pooled, desalted on Biogel P2 columns equilibrated with 0.5% formic acid, and lyophilised. The chains were re-dissolved in distilled water and the protein concentration determined by the Lowry method[29].

were reincubated in a medium containing unlabelled leucine (chase) for 1, 4 and 6 h. The specific activities of the α^{A} and β chains remained unchanged during the chase period as did that of the $\alpha^{Wayne\text{-}Asn}$ and $\alpha^{Wayne\text{-}Asp}$ chains (Table 2), suggesting that during the short period of the chase no detectable conversion or degradation of the abnormal chains relative to each other or to α^{A} occurs. If deamidation is a slow process, then a greater proportion of Hb Wayne-Asn relative to Hb Wayne-Asp should be present in reticulocytes when compared with the more mature red cells. A reticulocyte fraction was prepared from the peripheral blood of a heterozygote by means of a discontinuous Ficoll density gradient[4]. Isoelectric focusing in polyacrylamide gels of the reticulocyte haemolysate revealed a markedly decreased proportion of Hb Wayne-Asp when compared to the haemolysate prepared from unfractionated peripheral blood. Moreover, the proportion of Hb Wayne-Asn in reticulocytes was approximately twice as much as in peripheral blood (Fig. 1).

The above data support the hypothesis that $\alpha^{Wayne\text{-}Asn}$ chains are synthesised initially and subsequently converted to $\alpha^{Wayne\text{-}Asp}$ chains. Robinson and others[5–8] have shown that specific glutaminyl and asparaginyl residues have characteristic rates of deamidation that are determined by the nature of neighbouring amino acid residues in both the primary and tertiary structures. The discovery of Hb Providence, a β chain variant present as two components, one having asparagine instead of lysine at position 82 and the other having aspartic acid at the same position, indicates that deamidation is not unique to Hb Wayne[9–10]. It is also possible that Hb J-Singapore[11] is not a double substitution variant but a neutral substitution at α^{79} Ala → Gly followed by deamidation at α^{78} to yield aspartic acid[12].

It is interesting to note that $\alpha^{Wayne\text{-}Asp}$ chains account for a higher proportion of the total α-chain radioactivity in peripheral blood than in bone marrow (Table 1); the $\alpha^{Wayne\text{-}Asn}/\alpha^{Wayne\text{-}Asp}$ ratio was 0.5 in the former and 0.06 in the latter. A possible explanation for all of these findings is that the asparagine at residue 139 of $\alpha^{Wayne\text{-}Asn}$ forms a cyclic imide, as proposed by Bornstein[13], while the chains are in the nascent chain or monomer conformation. While in this conformation the cyclic imide is rapidly hydrolysed to the aspartyl form. Once the $\alpha^{Wayne\text{-}Asn}$ chain is incorporated into the Hb Wayne tetramer further cyclisation is blocked by steric hindrance but those cyclised chains that had formed are hydrolysed very slowly. In the bone marrow any $\alpha^{Wayne\text{-}Asp}$ monomer chains produced by deamidation of $\alpha^{Wayne\text{-}Asn}$ monomers might be rapidly degraded by proteolytic enzymes that are not active in mature erythrocytes[14,15].

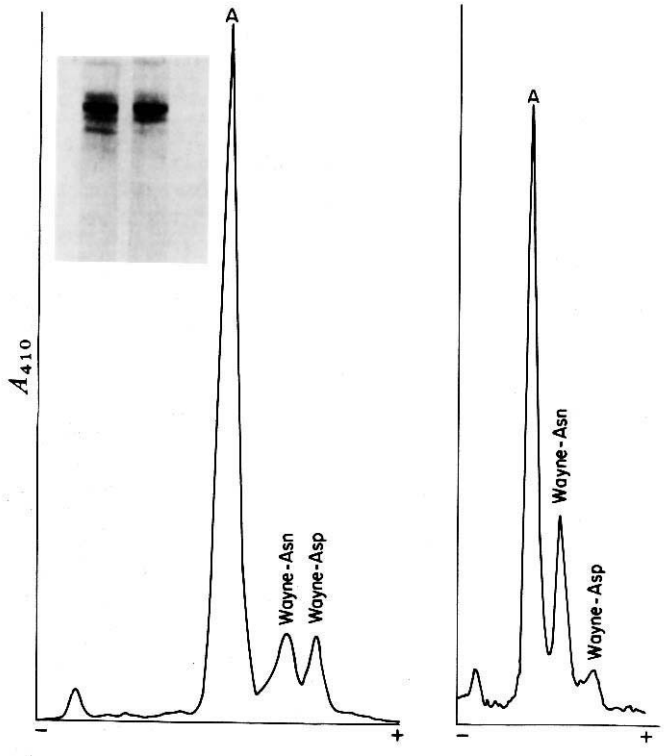

Fig. 1 Isoelectric focusing in polyacrylamide gels of peripheral blood haemolysate (left) and reticulocyte haemolysate (right). The densitometric tracings were made on unstained gels at 410 nm using a Gilford recording spectrophotometer.

Table 2 α Chain specific activities during a pulse chase experiment

Incubation time	Specific activity (c.p.m. per mg) $\alpha^{Wayne\text{-}Asn}$	$\alpha^{Wayne\text{-}Asp}$	$\frac{\alpha^{Wayne\text{-}Asp}}{\alpha^{A}}$	$\frac{\alpha^{Wayne\text{-}Asp}}{\alpha^{A}}$	$\frac{\alpha^{Wayne\text{-}Asn}}{\alpha^{Wayne\text{-}Asp}}$
15 min pulse	1,510	275	1.80	0.33	5.49
1 h chase	1,631	286	1.84	0.32	5.70
4 h chase	1,572	283	1.73	0.31	5.55
6 h chase	1,590	269	1.79	0.30	5.91

The presence of Hbs Wayne as minor components is shared by Hbs Constant Spring[16–18], Koya Dora[19], Icaria[20] and Seal Rock[21], other variants charaterised by elongated chains. Because the α^{Wayne} chains comprise only 11% of the total α chains synthesised during the period of incubation, instability of the chains does not fully account for the low proportion of Hb Wayne. A delay in translation of the α^{Wayne} chains as a result of a deficiency of tRNA for one or more of the amino acids corresponding to the abnormal sequence cannot be excluded, although such a delay has not been found in any of the abnormal haemoglobins that are present in reduced amount and that have been studied[22–25], including Hb Constant Spring[16]. Perturbation of termination of translation *per se* seems also an unlikely explanation since the synthesis of Hb Cranston, a frameshift mutant of the β chain, is not so severely impaired[26]. Yet another possible explanation, that the mRNA has been rendered unstable by the deletion responsible for the frame shift, has been excluded by the demonstration that no more Hb Wayne components are synthesised in bone marrow than in peripheral reticulocytes.

The expectation that an α-chain mutant would direct the synthesis of 25% of the α-chain mRNA is based on the unproven assumption that there are two loci that are equally active in directing α-chain synthesis. This assumption is partly based on the fact that individuals heterozygous for Hbs J-Buda and G-Pest[27] possess close to 25% of these variants, along with approximately 50% of Hb A, as well as the fact that a large number of other α-chain structural mutants comprise 20–25% of the haemoglobin of heterozygotes. Haemoglobin synthesis studies have not been performed on most of these variants. Consequently their true rate of synthesis is not known. Therefore, the reduced production of the Constant Spring and α^{Wayne} chains could also be explained on the basis of multiple α-chain loci that are not equally active in α-chain synthesis. Such a hypothesis has been invoked to explain a greater than expected amount of Hb J-Mexico in heterozygotes[28]. It is possible to envision one major locus, another of intermediate activity, and a third locus accounting for only a minor proportion of alpha chains. The mutation in α^{Wayne} would correspond to the intermediate locus and that of $\alpha^{Constant\ Spring}$ would correspond to the minor locus. A complete understanding of these complex abnormalities requires study of additional informative mutants.

This work was supported in part by USPHS grants GM15419 and NIH 5-T32-01723.

S. M. HANASH
W. P. WINTER
D. L. RUCKNAGEL

University of Michigan Medical School, Department of Human Genetics, Ann Arbor, Michigan 48109

Received 18 July; accepted 10 August 1977.

1. Seid-Akhavan, M., Winter, W. P., Abramson, R. K. & Rucknagel, D. L. *Proc. natn. Acad. Sci. U.S.A.* **73**, 882–886 (1976).
2. Huisman, T. H., Martis, E. A. & Dozy, A. *J. Lab. clin. Med.* **52**, 312–327 (1958).
3. Clegg, J. B., Naughton, M. A. & Weatherall, D. J. *J. molec. Biol.* **19**, 91–108 (1966).
4. DeSimone, J., Kleve, L. & Shaeffer, J. *J. Lab. clin. Med.* **84**, 517–524 (1974).
5. Robinson, A. B. *Proc. natn. Acad. Sci. U.S.A.* **71**, 885–888 (1974).
6. McKerrow, J. H. & Robinson, A. B. *Science* **183**, 85 (1974).
7. Lai, C. Y., Chen, C. & Horecker, B. L. *Biochem. biophys. Res. Commun.* **40**, 461–468 (1970).
8. Mideffort, C. F. & Mehler, A. H. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1816–1819 (1972).
9. Moo-Penn, W. J. *et al. J. biol. Chem.* **251**, 7557–7562 (1976).
10. Charache, S., Fox, J., McCurdy, P., Kazazian, H. & Winslow, R., *J. clin. Invest.* **59**, 652–658 (1977).
11. Blackwell, R. O., Wong Hock Boon, C. S. & Weng, M. I. *Biochim. biophys. Acta* **278**, 482–490 (1972).
12. Lehmann, H. & Huntsman, R. G. *Man's Haemoglobins* 2nd edn, 478 (Lippincott, Philadelphia, 1974).
13. Bornstein, P. *Biochemistry* **9**, 2408–2420 (1970).
14. Chalevelakis, G., Clegg, J. B. & Weatherall, D. J. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3853–3857 (1975).
15. Wood, W. G. & Stamatoyannopoulos, G. *J. clin. Invest.* **55**, 567–578 (1975).
16. Clegg, J. B., Weatherall, D. J. & Milner, P. F. *Nature* **234**, 337–340 (1971).
17. Clegg, J. B. & Weatherall, D. J. *Ann. N. Y. Acad. Sci.* **232**, 168–178 (1974).
18. Kan, Y. W., Todd, D. & Dozy, A. M. *Br. J. Haematol.* **28**, 103–107 (1974).
19. DeJong, W. W., Meera Khan, P. & Bernini, L. F. *Am. J. hum. Genet.* **27**, 81–90 (1975).
20. Clegg, J. B. *et al.* **251**, 245–247 (1974).
21. Bradley, T. B., Wohl, R. C. & Smith, G. J. *Clin. Res.* **23**, 131a (1975).
22. Rieder, R. F. *J. clin. Invest.* **51**, 364–372 (1972).
23. Rieder, R. F. *J. clin. Invest.* **50**, 388–395 (1971).
24. Farace, M. G. & Bank, A. *Biochim. biophys. Acta* **312**, 591–597 (1973).
25. Rieder, R. F. & James, G. W. *Blood* **47**, 489–494 (1976).
26. Franklin Bunn, H., Schmidt, G. F., Haney, D. N. & Dluhy, R. G. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3609–3613 (1975).
27. Hollan, S. R. *et al. Nature* **235**, 47–50 (1972).
28. Trabuchet, G., Pagnier, J., Benabadji, M. & Labie, D. *Hemoglobin* **1**, 13–25 (1976–77).
29. Lowry, O. H., Rosebrough, N. J., Farr, A. & Randall, R. S. *J. biol. Chem.* **193**, 265–275 (1951).

Red cell charge is not a function of cell age

REPORTS[1,2] that the oldest circulating human erythrocytes have an electrophoretic mobility up to 30% lower than the youngest cells have been widely accepted, particularly since such findings suggest a plausible mechanism for the removal of the oldest cells from circulation. For those studies[1,2] the red cells were separated on the basis of age by using the accepted relationship that older cells are denser on the average than younger cells. We report here that electrophoretic mobility studies conducted independently by conventional electrophoresis, streak width measurements and electrophoretic light scattering have shown the mobilities to be the same for red cell fractions of differing density and hence age *in vivo*. These data suggest that hypotheses which invoke a role for decreasing surface charge density in the mechanism of senescent red cell recognition *in vivo* are unsound.

The non-random nature of the red cell elimination process has led to suggestions that membrane determinants such as red cell surface charge[3] and/or antibody binding to the cell surface[4] may provide specificity as a result of age-dependent alterations which enhance cell adhesion to phagocytic cells.

The observation that various sialoglycoproteins are rapidly eliminated from the blood following removal of their sialic acid[5] has prompted considerable speculation on the role of sialic acid loss in the ageing and elimination of red cells[6,7]. In some instances, normal lifespans have been observed for sialic acid deficient red cells[8–10]. But, the sialic acid content of old (dense) red cells is lower than young (less dense) cells[11–13]. As the major source of negative surface charge on human red cells is the carboxyl group of membrane-bound *N*-acetylneuraminic acid[14], an age-related decrease in electrophoretic mobility[1,2] is consistent with a net loss of sialic acid per unit of membrane surface area.

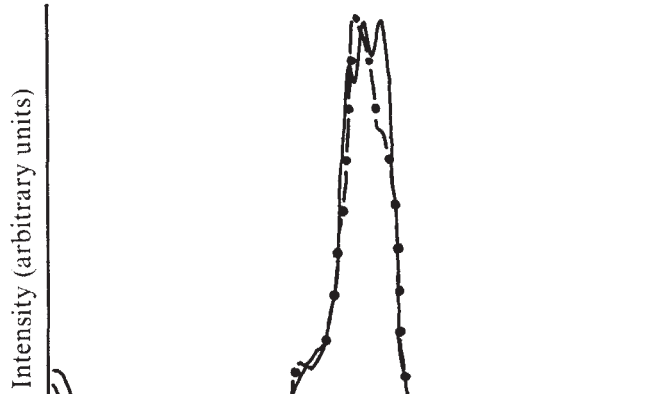

Fig. 1 Electrophoretic light scattering spectra[19] for least dense (top, ——) and most dense (bottom, — · — · —) human red cell subpopulations at 25 °C in 0.0145 M NaCl–4.5% w/v sorbitol–0.007 M *bis*-Tris buffer at *p*H 7.3. The top and bottom fractions each representing ~ 2% of the whole population were obtained by centrifugation of the cells in their own plasma for 30 min, 140,000*g* at 4 °C followed by three washes in the electrophoresis buffer. The mean Doppler shift for both spectra is 39.5 Hz, which corresponds to an electrophoretic mobility of $2.75 \pm 0.05\ \mu ms^{-1}\ V^{-1}\ cm$ after a correction for electro-osmosis (9.8 Hz). Experimental parameters were: scattering angle, 14.9°; laser wavelength, 514.5 nm; electric field, 16.2 V cm^{-1} and frequency resolution of ~ 0.03 Hz. In these conditions a 20% difference in mobility would produce a difference in shift frequency of ~ 6 Hz. The spectra shown for the subpopulations were indistinguishable from that of the whole population (not shown).