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THE ABNORMAL PHOSPHORYLATION OF SPECTRIN IN HUMAN HEREDITARY SPHEROCYTOSIS

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The phosphorylation of the proteins of the erythrocyte membrane of patients suffering from hereditary spherocytosis is investigated in intact erythrocytes by their incubation in the presence of radioactive inorganic phosphate. Examination of the phosphorylated components by high-resolution two-dimensional gel electrophoresis reveals only one defect in the pathological membranes, a depressed phosphorylation of the smaller polypeptide of spectrin; band 2. The phosphorylation of band 2 is measured with reference to the phosphorylation of syndein (2.1 + 2.2 + 2.3). In patients showing overt clinical symptoms and for whom splenectomy is advocated the phosphorylation of band 2 is depressed by approx. 70%. After splenectomy the phosphorylation of membrane proteins is restored to normal levels.

Introduction

The exploitation of mutant forms is a valuable asset to students of prokaryotic metabolism which is available in only a very restricted form to students of eukaryotes; however, those few eukaryotic mutants which are available can be of considerable value. In the investigation of the mechanisms controlling the biconcave shape of mammalian erythrocytes the human mutant which gives rise to the condition known as hereditary spherocytosis could be such an example. Hereditary spherocytosis has a dominant inheritance pattern, and is characterised by abnormally fragile erythrocytes many of which are spherostomatocytic; not, it may be noted, spherocytic, as the name suggests. The attraction of this mutant is 2-fold; not only are the cells misshapen, but phosphorylation of spectrin has been reported to be abnormal [1], and currently the relevance of spectrin phosphorylation to the maintenance of normal erythrocyte shape is contested, some workers concluding that it is crucial [2–5] and others reporting that it is not significant [6,7].

The phosphorylation of the erythrocyte membrane is a complicated process; it involves several kinases and phosphatases distributed between membrane and cytoplasm; potential protein substrates in the membrane respond differently to the various kinases; and the activities of the enzymes are dependent on cofactors and the ionic environment [8–11]. It is, therefore, perhaps unfortunate that the great majority of studies on the phosphorylation of erythrocytes have used isolated ghosts rather than intact cells. Although these studies have provided valuable information (e.g., that isolated ghosts have both cyclic AMP-dependent and independent protein kinases [12–15]), they involve a gross disruption of the normal physiological state of the intact erythrocyte. As the steady-state level of phosphorylation in the living cell will depend on the balance between the kinases and phosphatases of the membrane and cytoplasm there is an overwhelming case for examining intact cells.

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Abbreviations: SDS, Sodium dodecyl sulphate; Hepes; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

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Previous investigations on the putative defect in phosphorylation in hereditary spherocytosis have mostly been carried out on isolated ghosts [1,17,19]. We have studied the phosphorylation of intact pathological cells and examined the phosphorylated membrane proteins on high-resolution polyacrylamide gels, paying particular attention to the clinical status of the patients under study.

Materials

$^{32}PO_4$ (10 mCi/ml) as orthophosphate, carrier-free, in dilute HCl and [γ-^{32}P]ATP (1 mCi/ml, 3000 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, Bucks, U.K.), biochemicals from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and chemicals, all of analytical grade, from BDH Chemicals Limited (Poole, Dorset, U.K.).

Spherocytic blood was obtained from patients at the Blood Clinic at the Royal Infirmary, Edinburgh, normal blood from healthy adult volunteers.

Methods

Blood collection

Blood was collected by venipuncture using heparin as anticoagulant. White cells were removed by passing the blood through an α-cellulose : microcrystalline cellulose (2 : 1 w/w) column [24] in buffer A (133 mM NaCl, 4.5 mM KCl, 10 mM Hepes, pH 7.4) [25]. Filtration was carried out within 6 h of collection, and without further delay the blood was washed twice in buffer A and once in a buffer B consisting of buffer A containing 1 mM $MgCl_2$, 10 mM glucose, 1 mg/ml bovine serum albumin and penicillin and streptomycin (100 units/ml of each) (cf. Wolfe and Lux [22]).

Phosphorylation of the intact erythrocytes [22]

Red cells were suspended in buffer B at 10% hematocrit and 40 μCi carrier-free $^{32}PO_4$ added for each ml of suspension. Incubations (1–5 ml) were carried out at 37°C and the cells washed three times with 10 ml cold (4°C) buffer A. Ghosts were prepared by lysing the cells in 20 vol. 5 mM sodium phosphate buffer (pH 7.2) and centrifuging the resulting suspension at 38 000 × g for 25 min, all at 4°C. The pink ghosts were then washed twice in 20 vol. 5 mM sodium phosphate buffer (pH 7.5) at 4°C. The resulting off-white pellet was prepared for electrophoresis by addition of 1 vol. 0.125 M Tris containing 10% (w/v) SDS, 20% glycerol, 0.002% bromophenol blue, 1 mM EDTA and 100 mM dithiothreitol (final pH = 6.8) to 1 vol. ghosts. This mixture was then heated at 100°C for 5 min.

Phosphorylation of isolated ghosts

30 μCi γ-^{32}P-labelled ATP were dried down under vacuum, 30 μl 10 mM non-radioactive ATP added, followed by 150 μl ghost suspension (10 mg/ml) and 120 μl double-strength buffer A containing 2 mM $MgCl_2$ [26]. The mixture was brought from 4 to 37°C and incubated at 37°C. 50-μl samples were removed at various time intervals and 50 μl SDS buffer, described above, added.

Analysis of polypeptides

The polypeptide constituents of the ghosts were separated by SDS-polyacrylamide gel electrophoresis in the continuous buffer system of Fairbanks et al. [27], the discontinuous buffer system of Laemmli [28] and a two-dimensional system comprising both of these buffer systems [29]. The total polypeptide content of the ghosts was demonstrated by staining the gels with Coomassie brilliant blue R250 [27]. ^{32}P was detected by autoradiography of the dried gels. The wet Coomassie-stained gel was initially scanned on a Joyce Loebl recording microdensitometer to estimated protein and its autoradiograph subsequently scanned to estimate radioactivity. The areas under the peaks were measured and phosphorylation calculated with reference to the degree of Coomassie blue stain [30]. Direct comparison was only made between samples run together on the same gel. Ghosts were prepared at 4°C and SDS added immediately the preparation was complete, but degradation products of 2.1, namely 2.2 and 2.3, were always present [31]. Band 2.1 was therefore measured as the sum of the areas of 2.1 + 2.2 + 2.3. In ghosts from normal cells the amounts of 2.2 and 2.3 were small, but in pathological cells they were elevated, presumably because of the increased Ca^{2+} levels of these cells [32,33]. Addition of phenylmethylsulphonyl fluoride (PMSF) and EDTA from the time of lysis had not discernable effect.

Results

The phosphorylation of intact healthy erythrocytes

The phosphorylation pattern obtained after the incubation of intact cells with radioactive inorganic phosphate is essentially the same as that obtained by other workers [20–23]. When analysed by the Fairbanks system, polypeptides 2, 2.1, 3 (which is heterogeneous), 4.1, 4.5, 4.8 and some minor components are radioactive and a heavily radioactive, non-Coomassie blue staining region is present near the gel front. Better resolution of the polypeptides is obtained by the Laemmli system. The patterns differ in a number of respects, which are not of immediate relevance except for the anomalous mobility of band 2.1 which, in the Laemmli buffer, is coincident with the band 1. Further improvement of resolution is achieved by combination of the two buffers in a two-dimensional procedure [29], in order to amplify the search for defects in the pathological blood. (Figs. 1 and 2).

Cyclic AMP-dependent phosphorylation was studied by the addition of cyclic AMP (10–1000 μM) to the incubation. At concentrations as low as 10 μM, cyclic AMP (or dibutyryl cyclic AMP) has a pronounced effect on the phosphorylation pattern, markedly stimulating the phosphorylation of polypeptides 2.1/'1', 2.2, 2.3, 4.1, 4.8, 6.9 and 7.

The results (not shown) obtained from the phosphorylation of isolated ghosts are strikingly different and in general accord with those reported in the literature [14,26]. Incorporation is almost entirely confined to band 2.

The phosphorylation of hereditary spherocytic erythrocytes

The membranes of patients are illustrated in Figs. 1b and 2b. The Coomassie blue-stained gel is characterised by the increase of minor components, especially in the band 3 and 4.5 regions. Allen and Cadman [33] attribute this change to increased proteolysis and to the enhanced association of cytoplasmic components with the ghosts. There is considerable variation between patients in the amount of these additional components but their presence usually persists after splenectomy. Careful inspection of the two-dimensional gels of phosphorylated polypeptides reveals no abnormality in the spherocytic blood except for the depressed phosphorylation of component 2 in patients prior to splenectomy.

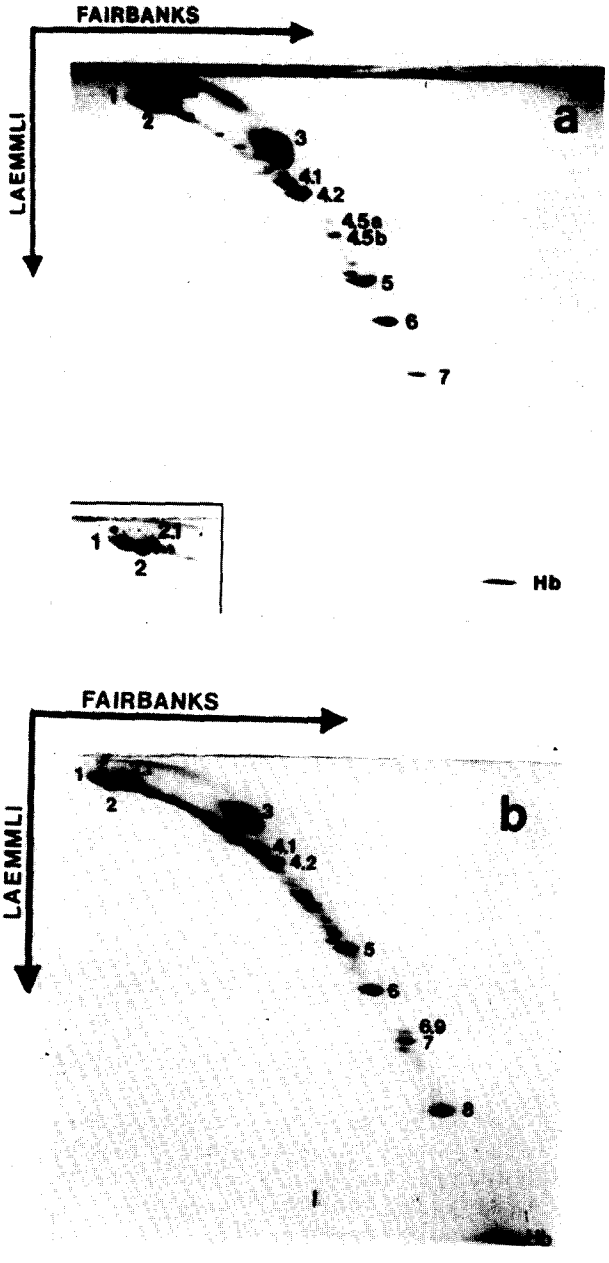

Fig. 1. Separation of erythrocyte polypeptides by two-dimensional gel electrophoresis; Coomassie blue staining. (a) Normal erythrocytes. (b) Erythrocytes of hereditary spherocytosis.

The depressed phosphorylation of this polypeptide was analysed with respect to the phosphoryl-

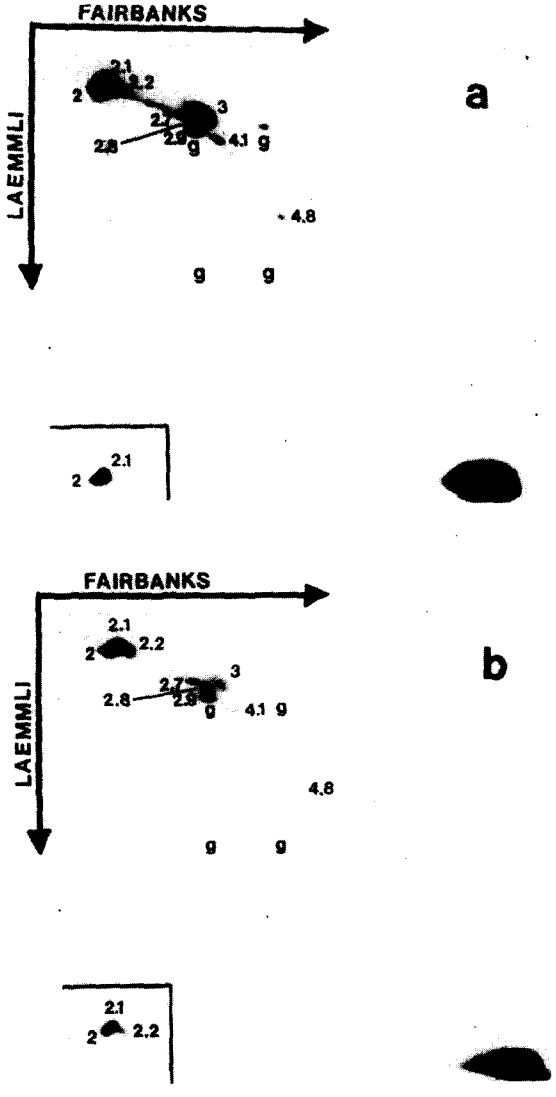

Fig. 2. Separation of erythrocyte polypeptides by two-dimensional gel electrophoresis; autoradiograph of phosphorylated polypeptides. g = glycophorin. (a) Normal erythrocytes. (b) Erythrocytes of hereditary spherocytosis.

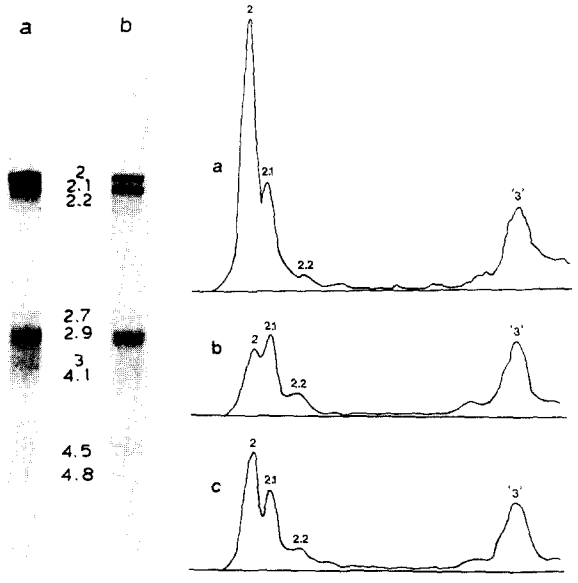

Fig. 3. (left) The defective phosphorylation of pathological erythrocytes prior to splenectomy; one-dimensional Fairbanks buffer. (a) Normal cells. (b) Cells of hereditary spherocytosis.

Fig. 4. (right) Densitometer tracing of radiocativity of bands 2, 2.1, 2.2, 2.3 and 3. (a) Normal cells. (b) Cells of hereditary spherocytosis prior to splenectomy. (c) Cells of hereditary spherocytosis 10 days postsplenectomy.

ation of band 2.1 by measuring the radioactivities of the two bands when separated by one-dimensional Fairbanks gels (Fig. 3). The results are expressed in terms of the ratio of the specific activities of 2/2.1, so as to be able to measure changes with respect to an internal control and avoid the problems and inaccuracies involved in the extraction and radioactive counting of closely migrating polypeptides. However, this protocol presents the dilemma of whether the change in ratio is due to an elevation of 2 or a depression of 2.1. Fig. 4 shows that band 2 phosphorylation is depressed not only with respect to band 2.1 but also with respect to the 'band 3' complex which is itself a composite band of several phosphorylated species. Phosphorylation of 2.1 appears to remain constant with respect to 3 and the minor phosphorylated peptides of lower molecular weight (e.g., 4.1 and 4.8) but a reliable measurement is not possible because of the great disparity in the degree of labelling and molecular weights.

In calculating the specific activities allowance must be made for the enhanced degradation of band 2.1 in spherocytic cells. The ratio of Coomassie blue staining of bands 2 and 2.1 is not abnormal in freshly prepared ghosts of diseased erythrocytes, but on incubation of the cells the degradation of 2.1 to 2.2 and 2.3 is more pronounced than in normal blood. Consequently the incorporation of phosphate per unit

protein for 2.1 is calculated by aggregating the Coomassie stain and radioactivity of 2.1 with the present in 2.2 and 2.3. After this correction the Coomassie staining of both diseased and normal cells remains constant throughout incubation. Further degradation to 2.6 and 3′ does not occur under the experimental conditions used.

Ideally the phosphorylation of the healthy and diseased cells should be compared under steady-state conditions. However, the kinetics are so complicated [22,34] that it is impossible to predict how long cells need to be incubated with radioactive phosphate before steady-state is achieved. The importance of the duration of incubation has previously been highlighted in hereditary spherocytosis by the experiments of other workers with isolated membranes (see Discussion) and, in order to minimise the danger of selecting an arbitrary incubation time, we measured the 2/2.1 ratio at various intervals. Initially the rate of phosphorylation of band 2 is greater than that of

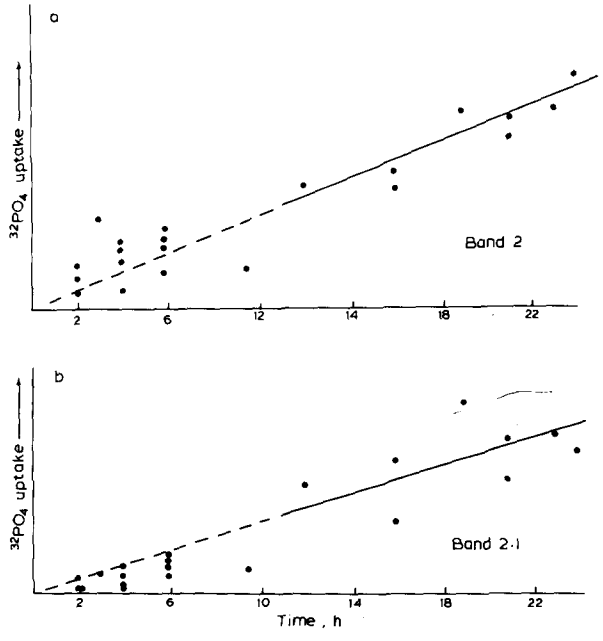

Fig. 5. Time course of the incorporation of radioactive phosphate into polypeptides of the erythrocyte. Data from several experiments have been normalised and pooled. Linear regression curves are calculated for uptake after 12 h when the relative uptake into 2 and 2.1 is constant. During the early stages incoporation into band 2 (5a) is elevated and incorporation into band 2.1 (+ 2.2 + 2.3) (5b) depressed.

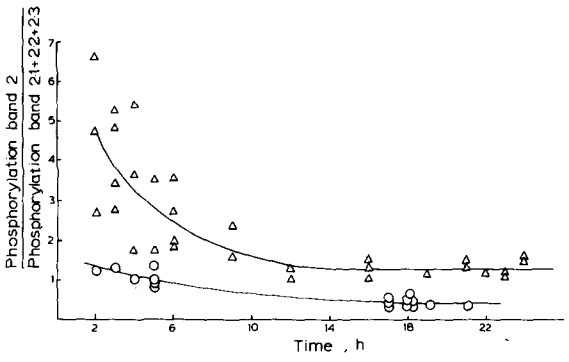

Fig. 6. The change in the relative phosphorylation of band 2 and band 2.1 (+ 2.2 + 2.3) with time. △, normal cells; ○, hereditary spherocytosis.

band 2.1 and consequently the ratio of specific activities of the two components is higher at short incubation times (Figs. 5 and 6). This effect is operative in normal and diseased cells but at any given time interval the ratio is always higher in normal cells. The defect is, therefore, not a function of incubation time. Once the ratio is stabilised, normal cells have a value of 1.3 (± 0.2) and abnormal cells 0.4 (± 0.1) so, if it is assumed that 2.1 is not affected by the disease, the inhibition of phosphorylation of band 2 approximates 70%.

The clinical status of the patient

The defective phosphorylation was present in all 11 cases investigated whose clinical condition advocated splenectomy. These patients had splenomegaly, microspherocytes in their blood smears, high reticulocyte counts, fragile erythrocytes of abnormally high density and membranes showing highly complex polypetide patterns. The results have been consistent with all cases where the diagnosis is unambiguous. One family which, over three generations, had fragile erythrocytes, but lacked the characteristic morphology of true hereditary spherocytosis and had spleens of normal size, had both normal phosphorylation and polypeptide patterns.

The abnormality was not present in six patients examined some considerable time after splenectomy (at least 1 year) and in three cases followed through the operation reversion to the normal state was observed. In two children a marked improvement was apparent 10 days after the operation (Fig. 4), and in

one adult female during the 3 weeks after the operation the ratio increased from a presplenectomy value of 0.48 to 1.15. One family is of particular interest. The original propositas (aged 38 years) was diagnosed after an episode of infectious mononucleosis. As the infection waned so did the general haematological pattern improve and phosphorylation revert towards normal. When relatives were examined both the 72-year-old father and the 13-year-old son were spherocytics by blood smear and fragility. The elderly father had no clinical manifestation of his condition and when the phosphorylation of his erythrocytes was tested it was virtually normal, the only trace of deficiency being detectable in old cells isolated on Percoll gradients [25]. By contrast, in patients in a critical condition with a high degree of reticulocytosis (greater than 10%), cells of all ages are affected. No sign of the defect is apparent in cells of any age obtained from healthy blood. These observations indicate that defective phosphorylation is not the primary defect in the disease but rather that it is the consequence of some splenic processing of the defective cell which can take place rapidly after the emergence of the cell into the circulation.

Metabolic depletion

Addition of glucose to the incubation medium normally prevents metabolic depletion of erythrocytes over a 24-h period, but it might be argued that because of the Na^+ leak [35] hereditary spherocytosis cells are more prone to metabolic depletion. This is improbable as (a) neither normal nor diseased cells show any morphological indication of depletion over 24-h incubation, (b) there is no clear evidence in the literature for a fall in ATP of circulating erythrocytes in hereditary spherocytosis and (c) when normal cells are incubated without glucose there is the predictable conversion to echinocytes and the level of phosphorylation is greatly diminished, but the 2/2.1 phosphorylation ratio is unchanged.

The effect of cyclic AMP

The defective phosphorylation in hereditary spherocytosis is not due to an abnormal level of cyclic AMP, for the defect is confined to the 2/2.1 complex and the other phosphorylation sites affected by cyclic AMP (bands 4.1, 4.8, 6.9 and 7) are not changed in pathological cells. Erythrocytes have been shown to be permeable to cyclic AMP [23].

Discussion

In the search for the molecular lesions which underlie pathological conditions it is desirable to locate defects that are effective under physiological conditions, since little confidence can be placed upon the biological significance of a defect which is only apparent under conditions that never pertain in living cells. Thus, when the lesion is thought to involve phosphorylation of erythrocyte membrane proteins, phosphorylation of the proteins by incubation of intact erythrocytes under physiological conditions in the presence of radioactive inorganic phosphate would appear to be preferable to the incubation of isolated erythrocyte ghosts with radioactive ATP. First, the incorporation into ghosts is short lived [14,18–26] while, when intact cells are used, incorporation into the proteins continues for at least 24 h. Second, the patterns of labelling produced by the two procedures differ quantitatively, the major difference being that when ghosts are phosphorylated the predominance of radioactivity in band 2 over the radioactivity of other bands is more marked [14,16,26].

Some of the perils of using isolated ghosts are illustrated by earlier studies of the suspected phosphorylation defect in hereditary spherocytosis. The defect has been reported and disputed by several groups. This dispute is largely a consequence of the assay procedure adopted [1,17–19,22,34,36]. Defective phosphorylation is only apparent after relatively lengthy incubation (approx. 1 h) of ghosts but the actual phosphorylation of the ghosts is linear for much shorter periods [1,22]. It appeared that the matter had been settled by Wolfe and Lux [22], who found no abnormality in phosphorylation when intact cells were studied. We were therefore surprised by our initial observation of the defect described above. Further cases confirmed our observations and on reflection our results are not necessarily discrepant from those of Wolfe and Lux. The defect is only detected in patients whose haematological condition is such as to warrant splenectomy and the defect is lost after splenectomy. Wolfe and Lux examined only one patient prior to splenectomy and his clinical condition was not described. Furthermore, Wolfe and Lux did not distinguish between the labelling of band 2 and 2.1. Our results do not substantiate the recent

claim of an elevated phosphorylation of band 2 in hereditary spherocytosis [34].

The observation that a defective phosphorylation of spectrin (i.e., band 2) is associated with a misshapen cell suggests that spectrin phosphorylation is important in the maintenance of normal cell shape. However, it would be facile to conclude a direct relationship. While splenectomy improves the general haematological condition and restores spectrin phosphorylation to normal, microspherocytes persist. The shape and stability of the cell must depend on a complex interplay of several factors, including spectrin phosphorylation and splenic processing. With respect to the disease, although depression of spectrin phosphorylation may lead to the demise of the cell, as phosphorylation is restored to normal levels by splenectomy it does not appear that the primary lesion is located in the phosphorylating systems. Evidence will be presented in a later publication that the changes in phosphorylation are caused by abnormal calcium levels in the diseased cell, but whether the primary defect might be a faulty calcium permeability remains to be established.

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