We have attempted to reproduce the IEFAG method specified by Wilson *et al.* (5) in our laboratory (7). Since the resolution obtained with their procedure appeared to be inadequate for identifying single protein bands in the relevant region, we have incorporated several methodologic improvements into the isoelectric focusing technique (4). These improvements have significantly enhanced the resolution and have thus enabled us to demonstrate a greater degree of heterogeneity of serum γ-globulins than is visible in the paper of Wilson *et al.* (5). Figure 1 is a comparison of the alkaline IEF patterns of serum proteins obtained in our laboratory by the method of Wilson *et al.* (5) and by one of our methods (3). Despite the significant increase in the number of bands resolved by our method, a difference among CF, heterozygote, and control sera in the pH 8.4–8.5 region could not be detected. In addition, the two-step IEFAG/disc electrophoresis technique of Altland *et al.* (1) has been tested in our laboratory. We have also modified this analytic procedure, enabling the fractionation of several small proteins from a portion of the IgG fraction. Again, despite an improvement in resolution which reveals more heterogeneity, no unique protein has been observed consistently in the CF and heterozygote sera.

The details of our analyses and our conclusions relative to the reports of Wilson *et al.* (5, 6) and Altland *et al.* (1) will be presented at a later time (3).

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Additional Notes on the Use of Analytic Isoelectric Focusing for the Detection of Cystic Fibrosis Protein in Serum

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In a previous communication (7), written in response to a letter by Smith *et al.* (3), we suggested several possible reasons for the inability of Thomas *et al.* (4) and other investigators (3) to detect cystic fibrosis protein (CFP) using electrofocusing techniques. Those investigators have reported that their techniques were analagous to those used in our laboratory (11) or claimed them to be improvements over our method (3, 4). Our explanation focused on the following three possible major differences between their techniques and ours: (*1*) the apparatus used for electrofocusing, (*2*) slight differences in the collection, processing, and storage of serum samples, and (*3*) the exact methodology used for electrofocusing (7). Also, we provided additional details concerning our methodology and the reagents employed (7).

Before the present exchange of letters, Thomas and coworkers communicated with us for the purpose of determining possible reasons for their inability to detect CFP by electrofocusing. Key points made during these conversations are reiterated below in detail in a firm attempt to alleviate future problems by inexperienced investigators in their attempts to use analytic electrofocusing to detect CFP.

SAMPLE COLLECTION

Extreme care in sample collection, processing, and storage must be exercised (7, 11). CFP and other diagnostic markers used to detect the CF gene and to distinguish homozygotes from heterozygotes for CF (*e.g.*, bands B, C, and D, and α_2Mf (9–12, 14)) are proteins whose biologic activity, structure, function, and isoelectric point (pI) depend on the manner of manipulating the blood before separation of the serum and subsequent serum analysis. In the case of CFP, we recommend the following procedures: (*1*) after removal from the vein, the blood should immediately be placed in glass tubes in an ice bath (if it must be transported from the clinic to the laboratory) or at 4° to clot (11). (*2*) After retraction of the clot has occurred, the blood should be centrifuged at 4°. (*3*) Subsequent removal of the serum should be performed employing plastic pipettes; aliquots should then be transferred to prechilled (in crushed ice) plastic or polypropylene tubes. The serum should then be stored at −70°; the serum should *not* be left at 4° awaiting analysis. When analytic procedures are performed, the serum should be thawed just before its application to the sample pads.

Failure to adhere to the above protocol could result in structural alteration of CFP (and also CF ciliary dyskinesia activity (13)) with a concomitant change in its pI. This structural alteration in CFP may be due to enzymatic degradation either by endogenous enzymes released from cells damaged during the process of clotting or by exoenzymes already present in the serum (especially if CFP coexists in bound and free forms at equilibrium in serum (9)). Degradation of CFP might be a problem, particularly in heterozygote carriers, since any enzyme defect in CF homozygotes appears to be only partly manifested in carriers. An example of a defect is the quantitative abnormalities noted in the ability of heterozygote carrier α_2-macroglobulin (α_2M) to regulate or modulate proteolytic enzymes as determined by the production of detectable α_2-macroglobulin subunits by electrofocusing or other assays (10, 14).

STANDARDIZATION OF ASSAY

As originally reported (11), all serum samples should be analyzed using a volume of serum standardized to contain 300 μg IgG. We have previously shown that CFP is a small molecule (9) associated with the serum IgG fraction (12). Based on the assumption that CFP has a stoichiometric relationship to IgG, we determined a level of IgG (the proposed carrier of CFP in serum) which, if used for analysis, would result in detectable levels of CFP in the majority of homozygotes and heterozygotes for CF (9, 11). One cannot simply run any given level of serum (*e.g.*, 50 μl) and expect to detect CFP reliably in *all* individuals who harbor the CF gene. Analysis of inadequate amounts of serum will result in false negatives. Use of too much serum will result in overloading the region between pH 8 and 9 and else-

where, producing dark background staining and heavy bands near CFP, which will collectively obscure CFP and make its detection impossible.

LOCALIZATION OF CFP ON GEL

In our 1973 report (12), we characterized CFP as a protein with a pI of 8.41 ± 0.10. Later (9, 11), we gave, in addition to a revised pI of 8.46 ± 0.05, the localization of CFP on the gel in centimeters from the anode. This was done for a specific purpose – to allow other investigators to localize CFP on a gel when the apparatus of Awdeh *et al.* (1, 6) is used, without having to worry about individual variations in the measurement of pH values by different laboratories. We also provided a photograph showing the entire stained serum protein patterns for two normal subjects, two heterozygote carriers, and one cystic sample (11), to demonstrate clearly that the CFP band was located in *the last centimeter of the stained serum protein patterns*.

In our communications with Thomas and coworkers we learned that, despite our suggestions, they had not analyzed the samples they screened by either using (*1*) the equipment of Awdeh *et al.* (1, 6) or (*2*) employing our standardized procedure (11). It is unknown whether or not they exercised the requirements for serum sample collection, processing, and storage. We feel the above reasons are sufficient to explain the reported failure of Thomas *et al.* (5) to reproduce our results and to detect consistently a CFP in sera from individuals with the CF gene. There is an additional reason for their failure which is clearly evident from the figure they presented.

Figure 1*A* of their communication (5) shows the results of analyzing one normal (N), one heterozygote carrier (H), and one cystic (C) sample by our method of electrofocusing but employing a Brinkman electrofocusing apparatus. A picture indicating the total serum protein pattern obtained is not given. A graph depicting the pH gradient as a function of centimeters from the anode is not given. A scale in centimeters from the anode (useful for the localization of any particular band) is not given. Therefore, Thomas *et al.* (5) do not present evidence that they reproduced either our pH 5–10 gradient, specifically made to enhance the detection of CFP, or our protein banding patterns, shown previously (11). We assume the picture in Figure 1*A* is provided as evidence that no banding differences exist between the N, H, or C samples shown. However, it is clear from an inspection of the uppermost centimeter of the patterns shown in Figure 1*A* that the cystic sample has obvious cationic protein bands not found in the normal sample (near pH 8.79 and 8.6); the H sample also shows these bands, although they appear fainter. The problem appears to be that Thomas *et al.* have based the evaluation of their results solely on attempts to locate a band with a pI at pH 8.41 (as stated in their abstract) (4). It is obvious from Figure 1*A* that the pH 8.41 band, as recorded in their lab, is not at all near the top of the stained serum protein patterns. Figure 1*B* of their communication epitomizes the problems of making accurate gradients and measurements. We and our colleagues (2) know of no normal serum protein focusing at pH 9.82 or higher which is detectable when 10–50 μl of serum is analyzed (4) in thin layer polyacrylamide gels by electrofocusing.

To summarize, in this communication and elsewhere (7, 11) we have alleviated some of the problems encountered by other investigators using electrofocusing to detect CFP by describing several guidelines to follow in collecting and analyzing sera. We feel that a major problem might have been the uniqueness of the apparatus of Awdeh *et al.* used in obtaining our results (9–13). The apparatus is a custom-built piece of equipment which requires a set of running conditions different from those noted by the manufacturers of commercially available apparatuses (*e.g.*, the LKB Multiphor and Brinkman electrofocusing apparatuses).

Previously (7, 9), we indicated our exploration of the LKB Multiphor for use in the detection of CFP and comparison of the results with those obtained using the apparatus of Awdeh *et al.* (1, 6). We have recently completed this study and, in addition, utilized the LKB Multiphor to follow the isolation of CFP (8, 15). Although the complete methodology and results obtained are the subject of another report (8), representative results are provided in Figures 1 and 2. The following basic modifications were necessary: (*1*) use of a pH 2.5–10 gradient instead of a pH 5–10 gradient and (*2*) use of constant power (instead of constant voltage) for focusing the serum proteins. Complete concordance was found between the results obtained with the LKB Multiphor

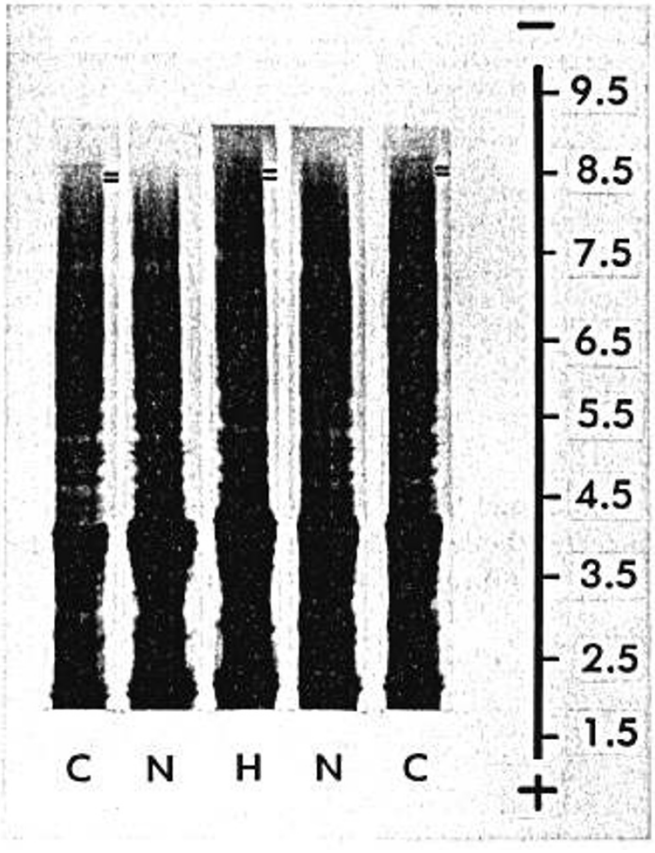

Fig. 1. Electrofocusing of whole serum in a pH 2.5–10 gradient using the LKB Multiphor. *N:* normal control subject; *C:* patient with cystic fibrosis; *H:* obligate heterozygote carrier for cystic fibrosis. Scale on right indicates centimeters from the anodal strip. The lines indicate the location of a band doublet found in cystic fibrosis protein-positive samples. The lower band is cystic fibrosis protein (pI about 8.57).

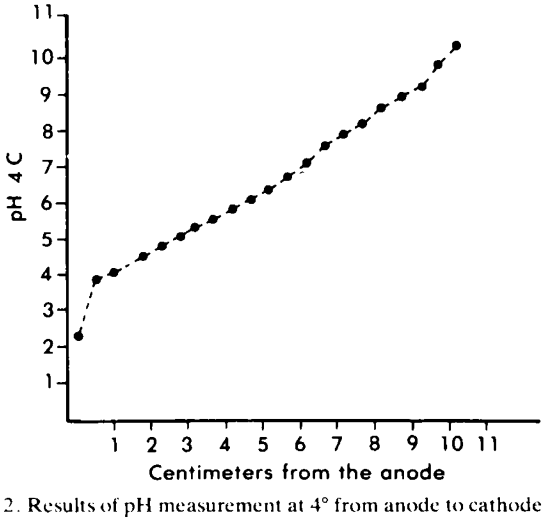

Fig. 2. Results of pH measurement at 4° from anode to cathode on the surface of a gel corresponding to the one shown in Figure 1.

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In a recent paper Richardson (3) reported a study of 6–10-year-old Jamaican children who had been severely malnourished in the first 2 years of life, and compared them with contrast children from the same neighborhoods. He found that small stature, disadvantageous backgrounds, and early malnutrition were each associated with low IQ. Multiple correlation revealed that the smallest contributor to the variance was early severe malnutrition. We feel that this may mislead some readers into minimizing the relationship of severe malnutrition to intellectual development.

The only information available about the nutritional status of the contrast group in early infancy was that they had not been hospitalized for severe malnutrition. Undernutrition by the Welcome Classification (4) is common in Jamaica and longitudinal studies have shown a prevalence of 20% in the 1st year of life in urban children (2) and 30% in the first 2 years of life in rural children (1). Experience has shown that infant malnutrition tends to occur in specific neighborhoods in Jamaica and the presence of known cases of severe malnutrition would indicate areas of higher prevalence. It is therefore probable that a significant number of the contrast children were underweight during the first 2 years of life. From the available evidence it would seem likely that even mild-moderate malnutrition in infancy is associated with the level of children's intellectual development. Consequently, the inclusion of such children in Richardson's study would minimize the proportion of the variance found to be due to early malnutrition.

At present we are studying young Jamaican children who are hospitalized with severe malnutrition and comparing them with well nourished children (over 80% expected weight for age) from similar socioeconomic backgrounds and hospitalized with other illnesses. We find that 1 month after discharge from hospitals there is an extremely large difference between the Developmental Quotients of the malnourished and the well nourished children. This difference is in the order of 25 points. From our work so far it would appear that at this age the relative importance of nutritional status in intellectual development is far greater than that found by Richardson.

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