SEPARATOR ISOELECTROFOCUSING : THE INFLUENCE OF BIOLOGICAL BUFFERS ON THE IEF PATTERNS OF TRANSFERRINS

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Genetic variation of human transferrin (TF) has remarkably increased, since isoelectrofocusing (IEF) procedures were applied for the investigation of this polymorphism in 1977.Until 1985, TF*C with a frequency of 0.99 in Caucasoids was split into 15 subtypes,C1 to C15,with C1,C2 and C3 encountered as common alleles.The identification of rare TF C subtypes, whose bands have pI values close to that of C1,C2 or C3 is complicated by too narrow corridors of heterozygotes in standard separation systems.

To overcome this problem,we applied the following five biological buffers ('separators') to polyacrylamide gels of various pH ranges:

1.ACES (N-2-acetamido-2-aminoethane-sulfonic acid ; pK 6.8)
2.HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid ; pK 7.5)
3.MOPS (3-(N-morpholino)-propane sulfonic acid ; pK 7.2)
4.MES (2-N-morpholino)ethane sulfonic acid ; pK 6.1)
5.EPPS (N-(2-hydroxyethyl)-1-piperazinepropane-sulfonic acid ; pK 8.9)

The total concentration of buffers ranged from 0.5 to 6.5 % (w/v).Sera were obtained from random blood donors from Hessen/Germany,from individuals tested in blood group expertises,and for reference typing purposes. Pretreatment of sera was performed with 0.15% ferrous ammonium sulphate 1:7 (v/v),incubated for 18hrs at 4^{o}C in a sealed plastic tube for Fe-saturation of TF.Neuraminidase treatment was used for desialysation of the TF molecule (clostridium perfringens neuraminidase type V,Sigma Co.),1:3 (v/v) in Fe-untreated or 1:9 in Fe-treated sera solutions.

Agarose gel electrophoresis (AGE) was performed with a DESAGA 202000 electrophoresis equipment in the barbital/sodium barbital/calcium lactate buffer system of pH 8.6 as originally designed for C3 typing by TEISBERG (1970).Isoelectrofocusing (IEF) procedures were carried out according to previously described issues with Multiphor/Ultrophor (LKB) equip-

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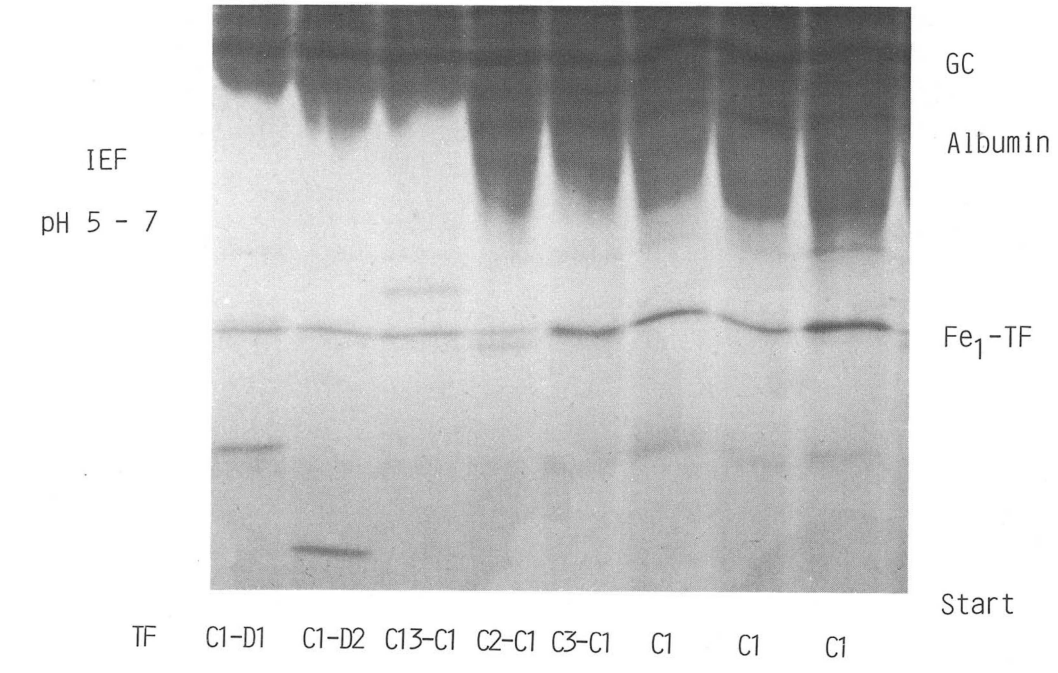
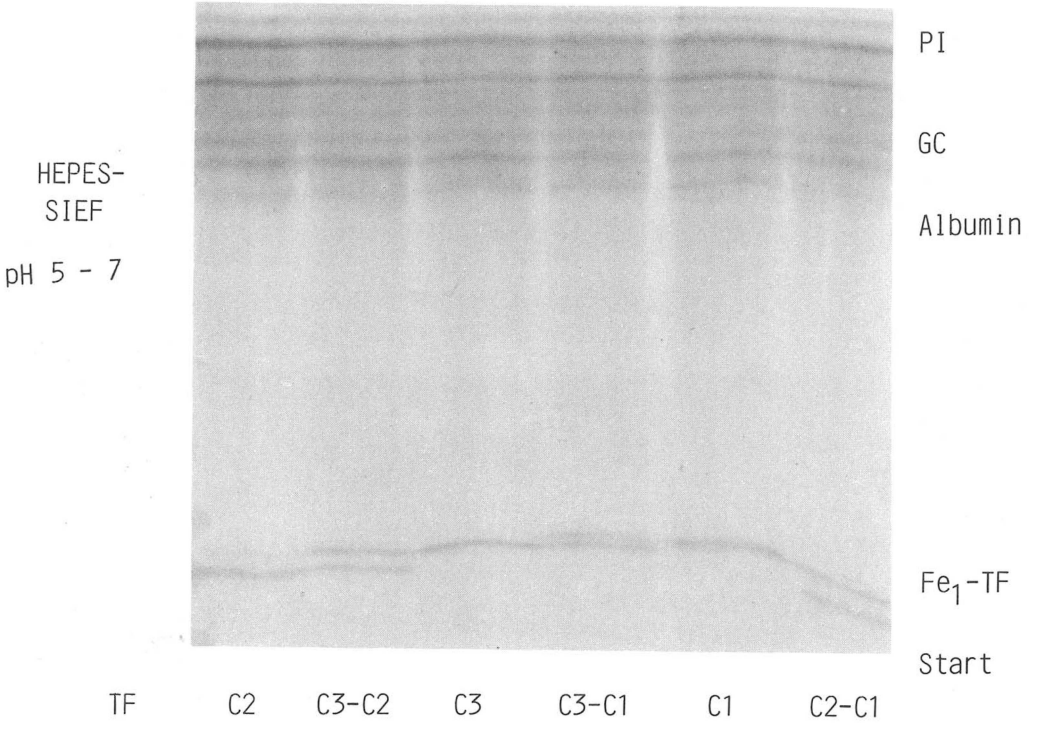
ment,Macrodrive 5 power supply,Multitemp II thermostat and the Ultromould gel casting apparatus.For the pH ranges 3.5-9.5,4-6.5,and 5-7 Ampholine PAGplates were used;own gels were prepared with the following specifications:T=5%,C=3%,ampholyte concentrations 3%;gel dimensions 245x110x0.5mm; ampholytes were chosen for the gradients pH 4-6,5-7 with 10% 3.5-10 (LKB), and 4.2-4.9 (Pharmalyte).Numerous modifications were tested for optimal separation conditions in separator isofocusing (SIEF).For standard gels of 0.5 mm thickness,1600V,25mA,and 20W were found suitable;for HEPES-SIEF, the combination which yielded the best separation of TF isoproteins,the following maximum settings and IEF parameters were selected:2000V,25mA,and 13W at a cooling temperature of 5°C for 5hrs total focusing time ; 30 min prefocusing,45 min with sample filter papers on the gel, and 225 min without filter papers.The size of the Whatman #1 filter paper samples was 7x10 mm,15 mcl of diluted serum were applied with a microliter syringe.Electrode wicks were soaked with 1% acetic acid as anolyte, and 1% ethanolamine as catholyte (Whatman #17 strips).The samples were applied in the center of the gel in a distance of 4.5 cm to each electrode.

The fixing of the gels after separation was performed in a PAG solution, consisting of 30 g of sulfosalicylic acid,330 ml of methanol, aq. dest. ad 2000 ml,for 10 min.The gels were then stained with a 0.1% Coomassie Brillant Blue R 250 dye dissolved in destaining solution (700 ml of 70% ethanol,160 ml acetic acid 96%,aq. dest. ad 2000 ml for 15 min.The subsequent destaining was oriented to the optimal visualization of the Fe_1-TF bands with a pI of approximately 5.9.

Compared with the standard IEF separation on commercially available 1mm gels or 0.5 mm gels cast on the Ultromould (Fig.1),wider corridors were obtained in the HEPES-SIEF ultra-thin-layer gels for the phenotypes C2-1 and in particular C3-1 (Fig.2). Whereas there is only a blurred,broad zone of Fe_1-TF activity,the corresponding isoprotein fractions are clearly distinct due to the flattening of the gradient caused by the biological buffer.Another remarkable side effect of these gels is the fact,that the area anodal to the region of the common C subtypes is devoid of the albumin trail,which reaches the TF B1-2 bands and may cover variants with even lower pI values than B1-2.





HEPES in a total concentration of 1.4% was found to be superior to the other substances added in comparable quantities (ACES, MES,EPPS and MOPS).ACES and EPPS led to drastic flattening of the area cathodal to the application zone and was found unsuitable for TF D differentiation;albumin,GC and TF were compressed to a gel area of 2 cm cathodal of the anode strip (ACES 0.06 g/gel) or even 1 cm (EPPS 0.06 g/gel), permitting no TF C1/C2 analysis.If MES was added in a concentration of 1 g/ 0.5 mm gel pH 5-7,albumin,GC, and TF were shifted to the cathodal half of the gel.The width of the C2-1 corridor reaches 2 mm,but no clearcut C3-1 types were seen.
Technical problems and drawbacks occurred due to inadequate maximum settings,salt concentrations and drying up of the ultra-thin gels during the application of the filter paper samples in the initial phase of our experiments.There were outweighed by considerably better separation results of TF C subtypes,smaller quantities of carrier ampholytes required and by shorter staining/destaining times.The results of the neuraminidase-treated samples were less satisfactory.Desialyzation led to the known cathodal shift of Fe_1-TF molecules,close to the application area.The sharpness of C-bands,in particular the C3-1 corridor however was second to that of the SIEF procedure described above.

Further improvements of the SIEF technique may render it an alternative to immobiline gels,which yield wider corridors between less distinct C subtype bands in our hands.Meanwhile routine and reference typing in our laboratory confirmed the existence of at least 16 different C subtypes,whose sequence in cathodal direction now is as follows: C14,13,15,12,9,4,11,1,8,3,5,2,10,6,7,and 16 (KORNHUBER et al.,1985,in preparation).Only three of them were confirmed to exist at polymorphic frequencies in the Caucasoid populations (Hessen/FRG,n=2000 : TF*C1= 0.76975;*C2=0.16350;*C3=0.05650),whereas *C6,*C7,and *C9 were only encountered as rare variants (0.00100,0.00025,and 0.00025 respectively. Based on these data and a combined allele frequency of non-C (B,D) of < 0.01,a single exclusion chance for non-fathers of 19.4% is calculated for the TF system,if sensitive and reproducible isofocusing procedures are applied.