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An Examination of Heart Proteins by Two-Dimensional Electrophoresis

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We examined specimens from explanted human hearts by two-dimensional electrophoresis. The protocol selected includes: (*a*) solubilization of the sample in a urea–detergent mix; (*b*) charge fractionation in the presence of urea and nonionic detergent on a pH 4–10 immobilized pH gradient; (*c*) size fractionation on a polyacrylamide concentration gradient in the presence of sodium dodecyl sulfate; and (*d*) staining with silver nitrate. The method is sensitive enough for analysis of biopsies in the 1–3 mg range (wet tissue). We saw, for explanted hearts, variations in the protein pattern with the site of sample dissection. Results are presented for 11 explanted human hearts: one control organ and 10 pathological samples. The recorded pathologies included dilatative cardiomyopathy (six cases), valvulopathy (one case), ischemic cardiopathy (two cases), and graft rejection (one case). The patterns for whole extracts as well as for cytoplasmic proteins and myofibril components are compared. Extensive individual variability was observed both between control and pathological cases and among the abnormal samples.

Additional Keyphrases: *organ transplants · heart disease · intra-individual variation*

Heart transplantation is now being done at eight cardiology units in Italy. This prompted a research effort by several groups (*1*) to study the pathological conditions that eventually necessitate transplantation, especially dilatative cardiomyopathy, the most frequent of such disorders and one whose etiology is still largely unknown. We undertook to map heart proteins by 2-D (two-dimensional) gel electrophoresis (*2*).[3] With this procedure as many as 1000 of the most abundant gene products in a given tissue extract can be detected (*3*), as can qualitative and quantitative differences between a control pattern and one representing the diseased state (*4*).

This report deals with the technical assessment of the protocol we have adopted in our investigation. It includes as a first-dimension separation a charge-fractionation step performed by isoelectric focusing (IEF), not with conventional carrier ampholyte (CA) buffers in thin polyacrylamide rods but with immobilized pH gradients (IPG) (*5*) in flat gel slabs. [We call this procedure "IPG-DALT," by analogy with the "ISO-DALT" acronym proposed by the Andersons (*6*)]. Advantages of this modification include: full control of the gradient shape and width (*7*); little influence from the volume and the ionic composition of the sample (*8*); and, most important, extremely high reproducibility of spot position (*9*), within and between batches.

The procedures for 2-D protein mapping detailed in this paper have been applied to analysis of heart specimens from nine patients undergoing heart transplantation between November 1985 and July 1986. These cases exemplify three different pathologies: dilatative cardiomyopathy, valvulopathy, and ischemic cardiopathy. In one case, the grafted heart had been rejected.

We report here our results, presenting the whole protein pattern after high-resolution analysis. We processed separately the components from two cellular compartments (myofibrils and cytoplasm) and have assessed the extent of contamination from residual blood. Examples of individual variability among the samples—both in a qualitative and in a semiquantitative sense—are given.

Materials and Methods

Samples

In toto *explants:* The apex was removed and the ventricles were cut open along the fore edges of the septum. The dissected organ was laid on a board and the walls were punched through with a cutting-rim cylinder, 1 cm in diameter. Eleven specimens were collected from each organ, as follows: left and right ventricle, front, upper and lower, and back, upper and lower; septum, upper and lower; and papillar muscle. After a 5-min rinse in isotonic saline, the samples were transferred to plastic containers chilled in liquid nitrogen. Table 1 lists some clinical data for nine of the patients whose explanted organs were analyzed in the present study. The recorded pathologies included dilatative cardiomyopathy (six cases), valvulopathy (one case), ischemic cardiopathy (two cases). Before surgery, all patients were treated with inotropic, vasodilatatory, diuretic, antiarrhythmic and, in some cases, anticoagulant drugs, either orally or by infusion (cases I and J). Patient D had to undergo a second transplantation eight months after the first surgery; the excised graft was designated case L. A normal heart (K) was explanted from a 46-year-old man, dying from cranial trauma, upon evidence of clinical death. The organ was maintained for 4 h according to standard protocols (*10*) in provision for grafting, then stored at −20 °C for 8 h before processing. In all cases specimens were sampled as described above; organs E–F, I–J, and L–M were dissected just after their explant, organs A–D—without thawing—after storage at −80 °C for up to two months.

Biopsies: Needle biopsies (1–3 mg) were taken with a Standford biotome through the inner jugular vein from the right ventricle of grafted hearts one week after surgery (during routine immunological checks).

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[3] Nonstandard abbreviations: IPG, immobilized pH gradient; IEF, isoelectric focusing; CA, carrier ampholytes; SDS, sodium dodecyl sulfate; 2-D, two-dimensional electrophoretic separation; 1st-d, first step of a two-dimensional separation, i.e., charge fractionation by IEF; 2nd-d, second step of a two-dimensional separation, i.e., size fractionation by SDS-electrophoresis; IPG-DALT, 2-D with IPGs for the 1st-d step; ISO-DALT = 2-D with CA-IEF for the 1st-d step; NEPHGE = nonequilibrium pH-gradient electrophoresis.

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Table 1. Clinical and Anatomic–Pathological Data for 11 Heart Samples

	Age, y	Diagnosis[a]	Duration, months[b]	NYHA class[c]	EF[d]	Heart tissue mass, g	Av. thickness of ventricle wall, mm		
							Left	Right	Septum
Control[e]					0.60	350	8–15	4–5	—
Cases[f]									
K	46	control				360	13	5	13
A	50	d	11	IV	0.20	450	18	12	14
C	23	d	7	IV	0.12	450	18	10	15
D	46	d	18	IV	0.22	500	17	15	17
F	23	d	6	III	0.18	450	17	7	17
I	29	d	36	IV	0.19	600	23	10	21
J	23	d	18	IV	0.16	500	18	10	17
B	42	v	14	III	0.20	900	24	18	20
E	45	i	12	IV	0.15	400	15	10	13
M	56	i	2	III	0.20	390	18	12	14
L[g]		r	6	IV	0.15	350	15	5	16

[a] d = dilatative cardiomyopathy; v = valvulopathy; i = ischemic cardiopathy; r = graft rejection.
[b] Elapsed time from allocation in NYHA class III.
[c] NYHA = New York Health Association (*36*).
[d] EF = ejection fraction, i.e., stroke volume to diastolic volume ratio.
[e] Control values according to Silverman and Schlant (*39*).
[f] All male subjects.
[g] Graft from a 23-year-old man who died from cranial trauma; graft was removed from recipient D eight months after transplant as heart failure developed.

Protein Extraction

Total protein: Tissue was homogenized in urea–detergent mixture, essentially according to Giometti et al. (*11*): 50–100 mg (or the whole biopsy) was homogenized for 3 min at full speed (*ca.* 24 000 rpm) in an ice-water bath with an Ultraturax T25 apparatus (Ika Labortechnik, Taufen, F.R.G.). The extraction medium included, per liter: 9 mol of urea, 20 g of carrier ampholytes for IEF in the pH range 8–10.5 (Pharmacia, Uppsala, Sweden; final pH = 9.5), 40 g of Nonidet P40 surfactant, 10 g of dithiothreitol (*11*), 2 mmol of phenylmethylsulfonyl fluoride, 20 μmol of Pepstatin A (4-aminomethylheptanoic acid, a pepsin inhibitor), and 2 mmol of benzamidine (all from Sigma Chemical Co., St. Louis, MO) (*12*). The buffer-to-tissue ratio was 8 (or higher); small samples were extracted once, then centrifuged for 20 min at 100 000 × *g* (55 000 rpm) with the TLA 100.2 fixed-angle rotor in a TL100 centrifuge (Beckman Instruments, Palo Alto, CA) at 2 °C. Larger samples were treated 3 min with one half of the buffer volume, and centrifuged 15 min at 3000 × *g*; each pellet was rehomogenized for 1 min with the remaining buffer, then centrifuged again at low speed. The pooled supernates were then centrifuged at 100 000 × *g*, as above, and stored at −80 °C in 50- to 100-μL aliquots.

Buffer-soluble proteins: To extract soluble proteins, we used a procedure similar to the one for the solubilization of total protein, except that the extraction medium contained only diluted carrier ampholytes and protease inhibitors.

Myofibrils: The purification scheme was performed according to Solaro et al. (*13*), with about 3 g of tissue per sample (B, D, K). Material was pooled from both the left and right atria; the front wall of the left ventricle was also extracted. Solubilization was then in urea–detergent, as above.

Two-Dimensional Electrophoresis

1st-d: Proteins were first fractionated by charge by IEF, either in the presence of carrier ampholytes or on immobilized pH gradients (IPGs). For the former the pH range was 3–11, for the latter 4–10 [either linear (*14*) or nonlinear (*7*); in a few cases, we used pH ranges of 4–7 and 5–10 (*15*)]. For both experiments the polyacrylamide matrix was T% = 3, C% = 4 (*16*), the slab thickness was 0.7 mm, and the gel contained 8 mol of urea and 20 g of Nonidet P40 per liter. The run lasted overnight at 2500 V for IPGs, with 10 g/L carrier ampholytes in the 3–10 range used as the electrodic solutions (*17*). In all, about 5000 V × h was delivered in CA-IEF (maximum voltage 2500 V). For silver staining we applied 25 μL per lane (40 μL for Coomassie Blue staining) near the anode on Paratex pads (LKB, Bromma, Sweden; no. 1850-901); the temperature was 12 °C. From each slab we obtained eight to 12 individual gel strips, 6–7 mm wide, 3–5 mm apart; they were cut apart before the run for the IPG assays, afterwards for conventional IEF.

2nd-d: The strips from the 1st-d were processed as described elsewhere (*18*), to remove most of the neutral detergent. Fractionation according to molecular size was then run in the presence of SDS on a 75–175 g/L polyacrylamide gradient, with the discontinuous buffer system of Laemmli (*19*). Further details on the IPG-DALT procedure are given in references *20* and *21*.

Staining: After fixation with a solution of 1 mL of formaldehyde, 500 mL of ethanol, and 499 mL of water per liter, we stained the protein pattern with either silver nitrate (*22*) or 2 g/L Coomassie Blue R250 (Serva, Heidelberg, F.R.G.) in acetic acid/ethanol/water (1/3/6 by vol).

Crossed Immunoelectrophoresis

Immunoelectrophoresis of normal human serum (pooled from 50 Red Cross male donors, ages 41–50 years) and of buffer-soluble heart proteins was performed according to Clarke and Freeman (*23*). The agarose medium was buffered with a barbital/glycine/Tris solution, according to Weeke (*24*). The antisera against whole human serum proteins and against albumin were from Dako, Copenhagen, Denmark. To decrease protein trailing, we allowed the heart sample to diffuse and be completely absorbed into the gel before starting the 1st-d electrophoresis. We then sealed the empty well with a drop of agarose sol (*25*).

Results

Technical Aspects

1st-d: IPG vs CA-IEF. The best site for sample application was tested both for CA-IEF and for IPGs. Cathodal applica-

tion always resulted in pronounced streaking of the alkaline bands and incomplete protein mobilization; the latter was so severe for CA-IEF (not shown) that the resulting pattern should be considered meaningless. When comparing the results obtained with an anodal sample application, for either IPG or CA-IEF, we found that no protein was lost at the application point with the former, but a vertical streak of precipitated material developed in the latter. This finding—plus the more general considerations mentioned earlier—motivated our selection of IPGs as support for 1st-d runs. With IPGs, varying the concentrations of the buffering monomers (β_{av} = 0.5 to 3 mmol L^{-1} pH^{-1}) in the presence of different concentrations of CAs (5 to 20 g/L in the IPG slab), as proposed by Hochstrasser et al. (*26*), could resolve the same number of spots (not shown).

Staining: Coomassie Blue vs silver nitrate. We observed strong differences in relative stainability for several spots, which suggests caution about quantifying mass on the basis of results with general protein stains.

Sample size. To extend its applicability to diagnostic procedures—both for assessing disease states and for follow-up after surgery—we needed to scale down the protocol, at the step of sample preparation, for use with samples the size of typical biopsies (1–3 mg). Figure 1 shows the protein pattern after 2-D from a 2.5-mg specimen (obtained one week after organ graft) homogenized with 100 μL of buffer. All major proteins can be detected, albeit with reduced intensity, and no loss of material is evident at the application point, as is sometimes the case when proteins are slowly removed from too large a sample. Taking into account the possibility of further enhancing the protein staining by recycling into silver nitrate (*27*), we consider the sensitivity of the method to be fully adequate for the proposed application.

Biological Aspects

Total proteins. Figure 2 shows the protein patterns for several heart samples, one control (K) and 10 pathological cases, after IEF on a nonlinear pH 4–10 IPG. All specimens were dissected from the front wall of the left ventricle. The technical quality of the separation—no precipitation at the application point, and the straight and neat migration of the bands—makes obvious some qualitative and quantitative differences among the various samples.

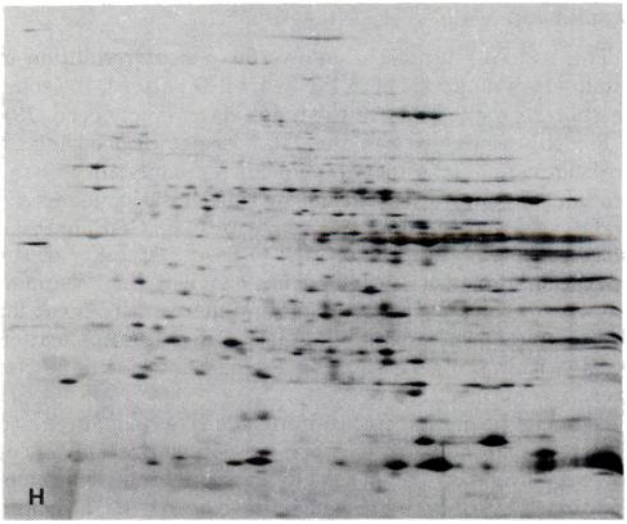


Fig. 1. 2-D electrophoresis of a 2.5-mg biopsy sample taken from the right ventricle of a transplanted heart one week after the heart graft

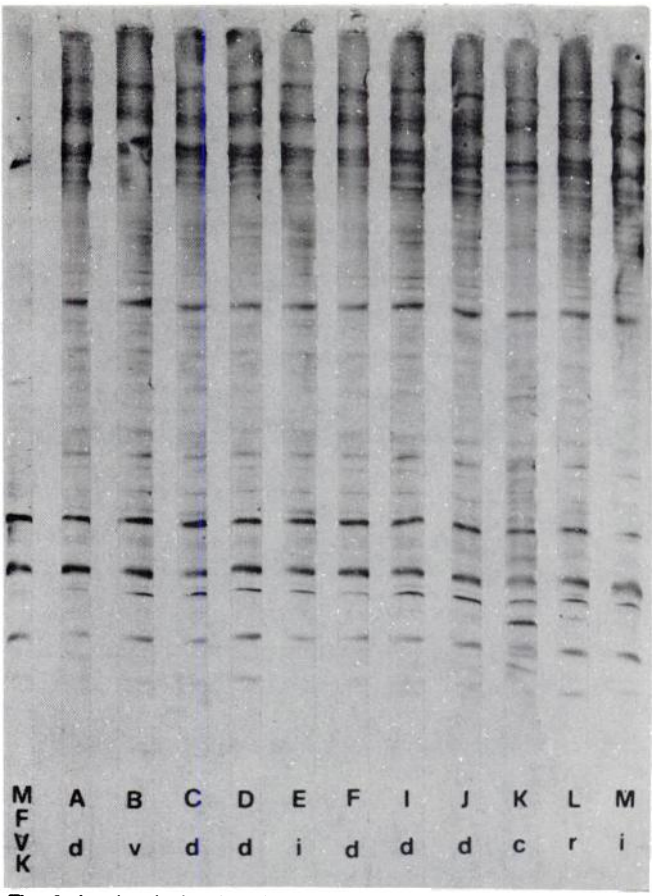


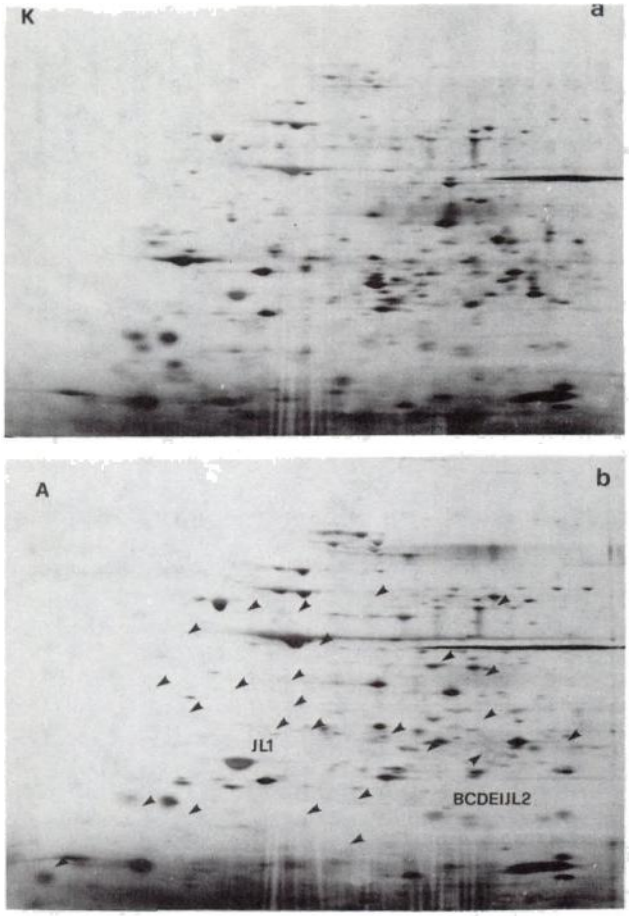
Fig. 2. Isoelectric fractionation of heart proteins, urea extracts from pathological and control (K) lower front left ventricles and from purified ventricular myofibrils from the control (MFVK) run on a nonlinear pH 4–10 IPG

The letters identifying the different samples and the symbols for the various diagnosis are as in Table 1

To evaluate the extent of such variations among samples, we fractionated the same samples in IPG-DALT. For increased resolution, we ran the 1st-d on both a 4–7 (Figure 3) and a 5–10 (Figure 4) pH gradient, and compared the results for the control with those for a pathological pattern. In Figures 3 and 4 the major differences between the control and pathological samples, e.g., presence/absence, relative intensity, position, either for single spots or clusters, are marked by arrowheads. Each Figure also displays several small close-up panels from several gels, depicting some of the individual variations among the pathological samples.

Buffer-soluble proteins. To dissect the whole pattern of proteins into specific compartments, we extracted separately the soluble proteins, the myofibril components, and membrane receptors (work in progress).

An alkaline, low-molarity buffer (such as 20 g/L carrier ampholytes) is expected to dissolve cytoplasmic proteins as well as extract residual blood. To sort out the relative contributions of the two classes of proteins, we subjected the heart extract (from the control) and normal human serum (not shown) to the same analytical conditions. Aside from albumin and transferrin, no serum component was detected in the stained pattern for the control. The extent of serum contamination varied by about twofold between specimens A through D (dissected from a frozen organ) and the other specimens (obtained just after heart explant); serum components thus correspond to 1/20 to 1/60 of the whole heart's



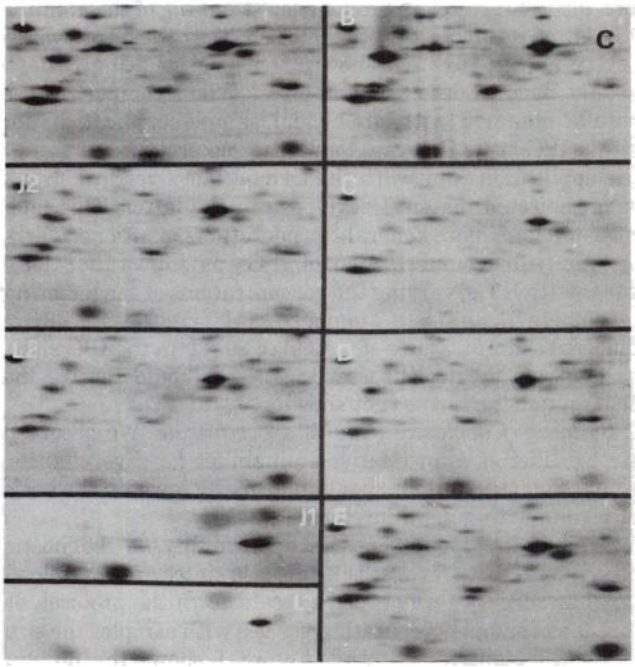


Fig. 3. 2-D mapping by IPG-DALT of heart proteins (1st-d on a 4–7 pH range)

Urea-detergent extracts from left ventricles: (*a*) control; (*b*) pathological sample, case A [proteins displaying qualitative and quantitative differences from the control are marked with *arrowheads*]; (*c*) composite of panels from different pathological maps, showing differences from (*b*), the corresponding areas from (*b*) being denoted by identical capital letters (in white in *c*)

protein content (i.e., the contamination amounted to 2%–5%).

By immunoelectrophoresis, we could identify albumin, transferrin, immunoglobulins, prealbumin, α_1-antitrypsin, and haptoglobin in heart tissue. Figure 5 shows that their proportions differ from the ones in control serum, as demonstrated by crossed immunoelectrophoresis (*23*) of sample volumes adjusted to contain the same amount of (immunoreactive) albumin [as evaluated by electroimmunodiffusion (*28*)]. Asymmetrical peaks [or arcs, by the immunoelectrophoresis procedure of Grabar and Williams (*29*)] are common in the heart-extract pattern.

As for the cytoplasmic components, their qualitative and quantitative variability among different samples was almost of the same order of magnitude as that for total proteins (not shown).

Myofibrils. Myofibril components were purified from both atria and from the left ventricle of three hearts (B, D, and K), as described by Solaro et al. (*13*). The acidic components of the solubilized material were then focused on a pH 4–7 IPG (Figure 6*c*), and the stained pattern was quantified by densitometry with a laser scanner (UltroScan; LKB). The relative intensities of the various bands—as marked along each lane—appear very similar for the three samples. To help identify the relevant spots in a 2-D pattern, Figure 6 (*a*, *b*) shows their position after IPG-DALT (1st-d on a pH 4–7 gradient).

Anatomical variations. Figure 7 compares the maps of heart proteins extracted from eight specimens dissected at different locations on the organ. Although the general pattern is obviously constant, some qualitative and quantitative changes are visible among the different samples (arrows). Thus the anatomical site of the dissection must be strictly controlled.

Influence of the extent of fibrosis. Figure 8 pictures maps of the acidic proteins (pH range 4–7, upper panels) and the basic proteins (pH 5–10, lower panels) extracted from specimens having various degrees of fibrosity. The application volumes of the samples were adjusted to contain identical protein loads.

Discussion

The IPG-DALT technique allows for a neat resolution of hundreds of major spots in the heart 2-D pattern; the zone-position reproducibility in the maps is extremely good (*19*).

For this work, we ran the 1st-d isoelectric focusing on horizontal gel slabs to improve the reproducibility of the procedure: samples are run side by side on the same matrix, and the gel may be supported on plastic (e.g., GelBondPAG; Marine Colloids, Rockland, ME) to assure stability of the migration dimensions. Moreover, one can load samples anywhere along the pH gradient and more easily check for the completion of protein migration from the application point (within the gel strip); 1st- to 2nd-d interfacing is also greatly simplified (*30*).

IPGs can be prepared containing additives (e.g., urea and detergents), either directly upon gel polymerization (*17*) or by reswelling a dry, empty gel in an appropriate solution of additive (*31, 32*). Both procedures have been tried; although the former gave somewhat better resolution (less trailing of the alkaline spots, data not shown), we routinely applied the

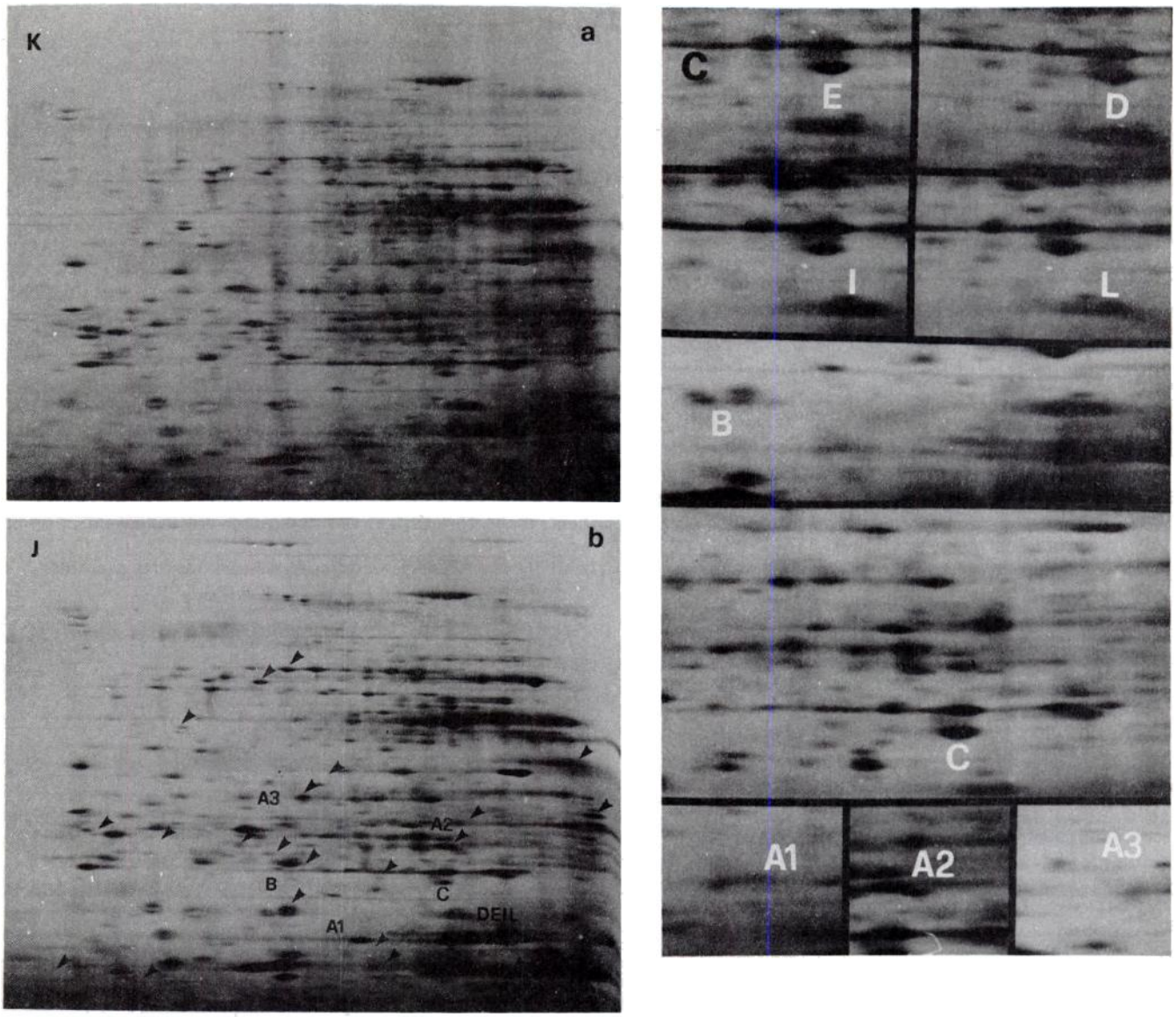


Fig. 4. 2-D mapping by IPG-DALT of heart proteins (1st-d on a 5–10 pH range)
Other details as in Fig. 3, except that the pathological sample in (*b*) is case J

latter, which allows one to cast in advance batches of homogeneous slabs.

The results we present imply only a preliminary, mostly qualitative evaluation of the information provided by 2-D protein mapping of the specimens. By visual inspection, at least 50 spots (or clusters thereof) appear to vary between the samples. For instance, in Figure 4c, panels from gels D-E vs I-L demonstrate a shift in pI, panels A1-2-3 the absence of some spots, and panels B and C quantitative differences in comparison with a reference map. Such variability may be caused by genetic polymorphism, histological heterogeneity, or some pathological condition of the organ.

Genetic polymorphism. Knowledge of the degree of variability in the population at large would require analysis of a large number of control hearts. In addition to obvious drawbacks, it is considerably difficult to obtain normal organs dissected under the same conditions as the pathological cases. A parallel study with animal models (swine) is thus underway to monitor the decay of the tissue components with time, which should demonstrate whether autopsy specimens would be suitable "control" samples.

Histological heterogeneity. The protein pattern varies with the site of sample dissection. However, an additional factor may influence the results, i.e., the degree of fibrosity of the specimen. For instance, specimens I and J had no (or negligible) fibrosis, but B and E were affected up to 40–50%. More important, the degree of fibrosis varied widely for adjacent areas (e.g., 15% in the upper front left ventricle vs 50% in lower front left ventricle of case B). The data presented above show that, indeed, many spots vary their relative concentration in specimens with different degrees of fibrosis.

Pathology. We expect to be able to correlate alterations in the protein pattern with altered functions for pathologies (e.g., infarction) that massively involve only one portion of the organ (in such a way that effects from genetic polymorphism or sample heterogeneity may be neglected or compensated for). For the other pathologies, we await quantitative data, such as those provided by computer-assisted image analysis, and a larger data base.

Previous reports on the protein alterations in dilatative cardiomyopathy imply an altered ratio between different

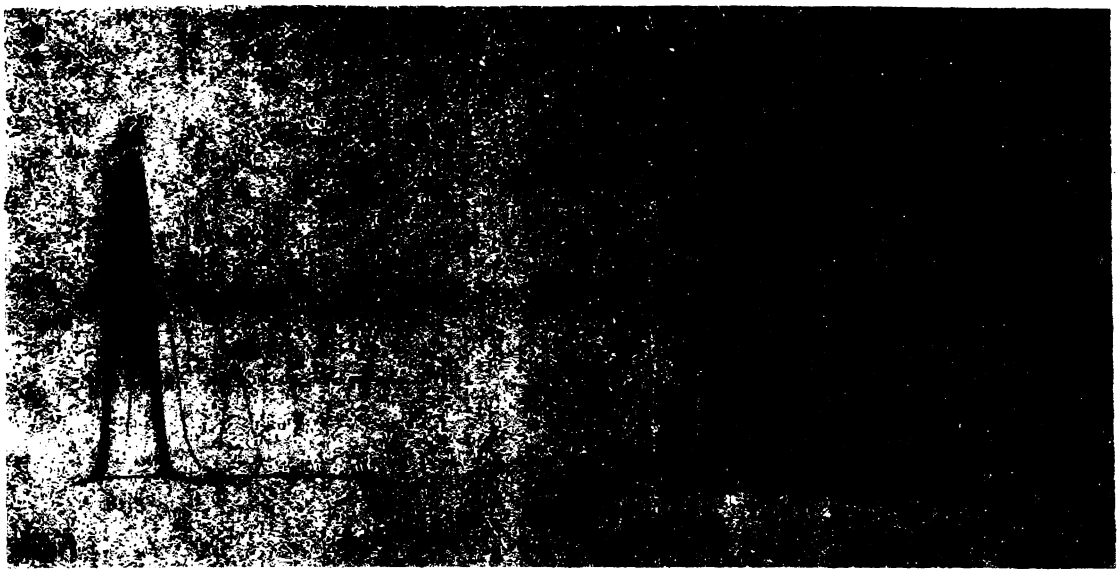
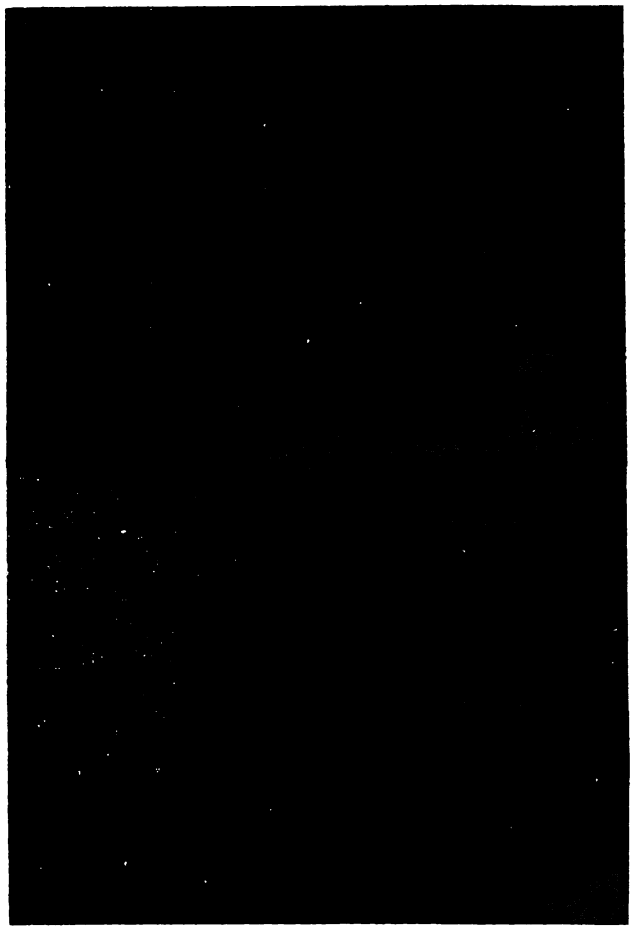


Fig. 5. Crossed immunoelectrophoresis of buffer-soluble heart proteins (from control case K, *panel a*) in comparison with the pattern for normal human serum (*panel b*)

Antiserum against whole serum proteins supplemented with antiserum against albumin. The tops of the faintest immunoprecipitates are marked by *arrowheads*



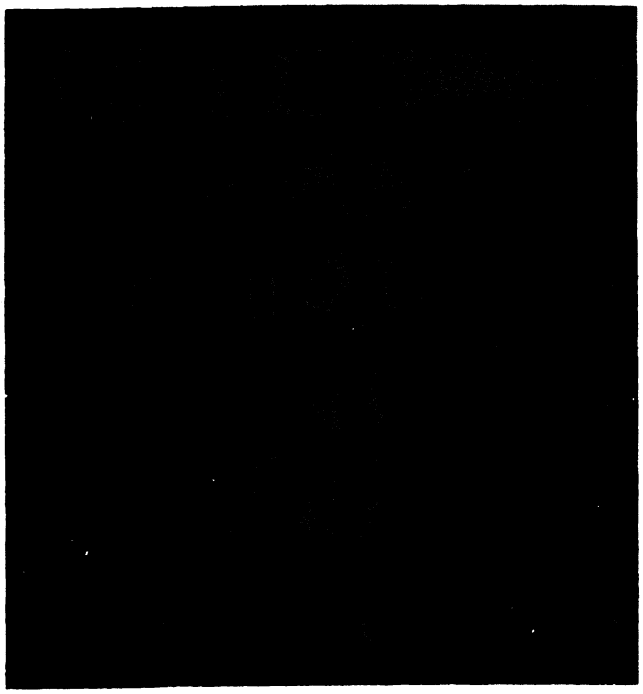


Fig. 6. 2-D pattern of the acidic components of purified myofibrils from atria (*a*) and ventricle (*b*) of heart K, with 1st-d run on a 4–7 pH gradient; (*c*) isoelectric fractionation of the acidic components of purified myofibrils from atria (*A*) and left ventricle (*V*) of hearts K, D, B (see Table 1)

The numbers above the ventricle lanes (*c*) refer to the relative percentage of each band as quantified by laser densitometry

types of myosin heavy chain in the affected atria (*33, 34*). Our selection of the ventricles as the main research subject—forced by the surgical technique for heart transplantation—doesn't allow us to correlate our present data with the published ones (*33, 34*).

Our data on the ventricle myofibrillar components are in contrast with the results presented by Hirzel et al. (*35*). With purified myofibrils we see no major differences (Figure 6) between pathological samples (dilatative cardiomyopathy, valvulopathy) and control samples. We stress that our experiment involved purified material, that the solubilization of the specimen was complete, and that no precipitation is seen in our IPG at the application point. In only one case (J; see panel J1 in Figure 3*c*) did the total protein map show

Fig. 7. Comparison of protein maps of samples from different anatomical sites of case K heart: *LV, RV* = left, right ventricle, *S* = septum, *P* = papillary muscle; *F* = front, *B* = back, *U* = upper, *L* = lower

Arrows mark the proteins exhibiting qualitative or quantitative variability among the samples

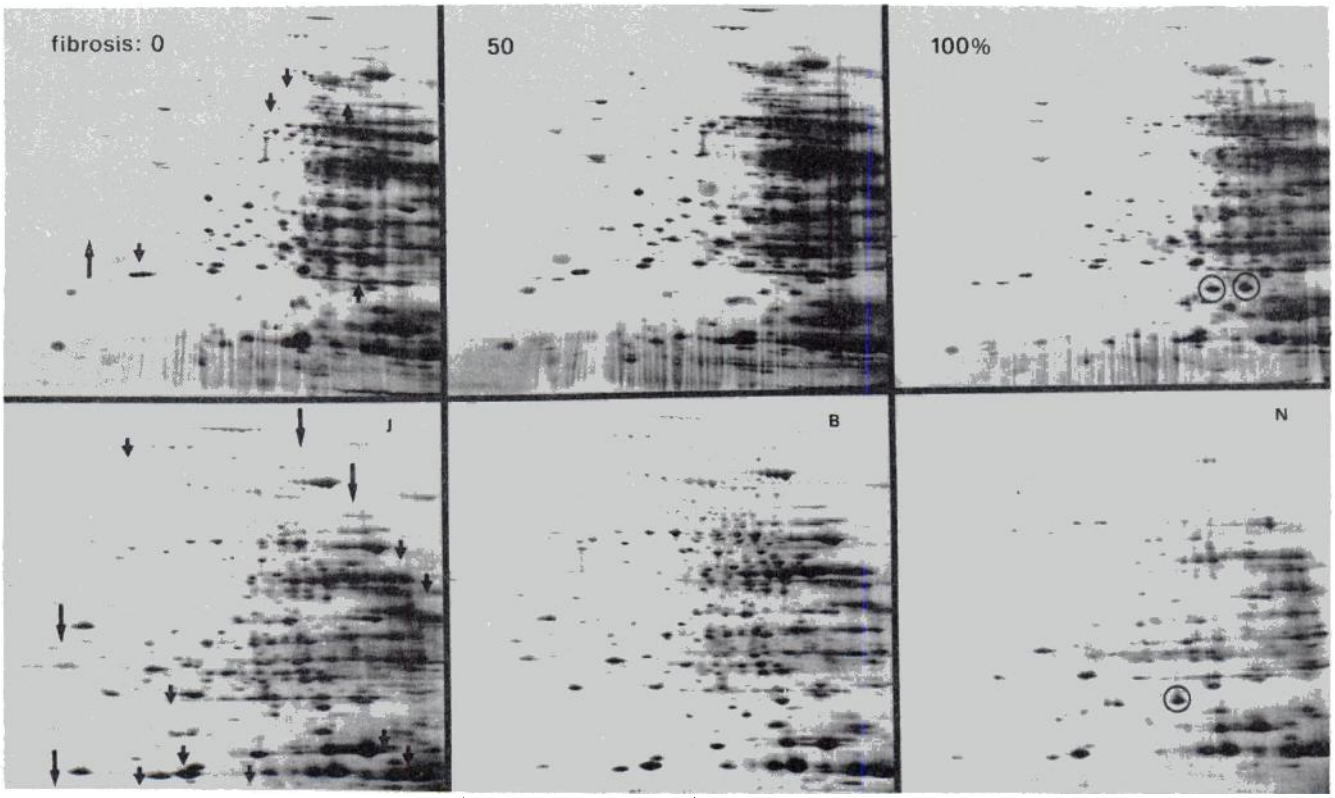


Fig. 8. Comparison of protein maps from samples with different extents of fibrosis: *left to right,* specimens with 0, 50%, and 80–100% fibrosis from cases J, B, and N, respectively

Upper row: IPG in the 4–7 pH range; *lower row:* pH 5–10. Downward and upward *arrows* mark proteins whose intensity respectively decreases or increases as the extent of fibrosis increases

an anomalous spot in the region of the myosin light chains. This cannot, however, be identified with an atrial-type polypeptide (as Hirzel et al. suggested), as shown by comparison with the purified material illustrated in Figure 6*a*.

In conclusion, we made the following critical findings:

• With typical sample loads, about 400 spots can be observed in the map of heart proteins when the pattern is silver-stained; only about 90 major spots can be detected after Coomassie stain. The relative intensities of several of the spots vary widely with either staining procedure.

• Direct extraction into a solvent homogeneous with the 1st-d separation medium (i.e., in urea–Nonidet) is preferable to detergent exchange after solubilization with SDS—as already discussed (*21*)—for the following reasons: (*a*) to respect IPG compatibilities (*18*); (*b*) in agreement with the

NEPHGE (*36*) or BASO-DALT (*37*) procedures, to which our 1st-d procedure, by its anodal sample application, is akin; (*c*) by analogy with the results of Giometti et al. (*11*) who, with a comparable sample (skeletal muscle), observed essentially identical patterns after either extraction procedure; (*d*) to minimize artifacts from incomplete solubility in the final solvent, both as background streaking and as spurious banding (*21*).

- The details of the protein pattern will change as the site of sample dissection varies; for comparable results all specimens should thus be obtained from the same area, e.g., the front wall of the left ventricle.
- The sensitivity achieved by the silver-staining procedures makes it possible to apply the proposed technique even to biopsies (or portions thereof) as small as 1–2 mg.

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References

1. Bevilacqua M, Vago T, Norbiato G, et al. Characterization of alpha$_1$ receptors in the sarcolemma from the myocardium of patients with dilated cardiomyopathy. J Cardiovasc Pharmacol, in press.

2. Anderson NG, Anderson NL. The human protein index. Clin Chem 1982;28:739–48.

3. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975;250:4007–21.

4. Young DS, Tracy RP. Two-dimensional electrophoresis in the development of clinical laboratory tests. Electrophoresis 1983;4:117–21.

5. Righetti PG, Gianazza E. Isoelectric focusing in immobilized pH gradients: theory and newer methodology. Methods Biochem Anal 1986;32:215–78.

6. Anderson NG, Anderson NL. Two-dimensional analysis of serum and tissue proteins. Anal Biochem 1978;85:331–40.

7. Gianazza E, Giacon P, Sahlin B, Righetti PG. Non-linear pH courses with immobilized pH gradients. Electrophoresis 1985;6:53–6.

8. Gianazza E, Astrua-Testori S, Righetti PG, Bianchi-Bosisio A. Urine analysis by two-dimensional gel electrophoresis with isoelectric focusing in immobilized pH gradients in the first dimension. Electrophoresis 1986;7:435–8.

9. Gianazza E, Astrua-Testori S, Caccia P, Giacon P, Quaglia L, Righetti PG. On the reproducibility of band position in electrophoretic separations. Electrophoresis 1986;7:76–83.

10. Watson DC, Dong Jr E, Shaumway NE. Distant heart procurement for transplantation. Surgery 1979;86:56–65.

11. Giometti CS, Anderson NG, Anderson NL. Muscle protein analysis: development of high resolution two-dimensional electrophoresis of skeletal muscle proteins for analysis of microbiopsy samples. Clin Chem 1979;25:1877–84.

12. Tracy RP, Wold LE, Currie RM, Young DS. Patterns of normal colon mucosa and colon adrenocarcinoma compared by two-dimensional gel electrophoresis. Clin Chem 1982;28:915–9.

13. Solaro RJ, Pang DC, Briggs FN. The purification of cardiac myofibrils with Triton X-100. Biochim Biophys Acta 1971;245:259–62.

14. Gianazza E, Astrua-Testori S, Righetti PG. Some more formulations for immobilized pH gradients. Electrophoresis 1985;6:113–7.

15. Gianazza E, Celentano F, Dossi G, Bjellqvist B, Righetti PG. Preparation of immobilized pH gradients spanning 2–6 pH units. Electrophoresis 1984;5:88–97.

16. Hjertén S. Electrophoresis in polyacrylamide gels. Arch Biochem Biophys 1962;suppl.1:147–51.

17. Gianazza E, Quaglia L, Caccia P, Righetti PG. Which electrodic solutions for immobilized pH gradients? J Biochem Biophys Methods 1986;12:227–37.

18. Gianazza E, Caccia P, Quaglia L, Righetti PG, Rimpiläinen MA, Forsén RJ. Two-dimensional analysis of membrane proteins with isoelectric focusing in immobilized pH gradients in the first dimension. Electrophoresis 1986;7:537–43.

19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) 1970;227:680–5.

20. Gianazza E, Astrua-Testori S, Giacon P, Righetti PG. An improved protocol for two-dimensional maps of serum proteins with immobilized pH gradients in the first dimension. Electrophoresis 1985;6:332–9.

21. Gianazza E, Rabilloud T, Quaglia L, et al. Additives for IPG-DALT of particulate material: comparison between commercial and new synthetic detergents. Anal Biochem, in press.

22. Guevara J, Johnson DA, Ramagali LS, Martin BA, Capetillo S, Rodriguez LV. Quantitative aspects of silver deposition in proteins resolved in complex polyacrylamide gels. Electrophoresis 1982;3:197–205.

23. Clarke HGM, Freeman T. Quantitative immunoelectrophoresis of human serum proteins. Clin Sci 1968;35:403–8.

24. Weeke B. Equipment, procedures, basic methods. In: Axelsen NH, Krøll J, Weeke B, eds. A manual of quantitative immunoelectrophoresis. Oslo: Universitetsforlaget, 1973:16–35.

25. Krøll J. Tandem-crossed immunoelectrophoresis. *Ibid.*:57–9.

26. Hochstrasser D, Angsburger V, Funk M, Appel R, Pellegrini C, Muller AF. Immobilized pH gradients in capillary tubes and two-dimensional gel electrophoresis. Electrophoresis 1986;7:505–11.

27. Merril CR, Goldman D. Detection of polypeptides in two-dimensional gels using silver staining. In: Celis JE, Bravo R, eds. Two-dimensional gel electrophoresis of proteins. New York: Academic Press, 1984:93–109.

28. Laurell C-B. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal Biochem 1965;10:358–66.

29. Grabar P, Williams CA. Méthode permettant l'étude conjugée des proprietés électrophoretiques et immunochimiques d'un melange de protéins; application au serum sanguin. Biochim Biophys Acta 1953;10:193–7.

30. Gianazza E, Frigerio A, Tagliabue A, Righetti PG. Serum fractionation on immobilized pH gradients with one- and two-dimensional techniques. Electrophoresis 1984;5:209–16.

31. Gianazza E, Artoni G, Righetti PG. Isoelectric focusing in immobilized pH gradients in presence of urea and neutral detergents. Electrophoresis 1983;4:321–6.

32. Gelfi C, Righetti PG. Swelling kinetics of Immobiline gels for isoelectric focusing. Electrophoresis 1984;5:257–62.

33. Gorza L, Mercadier JJ, Schwartz K, Thornell LE, Sartore S, Schiaffino S. Myosin types in human heart: an immunofluorescence study of normal and hypertrophied atrial and ventricular myocardium. Circ Res 1984;54:694–702.

34. Schiaffino S, Gorza L, Saggin L, Valfré C, Sartore S. Myosin changes in hypertrophied human atrial and ventricular myocardium: a correlated immunofluorescence and quantitative immunochemical study on serial cryosections. Eur Heart J 1984;75:95–102.

35. Hirzel HO, Tuchschmid CR, Schneider J, Krayenbuhel HP, Schaub MC. Relationship between myosin isoenzyme composition, hemodynamics, and myocardial structure in various forms of human cardiac hypertrophy. Circ Res 1985;57:729–40.

36. O'Farrell PZ, Goodman HM, O'Farrell PH. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 1977;12:1133–42.

37. Willard KE, Giometti CS, Anderson NL, O'Connor TE, Anderson NG. Two-dimensional electrophoretic analysis of basic proteins using phosphatidyl choline urea solubilization. Anal Biochem 1980;100:289–98.

38. Hurst JW, Spann Jr JF. Chapter 21 in: Hurst JW, ed. The heart. 6th ed. New York: McGraw-Hill, 1986.

39. Silverman ME, Schlant RC. *Ibid.* Chapter 2.