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ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL CARRIED OUT WITH A SIMPLE DEVICE FOR POWER REGULATION

APPLICATION TO MAMMALIAN GROWTH HORMONES

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SUMMARY

A unit is described to regulate the electrical power delivered to an isoelectric focusing cell. The device consists in a resistance set, in series with a power supply, which can be adjusted to a suitable resistance value in order to permit only minor changes in wattage. Simultaneous monitoring of voltage and current is also carried out with a pen-recorder adaptor.

The improvements in operative conditions achieved by these means are tested by the fractionation of mammalian growth hormones.

INTRODUCTION

Notable improvements in resolution and operational time can be achieved in isoelectric focusing in polyacrylamide gel (IFPA) experiments by maintaining a constant wattage [1—5]. Different methods have already been described for this purpose. Many employ time-consuming manual adjustment of the voltage [1, 2] or expensive power supplies which utilize an electronic control [2] or a servo motor for continuous wattage regulation [4]. Alternatively, power can be regulated by inserting an electrical resistance in series with the IFPA system [5].

In this report we describe a simple and inexpensive apparatus that exploits this technical procedure. The apparatus also includes a device designed to record either voltage or current during the IFPA run. The applicability of this procedure is demonstrated with the fractionation of mammalian growth hormones (GH).

EXPERIMENTAL

Reagents

Acrylamide and N,N′-methylene-bis-acrylamide of commercial grade (Eastman Chemical, Rochester, U.S.A.) were recrystallized from chloroform and acetone, respectively. Coomassie Brillant Blue R 250 was purchased from Serva (Heidelberg, G.F.R.) and Ampholine carrier ampholytes from LKB (Bromma, Sweden). N,N,N′,N′-Tetra methylethylenediamine and other chemicals were of analytical grade. Human haemoglobin, supplied by Dr. L. Rossi-Bernardi, was obtained as previously described [6]. The highly purified growth hormone preparations, supplied by the N.I.H. Endocrine Study Section as a gift from Dr. A.E. Wilhelmi and Dr. A.F. Parlow, were: bovine growth hormone (BGH) B1003A; ovine growth hormone (OGH) 0743B; porcine growth hormone (PGH) P526B; rat growth hormone (RGH) Rat GH B 2; canine growth hormone (CGH) D887A; and human growth hormone (HGH) H1648E.

Preparation of gel slabs

Acrylamide gel slabs were prepared according to the procedure described in the LKB application note [7]. Gel plates were prepared by combining acrylamide with N,N′-methylene-bis-acrylamide as cross-linker with the composition T*=5%, C=2.8% [8]. After the riboflavin had been added, the mixture was poured into the gel mould and photopolymerized for 30 min under the light of a mercury lamp (Osram HQL, 80 W) situated at a distance of about 200 mm from the gel. The final concentration of Ampholine in the gel (2—4%, w/v) was obtained according to the method of Vesterberg [9,10]. The gel plate was used directly and maintained during the focusing period at 4° as monitored on the gel surface by a thermistor probe (sensitivity ± 0.5; LISI, Milan, Italy).

IFPA of pituitary GH preparations

In a typical run, the focusing was performed in gels (250 × 115 × 2 mm) containing 4% (w/v) Ampholine mixture (pH 3.5—9.5).

A 19.8/kΩ resistance was inserted in series and the voltage was set at 100 V/cm (LKB 3371D, d.c. power supply). After 90 min 50—100 μg of protein sample were applied by filter paper (10 × 10 mm Whatman 3 MM) placed on the gel near the cathode. The focusing time under these conditions was 3 h. The end voltage was 70 V/cm.

The pH gradient was measured by cutting out discs of gel (diameter 4 mm) and eluting carrier ampholytes for 1 h into closed tubes with 0.5 ml solution of 20 m*M* KCl free from carbon dioxide [11]. Measurements of pH were performed at 4° in a cold room using a pH meter (model D.P. 100, Gibertini, Milan, Italy) equipped with a micro glass electrode (LOT 205 M3; W. Ingold, Zurich, Switzerland) [12]. Replicate gels were stained for proteins with Coomassie Brilliant Blue R 250 [13] and the isoelectric points of the bands were calculated from the corresponding pH curve.

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*Symbols used: T=g. acrylamide + g.N,N′-methylene-bis-acrylamide per 100 ml; C=(g/100 ml)N,N′-methylene-bis-acrylamide/T.

Apparatus

Gel isoelectric focusing was performed with an LKB Multiphor 2117 apparatus. Fig. 1A shows the basic wiring diagram of the resistances (R_1—R_4). The resistance values were: $R_2 = 2 \times R_1$, $R_3 = 4 \times R_1$, $R_4 = 8 \times R_1$, so that the total resistance between the terminals A_1 and A_2 ranges from the R_1 value to $15 \times R_1$, according to the positions of the on—off switches (S_1—S_4). In order to obtain a wider range of total resistance values, the basic circuit has been modified by the use of three-positions switches (K_1—K_4) and eight (20 W) additional resistances (Fig. 1B). These switches, when all in the same position, can change the values of R_1, R_2, R_3 and R_4 but keep constant their original relationship (Table I). From the resistance values in Table I, it can easily be seen that when the switches K_1, K_2, K_3 and K_4 are in position I ($R_1' = 0.5$ kΩ) it is possible to obtain total resistance values ranging from 0.5 to 7.5 kΩ with 500-Ω steps, in position II ($R_1'' = 2$ kΩ) from 2 to 30 kΩ with 2-kΩ steps, and in position III ($R_1''' = 8$ kΩ) from 8 to 120 kΩ with 8-kΩ steps. Furthermore, it is possible to obtain a wider range of total resistance values by the different combinations of the switches K_1—K_4. This operation allows the resistance value to be set very close to the one required (e.g. a 28.5-kΩ value is obtained by setting switches K_2, K_3 and K_4 in position II and the switch K_1 in position I).

Fig. 2 shows the wiring diagram of the pen-recorder adaptor, which allows an alternative recording of two different signals (Y_1 and Y_2) varying very slowly with time. D_1 and D_2 are suitable adaptor units to attenuate Y_1 and Y_2 in

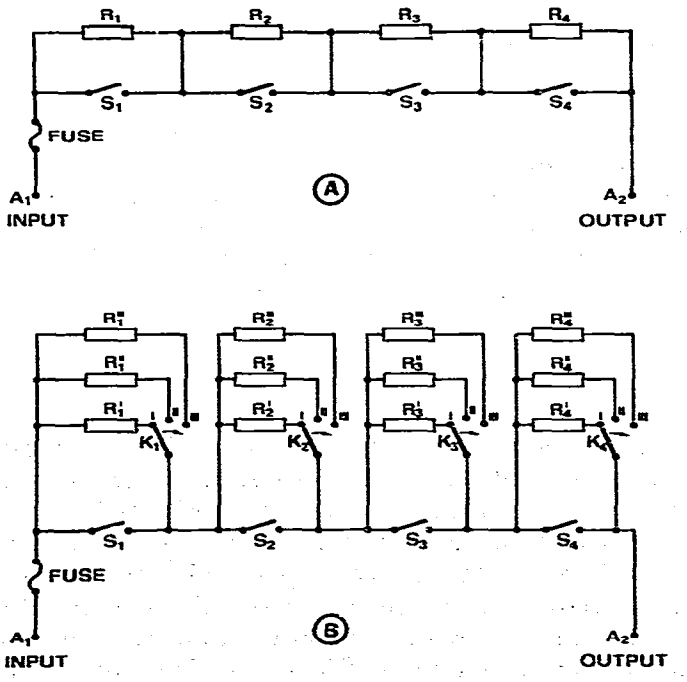

Fig. 1. Basic wiring diagram of the unit for power regulation. A, the resistance set with adjustable switches. B, detailed design of the resistance set of A.

TABLE I

RESISTANCE SET COVERING THE TOTAL RESISTANCE VALUES

Values in kΩ

Resistance	kΩ	Resistance	kΩ	Resistance	kΩ
R_1'	0.5	R_1''	2	R_1'''	8
R_2'	1	R_2''	4	R_2'''	16
R_3'	2	R_3''	8	R_3'''	32
R_4'	4	R_4''	16	R_4'''	64

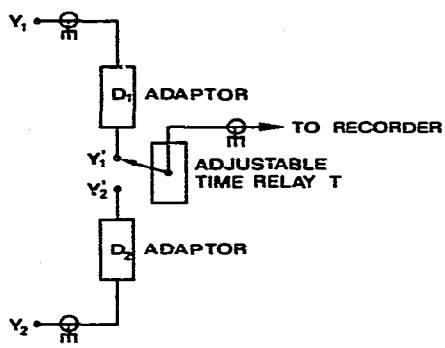

Fig. 2. Block diagram of the adaptor for one pen recorder.

order to maintain the pen of the recorder within the chart limits. An adjustable time relay provides that for a preset time, T, the unit is in position I and for a time T' (identical to T) the unit is in position 2. The time T must be long enough in comparison with the time required by the recording pen to pass from one position to the other. In such a way it is possible to have an alternate recording of two signals (i.e. voltage and current).

RESULTS

The developmental pattern of the voltage and the current during a typical experiment at pH 3.5—9.5, using a 19.8-kΩ series resistance, is shown in Fig. 3. At the start of the run, when a voltage of 100 V/cm is stabilized by the power supply, the effective voltage and current values, recorded between the electrodes, are 15 V/cm and 11 mA, respectively. The samples are applied 90 min after the start of the run when a condition of narrow power variation is achieved: voltage 60 V/cm, and current 5 mA. Four hours later the current is decreased to 4 mA and the voltage is raised to 70 V/cm. Therefore, the resistance inserted in series restrains the total power variation in the gel

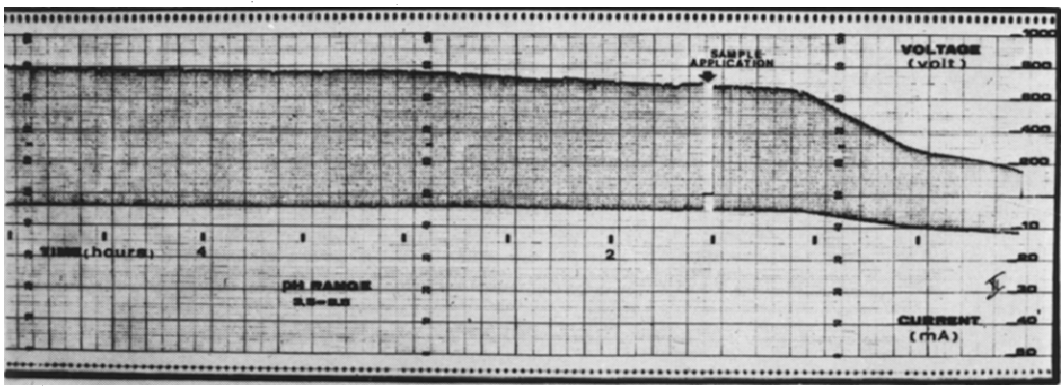

Fig. 3. Changes in the voltage (upper tracing) and current (lower tracing) during the IFPA experiment described in Fig. 4. The graph proceeds from right to left.

between 1.98 and 3.2 W. The IFPA patterns of six different GH preparations are shown in Fig. 4. Fig. 5 shows the band patterns of the same HGH preparation, refocused in a narrower pH gradient (Ampholine, pH 4.0—6.0 at 4% (w/v) concentration).

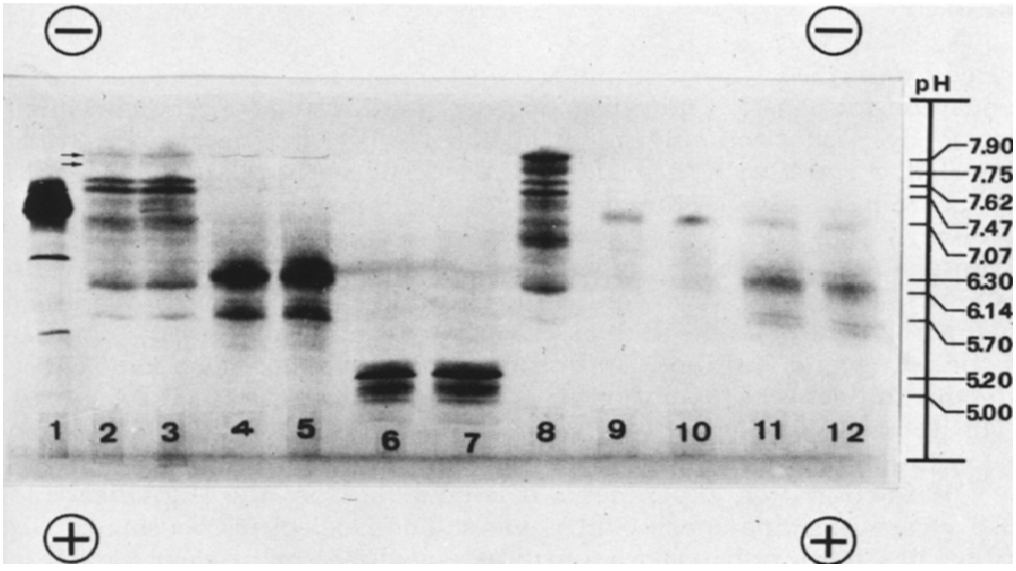

Fig. 4. IFPA of pituitary GH preparation of different animal species. Samples from left to right: (1) human haemoglobin; (2 and 3) ovine GH; (4 and 5) rat GH; (6 and 7) human GH; (8) bovine GH; (9 and 10) canine GH; (11 and 12) porcine GH. The arrows indicate the two more basic bands for OGH. The vertical scale shows the pH values along the gel, at 4°. Gel composition and electrolysis conditions are described in the text.

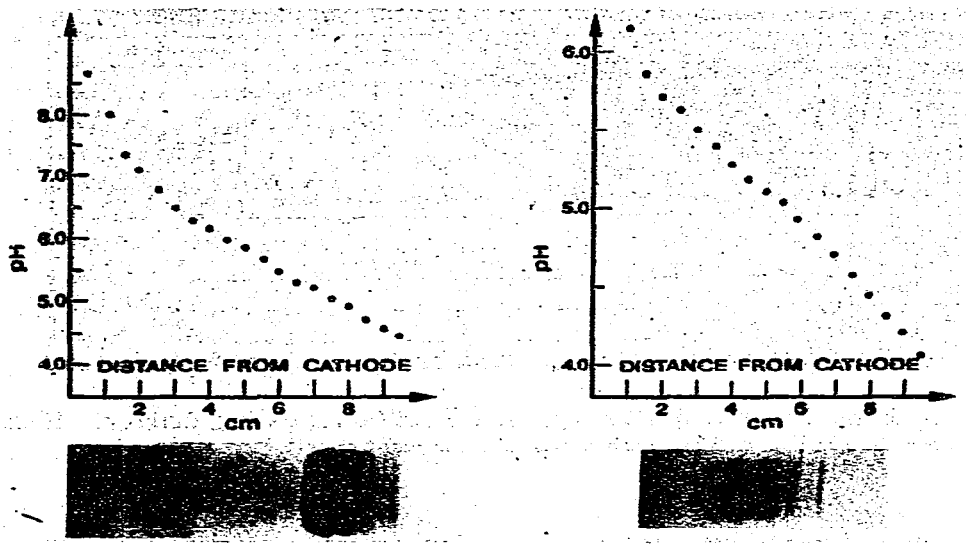

Fig. 5. Left: IFPA of HGH in the pH range 3.5—9.5 with a mixture of Ampholine in a final concentration of 3% (w/v) for a gel size of about 70x 115x 2 mm. A 35.4-kΩ resistance was inserted in series during the run. End voltage, 600 V; focusing time, 2 h. Right: IFPA of HGH in the pH range 4—6 with 4% (w/v) final concentration of Ampholine, for a gel size of about 120x 115x 2 mm, and with a 23.5-kΩ resistance inserted in series for power regulation during the run. End voltage, 500 V; focusing time, 3 h. The pH gradient was determined as described in the text, at 4°.

DISCUSSION

The circuit described is very simple and costs much less than a power supply built or adapted for constant power. It prevents heating and consequent sample denaturation by permitting only minor changes in power. The electrical resistances inserted in series with the electrophoretic cell can be quickly and simply adjusted according to the gel dimension, and the concentration and pH range of Ampholine.

By simultaneously monitoring voltage and current, a maximum power variation in the pH range 3.5—9.5 occurs within 90 min of the start-up operation. After this period it is possible to apply the sample and to control the development of the focusing conditions. By this means we have succeeded in considerably reducing the time of operation in medium-to-high voltage IFPA experiments. The focusing time can be estimated by determining the time required for the coalescence of samples applied at opposite ends of the slab.

In the GH fractionation experiments it is possible from the IFPA bands to distinguish clearly striking species differences. The isoelectric focusing of GH preparations has been previously reported using different procedures (IF in sucrose density gradient or IFPA in cylindrical tubes) [12—22].

We chose the flat gel as the analytical system because it allows comparison of very similar molecules side by side under the same experimental conditions.

The measured pI values of HGH and RGH reported are in good agreement with the data in the literature [14—19]. When HGH was refocused in a pH

gradient of 4.0—6.0 (Fig. 5) the isoelectric points of the major component and of the two successive bands were very similar to those obtained in the wider pH range. With regard to BGH and OGH the isoelectric point of banding patterns here reported are virtually identical to those obtained by Ellis et al. [14]. The resolution of our method appears to result in more distinct banding compared to other procedures. The higher pI values reported by Ellis for the two more basic components of either BGH or OGH are probably due to sucrose in the IF columns [21]. With regard to PGH, a pI value of 6.3 was first reported by Li and Liu [19], but a more recent paper [20] indicates that the apparent pI value for PGH is higher than 6.8. In our experiments PGH clearly shows a single major component at an isoelectric point of 7.08 as well as several minor components with acidic isoelectric points. On the other hand, as regards CGH, our isoelectric point for CGH is lower than that indicated by Hashimoto et al. [22]. However, the IFPA band pattern indicates that, in this case also, the native hormone consists of a single chain.

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