Journal of Chromatography, 264 (1983) 435–443
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,867

QUANTITATIVE ANALYSIS OF TWO-DIMENSIONAL ELECTROPHEROGRAMS WITH A TELEVISION CAMERA–MICROCOMPUTER SYSTEM

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(Received March 15th, 1983)

SUMMARY

A system to quantitate dye-stained proteins on two-dimensional polyacrylamide gels employing a television camera for data acquisition and a microcomputer for data analysis is described. All the equipment comprising the system and most of the software are commercially available. Therefore, minimal knowledge of computer software is needed to construct the system. The system is handy, low cost and useful for practical quantitation of protein spots, although it is semi-quantitative compared with the previously available systems. The system is especially suited for the analysis of micro two-dimensional gels since high resolution up to 30 μm is obtained.

INTRODUCTION

Previously we have reported a two-dimensional electrophoretic technique in which no denaturing agent is used[1], and demonstrated its applicability to clinical purposes[2,3]. A microscale-multisample version of the technique[4] enabled us to analyze eight to sixteen samples simultaneously and it facilitated comparative studies of multiple two-dimensional patterns[5]. During these comparative studies, we tried to analyze the changes in quantity of several specific proteins. Previously, we used a thin-layer chromatographic scanner, which is a flat bed x–y scanner, for the quantitation of a specifically increased serum protein in rat to which carbon tetrachloride had been administered[3].

However, for the quantitation of individual protein spots on a complex two-dimensional pattern, the scanner should be combined with a computer system for data analysis. Several reports have dealt with sophisticated systems for quantitation of individual proteins on two-dimensional gels and for comparisons of two-dimensional patterns, combining a flat-bed scanner or a drum scanner with a computer system[6–9], but these systems are costly and not handy for laboratory use.

In this report we describe a system for quantitation of two-dimensional electropherograms, which employs a television (TV) camera and a digitizer for data acquisition, a mini-floppy disk unit for data storage and a microcomputer for data analysis. The system is especially useful for quantitating proteins on micro two-dimensional electropherograms.

0021-9673/83/$03.00

MATERIALS AND METHODS

Two-dimensional electrophoresis

Two-dimensional electrophoresis of normal human serum proteins in the absence of denaturing agents using macro slab gels[10] or micro slab gels[4] was performed as described previously, except that the macro slab gel was 160 mm wide, 120 mm long and 3 mm thick. Micro two-dimensional electrophoresis of bovine brain soluble proteins was performed as described previously[11]. The macro slab gels were stained overnight in 0.025% Coomassie Brilliant Blue R-250–7% acetic acid–50% methanol and destained in 7% acetic acid. In the case of the micro slab gels, gels were stained in the above staining solution for 1 h and destained in 7% acetic acid. The micro slab gel was 38 mm wide, 40 mm long and 1 mm thick. Silver staining of micro slab gels was performed as described by Oakley *et al.*[12].

Instruments

An illustration of the TV camera–microcomputer system is shown in Fig. 1. The whole system is composed of commercial equipment. A stained two-dimensional gel was sandwiched between two glass plates, placed on a fluorescent light-box (Yodobashi Camera, Tokyo, Japan) and a black and white TV camera (NEMCO Model CN-120; Pax Electronica Japan, Tokyo, Japan) was set above the gel using a camera stand. The TV camera is equipped with a Cosmicar lens (f = 16 mm) and a Toshiba Y-2 filter (Tokyo Shibaura Electric, Tokyo, Japan). The magnification rate of the camera was adjusted by changing the length of extension tubes (Cosmicar extension tube kit; Asahi Precisin, Tokyo, Japan) attached to the lens. For example, by attaching a 15-mm extension tube, gel sections (8 × 6 mm) could be projected on the full screen of a TV display. The video signal is digitized by a digitizer (Computer Eye Model 4000; Pax Electronica Japan) which has an interface for an NEC PC-8801 microcomputer (Nippon Electric Co., Tokyo, Japan). The digitizer divides the video image into 256 × 256 dots and each dot has four levels of gray scale. The digitizer

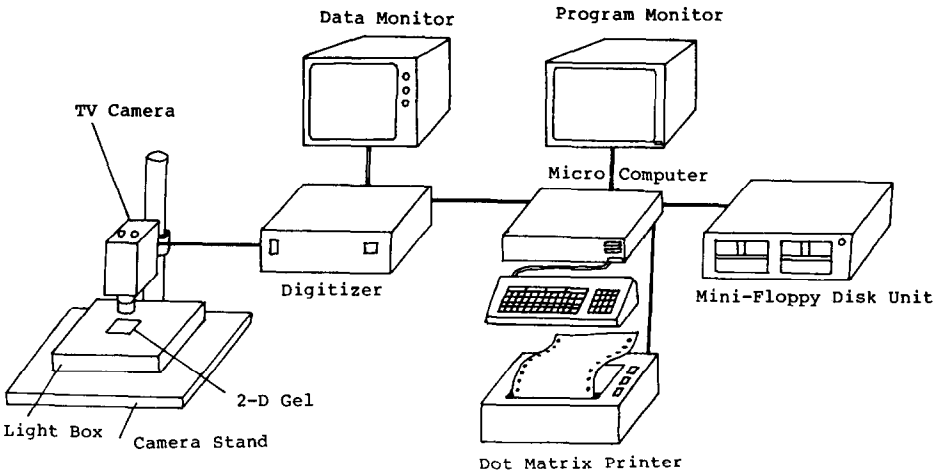

Fig. 1. The TV camera–microcomputer system.

is equipped with two variable resistors and these are adjusted when the gray levels are converted into dye density. The digitized image is acquired in the digitizer 1/60 sec after triggering a switch, and monitored by a TV display (NEC PC-8041 12-in. green display, Nippon Electric Co.). The digitized image can be stored in a 5-in. magnetic disk (143 kbytes of memory) by a floppy disk unit (NEC PC-8031 mini-floppy disk unit, Nippon Electric Co.). The floppy disk unit has two drives, one of which is used to set a 5-in. disk which stores the microcomputer programs to control the system and the other sets a disk for storage of the digitized images. One 5-in. disk can store seven digitized images. The programs to control the system were selected by monitoring a TV display (NEC PC-8851 14-in. monochrome display, Nippon Electric Co.). A dot-matrix printer (EPSON MP-82; Shishu Seiki, Shiojiri, Japan) was used for hard-copy of the digitized images.

Computer programs

The microcomputer programs, commercially available as a software package from Pax Electronica Japan, were used with some modifications. The software is written in BASIC language for the operator–computer interactions and in machine language for the computer–peripheral machine interactions. Our modifications were restricted to the BASIC programs. The software package is composed of the following programs: (1) "cecp", a program to select the subroutines described below; (2) "save", which reads the digitized image data in the digitizer and stores them in a magnetic disk file; (3) "load", which reads the image data in a magnetic disk and stores them in the memory of the digitizers; (4) "data match", which compares the digitized image in the digitizer with any of the images stored in a magnetic disk and displays the unmatched dot positions on the TV display; (5) "demo", which allows an operator to select any rectangular area of a digitized image using a cursor; [The number of dots in the selected area, in four gray levels ("white", "light gray", "dark gray" and "black"), is counted.] (6) "package II", which prints out the digitized image in the digitizer on a dot-matrix printer.

We added the following functions to the program "demo"; (a) after dot counting, the integrated density of the spot was calculated and printed out by the procedure described in the next paragraph: (2) after calculating integrated density, the selected area is distinguished from the remaining area by inversing the gray levels.

Settings of density levels and integration of density

A density standard tablet (Fuji Density Step Tablet; Fuji Photo Film, Tokyo, Japan), which is a photo-film representing stepwise thirteen levels of diffuse visual density, was used to convert gray levels into density. The tablet was put on the light-box and a digitized image of the tablet was shown on the TV display. By adjusting the two variable resistors of the digitizer, we set the four gray levels, white, light gray, dark gray and black, to the following diffuse visual density; 0.04, 0.28, 0.67 and 1.32, respectively. Since white corresponded to gel background, the density value of 0.04 was subtracted from the density value of each gray level. Then the integrated density of a spot was calculated by multiplying the dot counts in each gray level by the converted density and summing the products

$$\text{I.D.} = 0.24L + 0.63D + 1.28B$$

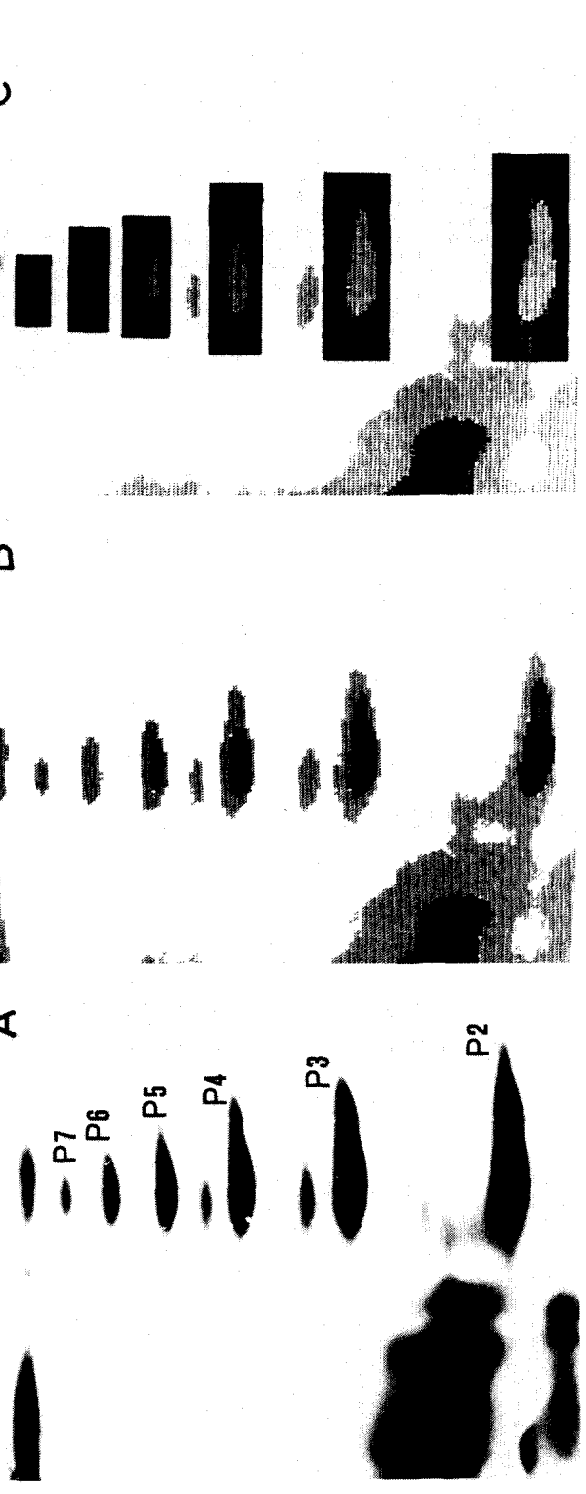

Fig. 2. The process of quantitating stained spots on two-dimensional (2-D) electropherograms. A, A section of a macro slab gel (160 × 120 × 3) pattern of a haptoglobin Type 2-1 serum. Haptoglobin polymer series, P2-P7, are indicated. B, A gray scale counter map of the gel section shown in A in 200-μm resolution. The print-out image is expanded 1.3-fold in the vertical axis. C, The gray scale contour map shown in B, after calculation of integrated density. By running the program "demo", a rectangular area which contained a spot of haptoglobin 2-1 polymer series was selected by keyboard control, the integrated density was calculated and the gray levels within the area were inverted.

where I.D. represents integrated density, L, D and B the number of dots in levels light gray, dark gray and black, respectively.

Comparison with a flat-bed scanner

A flat-bed x–y scanner, Shimadzu dual-wavelength thin-layer chromatographic (TLC) scanner CS-910 (Shimadzu, Tokyo, Japan), was used to compare the results of quantitation. The sample wavelength was set at 580 nm and the reference wavelength was 750 nm. The densitometer was operated in "zigzag scanning mode" and the protein amounts were quantitated by measuring the step height of the integrating signal.

Albumin quantity standard

In order to examine the sensitivity of the TV camera system, an albumin quantity standard slab gel was prepared. Varying amounts of bovine serum albumin (crystallized four times; Sigma, St. Louis, MO, U.S.A.) ranging from 0.05 to 3.2 μg were subjected to polyacrylamide SDS gradient gel electrophoresis. Micro polyacrylamide slab gels containing 1% SDS were prepared as described previously[11]. Electrophoresis was run in 0.05 *M* Tris–0.38 *M* glycine containing 0.1% SDS at 5 mA for 100 min.

RESULTS

Resolution

As the first step of quantification, we determined the degree of magnification of the image of a two-dimensional gel by selecting the lens focal length of the TV camera. Since the digitizer divides the video image into 256 × 256 dots, the resolution of the system depends on the degree of magnification. We routinely attached a 1.0-mm extension tube to the lens of the TV camera for the analysis of 50 × 40 mm sections of macro two-dimensional gels (160 × 120 × 3 mm), which corresponded to 200 × 160 μm resolution. In case of micro two-dimensional gels (38 × 40 × 1 mm), a 10-mm extension tube was attached and 12 × 9 mm sections were analyzed, which corresponded to 47 × 35 μm resolution.

Performance of the software

A haptoglobin Type 2-1 serum was subjected to two-dimensional polyacrylamide gel electrophoresis using a macro (160 × 120 × 3 mm) slab gel and a 50 × 40 mm gel section (Fig. 2A) which showed haptoglobin polymer series was analyzed by the TV camera system. The image data were acquired 1/60 sec after triggering a switch of the digitizer and the digitized image was displayed on the monitor TV as a gray scale contour map (Fig. 2B) without the aid of software. A spot to be quantitated was selected by keyboard control, the "integrated density" of the spot was calculated, and the gray levels in the selected area were inverted (Fig. 2C) by program "demo". The digitized image on the TV display could be copied on the printer by the program "package II". The time needed for the area selection and calculation for one spot was 10–20 sec, for hard-copy of one image it was 4 min. Routinely, we stored the image data in magnetic disks by use of the program "save" and then read the data from the disk by the program "load" when we wanted to quantitate spots of interest. The time needed to save or load one digitized image was about 20 sec.

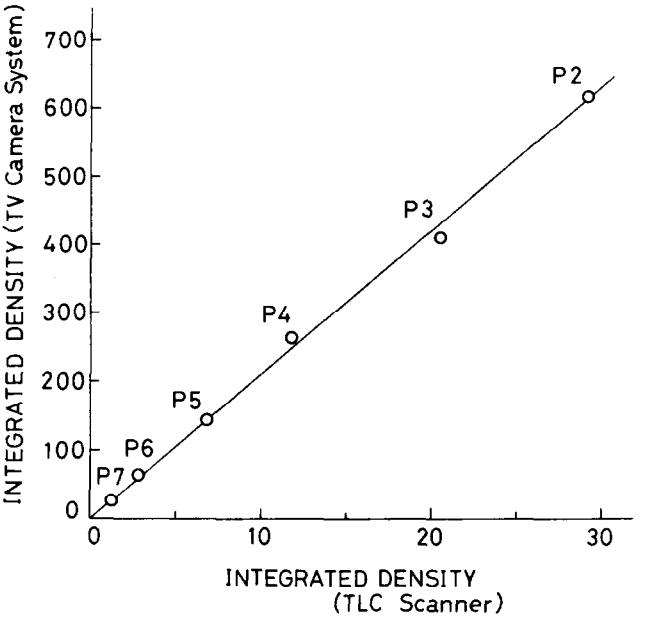

Fig. 3. Comparison of the values of integrated density calculated by the TV camera–microcomputer system with those obtained by an x–y scanner.

Quantitation of haptoglobin polymer series

The integrated density of each haptoglobin spot in Fig. 2A was calculated by the program "demo". A TLC scanner was also employed to measure the relative quantity of the haptoglobin spots. The values of integrated density calculated by the TV camera system were plotted against those obtained by the TLC scanner. As shown in Fig. 3, the two sets of results were in good agreement.

Application to micro two-dimensional gels

One of the advantages of the TV camera system is that high resolution is obtained simply by magnifying the video image. Fig. 4 shows a 12 × 9 mm section of a micro two-dimensional gel pattern of bovine brain soluble proteins, a print-out image at 47 × 35 μm resolution. The minimum spot size in the pattern was 500 μm in the direction of isoelectric focusing and 200 μm in the direction of gradient gel electrophoresis. Thus, the resolution of 47 × 35 μm was required for the analysis of spots on micro two-dimensional gels. The time needed to acquire image data is 1/60 sec, irrespective of the resolution. However, in the case of mechanical scanners, much longer time is necessary to obtain high resolution. Since the TV camera system can analyze stained gels directly, the time of data acquisition must be compared with that of flat-bed x–y scanners, which scan very slowly (at least 30 min being needed to scan a 40 × 38 mm slab gel in 50-μm steps).

Sensitivity of the system

As shown in Fig. 3, the integrated density obtained by the TV camera system correlated very well with that obtained by a densitometer equipped with an x–y

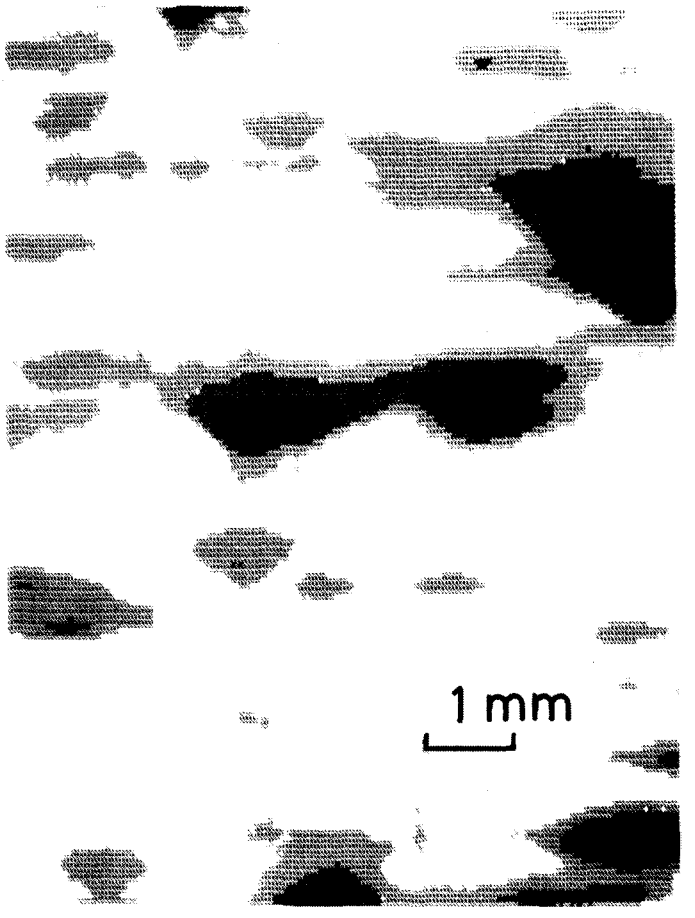

Fig. 4. A gray scale contour map of a gel section of a micro two-dimensional electropherogram, a print-out image. Bovine brain soluble proteins (20 μg) were subjected to micro two-dimensional gel electrophoresis, silver stained and analyzed by the TV camera system in 47 × 35 μm resolution.

mechanical scanner. Next we examined the sensitivity of the TV camera system. Various amounts of bovine serum albumin, ranging from 0.05 to 3.2 μg, were applied on the slots of a polyacrylamide gradient micro slab gel (40 × 38 × 1 mm), electrophoresed and the gel was stained with Coomassie Blue R-250. The gel was set on the light-box and the image data were acquired at 47 × 35 μm resolution. As shown in Fig. 5, the system could detect as little as 0.05 μg albumin. Since the albumin samples were only subjected to one-dimensional electrophoresis, the size of the 0.05-μg spot was about five times larger than the minimal spot obtained by two-dimensional electrophoresis, 0.1 mm^2. Therefore, the sensitivity of the system for two-dimensionally separated and Coomassie Blue-stained spots must be about 0.01 μg. The value of the integrated density was almost linearly correlated with the amount of albumin applied, up to 1.6 μg albumin.

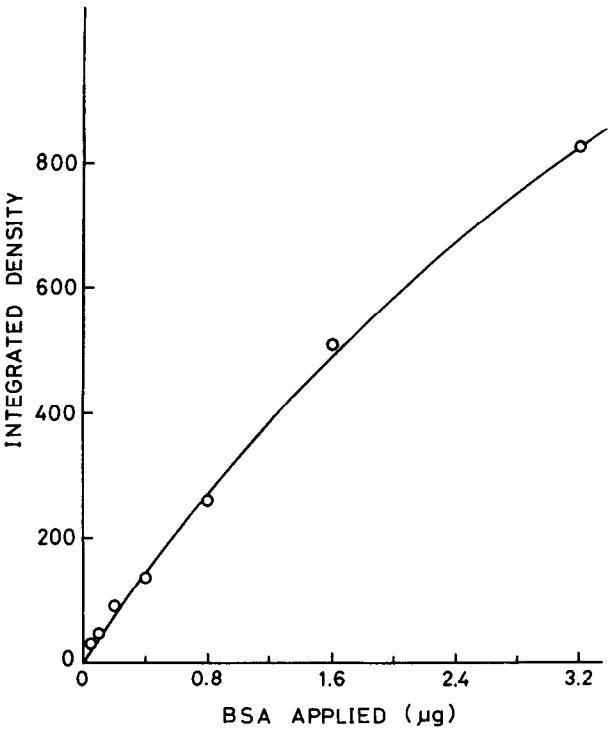

Fig. 5. Integrated density of albumin quantity standard spots. Various amounts of bovine serum albumin, ranging from 0.05 to 3.2 μg, were subjected to micro gradient gel electrophoresis, the gel was stained and the albumin spots were quantitated in 47 × 35 μm resolution.

DISCUSSION

Several reports have dealt with the quantitation of proteins on two-dimensional electrophorograms[6–9], each of which employed a mechanical scanner and a high-performance computer. These approaches are orthodox and should permit not only accurate quantitation of proteins but automated comparisons of two electropherograms. However, the quantitation systems are costly and not handy for laboratory use.

The advantages of the present system are as follows: (1) direct analysis of stained gels (not possible with a drum scanner); (2) rapid data acquisition (flat-bed scanners take time to scan especially when high resolution is required); (3) construction from commercially available equipment and software (although we slightly modified the commercial software, this is easy since the software is written in BASIC for the operator–computer interactions); (4) low cost, the whole system including all the hardware shown in Fig. 1 and the software being purchased for about $ 4000.

The sensitivity of the system was 0.05 μg protein per 0.5 mm^2 for Coomassie Blue-stained albumin spots on polyacrylamide micro slab gels. The minimum detectable quantity of proteins on two-dimensional electropherograms should depend on the minimum spot size on the slab gels (the resolution of the two-dimensional

electrophoretic technique) and the staining technique employed. The minimum spot size on macro two-dimensional gels (160 × 120 × 3 mm) was 2 mm^2 and that on micro gels (38 × 40 × 1 mm) was 0.1 mm^2. Therefore, the sensitivity of the system for the Coomassie Blue-stained proteins on macro and micro two-dimensional gels can be calculated to be 0.2 and 0.01 μg, respectively. Since our silver staining technique is at least twenty times more sensitive than Coomassie Blue staining, this system will have a sensitivity of 0.5 ng for proteins on silver-stained micro two-dimensional gels.

The values of integrated density were reproducible within 10% when a spot showed more than 100 units of integrated density per 600 dots of spot area, corresponding to 0.4 μg protein per mm^2. This level of reproducibility will be sufficient for most of biological studies, since the staining techniques have inherent limitations.

Recently, Mariash *et al.*[13] reported a system for quantitating two-dimensional radioautofluorograms employing a TV camera and a digitizer for data acquisition. However, their approach is quite different from ours. They aimed to attain an accuracy comparable with that of the mechanical scanner–high performance computer systems. They employed 64 levels of gray scale, scanned a whole 18 × 10 cm radiofluorogram at a relatively low resolution of 440 μm taking almost 1 h and calculated the quantitity of all the protein spots on the gel. In contract, we wanted to quantitate several spots of interest on multiple micro two-dimensional gels, for example, for comparisons of samples of different origin or to monitor quantities of substances after various sample treatments. Therefore, four gray levels were employed to digitize the video image, which facilitated easy programming and rapid calculation of integrated density. As shown in Figs. 3 and 5, the present system is useful in practical quantitation of stained spots on two-dimensional electrophorograms. Quantitative analysis of various changes in plasma proteins of experimental animals is now in progress.

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