

A Simplified Method for Computer Analysis of Autoradiograms from Two-dimensional Gels*

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A simple method is described for computer analysis of a discrete number of spots on autoradiograms from two-dimensional gels. The method involves digitizing the density data on an autoradiograph with a rotating drum densitometer and displaying the data on a graphics computer terminal. The software allows the operator to select the boundaries of the spots to be analyzed from the terminal, then integrates the density of the spots and tabulates the data. Graphics options allow the operator to display a computer-generated image of the area of the film being analyzed. Accurate integration of weak or overlapping spots is accomplished by a nonlinear least squares fit of the density data to normal Gaussian curves in the x and y dimensions followed by analytical integration of the equations. Since the software is written in Fortran IV and the equipment required to run the programs is available in most computing centers, this technique should allow laboratories of modest resources to quantitate information from two-dimensional gels.

ancillary services. For this reason, we have developed a simplified method for computer analysis of a small number of spots on autoradiograms from two-dimensional gels. The program displays a computer-generated image of any portion of an autoradiogram on the screen of a graphics computer terminal and allows the operator to select the regions of the x-ray film to be analyzed. This report describes the software and points out its advantages for quantifying the intensities of a small number of spots in a two-dimensional autoradiogram. The program is written in Fortran IV and is designed to run on equipment usually available in a university computing center. For these reasons, the method should be of general utility to laboratories that use two-dimensional electrophoresis to separate a few proteins of interest from complex mixtures.

MATERIALS AND METHODS AND RESULTS¹

Computer Display of the Autoradiograph—PROG-2 is used to locate, integrate, and generate an image of the density data from the autoradiogram. Its commands and their functions are listed in Table 1 (see "Materials and Methods" in the Miniprint). The sections below give examples of the use of the important commands in PROG-2 with actual data. The first step in the quantitative analysis of an autoradiograph is to locate the coordinates of the data to be integrated. This task is accomplished by having the computer display the entire autoradiograph on the Tektronix graphics terminal and then selecting the appropriate x and y coordinates needed for higher resolution images. The process of defining and resolving a series of spots is presented in Fig. 1, A-D. Fig. 1A presents an autoradiograph prepared by a 4-day exposure of Dupont Chronex 4 film to ³²P-labeled cytosolic proteins from glucagon-treated hepatocytes (12). The boxed area encompasses four major phosphoproteins ranging in $M_r = 80,000$ to 56,000 (top to bottom) and isoelectric point from about 6.0 to 6.5 (left to right). Fig. 1B presents a computer generated image of the boxed section obtained by selecting x coordinates of 359-516 and y coordinates of 289-386 with the SPOT command. For this figure, the LEVELS command was used to set the plot level at 0.04 absorbance above the film background of 0.35 absorbance. Only dots were plotted in order to obtain a rapid display (10-13 s). Each dot in this image represents the optical density reading of a 0.2-mm square section of the x-ray film (pixel) with a density of 0.39 absor-

The technique of two-dimensional electrophoresis followed by autoradiography is often used to resolve and display complex mixtures of proteins in homogenates or subcellular fractions (1-3). It is widely appreciated that the full power of this technique will be reached only when the gel systems are standardized and computer analysis of the autoradiograms is used to compile standard coordinates and intensities for a large number of proteins (4). At least four laboratories have directed their efforts to this end and have reported methods for computerized analysis of the entire autoradiogram (3, 5-7). Other workers have described computer programs for storing, transforming, or displaying the coordinates of proteins in the two-dimensional array (8, 9).

For some applications, each of the published approaches to data analysis presents shortcomings. In our work, two-dimensional electrophoresis is used to resolve the proteins whose phosphorylation state is altered following treatment of [³²P] PO₄³⁻-labeled, intact hepatocytes with hormones. Experience has shown that there are only 13 such proteins. While the computer software developed by Bossinger *et al.* (5) or Garrels (3) would allow quantitation of the phosphorylation changes, in practice these programs are unnecessarily complex and costly in terms of computer time, required hardware, and

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¹ The "Materials and Methods" and portions of "Results" (including Figs. 3 and 4 and Tables 1-3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD, 20014. Request Document No. 82M-562, cite the authors, and include a check or money order for \$4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

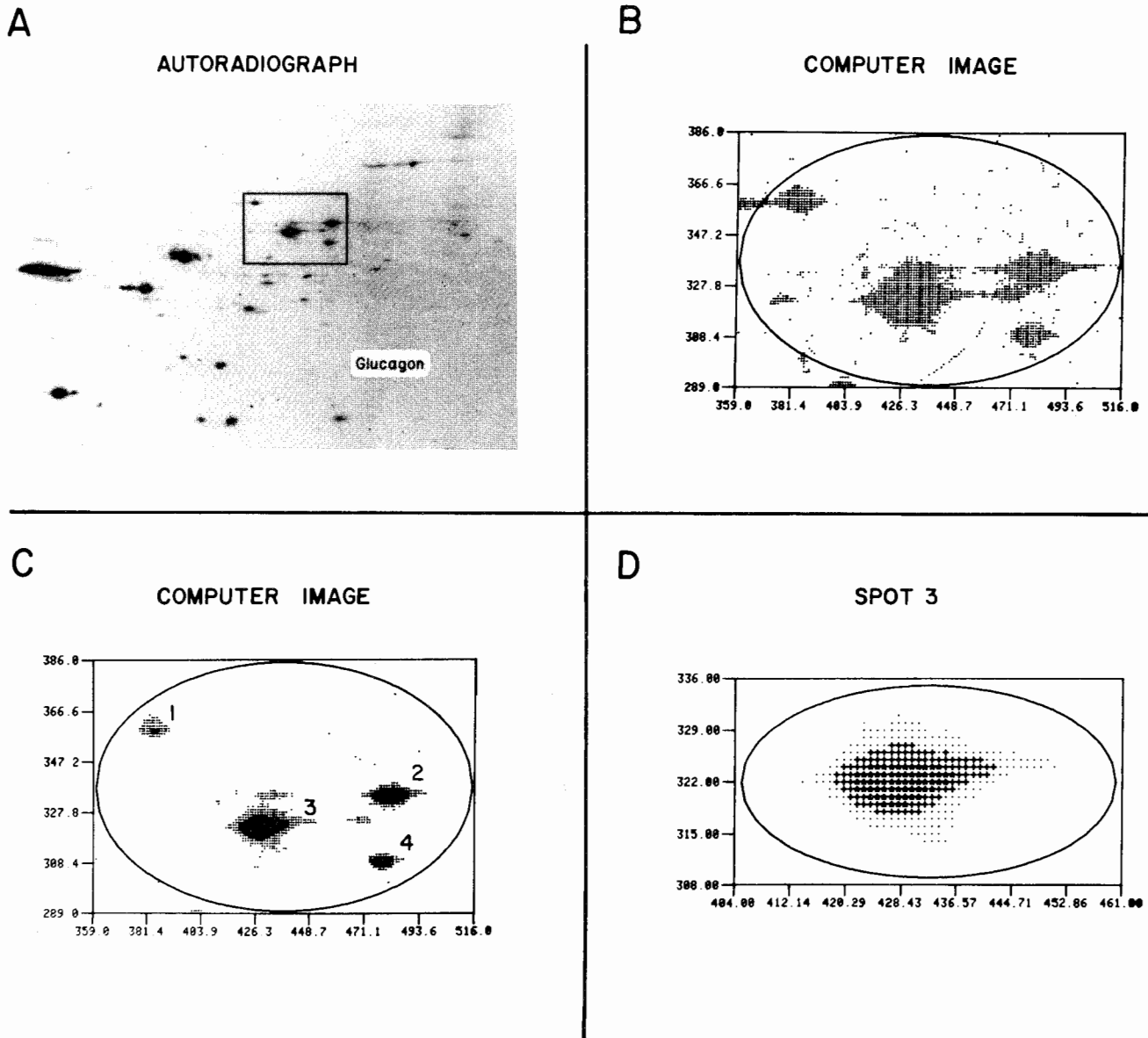


FIG. 1. Computer imaging of an autoradiograph. *A*, a two-dimensional autoradiograph made from ^{32}P -labeled cytosolic proteins isolated from glucagon-treated hepatocytes. The molecular weight dimension is vertical and the isoelectric focusing dimension is horizontal with the acidic end on the left. *B*, a computer-generated image of the boxed section of the autoradiograph. Each dot represents a 0.2-mm square area of film (pixel). The computer plots dots for those pixels with optical densities 0.04 or more above film background of

0.35 absorbance. *C*, computer image of the same film area shown in *B* with the plot level raised to 0.09 absorbance above background and crosses and stars plotted in place of dots for regions of higher density. *D*, a computer image focused on Spot 3 from *C* obtained by changing the *x* and *y* coordinates with the SPOT command. Dots are plotted for pixels with optical density values of 0.44–0.60, crosses for pixels with optical density values between 0.60 and 0.70 and stars for pixels with optical density values above 0.70.

bance or greater. Pixels with densities below 0.39 absorbance are not plotted on the screen. Fig. 1C shows the use of the LEVELS command to enhance resolution and contrast of the same film area. To obtain this image, the plot level was raised to 0.09 absorbance above film background with dots plotted for pixels corresponding to densities between 0.44–0.63 absorbance, crosses plotted for pixels with densities between 0.63–1.02 absorbance, and stars plotted for pixels with densities above 1.02 absorbance. Proper use of the LEVELS command produces an image closely resembling the original autoradiograph and rapidly provides a three-dimensional impression of the density contours on a two-dimensional screen. Substantial image enhancement of weak spots can also be obtained with this command. Fig. 1D shows further use of the SPOT command to resolve and integrate the density of one of the spots in Fig. 1C, Spot 3. For this image, the *x* and

y coordinates were narrowed around Spot 3 and slightly different contouring was provided using the LEVELS command, as outlined above. Note how the crosses and stars add a definite impression of density contour.

Integration of the density of the film is automatically provided for areas bounded by the square and the ellipse each time the SPOT command is used. The LEVELS command only affects the graphic output of the SPOT command; LEVELS does not affect the integration routines. Values printed with each image include: the square integral, its baseline, and the number of pixels summed; the elliptical integral, its baseline, and the number of pixels summed; the plot levels and the *x*, *y* coordinates (these details are not shown in Fig. 1). For Spot 3, these values were: square integral = 11,075 with a baseline of 0.35 absorbance and number of pixels = 1512; elliptical integral = 10,709 with a baseline of 0.35 absorbance

and number of pixels = 1116. For well separated spots, the values of the square and elliptical integrals usually agree to within 0-5%; however, the elliptical integral more closely approximates the true shape of the spot. Moreover, if necessary, the density contributions of closely adjacent spots can be minimized by selection of the x and y coordinates to cause the neighboring spots to fall outside the area of the ellipse. For strong, well separated spots, the above method of integration provides acceptable accuracy. However, weak spots and closely adjacent spots require the more complex methods of analysis available with PROG-3 (see below).

At this point, Spot 3 has been located, its image displayed, and its density values integrated. The operator may now save the information in one of three ways by: (a) making a permanent copy of the image and integral information displayed on the screen using the Tektronix 4631 Hardcopy Unit; (b) storing the square and elliptical integral values, their baselines, and the number of pixels integrated in a running table with the SAVE TABLE command; or (c) transferring the entire matrix of optical density data bounded by the x and y coordinates to a new disk file with the SAVE command. The new file can then be retrieved and analyzed using the more complex techniques available with PROG-3 (see below). Options (b) and (c) allow the operator to identify the stored data with numbers and names, respectively. Any combination of the three options can be chosen for each spot.

Use of Complex Analysis to Integrate the Density of Weak or Overlapping Spots—The above discussion presents a straightforward method of integrating spot densities. For strong spots (integrated densities greater than 200), the routines available with PROG-2 are quite accurate. Most spots on a properly developed autoradiogram fall into this category. However, weak spots (integrated densities of 100-200) and closely adjacent spots (strong or weak) cannot be accurately analyzed by PROG-2. Weak spots are difficult to integrate with PROG-2 because small changes in the film background subtracted from the integral will markedly affect the final value. (See Figs. 3 and 4 and Table 3 in the Miniprint for a

description of this problem.) Overlapping spots are not accurately integrated with PROG-2 because the algorithms cannot discriminate between the density contributions of adjacent spots. A third program, PROG-3, has been developed to integrate weak or overlapping spots accurately. This program uses nonlinear least squares curve fitting techniques to generate normal Gaussian curves describing the density distribution of a spot in both the x and y dimensions and then analytically integrates the equations. (See "Materials and Methods" in the Miniprint for a full description of this program.)

Fig. 2 describes the features of PROG-3 using Spot 4 of Fig. 1C as an example. Fig. 2A shows the image of Spot 4 generated with PROG-2. The integrated density of the elliptical integral from this analysis is 1301. The data in the area of the film bounded by the square is stored with the SAVE command, retrieved by PROG-3, and the density of the spot integrated. The value obtained is 1321, a result within 2% of that obtained with PROG-2. Fig. 2B shows the contour plot (one of PROG-3's plot routines) of the Gaussian curves fit to the data. The three ellipses represent constant optical densities of 0.40, 0.54, and 0.86, outer to inner, respectively. Fig. 2, C and D present the nonlinear least squares determined Gaussian curves (*solid lines*) and the actual data points (*stars*). Fig. 2C presents the distribution of the density values in the y dimension when the spot is scanned holding x constant at 475 or 478 (these x values are marked by *short arrows* in Fig. 2B). Note the excellent fit of the calculated curves to the actual data points. Fig. 2D presents a similar scan made in the x dimension. Scans at all other values of x and y across the spot revealed the same excellent fit (data not shown). The plots of Fig. 2, B-D are available with each use of PROG-3 and are important for determining the quality of the nonlinear least squares Gaussian fit to the experimental points.

Two important features of the algorithms used in PROG-3 provide accurate analysis of weak or overlapping spots. PROG-3 correctly integrates the density of weak spots because it determines film background as one of the fitting parameters

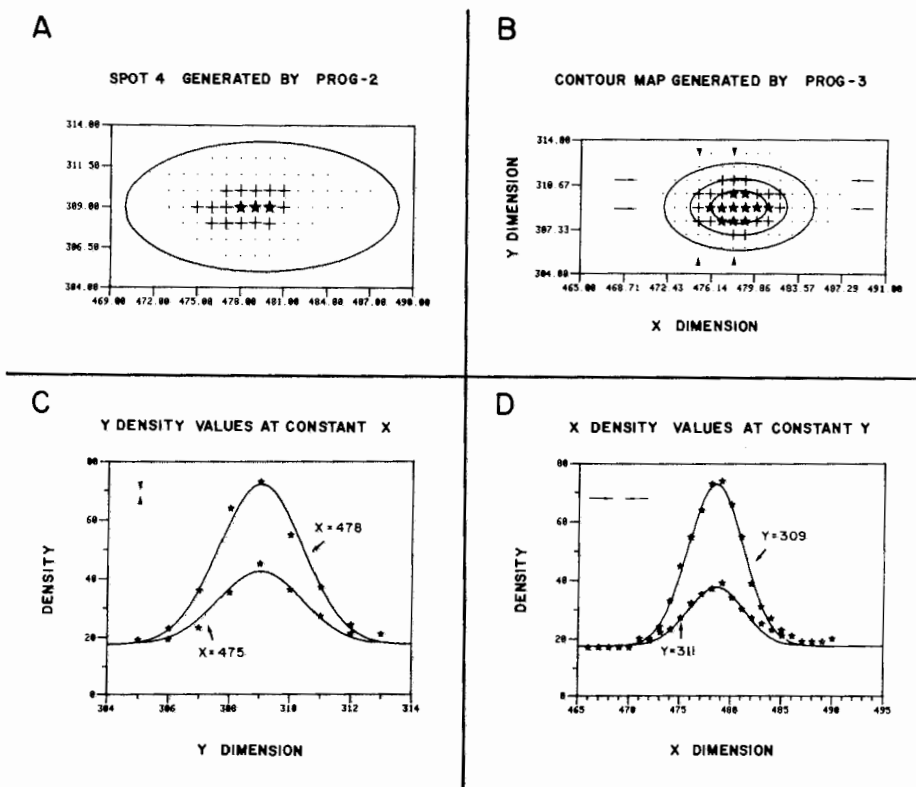


FIG. 2. An example of the use of PROG-3 to integrate a spot. A, the computer-generated image of Spot 4 from Fig. 1C after integration with the SPOT command of PROG-2. B, the same spot integrated with the algorithms of PROG-3. The figure shows a contour plot, one of the three plot routines in PROG-3. The elliptical areas represent contour lines of constant optical density. C, a plot of the y density values of Spot 4 holding x constant at 475 or 478. The solid triangles in B show the location of the sections through the spot. D, a plot of the x density values of Spot 4 holding y constant at $y = 309$ and 311. The arrows in B show the location of these sections through the spot.

required to make the spot have a Gaussian shape. This feature minimizes the background fluctuations that lead to inaccurate values for weak spots. (See Figs. 3 and 4 and Table 3 in the Miniprint for a more complete description of this function of PROG-3.) The algorithms of PROG-3 can separate the density contributions from overlapping spots because they assume that the density distribution of a given spot follows a single Gaussian distribution in the x dimension and a separate single Gaussian distribution in the y dimension. Thus, the algorithms will fit Gaussian curves to only one of a set of overlapping spots. The operator can direct the program to the spot of choice by proper selection of the spot boundaries when the data is stored for PROG-3 with the SAVE command. When the program is run, the algorithms ignore missing data or extraneous data from overlapping spots and provide the complete integral for a single Gaussian-shaped spot. Supporting data for this function of PROG-3 and other details about its uses can be found in the miniprint supplement.

DISCUSSION

While the technique of two-dimensional electrophoresis is widely used to resolve complex mixtures of proteins, quantitative analysis of the patterns obtained is not common. Among the main reasons for this situation is the large technical and financial investment needed for automatic analysis of the data in autoradiograms of two-dimensional gels (3-7). While this advanced technology is clearly required to be able to analyze and compare hundreds or thousands of spots, many laboratories use two-dimensional gels to separate a few proteins of interest from complex mixtures. The computer approaches described in this report offer many advantages that allow rapid, accurate quantitation of 10-50 spots in an autoradiogram. These advantages include simplicity, versatility, and low cost, yet the computer programs allow use of the full power of the two-dimensional technique. The obvious disadvantage of the approach is that the analysis is not automatic; operator intervention is required to select the area of the film to be analyzed. However, operator intervention also provides versatility that allows the program to be used for other purposes.

The main advantage of the software is that it is written in Fortran IV and is designed to run on a university computing center's main frame computer (a Control Data Corporation Cyber 730 in this case). Thus, the hardware required (disk drives, tape drives, central processor, and the graphics terminal) are owned and maintained by the computer center. The speed, memory capacity, and versatility of this type of system far exceed those of the mini-computer systems often used for analyzing two-dimensional gels (5, 7). Moreover, this type of facility should be available in most university or research settings for a nominal usage fee. The high speed densitometer needed to scan the autoradiographs is perhaps less widely available. However, since the output of this unit can be stored on magnetic tape, it is quite practical to travel to an available, off-site densitometer and scan many autoradiographs in one session. The tapes can then be analyzed on-site over a longer period of time. It should be noted that, while the software described in this report was developed using one particular hardware configuration, the main advantages of the method are independent of the computer equipment used. If the three basic items of hardware are available, the programs should allow laboratories of modest resources to use the full power of the two-dimensional technique.

A second advantage of the software described in this report is that the algorithms used to integrate the density of the spots can be tailored to meet the complexity of the integration. For well separated spots of moderate to high density, the simple summing routines of PROG-2 provide rapid, accurate

integration of the spot densities. Experience has shown that this mode of integration, combined with appropriate use of the BASE VALUE command to hold the subtracted background constant, can be used to integrate 80-90% of the spots in an autoradiogram. This mode of operation is very efficient because it uses little central processor time. Moreover, since the boundaries of the spots are selected interactively by the operator, the computer does not have to match or align the coordinates of spots in autoradiograms from different gels to compare two experiments. Thus, reproducibility of gel systems does not have to be as stringent as in totally computerized systems.

Weak or overlapping spots that require more complex analysis than is available with the SPOT command may be analyzed with PROG-3. This program contains algorithms that define the density distribution of a spot as Gaussian in the x and y dimensions and simultaneously estimate the appropriate background. In addition, since the equations do not require an entire spot for integration of density, PROG-3 is very useful for accurately integrating weak and/or overlapping spots (see "Results" for details). While the routines for PROG-3 could be used with all spots, they provide no improvement in accuracy for well separated spots of moderate to high density. Since PROG-3 requires more central processor time than PROG-2, its use is best reserved for weak or overlapping spots. The combined use of PROG-2 and PROG-3 can quickly and accurately process 10-50 spots of all types on a typical autoradiogram.

The final advantage of the software presented in this report is its versatility. Since the SPOT command of PROG-2 makes no assumptions about the area selected to be integrated, it can be used to integrate densitometric data from sources other than two-dimensional gels. As noted in the Miniprint, PROG-2 has been used to integrate direct positive transparencies made from the stained proteins on two-dimensional gels, long thin areas (1 × 15 mm) in one-dimensional gels used in nucleic acid research and autoradiographs from two-dimensional peptide maps made on thin layer cellulose plates. Other uses are certainly possible.

The authors will provide documented copies of the program to interested parties on request.

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SUPPLEMENTARY MATERIAL TO

A Simplified Method for Computer Analysis of Autoradiograms from Two-Dimensional Gels

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MATERIALS AND METHODS

Required Hardware: The computer programs described for analyzing autoradiograms from two-dimensional gels require access to three major pieces of equipment: 1) a high speed densitometer capable of scanning and recording the optical density of the X-ray film at raster sizes of 200 μ or smaller; 2) a large mainframe computer; 3) a graphics computer terminal. The actual equipment used in this work included: 1) an Optronics P-1000 densitometer and its integral tape drive; 2) the Control Data Corporation Cyber 730 computer at the University of Virginia Academic Computing Center; 3) a Tektronix 4006 graphics terminal linked to a Tektronix 4631 Hardcopy Unit. The Hardcopy Unit is not actually necessary but greatly facilitates storing the results of the analysis. It should be noted that neither the particular models of film scanner, computer or graphics terminal nor the mode of data transfer are important. The software described could be adapted to run on a variety of different equipment.

The Optronics densitometer scans the X-ray film with a 200 μ raster size and translates optical density readings of 0.2-5 O.D. into numerical readings ranging from 0-255 at a 0.2 mm square area of the film (pixel) with an optical density of 1.00 would be recorded as a reading of 102. The autoradiograms usually measure 160 mm x 115 mm translating to 475,000 pixels per film. The primary purpose of the computer programs is to allow the investigator to manipulate the large quantity of data generated by the densitometer.

Programs and Computational Methods: The software for analysis of autoradiograms comprises three separate programs written in Fortran IV. These have been named PROG-1, PROG-2, and PROG-3. PROG-1 is a utility program and PROG-2 and PROG-3 are used to integrate the density of spots on the autoradiograms.

PROG-1 is necessary because the Optronics P-1000 scanner stores the densitometric data on a 9-track magnetic tape and our CDC Cyber 730 does not allow a time sharing user to manipulate data stored on magnetic tapes. Consequently PROG-1 transfers the data from the magnetic tapes to disk files which can be used by the interactive program PROG-2. This configuration has utility since data from many films can be left on the disk and analyzed during periods when operators are not present to load magnetic tapes.

PROG-2 is the program which is used for routine analysis of two-dimensional autoradiograms. It comprises eleven commands which allow the user to select, integrate, store and retrieve data from the autoradiograms. A listing of these commands and their uses is presented in Table I (below). The SPOT command is used to define and integrate the data in an autoradiogram by recalling regions of the film according to an X-Y coordinate system. PROG-2 assigns an X-Y coordinate to each pixel beginning at the lower left corner of the film. Increments in the X coordinates represent successive data points from a single rotation of the Optronics drum and increments in the Y coordinate correspond to each successive rotation of the drum. With each use of the SPOT command the optical density of the area selected is integrated by numerically summing the optical densities of each pixel in the chosen field. The integrated density is calculated as the total of the optical density readings in the field minus the calculated background (see below) for that field. The data are not averaged (5) as we generally use a fine grain X-ray film such as Dupont Chronex 4 or Kodak Min R. The output of SPOT is presented on the graphics terminal as symbols within the square encompassing the X-Y area selected and an ellipse bounded by the square. The ellipse approximates the spot shape and can be used to remove the density contribution of closely adjacent spots (See Results). Three symbols are used by SPOT to represent the density of a pixel; dots, crosses or stars. The density level corresponding to each symbol can be selected by the LEVELS command (See Results). The graphic output also includes the integrated densities for the square and elliptical areas, the background, the X and Y addresses bounding the square, and the number of pixels used in the integration.

TABLE I - The Commands of PROG-2

Command	Use
SPOT	Selects and integrates the region of the X-ray film to be analyzed
LEVELS	Selects the symbols printed for the optical density of each pixel
SAVE TABLE	Tabulates the spot number and integrated area after each analysis
GET TABLE	Retrieves the table generated by the SAVE TABLE command
SAVE	Transfers the data from a selected film region to a new file for PROG-3
NEW FILE	Selects another data file on the disk
BASELINE	Graphically presents the histogram used to calculate film background
BASE VALUE	Allows the operator to insert the value for film background used by SPOT
CLEAR	Resets LEVELS, BASELINE, and FILE to the default values
DUMP	Prints the original density readings for each pixel of a given area of film
END	Transfers from PROG-2 to CDC operating system

Since film background can vary in different regions of the film (5), the background level subtracted by SPOT is calculated by generating a histogram of the number of readings at each density level within the X-Y coordinates recalled by SPOT vs the density reading (0-255). The film background for the region recalled by the SPOT command is taken as the weighted average of the maximum of the histogram and the two values immediately on either side of the maximum. This calculation is similar to that used by Bossinger et al. (5). Proper functioning of this background routine requires that the area recalled by the SPOT command contains a large number of background readings. With each use of the SPOT command, PROG-2 allows a graphic display of the background histogram with the BASELINE command. BASELINE allows the operator to determine the quality of the background value subtracted with each use of SPOT. If necessary, the automatic baseline calculation can be overruled and an operator selected baseline can be inserted using the BASE VALUE command.

The above method of background calculation works well for spots with densities about 10 times the film background. However, when used with weaker spots, this background routine gives erroneously high readings. The reason for this problem is that the density readings of the spot and those of the background do not differ enough to give a sharp histogram and the calculated background is increased a small amount by the spot readings. This problem is exacerbated by the small size of weak spots and the ease with which one can select boundaries too close to the spot being analyzed resulting in a smaller number of true background readings. The consequences of small changes in background on the integration of weak spots are outlined in Fig. 4. When analyzing weak spots, problems with the background routine can be overcome by manually setting background with BASE VALUE (see above) or by using PROG-3 (see below).

Complex Analysis: To obtain an accurate integration of the density of weak or overlapping spots, one can use the more complex routines of PROG-3. To use Prog-3 the operator creates a new disk file comprising the data for one spot with the SAVE command of PROG-2. PROG-3 assumes that the distribution of densities comprising this spot in the X and Y directions can be represented by a normal (Gaussian) distribution in each direction with an additive constant representing the film background: i.e.

$$\text{density}_{X,Y} = \text{density}_{\max} \left[e^{-\frac{(X-X_{\max})^2}{\sigma_X^2}} + e^{-\frac{(Y-Y_{\max})^2}{\sigma_Y^2}} \right] + \text{Background}$$

where X_{\max} , Y_{\max} and density_{\max} are the values corresponding to the maximum point and σ_X and σ_Y are the corresponding standard deviations. Garrels and Taylor et al. have also described the fit of data from two-dimensional gels to Gaussian curves (3,7). The assumption of a Gaussian shape allows PROG-3 to generate an equation that describes the spot in the X and Y dimensions. The equation is generated by a nonlinear least-squares fit (10) of the data to the Gaussian equation to find optimal values of X_{\max} , Y_{\max} , σ_X , σ_Y , density_{\max} and Background. The equation is then integrated analytically from minus to plus infinity in the X and Y directions to find the spot density (11).

PROG-3 determines the film background as one of the fitting parameters of the least-squares analysis. Consequently, film background is determined as the optimal value required to make the spot have a Gaussian shape. This procedure employs all of the data for its determination instead of only the data around the edges of the SPOT as in PROG-2, and thus minimizes the problems experienced with the background routines of PROG-2. Since the equations described for the Gaussian distribution can be generated from a minimum of one-fourth of a spot (including the peak values), PROG-3 can also be used to integrate closely adjacent or overlapping spots. PROG-3 contains three graphics options to allow the operator to monitor the least-squares fit of the calculated curves to the experimental data. The use of these routines-PLOT X, PLOT Y, and CONTOUR PLOT is described in RESULTS.

Two-Dimensional Gel Electrophoresis: The methodology used to prepare samples, run two-dimensional gels, expose and develop autoradiograms is described in the accompanying report (12). All autoradiograms were made with Dupont Chronex 4 or Kodak Min R X-ray film having low (0.2-0.35 O.D.) and consistent backgrounds.

Expression of Results: Spot densities in RESULTS are presented as unitless numbers that represent the background corrected sum of the readings from all pixels (0.2 mm areas) in the chosen field. These readings are directly proportional to optical density. Each unit is equal to 0.0098 O.D. (1 O.D. = 102). In appropriate instances, the readings have been converted to O.D. units for clarity.

RESULTS

Validation of the Method: The programs described in this report were developed to quantitate the effect of hormones on the phosphorylation of proteins in intact hepatocytes whose ATP pools had been pre-labeled with [32 P]ATP. Within limits, changes in the phosphorylation state of proteins in these cells can be visualized via autoradiography of the 32 P-labeled proteins resolved in an acrylamide gel system (13). However, the software can be used to integrate the density information of a piece of X-ray film produced by any source, including X-rays or other B emitters such as 14 C, 35 S, or 3 H.

Primary concerns in quantitating the data from 32 P-labeled phosphoproteins are consistency and linearity of the response of the X-ray film to changes in radioactivity. To test the linearity of some X-ray films to increasing amounts of isotope, eight aliquots of 5 μ l of H₂O containing 0-3400 cpm of [32 P]PO₄ were dried on a piece of hard surfaced cardboard (to minimize absorption spreading) and exposed to 4 types of X-ray film for 20 hours. The films were developed in an automatic processor, scanned on the Optronics P-1000 and the densities of the eight spots integrated using the SPOT command. When the integrated densities of the eight spots were plotted versus the actual counts per minute as determined in a scintillation counter, a straight line was obtained (correlation coefficient = 1.00) using Dupont Chronex 4 film. The optical density of the spots ranged between 0.2-5 O.D.; however, application of more radioactivity or longer exposure times saturate the film and cause the response to become nonlinear. Three Kodak films, SB, XAR, and Min R also gave linear responses but with different slopes. The slopes (expressed relative to Chronex 4) and backgrounds (BKG) of these films are: Min R, slope = D.38, bkg = 0.25 O.D.; Chronex 4, slope = 1.00, bkg = 0.38 O.D.; SB, slope = 2.7, bkg = 0.53 O.D.; XAR, slope = 4.13, bkg = 0.52 O.D. The use of intensifying screens makes the response non-linear. In actual practice, it is not difficult to adjust the exposure times of experimental, 32 P-labeled proteins to keep film response within the linear range.

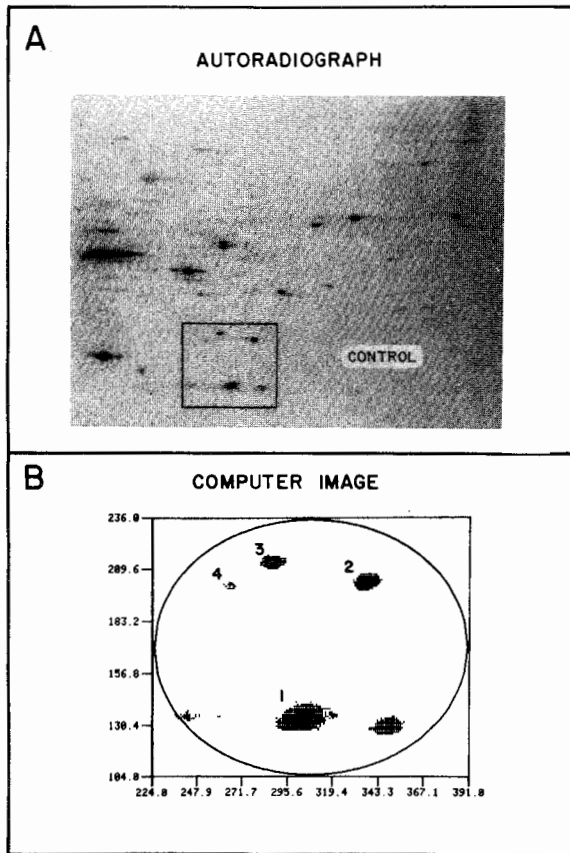
The reproducibility of the Optronics P-1000 scanner has been checked by scanning an autoradiogram 3 consecutive times and analyzing the same six spots in each scan. The six spots were chosen to represent a 15 fold variation total integrated density. The differences in integrated density between replicate scans varied between 0.07-0.5% of the total spot density with an average of 0.3% (n=18).

Table 2 uses the densities of the spots labeled 1-4 in Figure 1C to provide a typical example of the use of PROG-2 to analyze the effect of hormones on protein phosphorylation. The integrated densities were obtained for each spot using the SPOT command. The data was tabulated with the SAVE TABLE command and retrieved with the GET TABLE command. The table shows that the density (phosphorylation state) of Spots 1,3 and 4 increased 7-13 fold following the treatment of intact cells with glucagon. These four spots were also analyzed by the more complex techniques available in PROG-3 (see below) with essentially identical results. Note that the phosphorylation state of Spot 2 is not changed by treatment of the cell with hormones. There are many such proteins in a typical autoradiographic pattern providing important internal controls to demonstrate that control and hormone treated cells are labeled to equal specific activities; equal amounts of protein are loaded onto the two-dimensional gel system; and film and scanner responses are constant in each experiment. It is important to analyze several such proteins in each experiment to provide these assurances.

Table 2. Example of the use of PROG-2. The cytoplasmic proteins from control and hormone treated cells were resolved in two-dimensions as described (12). The autoradiograms from each gel were analyzed with PROG-2 and the SPOT command as described in the text. The integrated spot densities correspond to the density readings summed for all pixels in the spot (see Methods for Details).

Spot Number	Spot Density Control	Spot Density 100 nM Glucagon	Density Glucagon/Control
1	133	986	7.41
2	3421	3583	1.05
3	1614	10709	6.63
4	99	1301	13.14

Analysis of Weak Spots: If at all possible, the analysis of weak spots should be avoided by altering the protein loads, the specific activities of radioactive proteins, or the exposure times to intensify weak spots on the autoradiographs. However, because of the short half life of [32 P] some weak spots will probably require accurate analysis. This situation occurs because low levels of isotope decay before they darken the higher resolution X-ray films. The following sections demonstrate the utility of PROG-3 in the analysis of weak spots. Figure 3 shows an autoradiograph of 32 P-labeled proteins obtained from unstimulated hepatocytes. The boxed area in Figure 3A encompasses six phosphoproteins that vary 24 fold in integrated density. Figure 3B presents the computer-generated image of the area. This region of film was chosen to demonstrate the utility of PROG-3 in the analysis of weak spots. Figure 3C shows the computer-generated image of the boxed area in Figure 3A. 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The main problem with integrating spots such as Spot 4 is that small changes in the background level subtracted from the integral will markedly affect the final value (see below). The magnitude of the effect of a change in background on the integrated value also depends on the spot shape. The integral for a sharp strong spot (for example Spot 2) will be affected little by a small change in background, while that for a more diffuse spot (for example Spot 1) will be more greatly affected. However, no matter what the spot shape, the effect of a small change in background will have the greatest effect on weak spots.

The effects of small changes in the film background on the integrated density of strong and weak spots are clearly shown in Figure 4. The actual densities of Spots 1-4 in Figure 3 were used for this calculation and the assumption is made that all spots have the same geometric shape for the sake of the illustration. The position of the integrated densities of Spots 1-4 is shown at the top of Figure 4 along the X-axis by the arrowheads labeled S-1 through S-4. The solid lines represent the loss of density that results as the baseline is raised by increments of 0.5%. Note that a 1-2% increase in baseline does not have a large effect on strong spots such as Spot 1. However, a 2% change in the baseline can reduce the density of a weak spot such as Spot 4 to 40% of its original value. Experience has shown that 0.5-1% increases in the baseline do occur when analyzing weak spots with PROG-2 because the slow rise above film background slightly skews the background calculation to higher values.

Table 3 compares the use of PROG-2 and PROG-3 to integrate the four spots labeled 1-4 in Figure 3. The baseline in this area of the film is 35.72 (or 0.35 O.D.) measured in large blank areas of the film. The table shows the integrated densities calculated by each program, the baselines obtained and the deviation from the film background of the actual yield equivalent densities with minimal (0.3-0.9%) deviations of the calculated background from the established film background. However, as the spots become less dense, the baseline calculated with PROG-2 begins to rise and the integrated densities fall to 85% (Spot 3) and 50% (Spot 4) of those calculated with PROG-3. Note the close correspondence between the actual loss of density demonstrated with weak spots in Table 3 and the losses predicted from the calculations presented in Figure 4. The integrated densities of weak spots obtained with PROG-3 are higher (and more correct) than those obtained with PROG-2 because PROG-3 gives a more accurate (lower) calculation of background.

TABLE 3. Comparison of the Integrated Spot Densities of Spots 1-4 in Figure 3 obtained using PROG-2 and PROG-3. The integrated densities of the four spots labeled in Figure 3B were obtained using PROG-2 and PROG-3 as described in the text. The column labeled % deviation from film background is calculated as the difference in the program calculated background for each spot from the "true" film background of the area, 35.72 (0.35 O.D.) obtained from large blank areas of the film.

Spot No. Fig. 3B	Integrals Calculated with PROG-2			Integrals Calculated with PROG-3			
	Density	Base line	% Deviation from film bkg	Density	Base line	% Deviation from film bkg	Density PROG-2 / Density PROG-3
1	4804	36.04	0.9%	4414	36.04	0.9%	1.08
2	1777	35.79	0.2%	1769	35.82	0.3%	1.00
3	783	37.57	5.2%	932	36.04	0.9%	0.84
4	129	36.64	2.6%	253	35.72	0.0%	0.51

If PROG-2 is used for analysis and background level is held constant at 35.72 with the BASE VALUE command, the integrated spot densities obtained with the SPOT command are within 5% of those obtained with PROG-3 for all 4 spots (data not shown).

Analysis of Overlapping Spots: The methods of calculation used by PROG-3 to fit Gaussian curves to the data points do not require data from the entire spot. As long as the peak of the spot is in the field stored by the SAVE command, PROG-3 will generate the correct value for the integrated density. This feature allows the operator to calculate the integrals of overlapping spots. The use of PROG-3 in this manner can be demonstrated using Spot 2 from Table 3 as an example. The integrated value of Spot 2 is 1769. Using the SPOT command, approximately 1/4 of the density data comprising the spot (including the peak) was displayed and then stored with the SAVE command. The integrated density of this area was 492. The stored data were indexed by PROG-3 and the spot integrated by the least-squares fit giving an area of 1706. This value agrees to within 3-4% of that obtained by integrating the entire spot. In a more demanding example, the areas of two overlapping spots were analyzed using PROG-3 and then compared with the area of the entire complex analyzed by PROG-2. In this case the areas of the two spots were 1459 and 8981 after analysis by PROG-3. The summed areas of the two spots obtained with PROG-3, 10,440 agrees to within 5-6% of the area of the complex obtained with PROG-2, 11,051.

Other Uses: While the sections outlined above describe the use of the program to analyze autoradiograms from two-dimensional gels, the programs are fully capable of analyzing other types of densitometric data. For example, we have used PROG-2 to analyze the spot densities of Coomassie blue stained gels by making direct positive transparencies of the gels and scanning the photographic film. PROG-2 has also been used to integrate the density of very long thin bands (1 mm by 15 mm) on one-dimensional gels used in nucleic acid research and to analyze autoradiograms from two-dimensional peptide maps. In each of these instances, the SPOT command of PROG-2 was used to enclose the area to be integrated. Since the integration routines in this mode do not make any assumptions about the shape of the spot, PROG-2 is very useful for this type of analysis.

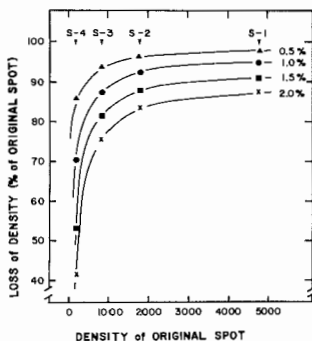


Figure 4. The effect of raising film background on the integrated density of strong and weak spots. The computer calculated densities of Spots 1-4 in Figure 3B are indicated on the X axis as the arrowheads (▼) labeled S-1, S-2, S-3, S-4. (see Table 3 for the actual integrated values). The lines show the calculated loss of density that occurs if the film background subtracted in the original calculation were to be increased by 0.5% (▲), 1.0% (●), 1.5% (■), or 2.0% (X). The loss of density observed as the background increases is plotted on the Y axis. All spots are assumed to have the same geometry for the purpose of this illustration.