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COMPUTER-ASSISTED OPTIMIZATION IN ANALYTICAL CHEMISTRY

An Abstract of a Dissertation
Presented to
the Faculty of the Department of Chemistry
University of Houston

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Lloyd R. Parker, Jr.

August 1978

ABSTRACT

Optimization of a response from an experimental chemical system is shown to be an important tool in analytical chemistry. Both the improvement of the response (through the use of the sequential simplex algorithm) and the understanding of how the factors or input variables affect that response (through the use of multivariate and univariate mapping designs and regression analysis) are important parts of the optimization. Three studies are included: the optimization of resolution in a gas chromatographic system (within a given time constraint); the optimization of sensitivity in a colorimetric continuous-flow method for calcium (while maintaining other responses within certain limits); and the investigation into a method for analysis of phenylephrine, a pharmaceutical compound.

Automation and computer control of experimental systems are shown to be useful in optimization procedures. The interface built to allow computer control of laboratory instruments is described, and BASIC programs illustrating the use of the computer and interface in the three studies are included as Appendices.

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CHAPTER I

INTRODUCTION TO OPTIMIZATION

In most analytical methods, there are a number of responses, or outputs, which are important. For a spectrophotometric method, sensitivity and linearity are two responses of interest; in chromatography, important responses would include resolution and retention time. Optimization of these responses can be valuable to the chemist, since the responses affect the quality and usefulness of the method.

Optimization is usually taken to mean an increase in some quantity. Thus, optimization of the sensitivity of a method to an analyte indicates an increased sensitivity. This is usually desirable, because increased sensitivity usually means lower detection limits and the ability to detect smaller changes in the concentration of the analyte. Similarly, optimization of resolution in chromatography indicates an increased separation of components in a mixture, which means qualitative and quantitative evaluations are easier.

Optimization can also be minimization, however. To improve linearity, the sum of squares of residuals is minimized, leading to a more reliable method. In chromatography, a minimum retention time is desirable, not only for operator convenience and greater sample throughput, but also to reduce peak broadening (1). Often, an optimization will be performed to simultaneously maximize one

response and minimize another response; for example, to maximize the sensitivity to the analyte and reduce the sensitivity to an interferent.

An overall optimization scheme in shown in Figure 1. The first task is represented by the apex of the triangle; obtain a response. This task must be performed by the chemist, relying on theory, intuition, trial-and-error experimentation, established methods, or any other means (2). The response can be thought of as the output of a transform (Figure 2). Various factors, or inputs, are acted upon by the transform, which may be known or unknown. A simple example is shown in Figure 3. Here, a mathematical equation, $z = 3x + 2y$, is the transform, x and y are inputs, and z is the output. The transform takes, as inputs, values of x and y, and transforms them into values of z, the output. The input(s), transform(s), and output(s) are collectively referred to as the system (3).

More complicated methods can also be visualized in this system approach. For example, Figure 4 shows an organic synthesis system. The inputs are the levels of the various reagents, temperature, and pH; the outputs are the per cent yield of product and the per cent yield of an undesirable impurity. The transform itself need not be known to the chemist.

Once a method for obtaining a response has been se-

FIGURE 1

OVERALL OPTIMIZATION SCHEME

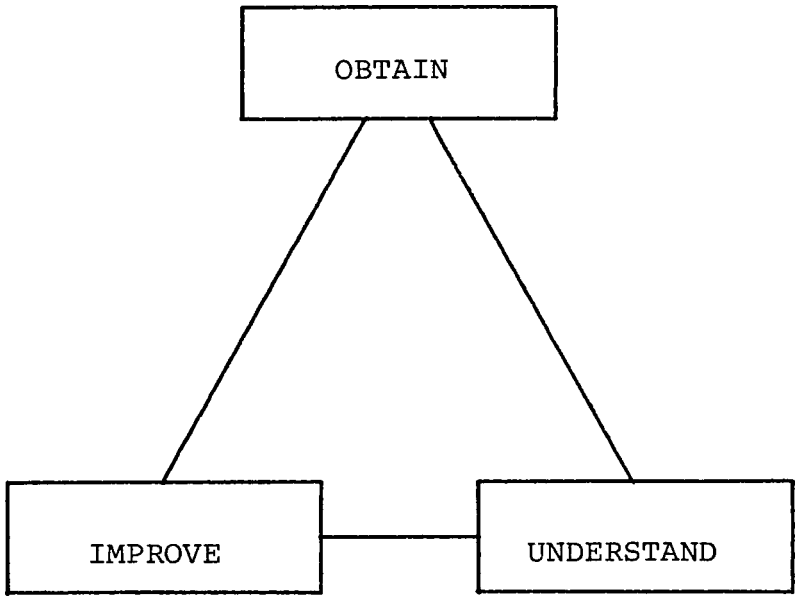

OBTAIN
IMPROVE
UNDERSTAND

FIGURE 2

SYSTEMS THEORY DIAGRAM

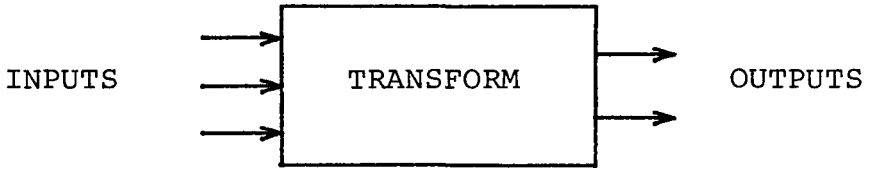

INPUTS
TRANSFORM
OUTPUTS

FIGURE 3

MATHEMATICAL TRANSFORM

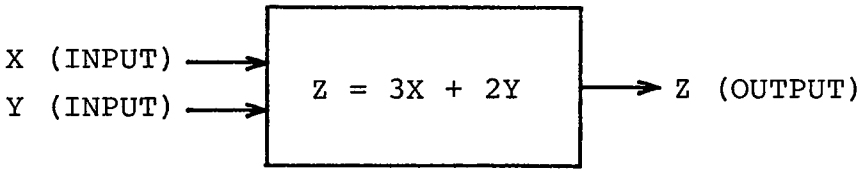
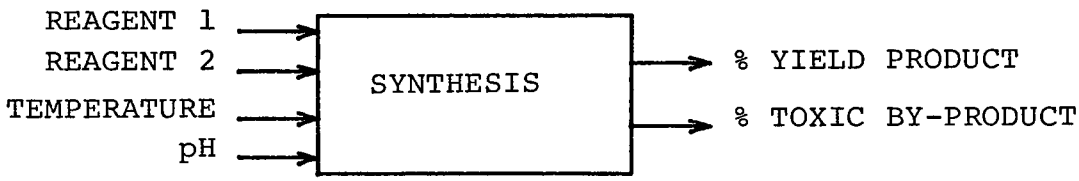

X (INPUT)
Y (INPUT)
Z = 3X + 2Y
Z (OUTPUT)

FIGURE 4

ORGANIC SYNTHESIS TRANSFORM


REAGENT 1
REAGENT 2
TEMPERATURE
pH
SYNTHESIS
% YIELD PRODUCT
% TOXIC BY-PRODUCT

lected, the next step is at the lower left corner of the triangle (see Figure 1): improve the response. There are a number of algorithms which will perform this task.

The third stage is the understanding of the response or responses. This step is important for three reasons: (1) Tolerances can be specified for each factor (4); maintaining the factors at their optimum levels within certain tolerances will maintain the optimum response. If it is found that a response is relatively insensitive to a factor at the optimum, that factor need not be tightly controlled. (2) Trade-offs may be possible among certain factors or responses (5). Thus, if a certain level of factor A and a level of factor B combine to give an optimum response, and factor A is more expensive, it might be that decreasing the level of A and increasing the level of B would give essentially the same response at a lower cost. Also, if the sensitivity of a method is more than adequate, it might be possible to adjust the factors for less sensitivity but improved linearity. (3) In chemistry, as in science in general, mere improvement of a response does not really contribute to the advancement of knowledge; on the other hand, if the optimization leads to a more complete understanding of how the factors affect the response, then true chemical knowledge has been gained (6). In fact, understanding a response after it has been improved and the im-

provement stage should be considered as integral parts of optimization.

METHODS FOR IMPROVING THE RESPONSE

There are a number of strategies employed in optimization (7). The most common is the univariate search. Figure 5 shows such a search routine on a response surface. (The lines shown are contour lines; these are intially unknown.) Although a two-factor response surface is shown, the method is general and can be applied to any number of factors. All the factors are set to predetermined levels, and one factor is varied. The level of that factor which gives the best response is found and that factor is fixed at that "best" level. A second factor is then varied (hence the other name for this technique, "one-factor-at-a-time"). Often, after searching each factor once, the search is halted and the resulting set of conditions labelled as the "optimum." If the strategy is more sophisticated, more iterations of the search may be employed, which, as in Figure 6, move the set of conditions closer to the true optimum.

However, Figures 5 and 6 present a very idealized situation. Here, there is no interaction among the factors (the contours are either circular, or, if elliptical, have their axes parallel to the factor axes). This is not usually the

FIGURE 5

UNIVARIATE SEARCH

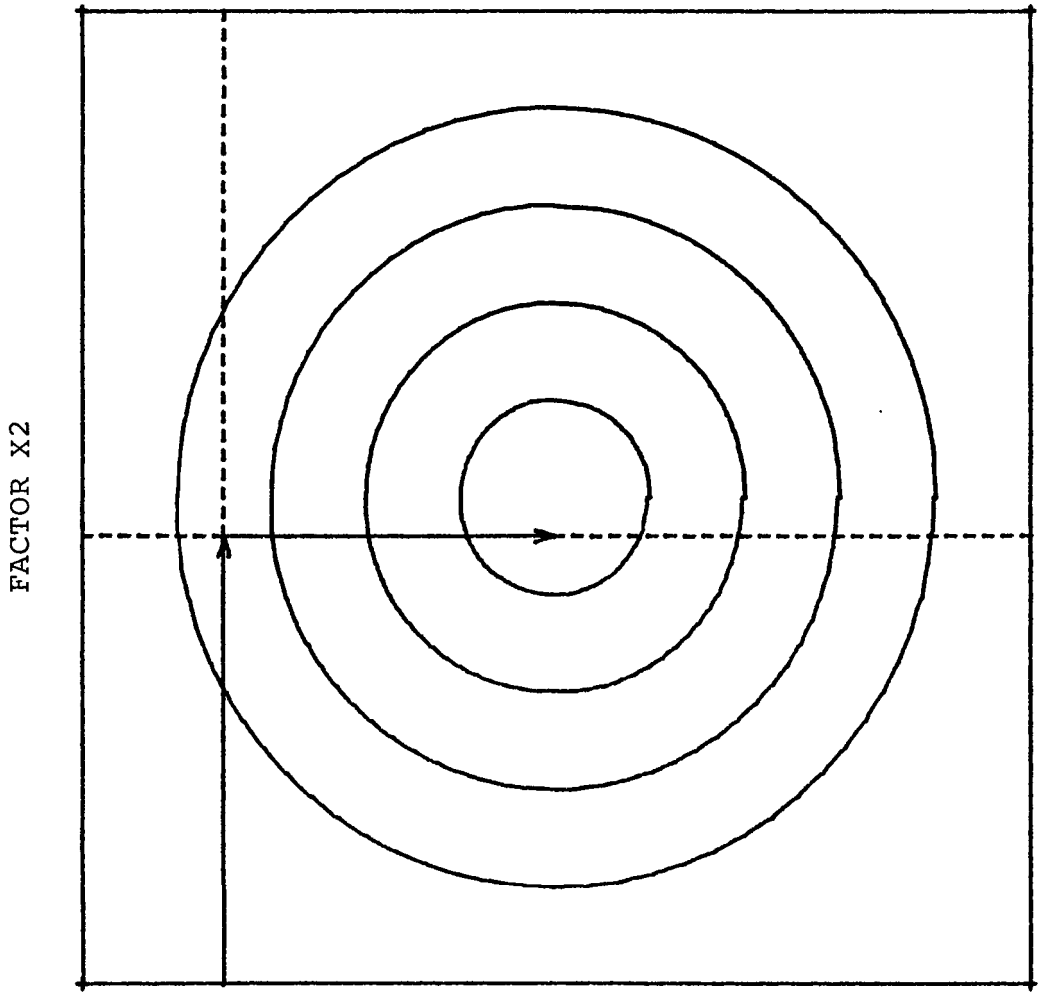
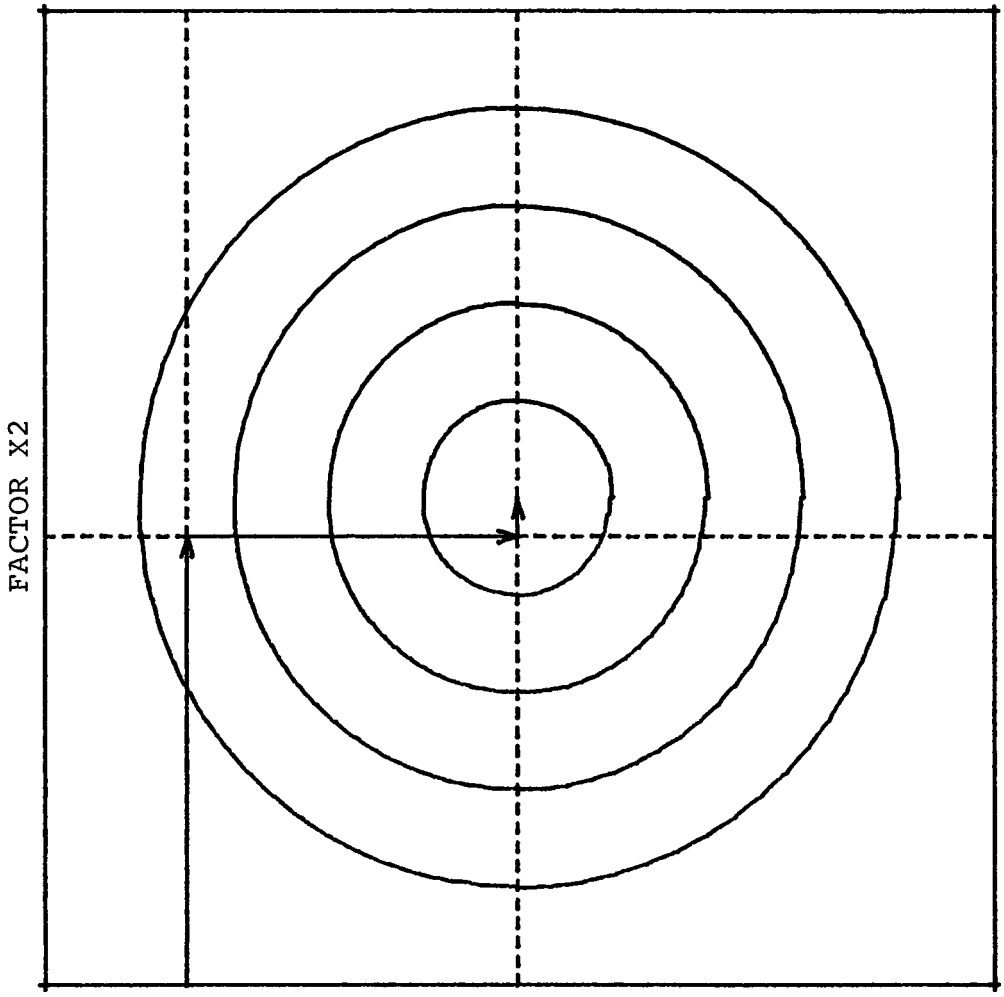

FACTOR X2
FACTOR X1

FIGURE 6

UNIVARIATE SEARCH WITH SECOND ITERATION


FACTOR X2
FACTOR X1

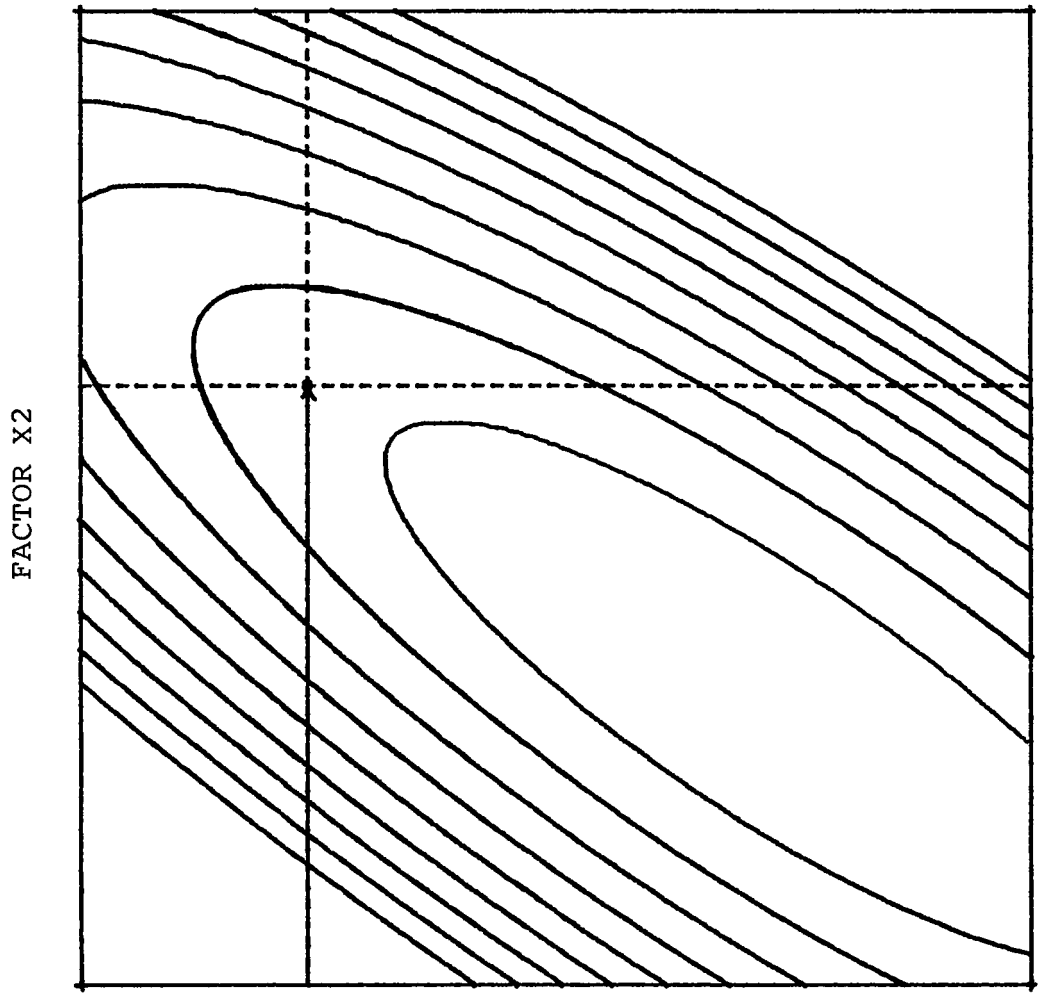
case in a real-world, experimental situation, where interactions often occur among factors. Figure 7 shows this type of response surface. The contours are rotated with respect to the factor axes; this procudes a diagonal ridge in the response surface. (An example of such a ridge occurs in atomic absorption spectroscopy, where the air flow rate and fuel flow rate interact to produce a ridge corresponding to an optimum air-fuel ratio (8).) A univariate search will fail on this type of response surface as the Figure shows: As factor 2 is held constant and factor 1 varied, the "best" response will occur at the point which the search crosses the ridge. If factor 1 is held constant at this level and factor 2 varied, the "best" response will occur at the same point. The "optimum" will be found at the point where the first search crosses the ridge, although this point is not necessarily near the true optimum. The univariate search, then, becomes stranded and fails when applied to a system with factor interactions (5).

The alternative to a univariate search is a multivariate search, in which the levels of all the factors are varied siumltaneously. There are three principal multivariate search strategies which are well-suited for experimentation (as opposed to numerical problem solving):

(1) Method of steepest ascent (9). Using this method, a small number of experiments (or treatment combinations)

FIGURE 7

UNIVARIATE SEARCH ON A RESPONSE SURFACE WITH INTERACTION


FACTOR X2
FACTOR X1

is performed in a limited region of the available factor space. A plane is fit to the responses from these experiments:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n \qquad (1)$$

where y is the observed response, x_i is the level of the i-th factor, and β_i is a regression coefficient. A total of (n+1) points must be evaluated, and the plane is fit as follows:

$$\underline{X} = \begin{bmatrix} 1 & x_{11} & x_{21} & \cdots & x_{n1} \\ 1 & x_{12} & x_{22} & & x_{n2} \\ \vdots & \vdots & \vdots & & \vdots \\ 1 & x_{1n} & x_{2n} & & x_{nn} \\ 1 & x_{1(n+1)} & x_{2(n+1)} & & x_{n(n+1)} \end{bmatrix}$$

$$\underline{Y} = \begin{pmatrix} y_1 \\ y_2 \\ \cdot \\ \cdot \\ \cdot \\ y_n \\ y_{(n+1)} \end{pmatrix} \qquad \underline{\beta} = \begin{pmatrix} \beta_0 \\ \beta_1 \\ \beta_2 \\ \cdot \\ \cdot \\ \cdot \\ \beta_n \end{pmatrix} = (\underline{X})^{-1}\underline{Y} \tag{2}$$

where $x_{\underline{jk}}$ is the level of the $\underline{j}$-th factor at the $\underline{k}$-th experiment. The partial derivatives of y with respect to the factors give the relative steepnesses of the plane in the factors, and are given by the $\underline{\beta}$ matrix:

$$\begin{aligned} \delta y/\delta x_1 &= \beta_1 \\ \delta y/\delta x_2 &= \beta_2 \\ &\cdot \\ &\cdot \\ &\cdot \\ \delta y/\delta x_n &= \beta_n \end{aligned} \tag{3}$$

The direction to move is then given by the vector:

$$[\beta_1 \quad \beta_2 \quad \cdot \quad \cdot \quad \cdot \quad \beta_n] \tag{4}$$

After moving along the vector, a new set of experiments is conducted and the algorithm performed again. There are

three problems with this technique: (1) A model must be fit to data -- with several factors, a computer becomes a necessity; (2) the method gives no indication of how far along the vector to move; and (3) the method ignores curvature in the response surface.

(2) Factorial-type design search (10,11). A factorial design encompasses usually either 2 or 3 levels of each factor, and consists of all combinations of the levels of all the factors. The number of experiments is given by k^n, where k is the number of levels and n is the number of factors. Figure 8 shows a 2-level, 2-factor design with 4 (2^2) treatment combinations; Figure 9 shows a 3-level, 2-factor design having 9 (3^2) points. In an optimization procedure, an experiment would be conducted at each point in the factorial design; based upon the responses, the location of the next design is determined. The next factorial design is usually situated so as to share a point or a face (hyperface in multidimensional space) with the previous design. In this manner, then, the factorial design "moves" through factor space (12).

The factorial design search algorithm specifies how far the next design goes beyond the present one, for the spacing between experiments remains the same for each design. However, the main disadvantage is that the required number of experiments increases exponentially for each added fac-

FIGURE 8

2-LEVEL 2-FACTOR FACTORIAL DESIGN

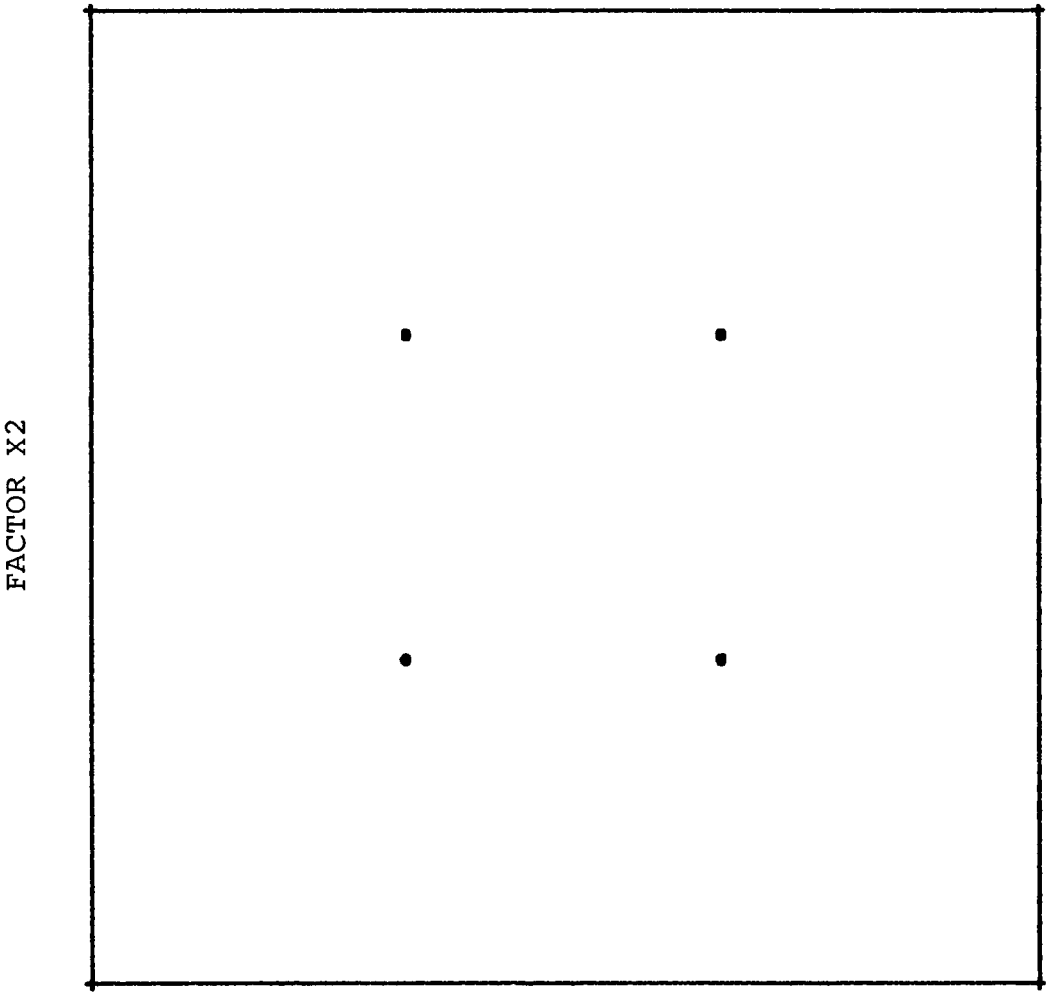
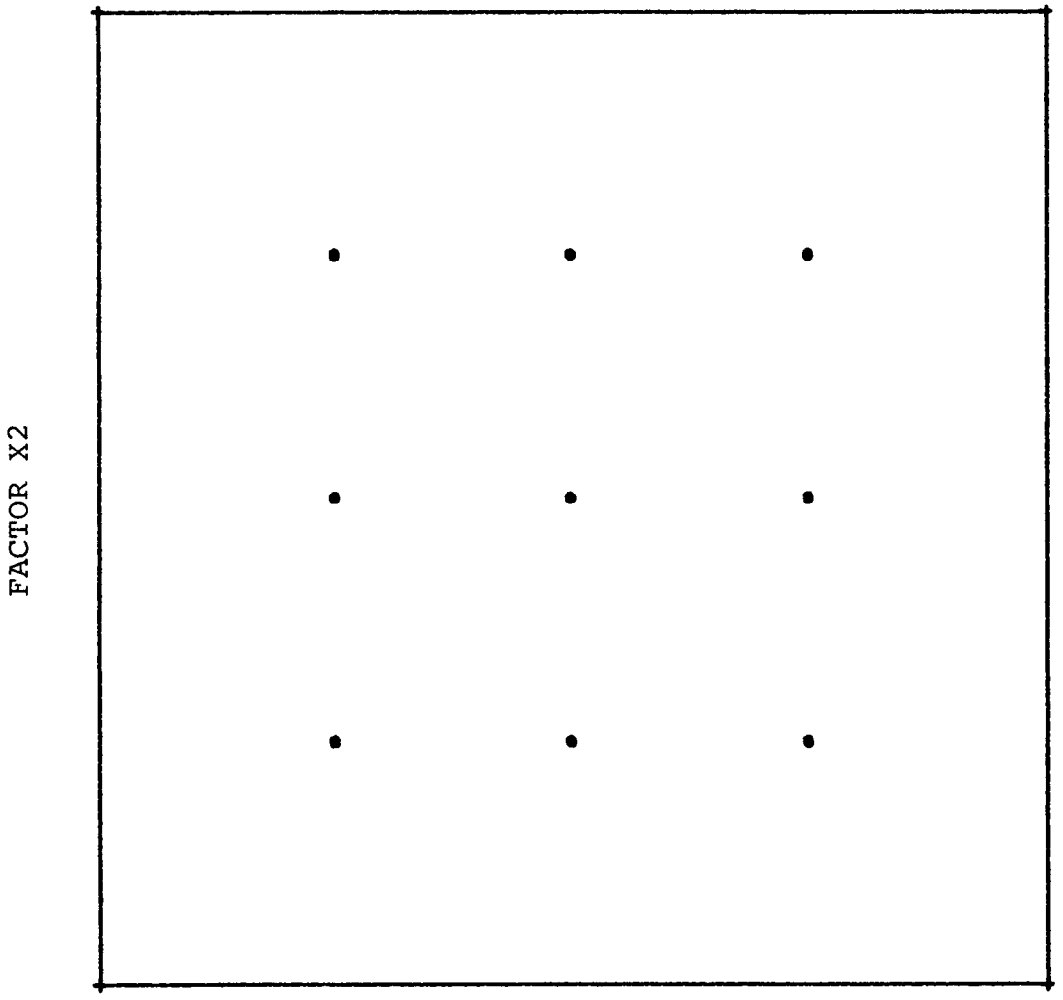

FACTOR X2
FACTOR X1

FIGURE 9

3-LEVEL 2-FACTOR FACTORIAL DESIGN


FACTOR X2
FACTOR X1

tor -- whereas a 2-level, 3-factor design requires 8 experiments (2^3), a 2-level, 4-factor design would require 16 experiments (2^4), an increase of 8 experiments per iteration. Although fractional factorial designs (13,14) do reduce the number of experiments, the factorial design still remains an inefficient optimization strategy.

(3) Simplex optimization (15,16,17,18). Simplex optimization is an efficient process: a simplex necessitates only $n+1$ experiments (where n, again, is the number of factors) to set up the initial simplex, and only 1 or 2 additional experiments for each subsequent simplex. Definitive rules specify where to move (both in direction and distance), and, unlike the method of steepest ascent, no complex calculations are involved.

CHAPTER II

SIMPLEX OPTIMIZATION

A simplex is a geometric figure defined by n+1 points. In two-dimensional space (2 factors), a simplex is a triangle; in three-factor space, the simplex is a tetrahedron. (In higher dimensional space, the simplex cannot be so visualized.) Figures 10 and 11 show 2- and 3-factor simplexes, respectively. The vertexes (each corresponding to a treatment combination, and thus an experiment) are labelled according to the following convention: B indicates the vertex with the best response (which may be either the largest or the smallest value) in the simplex, N indicates the vertex with the next-to-worst response, and W indicates the vertex with the worst response. No designation is given to the remaining (for three or more factors) vertexes.

The initial simplex must be specified by the user. The simplex vertexes can be chosen at random, although care must be taken to ensure that the vertexes are not collinear (or co-planar in the case of three dimensions), as this will restrict the movement of the simplex (in Figure 12, for example, the simplex has been reduced to a one-dimensional search along the line WNB). It is important to note that a simplex does not have to be equilateral; in fact, in an experiment where one factor may be in degrees Celsius (temperature) and another in ml min^{-1} (flow rate), the idea of "equilateral" has no validity. If the simplex was chosen so as to be an acute triangle, the two factors

FIGURE 10

TWO-DIMENSIONAL SIMPLEX

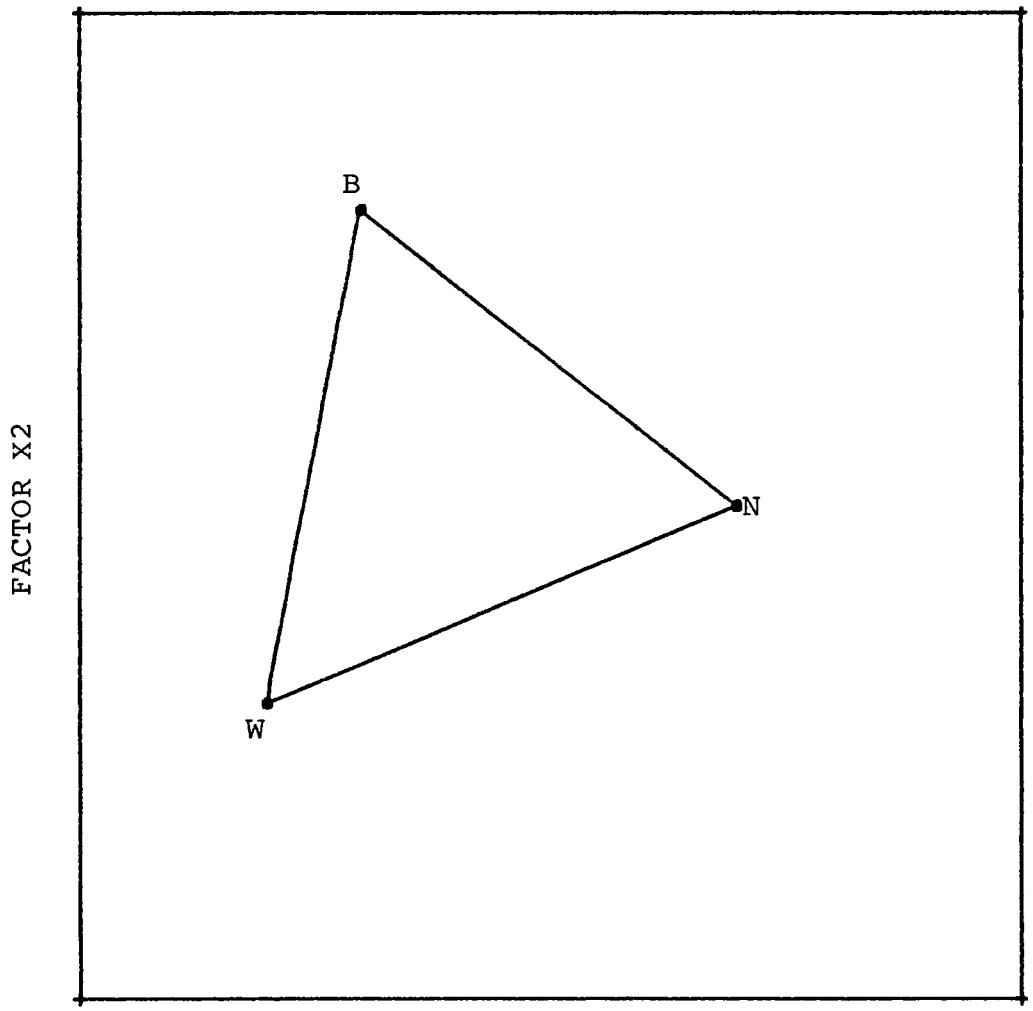

B
N
W
FACTOR X2
FACTOR X1

FIGURE 11

THREE-DIMENSIONAL SIMPLEX

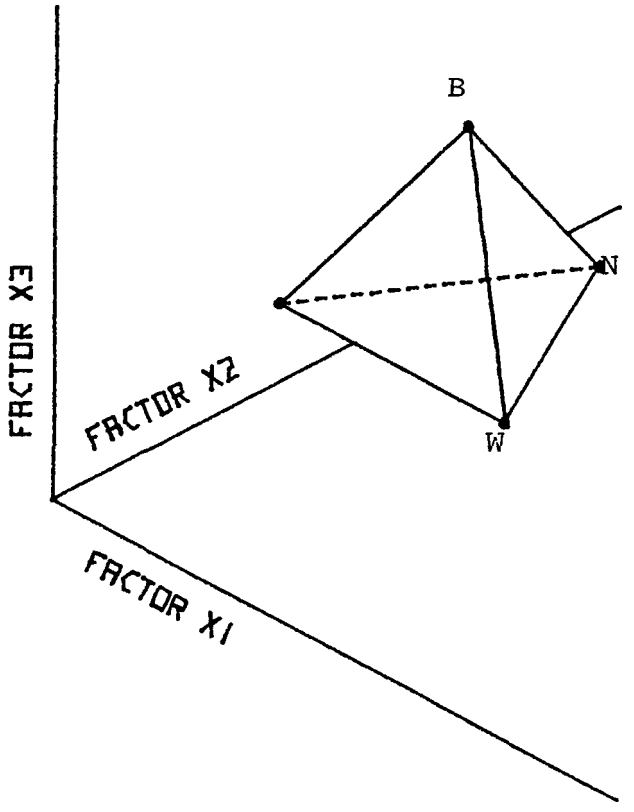
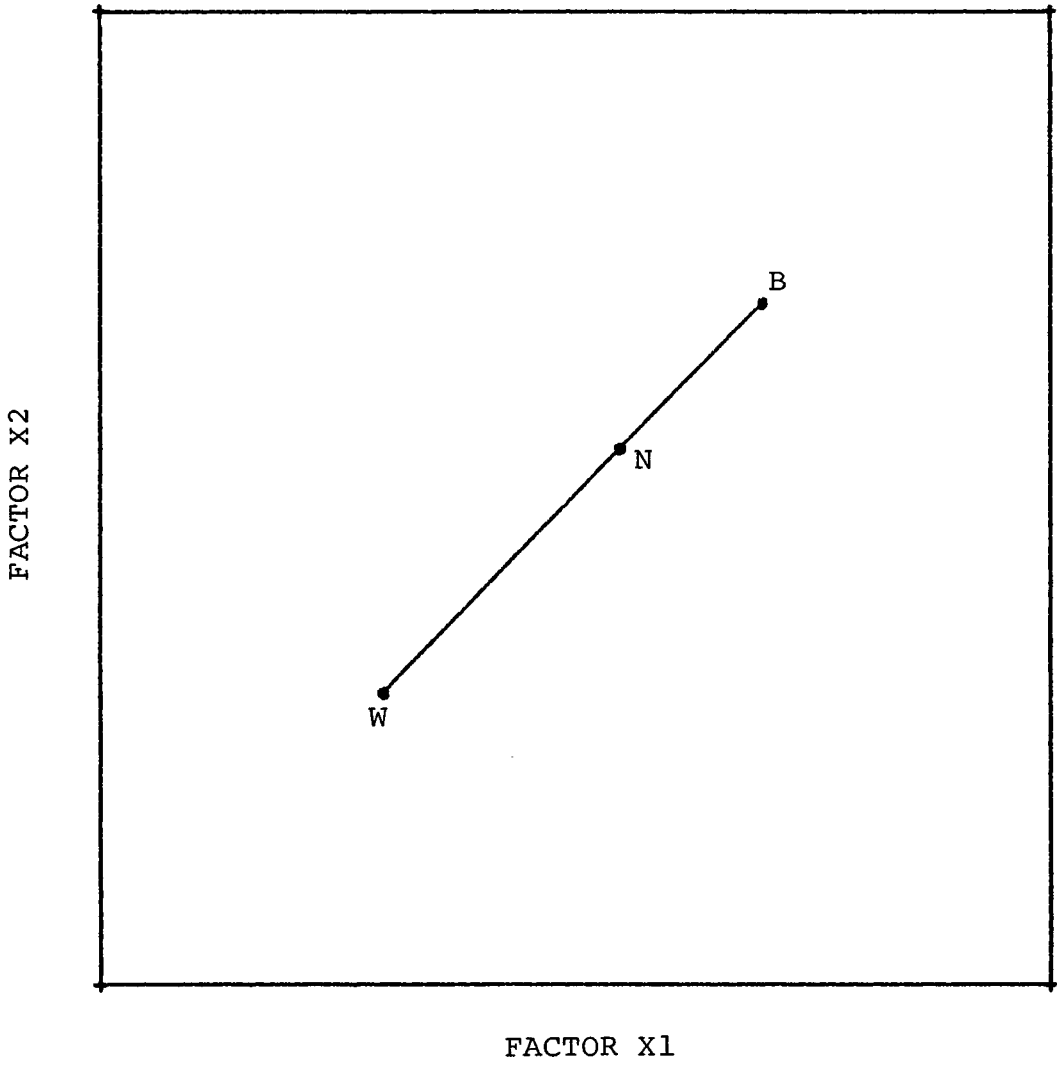

B
N
W
FACTOR X3
FACTOR X2
FACTOR X1

FIGURE 12

TWO-DIMENSIONAL SIMPLEX

WITH COLLINEAR VERTEXES


B
N
W
FACTOR X2
FACTOR X1

can be scaled so as to produce the "equilateral" simplex shown in the Figures.

To help eliminate the problem of collinearity, an algorithm was developed (16) to facilitate setting up the initial simplex. The coordinates of one of the vertexes and the step size in each factor are specified by the user, and the algorithm computes the other vertexes.

The simplex, as originally described by Spendley, Hext, and Himsworth (16), moves by eliminating the vertex with the worst response ($\underline{W}$) and creating a new vertex (experiment) designated as $\underline{R}$ (for reflection). Thus, Rule 1 states: the new vertex is created by reflecting $\underline{W}$ through the centroid of the remaining hyperface, $\bar{\underline{P}}$ (see Figure 13). The question of how far to move is thus answered: the new vertex is placed as far from the centroid of the hyperface as the old vertex, but on the opposite side. (Although only a two-dimensional simplex is pictured, and only simplexes in two factors will be used for illustrations, the algorithm is general and can be used for any number of factors.)

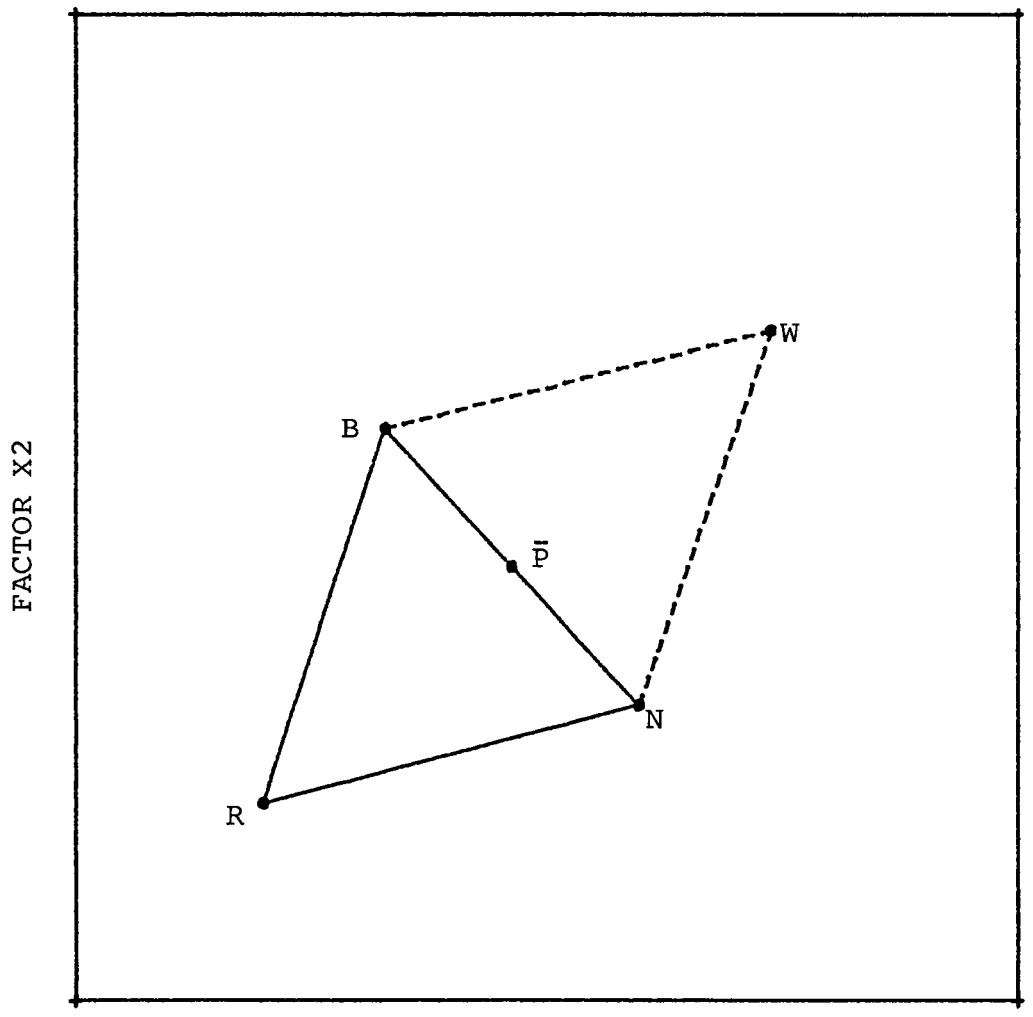
If the coordinates of $\underline{B}$, $\underline{N}$, and $\underline{W}$ are represented as vectors:

$$\underline{B} = [x_{1b} \quad x_{2b}]$$

$$\underline{N} = [x_{1n} \quad x_{2n}]$$

FIGURE 13

FORMATION OF NEW SIMPLEX VERTEX


W
B
P̄
N
R
FACTOR X2
FACTOR X1

$$\underline{W} = [x_{1w} \quad x_{2w}] \tag{5}$$

then the centroid of the remaining hyperface is simply the average of the coordinates of the remaining points:

$$\bar{\underline{P}} = \frac{1}{2}(\underline{N} + \underline{B}) = [\frac{x_{1n} + x_{1b}}{2} \qquad \frac{x_{2n} + x_{2b}}{2}] \tag{6}$$

In the more general case of $\underline{k}$ factors:

$$\bar{\underline{P}} = \frac{1}{\underline{k}} (\underline{N} + \ldots + \underline{B}) \tag{7}$$

The vectors $\bar{\underline{P}}$ and $(\bar{\underline{P}} - \underline{W})$ are summed to give the coordinates of the new vertex, $\underline{R}$:

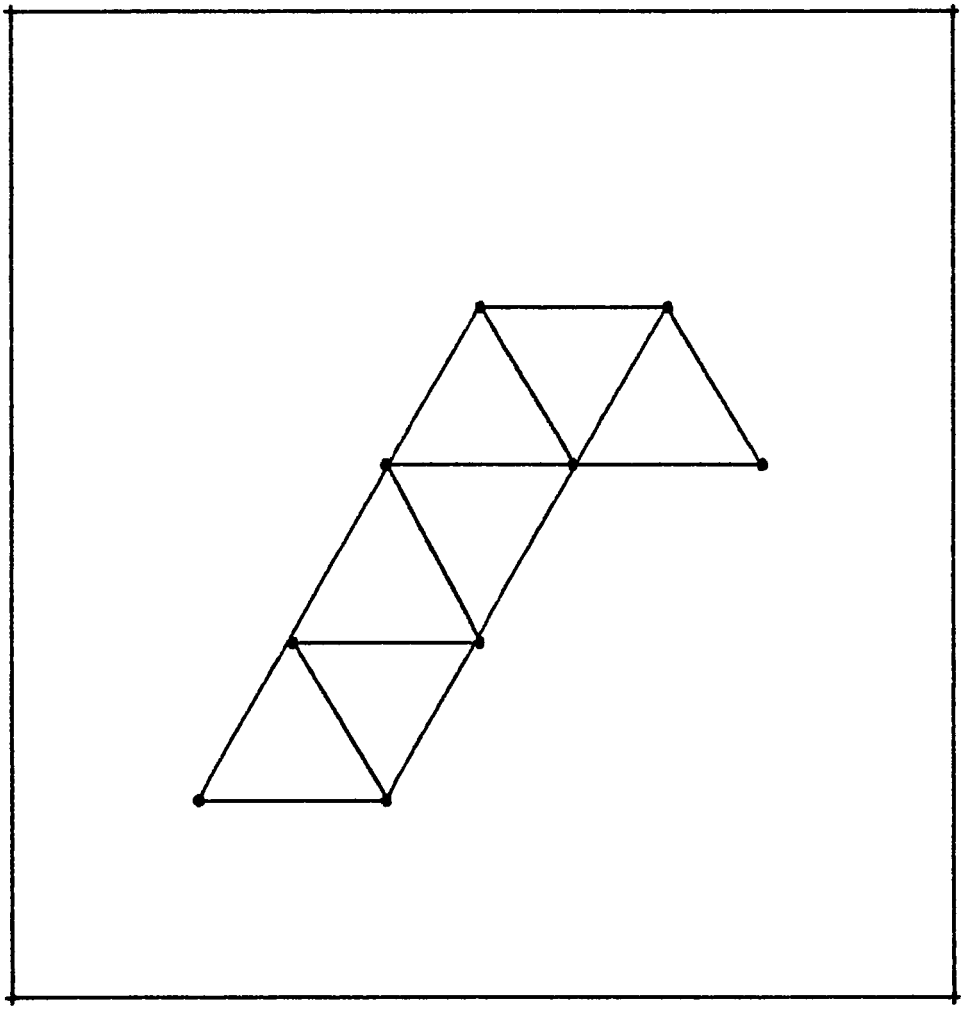
$$\underline{R} = \bar{\underline{P}} + (\bar{\underline{P}} - \underline{W}) \tag{8}$$

Thus, as $\underline{W}$ is eliminated and $\underline{R}$ is created, a new simplex ($\underline{BNR}$) is formed. An experiment is conducted at the set of factor levels given by the point $\underline{R}$ and the response is measured. The vertexes in the simplex can once again be ranked as $\underline{B}$, $\underline{N}$, and $\underline{W}$. In this approach, only one new experiment needs to be conducted to create a new simplex, and thus a "move." Figure 14 shows a succession of simplex moves in factor space.

However, a situation can arise in which the simplex

FIGURE 14

SIMPLEX MOVES IN FACTOR SPACE


FACTOR X2
FACTOR X1

becomes stranded. Figure 15 shows such a case: the new vertex, R, has the worst response in the new simplex; application of Rule 1 would reflect R through $\bar{P}$ to regenerate W as the new vertex. Thus, the simplex would oscillate between the points R and W. An exception to Rule 1 is necessary:

Rule 2 (next-worst reflection rule): If the new vertex has the worst response in the new simplex, reject the next-worst vertex (N). Figure 16 shows how application of this rule allows the simplex to move toward the optimum, alleviating the oscillation of Figure 15.

A second exception to Rule 1 becomes necessary when simplex optimization is used on real, experimental response surfaces (containing noise). In this case, if one vertex, when evaluated, has a large positive contribution from the noise, the simplex will become stranded around the coordinates of that vertex and will not be able to move away.

Rule 3. If a vertex has remained in n+1 simplexes and is not due to be rejected on the next move, re-evaluate that vertex before making the next move. The response for that vertex is then the average of the original experiment and the re-evaluation. In this manner, the simplex can be directed away from falsely "good" responses.

In real systems, there are usually boundaries on factors. For the temperature of a solvent, the boundaries

FIGURE 15

SIMPLEX STRANDED ON A RIDGE

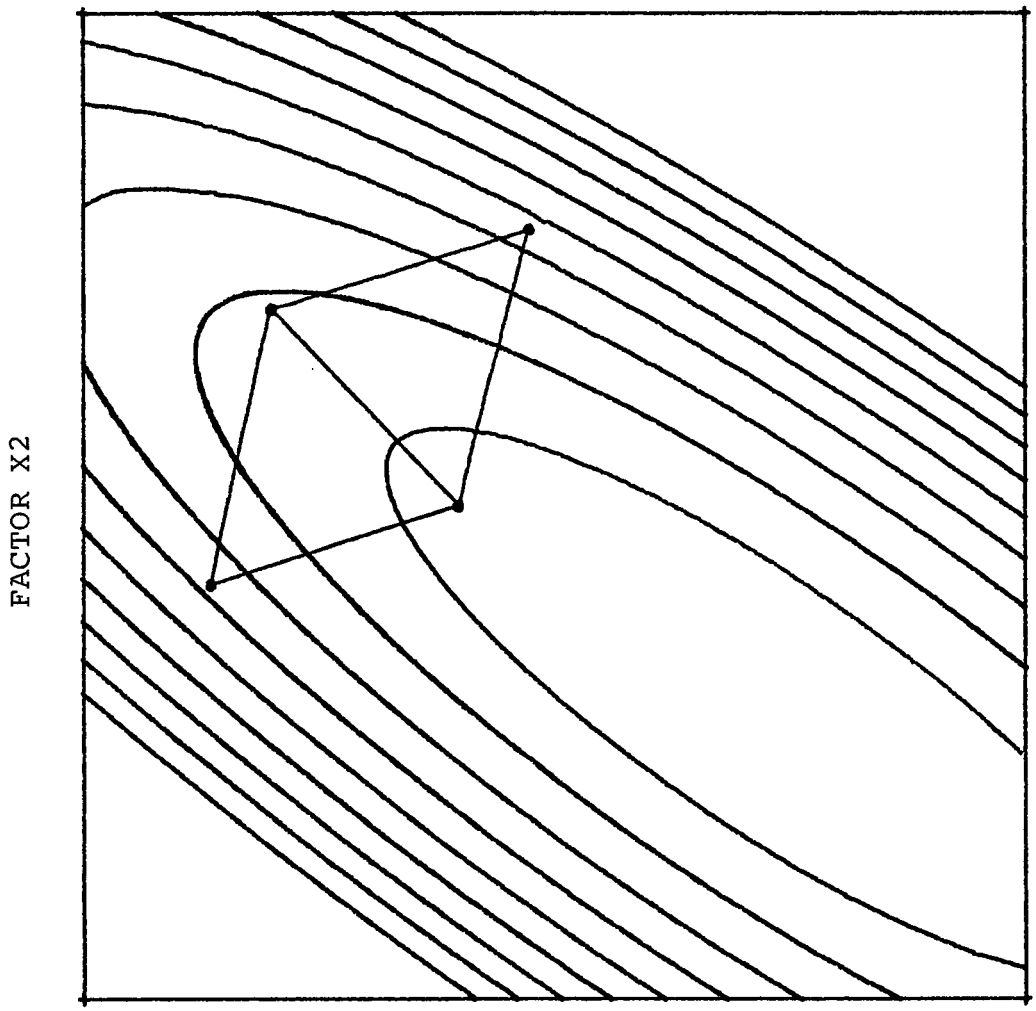
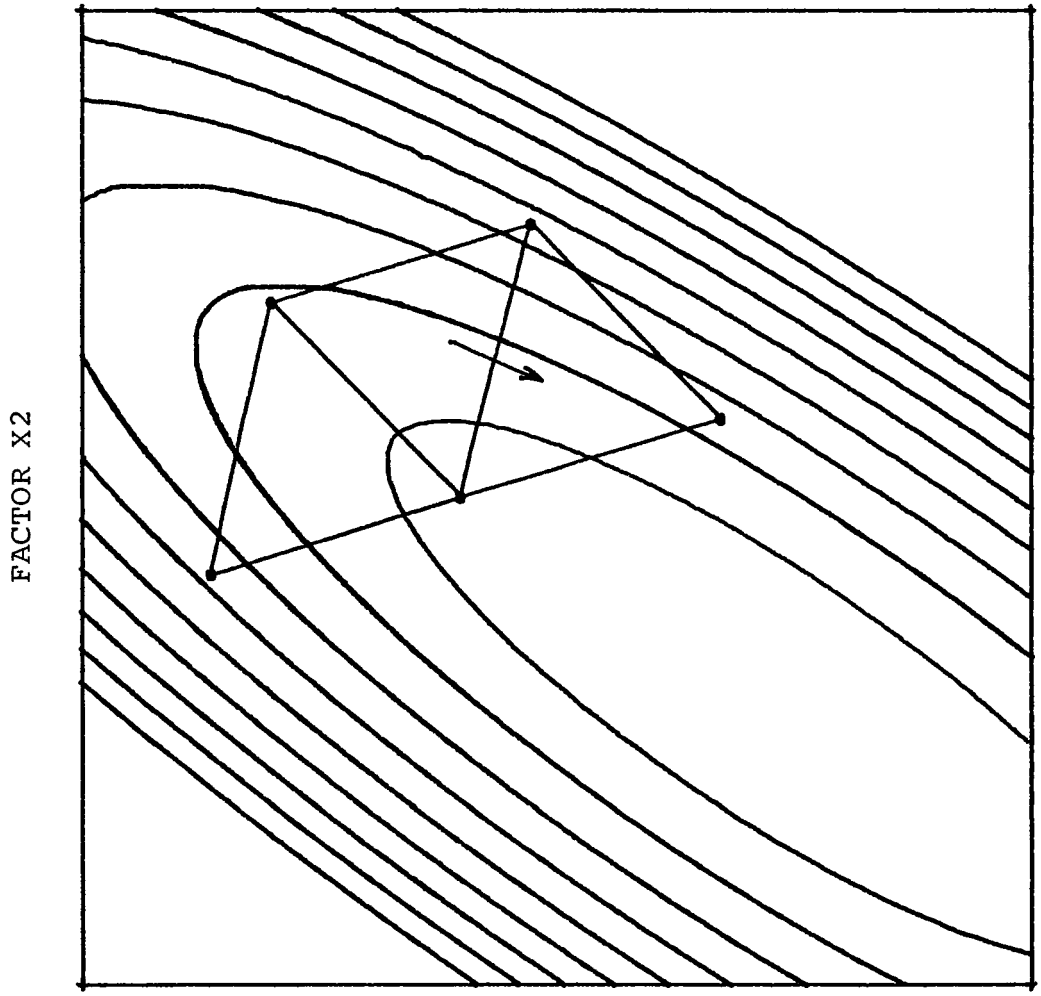

FACTOR X2
FACTOR X1

FIGURE 16

APPLICATION OF NEXT-WORST

REFLECTION RULE


FACTOR X2
FACTOR X1

might be the freezing and boiling points; for mole percent of a solute, the boundaries would be 0 and 100. If the simplex algorithm attempts to locate a vertex outside of a boundary (e.g., -5% solute), obviously the experiment cannot be conducted. Instead, a very bad (undesirable) response should be assigned to that vertex, thus forcing the simplex back within the bounds.

There are four disadvantages to the above, fixed-size simplex algorithm. First, it is difficult to know when the optimum has been reached. In two factors, the simplex will circle the region of the optimum, as triangles will close-pack. However, tetrahedra and higher-dimensional simplexes will not exhibit close-packing.

Second, the simplex wastes time and experimentation when it is climbing up a ridge; the size and orientation are often such as to require a zig-zag path along the ridge as well. This is because the fixed-size simplex has no provision for the adjustment of its size.

Third, if a large initial simplex is chosen, the optimum may not be well-defined, as there will be a relatively large region of factor space between vertexes. (A small simplex, of course, is very inefficient as discussed above.) A second, smaller simplex can be started around the best vertex found, but again, this is somewhat inefficient.

Fourth, the simplex can become stranded on a very

steep ridge. Rotation would allow the simplex to continue to move, but this requires additional calculations and experiments.

An alternative simplex algorithm which avoids the above difficulties was devised by Nelder and Mead (17). Although originally intended for numerical optimization (least squares, roots of polynomials, etc.), it is well-suited for experimental use as well.

Figure 17 shows the Nelder and Mead, or variable-size, simplex. As in the case of the fixed-size simplex, the vertexes are ranked $\underline{B}$, $\underline{N}$, and $\underline{W}$; $\underline{W}$ is rejected; a new vertex, $\underline{R}$, is created by the reflection of $\underline{W}$ through $\bar{\underline{P}}$; and the response at $\underline{R}$ is evaluated. The following four possibilities exist:

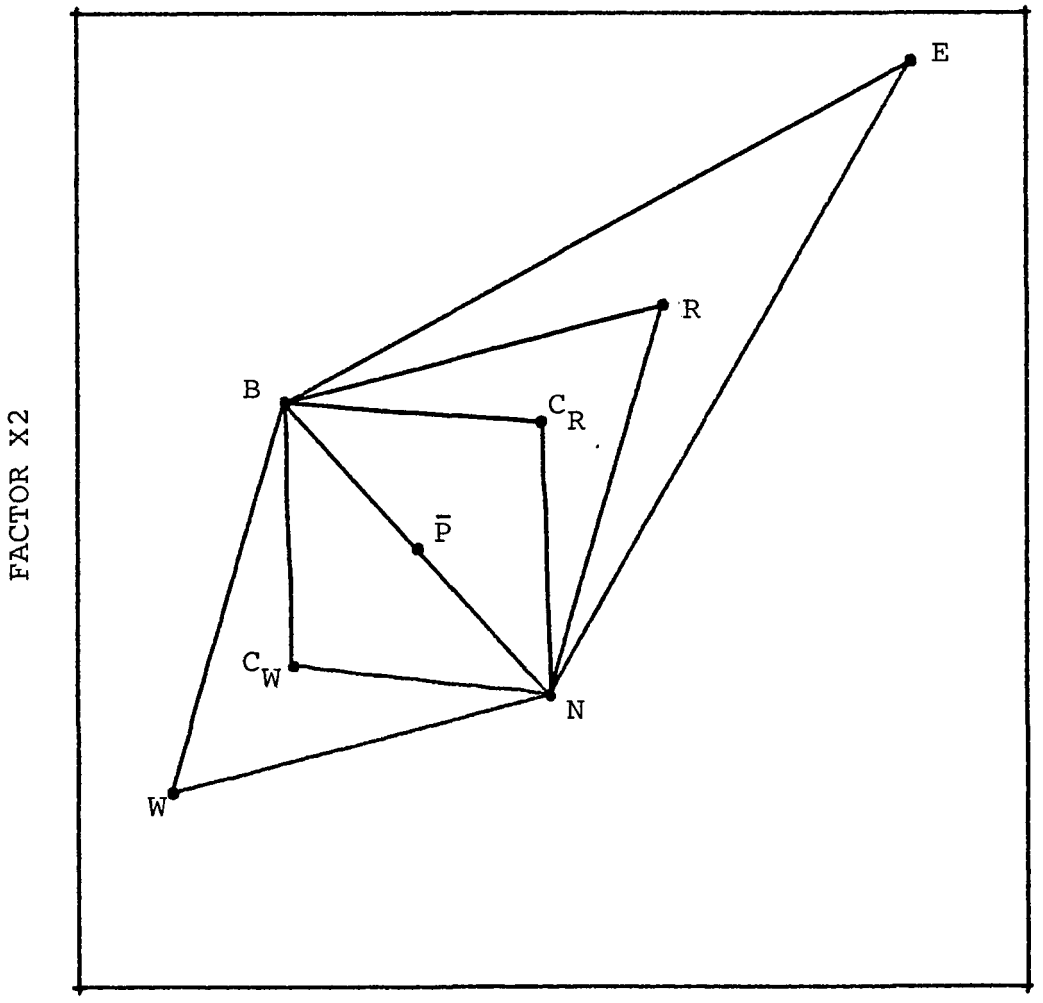
(1) The response at $\underline{R}$ is better than that at $\underline{B}$. This indicates that moving in the direction of $\underline{R}$ is advantageous, so an attempt is made to move even further in that direction. A new vertex, $\underline{E}$ (for expansion), is generated:

$$\underline{E} = \bar{\underline{P}} + 2(\bar{\underline{P}} - \underline{W}) \tag{9}$$

and the response evaluated. If the response at $\underline{E}$ is also better than the response at $\underline{B}$, the new simplex is taken as $\underline{BNE}$; if the response at $\underline{E}$ is not better than the response at $\underline{B}$, the expansion has failed and the new simplex remains

FIGURE 17

VARIABLE-SIZE SIMPLEX


E
R
B
C_R
$\bar{P}$
C_W
N
W
FACTOR X2
FACTOR X1

$\underline{BNR}$.

(2) If the response at $\underline{R}$ is worse than that at $\underline{B}$ but better than the response at $\underline{N}$, the reflection is retained and the new simplex becomes $\underline{BNR}$.

(3) If the response at $\underline{R}$ is worse than the response at $\underline{N}$, but better than the response at $\underline{W}$, a contraction is indicated. The simplex has improved the response by moving from $\underline{W}$ through $\bar{\underline{P}}$ to $\underline{R}$, but may have gone too far past $\underline{N}$. The contraction vertex, $\underline{C_R}$, is placed between $\underline{R}$ and $\bar{\underline{P}}$:

$$\underline{C_R} = \bar{\underline{P}} + \frac{1}{2} (\bar{\underline{P}} - \underline{W}) \tag{10}$$

The new simplex has become $\underline{BNC_R}$.

(4) If the response at $\underline{R}$ is worse than the response at $\underline{W}$, the simplex is moving in the wrong direction from the hyperface. A contraction vertex, $\underline{C_W}$, is placed between $\underline{W}$ and $\bar{\underline{P}}$:

$$\underline{C_W} = \bar{\underline{P}} - \frac{1}{2} (\bar{\underline{P}} - \underline{W}) \tag{11}$$

The new simplex becomes $\underline{BNC_W}$.

If the response at $\underline{C_R}$ or $\underline{C_W}$ is worse than the response at $\underline{N}$, a "failed contraction" has occurred. The simplex could oscillate about the hyperface in a series of contractions, with the result being a virtually-collinear simplex.

To avoid this, Nelder and Mead (17) suggested a massive contraction as shown in Figure 18. This approach has two drawbacks: $\underline{n}$ new simplex vertexes are required (and thus $\underline{n}$ new experiments must be conducted) before the next move; and the volume of the simplex is reduced to $(\frac{1}{2})^{\underline{n}}$. Ernst (19) suggested a translation of the simplex instead (Figure 19); this avoids the shrinkage, but requires $\underline{n}+1$ additional experiments before moving. King (20) suggested applying the next-worst reflection rule of Spendley, Hext, and Himsworth (16): If the response at $\underline{C}_{\underline{R}}$ or $\underline{C}_{\underline{W}}$ is worse than that at $\underline{N}$, reject the next-to-worst vertex ($\underline{N}$) on the next move. This last approach has been used successfully, and is the strategy employed in the work described in this dissertation.

The four drawbacks to the fixed-size method are thus overcome: the simplex will continually contract (collapse) about the optimum, giving a better indication as to when it has been reached and a more precise location; the simplex can accelerate (expand) when moving toward better response (as up the side of a ridge or along the top of a ridge) and can decelerate (contract) around the optimum and to re-orient itself to move along a ridge; and the simplex will not become stranded.

In the fixed-size simplex, the step size is a compromise: if too small, the simplex will take an excessive amount of time to reach the optimum; it too large, the sim-

FIGURE 18

MASSIVE CONTRACTION OF SIMPLEX

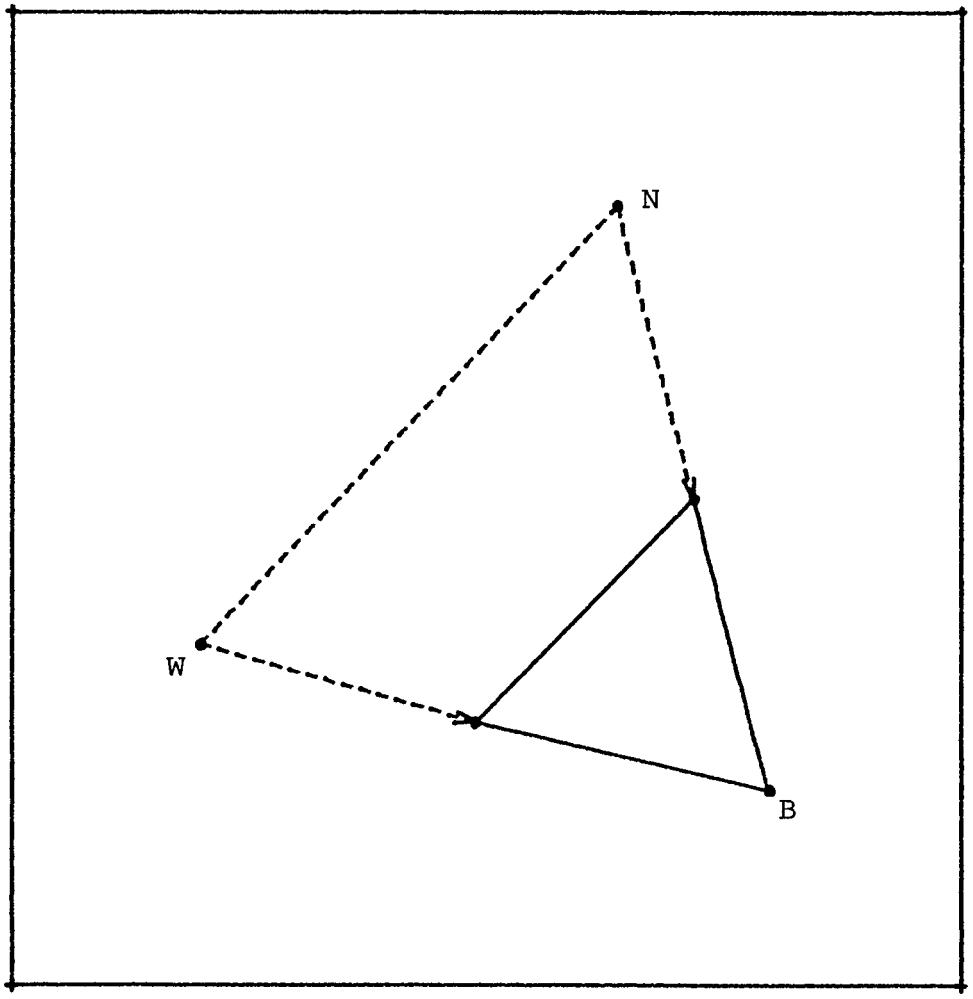
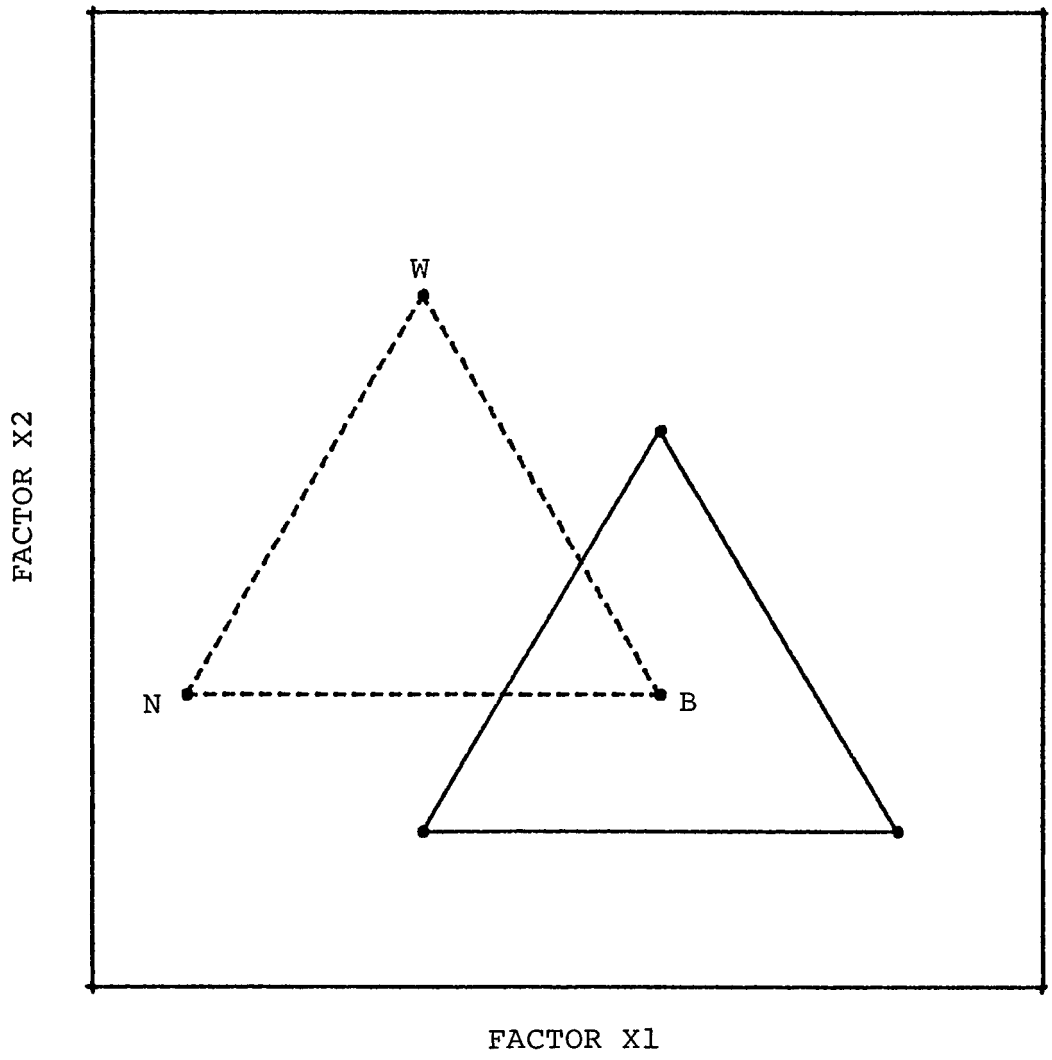

N
W
B
FACTOR X2
FACTOR X1

FIGURE 19

TRANSLATION OF SIMPLEX


W
N
B
FACTOR X2
FACTOR X1

plex may not be able to move at all. Since the variable-size simplex adjusts the step size as it moves, there is more freedom in the choice of the initial step size. A case can be made for starting with a large initial simplex (which will then collapse toward the optimum) (21): widely different regions of the factor space are investigated at the beginning of the optimization process. This would not be desirable, however, if any region of factor space was potentially dangerous (as might be the case with some synthesis reactions and flame-type instruments).

There are numerous convergence criteria for terminating the simplex algorithm. These include minimum change in response, minimum simplex size, minimum change in response per change in factor levels, region of acceptable response reached, and maximum number of vertexes reached.

CHAPTER III

A REVIEW OF EXPERIMENTAL SIMPLEX OPTIMIZATION

The material contained in this chapter will appear as part of an invited paper by S. N. Deming and L. R. Parker, Jr., in CRC Critical Reviews in Analytical Chemistry.

One of the earliest applications of the sequential simplex in analytical chemistry was by Ernst (19), who used the variable-size algorithm to improve NMR magnetic field homogeneity: linear y-gradient and quadratic y-gradient magnetic shim coil currents were controlled. The experiment was interfaced to a computer which performed the simplex calculations and varied the currents supplied to the coils by means of digital-to-analog converters (DAC's). In this work, the performance of the simplex method was compared to the performance of the steepest ascent, or gradient, method; it was concluded that the simplex algorithm converges faster and is the more efficient of the two. The need for repeating measurements on vertexes (the $n+1$ rule) was re-emphasized.

In a 1969 paper, Long (22) discussed many aspects of simplex optimization as applied to analytical chemistry. Among the points included were selection of factors and response, step sizes, boundaries, starting coordinates, the effect of error, and a shifting optimum. Specific cautions were not to use the concentration of the substance being determined as a factor and to combine two interdependent factors into a single factor. Long pointed out the risk that too large an initial step size could cause the simplex to miss the optimum; this is a problem only with the fixed-size algorithm. Long also recommended initial screening

experiments (e.g., factorial designs) to determine which factors were important enough to be included in the simplex optimization; later work (8) has shown that this is not necessary and that any potentially important factors can be included -- factors of apparently small importance do not adversely affect the simplex. The concept of response surfaces, especially in regard to ridges, was also discussed.

Finally, to illustrate these points, Long gave an analytical example: optimizing the absorbance in the p-rosaniline test for SO_2, where the factors were volume of HCl and volume of formaldehyde used. A fixed-size simplex was used, which soon circled the optimum. A smaller simplex was then begun and the optimum was located more precisely.

Long's cautions were not heeded in a 1970 paper by Houle, Long, and Smette (23). Houle et al. were attempting to optimize the sensitivity of the chromotropic acid (CTA) method for the colorimetric determination of formaldehyde. The factors chosen were sample volume, reagent concentration (% CTA in sulfuric acid), and reagent volume; the response chosen was absorbance. Sensitivity (the quality the authors claimed to optimize) is usually defined as the change in absorbance per unit change in concentration of the analyte; if Houle had not used sample volume as a factor (but had fixed it at some level), sensitivity would have been propor-

tional to absorbance (assuming a linear relationship between absorbance and concentration); or if the absorbance had been divided by the sample volume and the result used as the response, sensitivity might have been optimized. However, by using absorbance as the response and sample volume as a factor, the simplex simply moved to higher relative sample volume which, as would be expected, yielded higher absorbance. If the simplex had been allowed to continue until an apparent optimum was found, the sample volume would probably have increased towards whatever was set as its upper limit. This is in conflict with Long's caution (22) not to use the concentration or amount of the substance being determined as a factor. In addition, the other two factors used by Houle, reagent concentration and reagent volume, are interrelated and could be expressed as one factor -- amount of reagent added. Again, Long (22) recommended combining interdependent factors. Finally, the criterion used by Houle for stopping the simplex and going to a second, smaller simplex was that a reflection gave a lower response. This is highly suspect, as the simplex might have passed over a ridge (not an optimum) and, if allowed to continue, could have re-oriented itself and moved towards a different region (24).

Olansky and Deming (25) in a later work re-optimized this wet chemical method for formaldehyde. The sample volume of formaldehyde was fixed at 2.00 ml, and the factors

varied were volume of CTA and volume of sulfuric acid added (in order to investigate these factors independently). Absorbance was used as the response, and, since the amount of analyte was held constant, the authors were able to optimize sensitivity (again, assuming a linear relationship between absorbance and concentration). An important finding was that an optimum exists in the ratio of sulfuric acid volume to total volume. Later regression analysis used a model which, among other things, accounted for the effect of dilution upon response (increasing the volume of added reagents may produce more of the colored species, but it also increases the total volume, which tends to lower the absorbance).

Two applications of simplex have been published by Czech (26,27). In the first paper, the optimization of the J-acid method for the determination of formaldehyde was described. However, the procedures of Houle et al. (23) were repeated here. The author claimed to optimize sensitivity, yet the response used was absorbance and the sample volume was one of the factors. Also, two interrelated factors, reagent concentration and reagent volume, were used. The second application by Czech (27) was the acetylacetone method for the determination of formaldehyde. The factors and response were the same as in the J-acid method. Czech also questions the need for replicating any experiments

(27); however, as Spendley *et al.* (16) pointed out and Long (22) re-emphasized, failure to replicate in accordance with the *n*+1 rule may lead to the simplex converging to a false optimum on an experimental system with noise.

Further, Czech made the statement that simplex optimization can increase productivity (or, in an analytical method, sensitivity) from 6- to 8-fold (27). Such a statement is misleading, as some processes or methods may already be near their optima; there is no guarantee that simplex optimization can improve a process by any arbitrarily selected amount.

A paper by Deming and Morgan (18) outlined the simplex rules, including the next-worst reflection and *n*+1, and also discussed handling vertexes which lie outside boundaries. The advantages of the variable-size simplex over the fixed-size simplex were also discussed. Two examples of the use of simplex techniques were given: a numerical example in which an exponential model was fit to absorbance *vs.* time data (non-linear least squares) and an experimental example in which the absorbance of the Liebermann-Burchard method for cholesterol determination was optimized.

A paper by Morgan and Deming (28) further discussed the moves of the simplex, and showed, as Box had previously (5), how traditional, univariate optimization procedures can fail to find the optimum. The cholesterol optimization

study briefly mentioned earlier (18) was presented in greater detail. The sample volume was fixed, and 4 factors [volume per cents of solvent (acetic acid), dehydrating reagent (sulfuric acid), and color reagent (also sulfuric acid), and color development time] were varied. A preliminary screening factorial experiment, as recommended by Long (22), indicated that a potential fifth factor, dehydration time, was not important. The response used was a weighted combination of the absorbance and the stability of the colored species. The use of derivatives as an indication of when the optimum has been reached (in the absence of factor interactions) was discussed. A univariate mapping study was performed in the region of the optimum, and the data from the initial screening experiments, simplex optimization, and mapping study were used to fit a full second-order polynomial model by regression analysis.

The use of a preliminary 2-level factorial design to screen for potentially significant factors was deemed by the authors to be suspect. As the authors stated, "Ideally, the question that should be asked is not, 'What factors are significant at the x level of probability?', but rather, 'What factors are insignificant at the x level of probability?' The number of factors retained when using the second criterion will in general be larger than the number retained when using the first; the investigator will, however, be

assured that he is probably not omitting from investigation any factors that are important." (28)

Parker, Morgan, and Deming (8) applied simplex optimization to a five-factor atomic absorption system. Here, no initial screening experiments were performed. Four factors thought to be important were included in the study: air flow rate, fuel flow rate, hollow cathode lamp current, and burner height. Because this strategy can lead to the inclusion of insignificant factors, it was also desired in this work to investigate what effect such a factor would have upon the simplex. Therefore, a fifth, insignificant factor was included -- the volume of water in a graduated cylinder far removed from the instrument. The response used was the absorbance for a fixed concentration of calcium.

The authors reached three major conclusions: (1) The inclusion of an insignificant factor did not appear to affect the progress of the simplex toward an optimum in the other factors, although this was not confirmed since a second optimization omitting the fifth factor was not conducted. However, a 1/3-fractional 3^5 factorial experiment did indicate that the simplex had achieved an optimum in the three factors found to be significant (air flow rate, fuel flow rate, and burner height). (2) Convergence of a factor (in the variable-size simplex) does not necessarily mean that the factor is at an optimum; as the simplex contracts, it

shrinks in all factors simultaneously; therefore, additional experiments (mapping, factorial) are needed in the region of the optimum to verify whether each factor is at an optimum. (3) The system studied had a major ridge (air-fuel ratio), over which the simplex moved extremely well; replotting the data for air and fuel as the air-fuel ratio showed tight convergence.

Johnson, Mann, and Vickers (29) also applied simplex optimization to atomic absorption, using pulsed hollow cathode lamps. The authors conducted several studies optimizing peak intensity and integrated intensity of the output from various lamps. Factors varied included pulse height (peak current), DC level (background current between pulses), pulse width, average current, and duty factor. Rippetoe, Johnson, and Vickers (30) used simplex optimization to tune a spectrophotometer before undertaking a study of a DC plasma arc. Variables used were arc current, slit sidth, slit height, and the arc optical path; the response used was the signal-to-noise ratio of the emission intensity for calcium. The optimum found was then used for all the elements in the study. This practice should be used with caution, for response surfaces often change when a certain discrete factor (such as element being determined) is changed.

Michel, Coleman, and Winefordner (31) have used simplex

optimization in the construction of electrodeless discharge lamps for atomic absorption using ten factors. A large initial simplex, as recommended by Yarbro and Deming (21), was used; this resulted in nine of the eleven initial vertexes giving no response. Michel et al. adjusted the coordinates of the vertexes to obtain responses from all eleven lamps. This must be done carefully, however: arbitrary changes to the initial simplex may result in two or more of the vertexes lying in the same hyperplane, thus removing a degree of freedom of movement from the simplex.

Smits, Vanroelen, and Massart (32) applied simplex to the optimization of information in cation exchange chromatography. The concentrations of two compounds (HCl and dimethylsulfoxide) in the eluting agent were the factors; the response was a summation of the amount of overlap of each peak with the two adjoining peaks. The response was divided by the time of elution in an attempt to maximize the information content per unit time. However, the simplex simply moved towards shorter time: as the time (denominator in the response function) decreased, the response increased. This, then, points to an additional caution: dividing the response by a factor (such as absorbance divided by concentration for sensitivity) or by a secondary response (such as division by time) might lead the simplex to decrease that factor or secondary response; the response function will be

at a maximum when the denominator is zero. Smits et al. repeated the optimization, but with an upper boundary on the amount of overlap (and thus a lower boundary on the time); this resulted in an optimum in informing power within the given restraint.

Morgan and Deming (33) also applied simplex optimization to separation, using gas chromatrogaphy. Here, two instrumental parameters (column temperature and carrier gas flow rate) were the factors varied; the response was a measure of the separation between pairs of adjacent peaks. Two modifications to the variable-size simplex were used here: (1) After a failed contraction, a massive contraction (Nelder and Mead (17)) was not performed; instead, a next-worst reflection (Spendley et al. (16)) was carried out. (2) A limit was set on the minimum simplex size; if a contraction attempted to shrink the simplex below the limit, the contraction was disallowed (this was to prevent the simplex from becoming too small to be able to move on a response surface with noise). Two-, three-, and five-component mixtures were run, each followed by a factorial experiment and regression analysis. Morgan and Deming set an upper limit on elution time; this is a means of controlling an undesirable secondary response (long elution time) while keeping the actual response function simple. Morgan and Deming (34) have also discussed optimization in chromatography

in a more general sense.

Vanroelen, Smits, Van den Winkel, and Massart (35) carried out a factorial design with replication on the optimization of the absorbance in an extraction procedure for the determination of phosphate (using as factors concentration of $HClO_4$, concentration of ammonium molybdate, and isobutanol-to-benzene ratio in the extracting agent). Although the factorial study was well-designed, the authors concluded that additional factorials (each consisting of 81 experiments) would be needed to more closely define the optimum. A simplex optimization was then undertaken and proved far more efficient, as it converged to an apparent optimum after only 19 experiments. This apparent optimum agreed well with the optimum predicted by the factorial study.

King and Deming (36) gave the name UNIPLEX to the special case of simplex optimization where only one factor is varied. The system reported on was the maximization of absorbance of dichromate; the factor varied was the amount of chromate which reacted with a fixed amount of acid to produce the dichromate. The system described was automated, with computer interfacing and stepper motors driving pumps for the chromate and acid. A later work by Cantor and Jonas (37) also used UNIPLEX; the goal was to optimize first the phase and then the length of pulses in pulsed NMR spectrometry.

Deming and King (38) used the same automated system as described above (36) in a simplex optimization of the acetylacetone method for the determination of formaldehyde; factors varied were acetylacetone and ammonium ion. These reagents were pumped from solutions of fixed concentrations; a make-up reagent (water) was added via a third pump to keep the total flow constant, thus achieving independent variation of the two factors. The response was the absorbance for a fixed concentration of formaldehyde, added via a fourth pump.

Mieling, Taylor, Hargis, English, and Pardue (39) also used an automated system in a simplex optimization. The study was performed on the reaction of H_2O_2 with Ti(IV) in the presence of EDTA. Mieling et al. optimized a response function containing both the absorbance and stability of the complex; the factors chosen were the concentrations of the Ti(IV) and EDTA. The optimal levels of these two factors agreed well with earlier studies by the same authors.

Krause and Lott (40) used a commercially-available automated instrument, the Technicon AutoAnalyzer^{TM}, for simplex optimization studies. Two studies were undertaken: (1) The interaction between samples in the system for the determination of copper was minimized by varying the sample-to-wash ratio and flowcell pull-through rate. The authors noted that these factors were not truly continuously varia-

ble due to the physical limitations of the system (changing flowcell pull-through, for example, required changing the size of tubing, a discrete factor). (2) The interaction between samples in the determination of glucose was minimized by varying the percent sample (determined by the sample-to-wash ratio), percent pull-through, and percent air in the sample stream.

Krause and Lott then performed two additional simplex optimization studies in the area of clinical chemistry: (1) In a kinetic (the LD-catalyzed pyruvate-to-lactate reaction), four factors (pH and concentrations of Tris buffer, pyruvate, and NADH) were varied to find the optimal rate of conversion. (2) Using the GEMSAEC centrifugal analyzer, the optimal conditions found for the pyruvate-to lactate reaction were set, and the times between readings and the number of readings were varied; the response was the coefficient of variation (CV). The simplex optimzation reduced the CV from 3.1 at the manufacturer's recommended settings to 1.6 at the optimum.

Lott and Turner (41) applied simplex optimzation to an automated continuous-flow method; the object was to maximize the absorbance in the glucose oxidase method for determining glucose in serum. The factors chosen were glucose oxidase and peroxidase activities and concentrations of two reagents (4-aminoantipyrine and phenol). The simplex was terminated

upon reaching an adequate level of response.

Basson, Pille, and DuPreez (42) have also worked with the simplex technique on a Technicon AutoAnalyzer. The goal was to optimize the absorbance in the method for the determination of boron. Because the indicator, azomethine H, deteriorated rapidly, the authors decided to synthesize it in situ from H-acid and salicylaldehyde. The factors chosen were concentrations of salicylaldehyde and H-acid and pH. No details of the method or the optimization were given.

CHAPTER IV

UNDERSTANDING THE RESPONSE

Once the simplex has located an (or the) optimum, additional experiments are necessary to verify that an optimum has indeed been reached and to understand how the factors affect the response. This understanding is necessary only in the region of the optimum, since presumably this is where the method (or synthesis, etc.) will be important (43).

Over a limited region of the response surface, an optimum can be approximated by a quadratic model:

$$y = \beta_0 + \beta_1 x_1 + \beta_{11} x_1^2 + \beta_2 x_2 + \beta_{22} x_2^2 + \ldots + \beta_n x_n + \beta_{nn} x_n^2 + \beta_{12} x_1 x_2 + \ldots + \beta_{1n} x_1 x_n + \beta_{23} x_2 x_3 + \ldots + \beta_{(n-1)n} x_{(n-1)} x_n \qquad (10)$$

There are four types of regression coefficients (β's): the offset, β_0; linear parameters, $\beta_{\underline{i}}$; quadratic parameters, $\beta_{\underline{ii}}$; and interaction parameters, $\beta_{\underline{ij}}$. In a sense, β_0 gives the response at the origin (all factors at zero), $\beta_{\underline{ii}}$ the relative steepness (curvature) of the response surface in the direction of each factor, $\beta_{\underline{ij}}$ the rotation of the response surface in the $x_{\underline{i}}$-$x_{\underline{j}}$ plane, and $\beta_{\underline{i}}$ (along with the other coefficients) the relative position of the optimum

in each factor. There will be 1 β_0, $\underline{n}$ $\beta_{\underline{i}}$'s, $\underline{n}$ $\beta_{\underline{ii}}$'s, and $\underline{n}(\underline{n}-1)$ $\beta_{\underline{ij}}$'s, for a total of $(\underline{n}+2)(\underline{n}+1)/2$ terms.

To fit such a model, a factorial-type design must be used. A two-level factorial design (as in Figure 8) will not suffice, since it gives no indication of curvature. There are two principal types of designs which can be used to map the region of the optimum:

(1) Three-level factorial designs (as in Figure 9). This design will provide the necessary curvature and interaction information for the model. However, the number of experiments is equal to 3^n; as the number of factors increases, the number of experiments increases exponentially. Thus, for 3 factors, 27 experiments are required; for 4 factors, 81 experiments are necessary.

Fractional factorial designs are possible (e.g., a $\frac{1}{3}$-fractional three-level design can be used with 4 or more factors); careful selection of the treatment combinations to be eliminated can allow the model to be fit. Since data is eliminated, some information has to be lost, but it can be the higher-order interaction terms (e.g., β_{1223}, corresponding to the $x_1x_2^2x_3$ interaction) that are not present in the model. (Actually, these terms are aliased with terms in the model; the assumption is that the higher-order terms are relatively minor.) A special type of fractional factorial design, the Box-Behnken design, may also be used.

(2) Central composite designs. These designs are more efficient than full or fractional factorial designs. A two-factor example is shown in Figure 20. The central composite design consists of a 2-level factorial design (which provides information on β_0, $\beta_{\underline{i}}$, and $\beta_{\underline{ij}}$) and a "star" design (which provides information on $\beta_{\underline{ii}}$, as well as β_0 and $\beta_{\underline{i}}$). The number of terms is equal to 2^n (from the factorial) plus 2n+1 (from the star). In the 2-factor case, a three-level factorial design and a central composite design will require the same number of points (nine); for three factors, the factorial requires 27 points and the central composite 15; for four factors, the number of points are 81 and 25, respectively. Thus, the greater the number of factors, the greater the advantage of the central composite design in terms of efficiency.

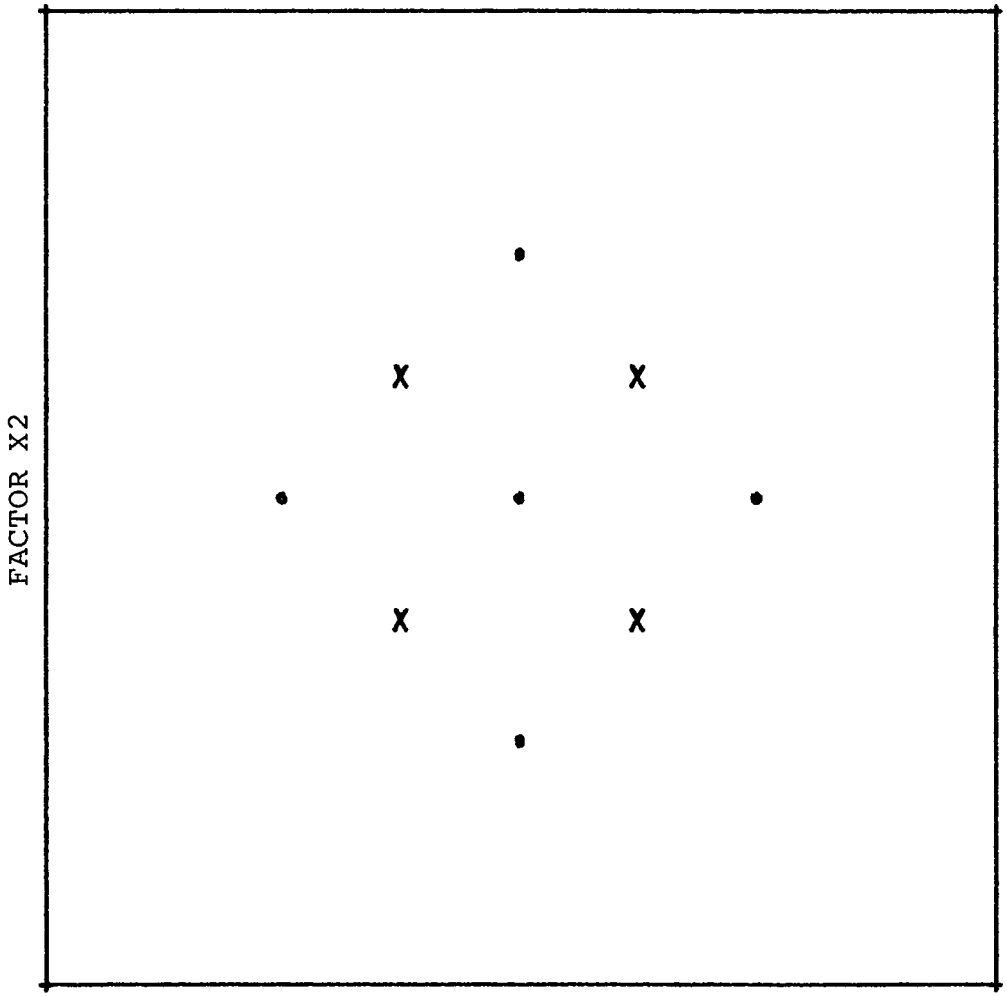
Once the experiments have been conducted and the responses obtained, the model must be fit. In two factors, this can be done using least-square equations; however, a more general approach is to use matrices:

FIGURE 20

CENTRAL COMPOSITE DESIGN

IN TWO FACTORS

X corresponds to factorial points
• corresponds to "star" points


FACTOR X2
FACTOR X1

$$\underline{X} = \begin{pmatrix} 1 & x_{11} & x_{11}^2 & x_{21} & x_{21}^2 & x_{11}x_{21} \\ 1 & x_{12} & x_{12}^2 & x_{22} & x_{22}^2 & x_{12}x_{22} \\ 1 & x_{13} & x_{13}^2 & x_{23} & x_{23}^2 & x_{13}x_{23} \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ 1 & x_{1n} & x_{1n}^2 & x_{2n} & x_{2n}^2 & x_{1n}x_{2n} \end{pmatrix}$$

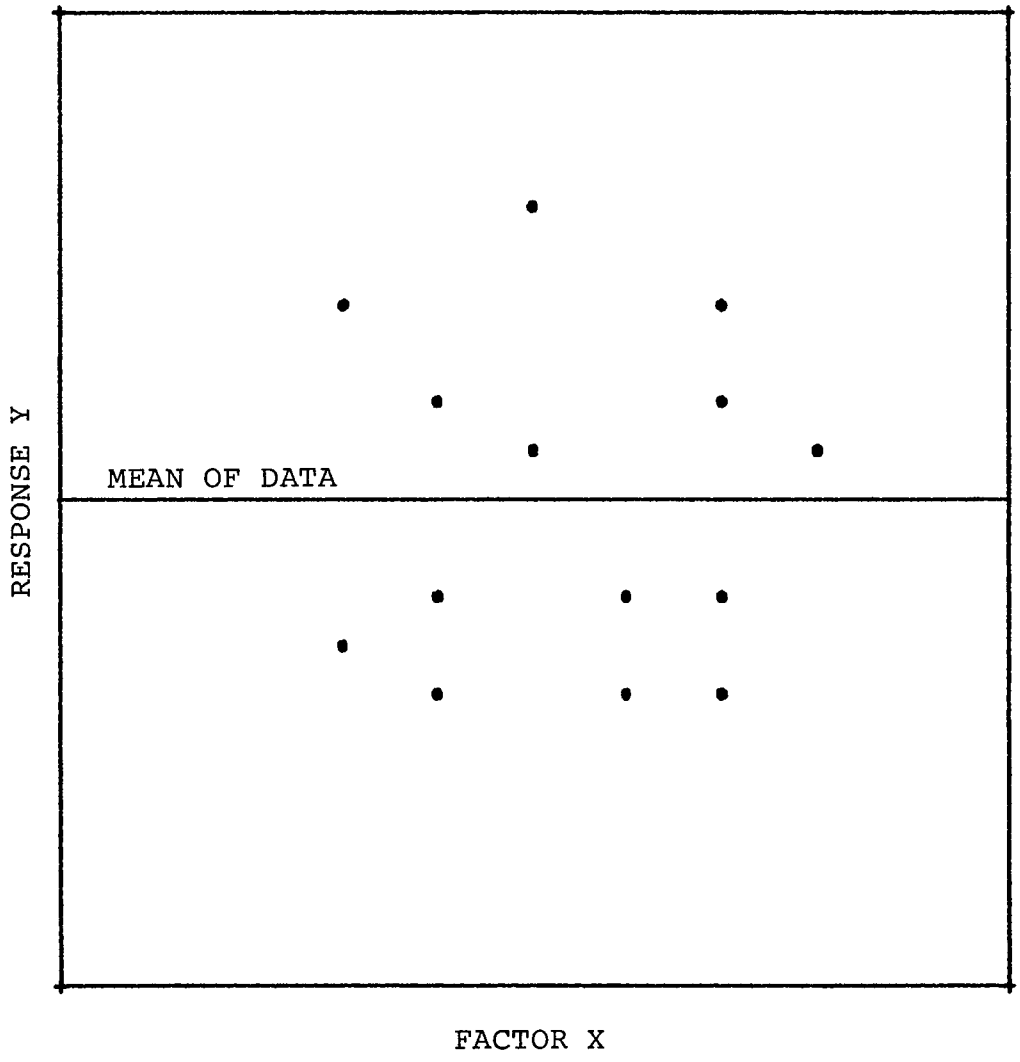
$$\underline{Y} = \begin{pmatrix} y_1 \\ y_2 \\ y_3 \\ . \\ . \\ . \\ y_n \end{pmatrix} \qquad \underline{\beta} = \begin{pmatrix} \beta_0 \\ \beta_1 \\ \beta_{11} \\ \beta_2 \\ \beta_{22} \\ \beta_{12} \end{pmatrix} = (X'X)^{-1}X'Y \tag{11}$$

for a two-factor case. x_{ij} represents the level of factor i in the j-th experiment; y_j is the response in the j-th experiment. (In a central composite design with no replication, n would equal 9.)

The overall system is depicted in Figure 21. Data can be visualized as varying about its mean. In the absence of factor effects and experimental uncertainty, the expected value for each experiment is the mean, $\bar{x}$. Variation from this mean can be due to two sources: factor effects and

FIGURE 21

VARIATION OF DATA ABOUT ITS MEAN


RESPONSE Y
MEAN OF DATA
FACTOR X

pure experimental uncertainty. Thus, the sum of squares about the mean can be divided into the sum of squares due to pure (experimental) error (SS_{PE}) and the sum of squares due to the factors (see Figure 22). The sum of squares due to the factors can be further divided into the sum of squares due to the factors and explained by the model (sum of squares due to regression) and the sum of squares due to the factors but not explained by the model (sum of squares due to lack of fit, or SS_{LOF}). The sum of squares due to lack of fit and the sum of squares due to pure error are often combined into the sum of squares of residuals (SS_R).

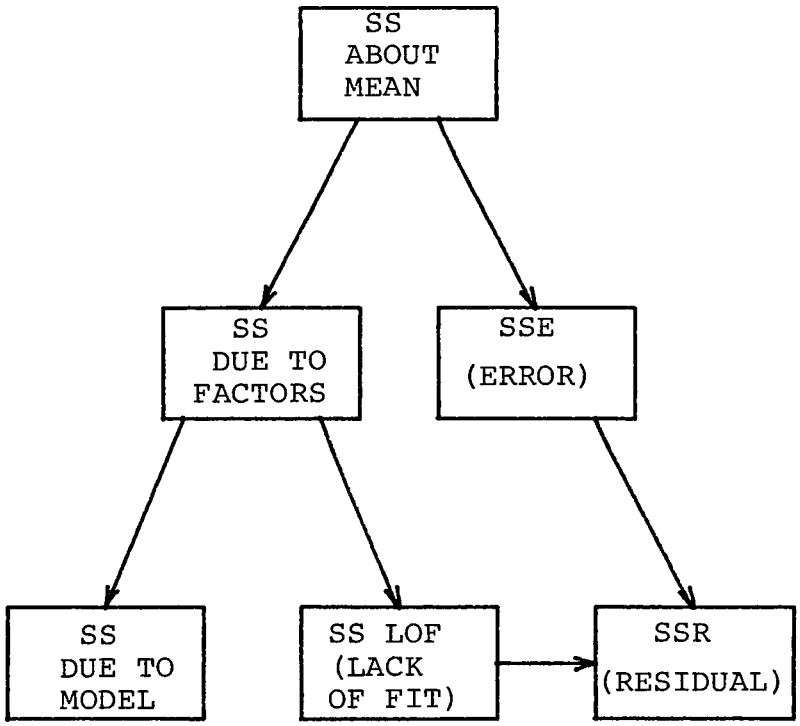
The sums of squares can be divided by the appropriate number of degress of freedom (d.f.), thus yielding the variance (or mean square). The degrees of freedom for the sums of squares are : SS due to regression, p-1; SS_R, n-p+1; SS total corrected for mean, n-1 (where p is the number of parameters in the model and n is the number of experiments). The degrees of freedom associated with the SS_{PE} will be equal to the number of replicate experiments performed; the number of degrees of freedom for the SS_{LOF} will be the number for the SS_R minus the number for the SS_{PE}.

The following tests can now be made about the regression:

(1) Significance of each of the parameters. A t-test can be performed, referenced to either the pure error

FIGURE 22

A PARTITIONING OF THE SUM OF SQUARES


SS
ABOUT
MEAN
SS
DUE TO
FACTORS
SSE
(ERROR)
SS
DUE TO
MODEL
SS LOF
(LACK
OF FIT)
SSR
(RESIDUAL)

variance or the residual variance, to test if each parameter is significantly (at some given level of confidence) different from zero. The pure error variance is the preferred comparison, but this test is possible only when there is replication of experiments.

(2) Significance of the regression. An F-test can be performed to test whether the variance the model explains is significantly different from either the pure error variance or the residual variance. If the regression is not highly significant (95% level of probability or higher), this is an indication that the assumed model does not explain, or account for, a significant portion of the total variance of the data about the mean.

(3) Significance of the lack of fit. This test is also an F-test, comparing the variance due to lack of fit and that due to pure error. If the lack of fit is highly significant (variance due to lack of fit is significantly greater than the variance due to pure error), the indication is that the model is inadequate. This test and the test for the significance of the regression are not redundant: if both the lack of fit variance and the pure error variance are small, the lack of fit can be significant, yet so can the regression.

In the absence of significant interactions among the factors, the significance of the regression coefficients

gives direct, meaningful information about the response surface. If a β_{ii} term is not significant, this indicates that the response surface has little curvature in the i-th factor, for example. If neither the β_i nor the β_{ii} term is highly significant, this would indicate that, within the region of the response surface covered by the design, the level of the i-th factor has little effect upon the response.

If the interactions are significant, this indicates that the response surface contours are rotated; a highly significant β_{ij} term indicates rotation of the surface in the x_i-x_j plane. Because of this, direct interpretation of the regression coefficients is difficult. A technique which overcomes this difficulty is canonical analysis. Basically, canonical analysis:

(1) Translates the origin to the stationary point in the response surface, thus removing the β_i terms; and

(2) Rotates the axes so as to align them with the principal axes of the elliptical contours, thus removing the β_{ij} interaction terms. The result is an equation of the form (for 2 factors):

$$Y = \beta_0' + \beta_1'(X_1')^2 + \beta_2'(X_2')^2 \qquad (12)$$

where X', β_0', β_1', and β_2' do not have the same values as the

original terms x, β_0, β_1, and β_2. In this form, β_0' gives the response at the stationary point and β_i' indicates the steepness of the curvature in the X_i' direction. The sign of β_i' indicates whether there is a maximum (- sign) or a minimum (+ sign) in factor X_i' at the stationary point. Thus, if both (β')'s are positive, the stationary point is a minimum; if both (β')'s are negative, the stationary point is a maximum; if the (β')'s differ in sign, the stationary point is a saddle point (moving in the X_1' direction away from the stationary point might increase the response; moving in the X_2' direction would then decrease the response).

CHAPTER V

AUTOMATION

Note: This chapter describes an interface which was built in our laboratory by Lloyd R. Parker, Jr. and Ad S. Olansky, under the direction of Dr. S. N. Deming.

Optimization of a method (both improving and understanding) can be an involved and time-consuming process if performed manually. Computerization of the experimentation can increase the efficiency (work per time) of the optimization process. The computer would calculate the coordinates of each simplex vertex, set the experimental conditions corresponding to these coordinates, conduct the experiment, measure (and/or calculate) the response, and decide where the next vertex should be located. The computer could also decide when to terminate the simplex and where to locate the mapping study, and could then conduct the mapping experiments.

This routine requires a computer interface that is a closed-loop system: the computer controls the instrumentation and acquires the data. The computer used in this work is a Hewlett-Packard (HP) 9830A with hard-wired BASIC programming language, 8K words of memory, dual on-line disc mass memory storage, thermal printer, and plotter. The interface used consists of the following major parts: computer rack, transmission cable, remote rack (containing motor controllers, a relay controller, and a data acquisition device (analog-to-digital converter, or ADC)), a programmable clock, and a Manual Interface Data Input Module (hereinafter referred to as "MIDIM").

(1) Computer rack. A block diagram is shown in Figure 23. The word length used in the interface is 32 bits (four 8-bit bytes); the word is formed 8 bits at a time. The leftmost 8 bits (byte) contain 2 framing bits and 6 bits for control and sub-device select. These bits are used for determining which motor at the remote rack is to receive data and for actuating other peripherals. The middle 16 bits (2 bytes) contain the actual data. The right 8 bits (final byte) contain the device address and 2 framing bits. The framing bits, 2 at each extrema of the word, are designed to indicate to any device reading the word that the 28 bits between the framing bits are, indeed, a word and not noise. The device address is used to select certain features of the interface (see Table I and Figure 24). The four bytes are sent, in sequence, from the computer by writing to channel 2, and are applied to each of the four 8-bit shift registers simultaneously. Also, the computer transmits a select code to a demultiplexer by writing to channel 1, thereby activating <u>one</u> of the four shift registers, allowing the data to be loaded in. By activating each shift register when the data meant for it has been sent, the entire 32-bit word is formed. (The framing bits are wired in and do not need to be transmitted by the computer.) The word is then sent out on the interface cable by sending the value 300_8 to channel 1. A computer subroutine that

FIGURE 23

BLOCK DIAGRAM OF COMPUTER RACK

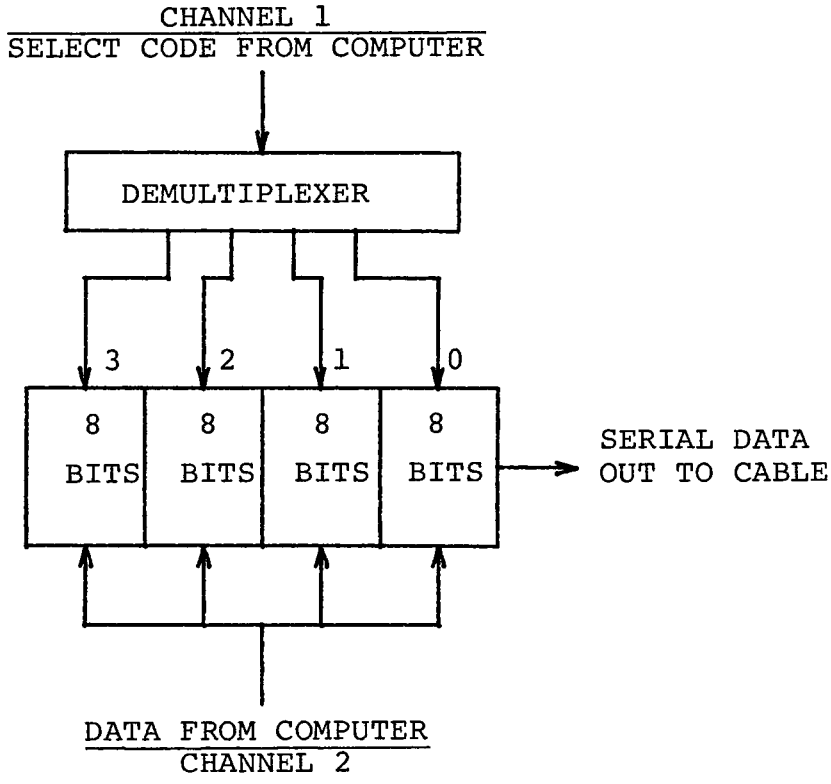

CHANNEL 1
SELECT CODE FROM COMPUTER
DEMULTIPLEXER
3
2
1
0
8 BITS
8 BITS
8 BITS
8 BITS
SERIAL DATA OUT TO CABLE
DATA FROM COMPUTER
CHANNEL 2

TABLE I

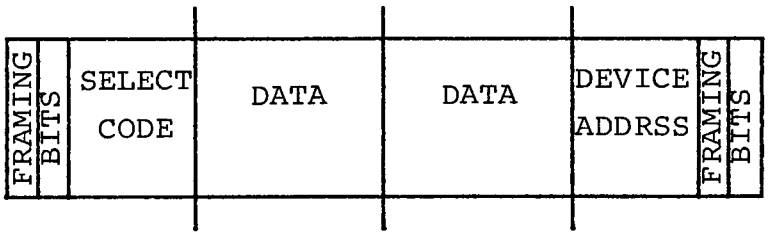
INTERFACE ADDRESSES AND SELECT CODES

Device	Address (Octal)
Computer	00
Programmable clock	76
Remote rack	77

Sub-devices in Remote Rack	Select Code (Octal)
Motors (12)	00 - 13
Turn on relay	57
Turn off relay	17
Actuate ADC	20

FIGURE 24

32-BIT COMPUTER WORD


FRAMING
BITS
SELECT
CODE
DATA
DATA
DEVICE
ADDRSS
FRAMING
BITS

performs the entire task is given in Appendix A.

There are 2 separate 8-bit buffers from which the computer reads data sent to it by the interface.

Also in the computer rack is the master timing clock. A 100-KHz clock pulse train is continuously transmitted to the remote and computer racks where it is used for clocking data into and out of shift registers and other timing functions.

(2) Interface transmission cable. The cable used is a dual differential-signal twisted-pair cable. Both wires of a pair are at the same potential when no information is being transmitted. A potential is forced on one line when a logical "0" is being transmitted and a potential is forced on the other line when a logical "1" is transmitted. One of the twisted pairs transmits data while the other pair continuously transmits the master clock signal.

(3) Remote rack. A word with an address of 77_8 has its data latched in at the remote rack. There are three types of equipment located here:

(a) Motors. The interface can individually control 12 stepper motors, each with a motor controller circuit card. If the select code corresponds to 00_8 through 13_8, the appropriate motor controller circuit card is actuated. The motors themselves may be attached to pumps, valves, potentiometers, etc. To be able to work these different

peripherals, the motors are capable of running in frequency mode (turning at a fixed, programmable rate) or in position mode (turning to a specified position and then stopping).

Figure 25 shows a block diagram of the entire motor control circuit which was constructed. The circuit consists of two major portions, one for frequency control and one for position control. The only elements of the circuit which are common to both modes are the 12 bits of input data (see Figure 25) and the two circuit outputs: 1) a level that indicates which direction the motor is to turn (forward or reverse); and 2) drive pulses which determine the number and/or rate of steps executed by the motor.

The mode control bit determines whether the signal from the frequency portion of the circuit or from the position portion will be output by the mode selector. (See Figure 26 for the function of all the bits.) In frequency mode, an additional bit of data (F/R) is used to indicate the direction of motor rotation. For position mode, a comparator determines the direction of motor rotation necessary to move from the present position to the desired position.

When the frequency portion of the circuit is activated, drive pulses are output at a rate determined by the value of the least significant 12 bits in the data latch. Pulses will be output at this frequency until a new value is latched in. In position mode, a certain number of pulses

FIGURE 25

BLOCK DIAGRAM OF MOTOR CONTROLLER CIRCUIT

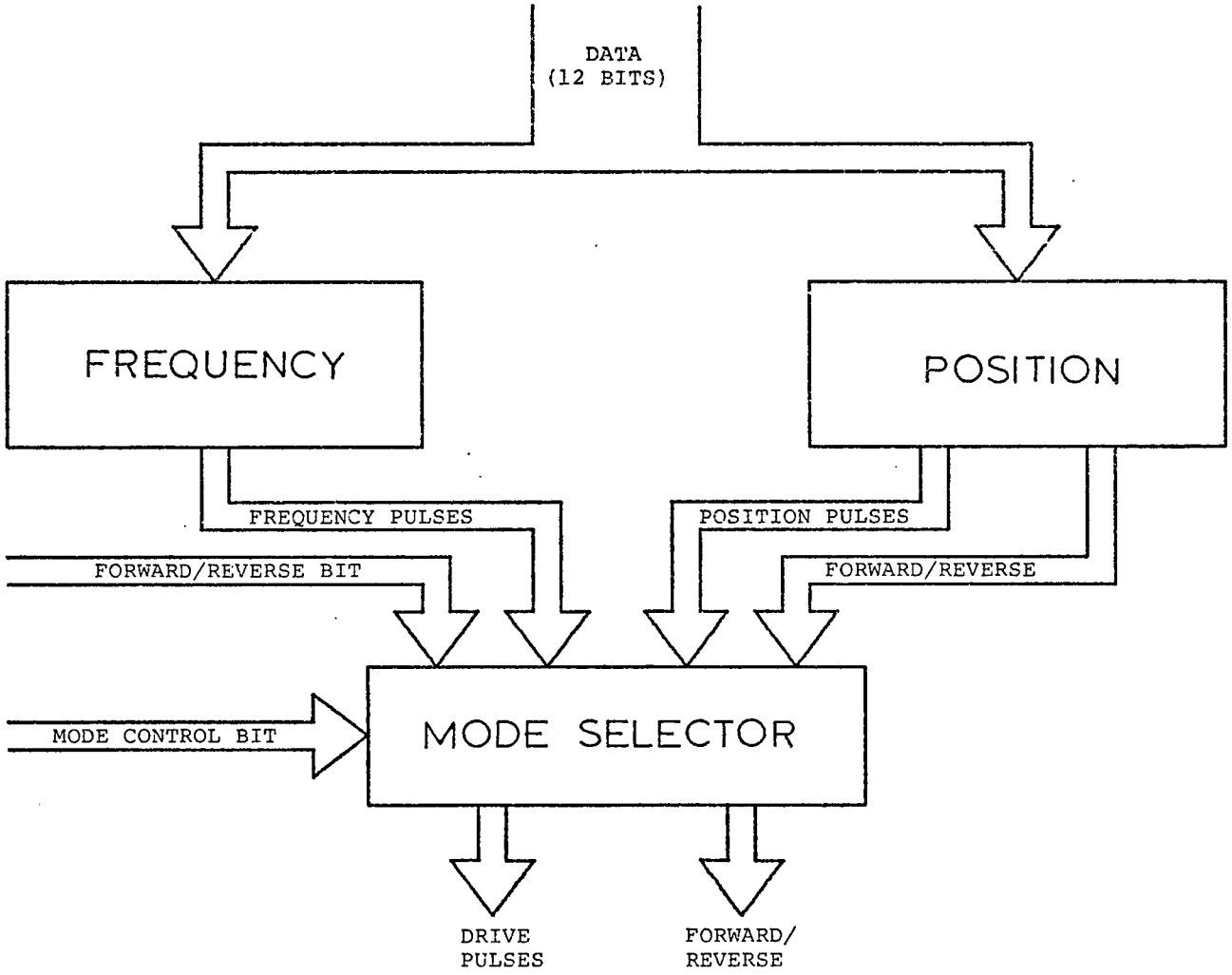
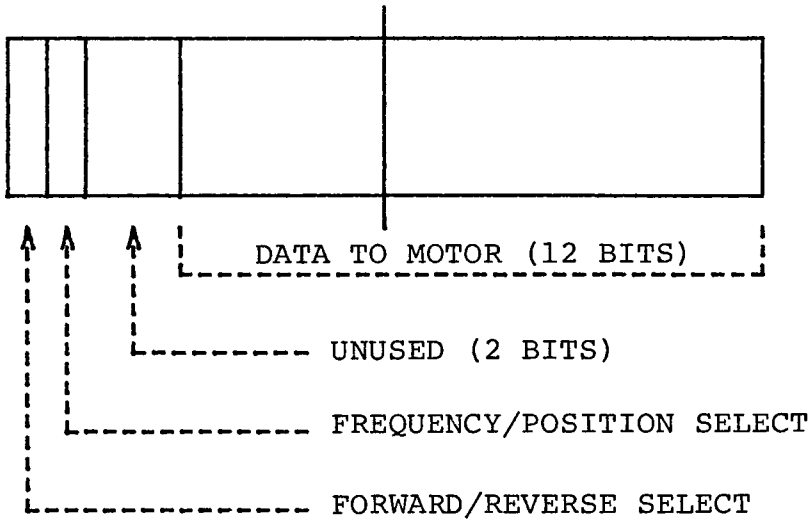

DATA
(12 BITS)
FREQUENCY
POSITION
FREQUENCY PULSES
POSITION PULSES
FORWARD/REVERSE BIT
FORWARD/REVERSE
MODE CONTROL BIT
MODE SELECTOR
DRIVE
PULSES
FORWARD/
REVERSE

FIGURE 26

16 BITS USED TO CONTROL MOTORS


DATA TO MOTOR (12 BITS)
UNUSED (2 BITS)
FREQUENCY/POSITION SELECT
FORWARD/REVERSE SELECT

is output, corresponding to the difference in the present position (stored in an up/down counter) and the desired position (stored in the data latch).

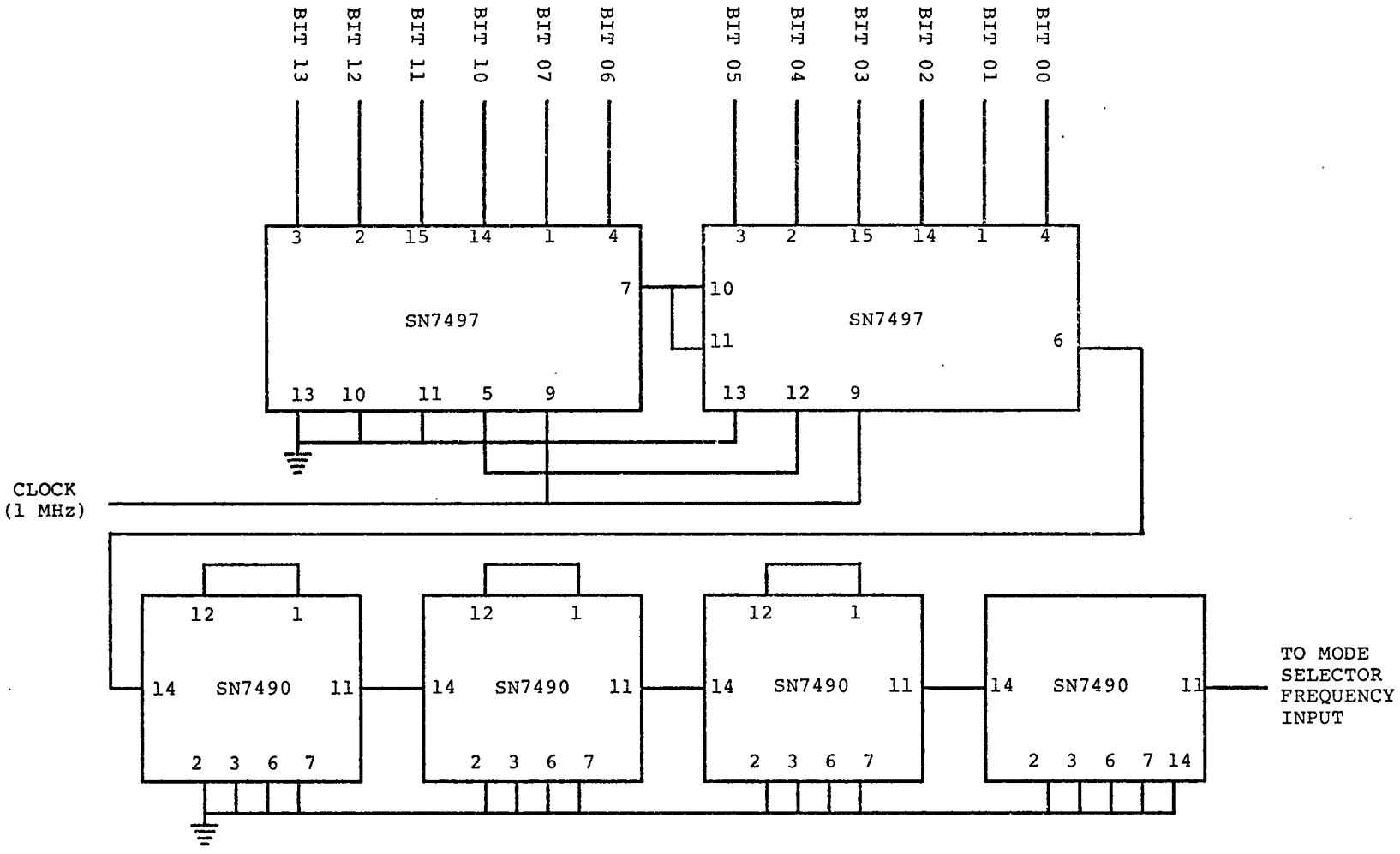
Frequency mode. The frequency portion of the circuit (Figure 27) utilizes a 1-MHz clock frequency input, which is subjected to two division processes. The first of these is carried out by two SN7497 synchronous 6-bit binary rate multipliers which multiply the input frequency by a fraction less than unity (in effect, a division process). The combination of the two 6-bit rate multipliers sets the denominator of the multiplier fraction to the value 4096. The numerator value corresponds to the value of the input data (stored in the latch) and has a range of 0 to 4095. The frequency output of the rate multipliers is accurate over the long term, but the period between pulses can vary by a factor of two. The use of four SN7490 dividers in the second stage of frequency division assures that a uniform square wave output will be delivered to the stepping motor. Three of the four counters are used as full divide-by-tens, while the fourth is used as a divide-by-five. The combination thus divides the frequency output of the rate multipliers by 5000, and provides a range of output frequencies selectable in frequency mode from 0 to 200 Hz, in increments of approximately 0.05 Hz.

Position mode. The position portion of the circuit

FIGURE 27

FREQUENCY PORTION

OF MOTOR CONTROLLER CIRCUIT

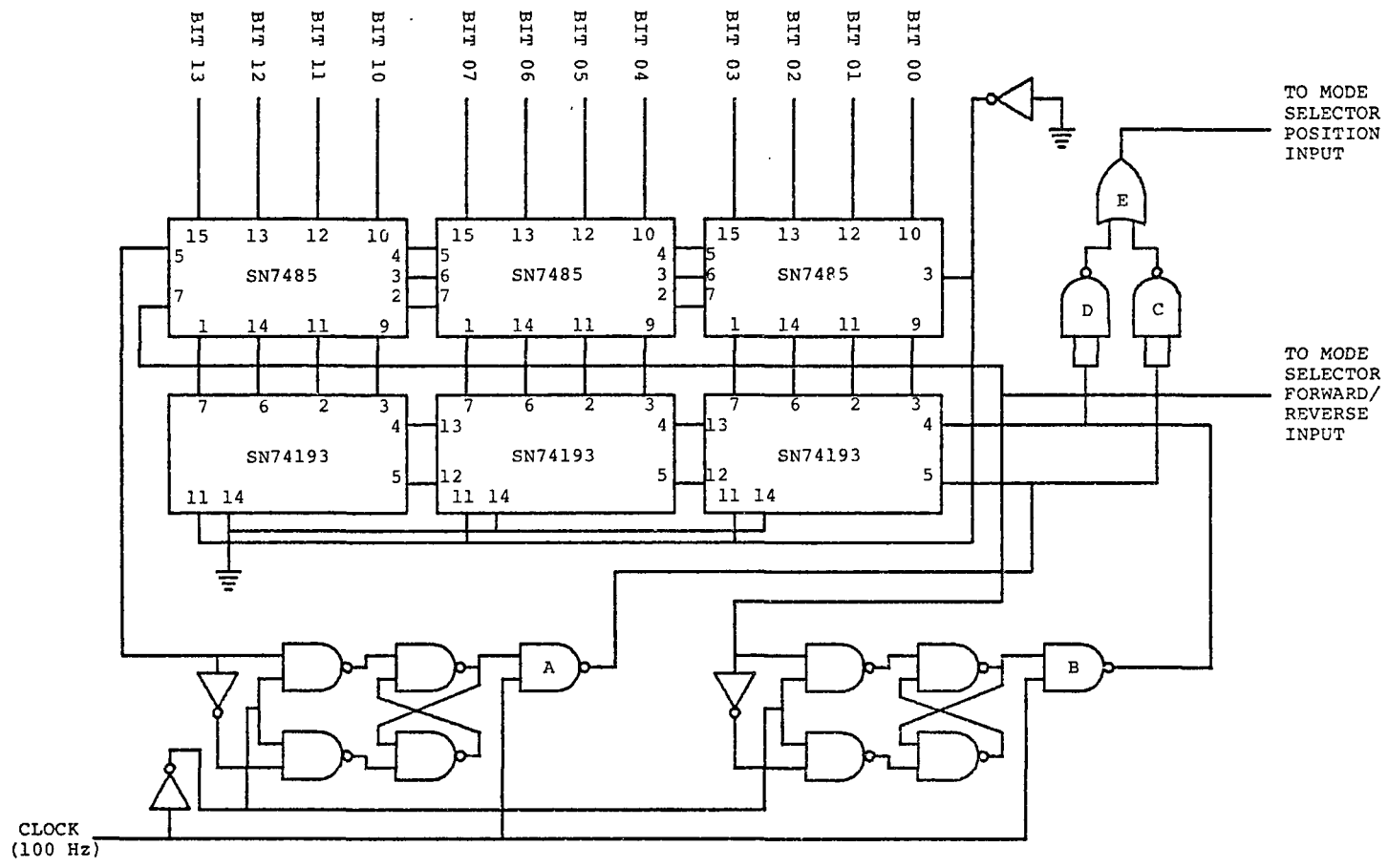

BIT 13
BIT 12
BIT 11
BIT 10
BIT 07
BIT 06
BIT 05
BIT 04
BIT 03
BIT 02
BIT 01
BIT 00
SN7497
SN7497
CLOCK
(1 MHz)
SN7490
SN7490
SN7490
SN7490
TO MODE
SELECTOR
FREQUENCY
INPUT

(Figure 28) applies 12 bits of binary data to one set of inputs (P inputs) of a series of SN7485 4-bit magnitude comparators. The other set of inputs (Q inputs) is connected to the outputs of a group of SN74193 synchronous 4-bit binary up/down counters. The number on the output of these counters represents the present motor position. If the desired position is greater than the present position, the P>Q line of the comparators (pin 5) goes high and the P<Q line (pin 7) remains low. The inverted 100 Hz clock and the P>Q line form the inputs for the lower left series of gates. Use of the inverted clock ensures that the flip-flop will not change states during the logical "1" half of a clock pulse. If the P>Q line is high, the gated flip-flop changes state when the clock line is low. This enables the NAND gate (A); the clock pulses then pass through it with inversion and into the count-up input of the counters, as well as through gate (C) (with inversion again) and OR gate (E) into the mode-selector portion. The counters increment on the trailing edge of each clock pulse (rising edge of each inverted clock pulse). When the counter output becomes equal to the data input to the comparators, the P>Q line drops low, immediately changing the state of the flip-flop and inhibiting the NAND gate (A), thus preventing further clock pulses from reaching the counters or the mode selector. The P<Q line is connected to the lower-right flip-flop cir-

FIGURE 28

POSITION PORTION

OF MOTOR CONTROLLER CIRCUIT


BIT 13
BIT 12
BIT 11
BIT 10
BIT 07
BIT 06
BIT 05
BIT 04
BIT 03
BIT 02
BIT 01
BIT 00
SN7485
SN7485
SN7485
SN74193
SN74193
SN74193
A
B
C
D
E
TO MODE SELECTOR POSITION INPUT
TO MODE SELECTOR FORWARD/ REVERSE INPUT
CLOCK (100 Hz)

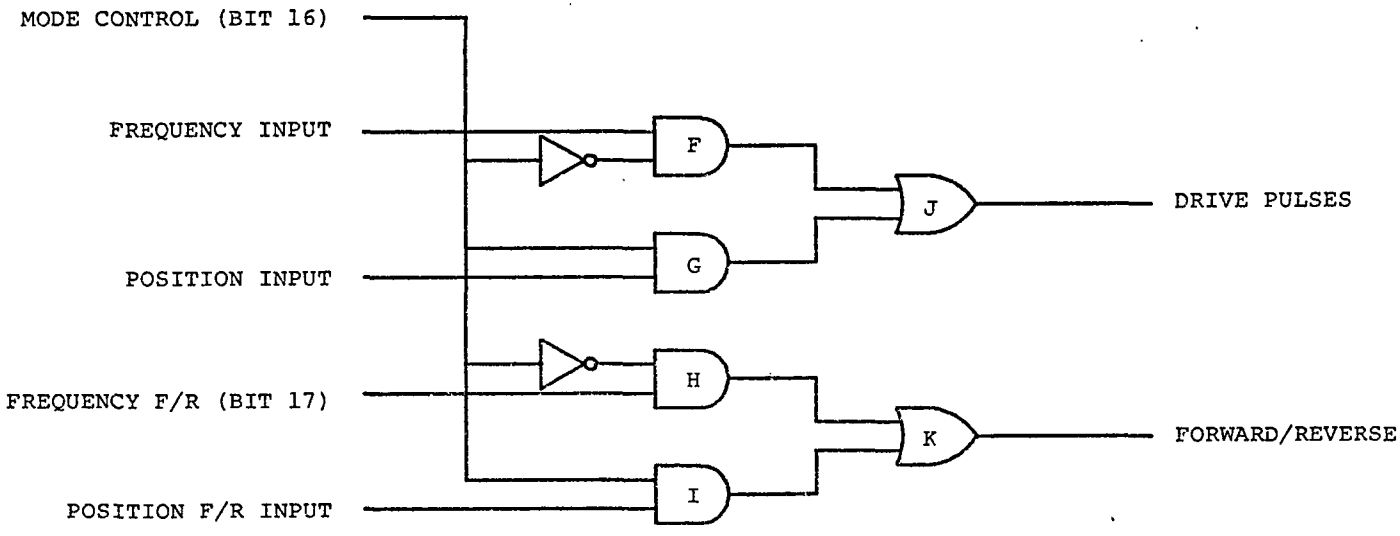
cuitry which is connected to the count-down input of the counters. This functions identically to the count-up portion, using gates B and D and the OR gate (E). The P<Q line also determines the direction of motor movement. If the desired position is greater than the present position, the P<Q line is low, signifying forward motion. If the desired position is less than the present position, the P<Q line is high, signifying forward motion. This line is sent to the mode selector also.

Mode selector. The mode selector portion of the circuit (Figure 29) selects which set of pulses (from the position or frequency circuitry) and which direction command (from the forward-reverse bit or the position circuitry) will be sent to the motor driver. The mode control bit (0 = frequency, 1 = position) is input to all four NAND gates, with inversion for gates F and H. Thus, for frequency mode, gates F (pulses from frequency portion) and H (direction from forward/reverse bit) are activated; in position mode, gates G (pulses from position portion at fixed frequency) and I (direction from position portion) are activated. The two AND gates for pulses (F and G) are connected to an OR gate (J) which sends the pulses to the motor driver, and the two AND gates for direction (H and I) are connected to an OR gate (K) which sends the proper level to the motor driver.

FIGURE 29

MODE SELECTOR PORTION

OF MOTOR CONTROLLER CIRCUIT


MODE CONTROL (BIT 16)
FREQUENCY INPUT
POSITION INPUT
FREQUENCY F/R (BIT 17)
POSITION F/R INPUT
F
G
H
I
J
K
DRIVE PULSES
FORWARD/REVERSE

The circuit is used with an interface to the HP9830A computer. The outputs of the circuit (drive pulses and forward/reverse) are sent to one of twelve 9904-131-03003 four-phase motor driver cards (North American Philips, Chesire, CT) which, in turn, is connected to one of twelve K82816-P1 stepping motors (North American Philips). Again, the frequency portion is used to drive devices such as pumps, while the position mode is used for devices such as potentiometers and valves. With the internal gearing of the motors, each revolution corresponds to 200 steps; thus, in position mode, up to 20 turns of a device are possible.

(b) Relay control. The interface is capable of controlling the power to any device which can be plugged into an AC outlet, via a solid-state relay. Using address 77_8 and select code 57_8 will turn on the device plugged into the relay; sending address 77_8 and select code 17_8 will turn off the device (the middle 16 bits, the data, do not matter in this process).

(c) ADC. A 12-bit analog-to-digital converter (model ADC-12QZ, Analog Devices, Norwood, MA) can sample any analog voltage (as from a colorimeter or other detector) via an instrumentation amplifier (model AD-521J, Analog Devices). When an address of 77_8 and a select code of 20_8 is issued (again, the data bits are irrelevant), the ADC converts the analog voltage present at its input to a digital value,

which is then automatically transmitted back to the computer rack. A separate remote transmitter handles this task, setting an address of 00_8. The data is then latched into the two 8-bit buffers for the computer to read.

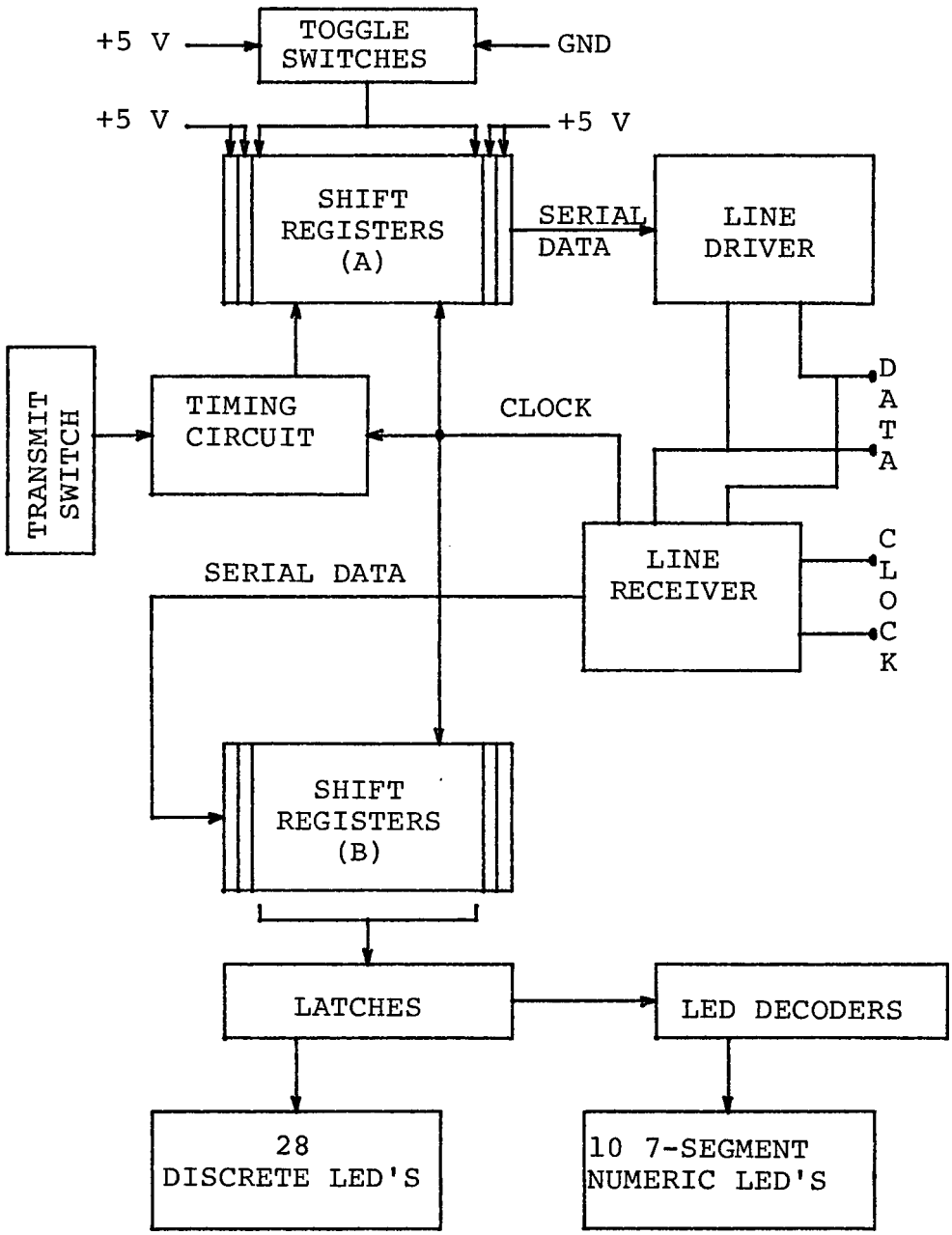
(4) Programmable clock. Located in the computer rack, the programmable clock derives its timing from the AC power line (60 Hz). The clock can be programmed in 1/60 sec increments by placing the number of increments desired in the 16 data bits and adressing device 76_8. The computer can query the clock by "writing" to channel 3. The computer will not continue to the next line in its programming until the clock indicates, via channel 3, that one of the pre-selected increments has passed. Thus, if the clock is programmed with 60_{10}, it will indicate to the computer when 1 second has passed. In practice, the clock is programmed with 60 1/60-ths of a second (or 1 second) and the computer writes to channel 3. The next step in the program would be the correct transmission to actuate the ADC, and the computer would then read its two buffers. In this manner, the computer will take data at 1-sec intervals.

(5) MIDIM. MIDIM is a data input and reception module (see Figure 30). A series of shift registers (A) allows the input of data (28 bits; the four framing bits are hard-wired to +5 volts). The data is input from a row of 28 toggle switches which direct either +5 volts (logical "1")

FIGURE 30

BLOCK DIAGRAM OF MIDIM

(MANUAL INTERFACE DATA INPUT MODULE)


+5 V
TOGGLE SWITCHES
GND
+5 V
+5 V
SHIFT REGISTERS (A)
SERIAL DATA
LINE DRIVER
TRANSMIT SWITCH
TIMING CIRCUIT
CLOCK
DATA
LINE RECEIVER
CLOCK
SERIAL DATA
SHIFT REGISTERS (B)
LATCHES
LED DECODERS
28 DISCRETE LED'S
10 7-SEGMENT NUMERIC LED'S

or ground (0 volts, logical "0") to each of the 28 inputs. A transmit switch is then pressed, and a timing circuit allows a clock to shift all 32 bits of data, which are directed (as serial data) to a line driver. The line driver converts the serial (one-line) data to the serial differential (two-line) data and transmits it on the interface cable.

A line receiver is hooked to the clock pair of the interface cable, and the differential clock signal is converted to a serial clock. This clock is used to shift the data into and out of the shift registers and to count the 32 bits in the transmission timing circuit.

The second half of the line receiver is hooked to the data pair of the interface cable, and the differential data is converted to serial data corresponding to the transmitted 32-bit word. The serial data is strobed into a set of shift registers (B), and, if the four framing bits are detected (indicating a complete word), the 28 non-framing bits are latched. The outputs of the latches are directed to two displays:

(a) A set of 28 discrete LED's is used to show the word in binary form. (All that is necessary is that the outputs be sent through a resistor, the LED, and then to +5 volts.) The LED's are color-coded: the 6 right-most (address) are green; the middle 16 (data) are red; and the 6 left-most (select code) are yellow.

(b) The latch outputs also go through a set of LED decoders, resistors, 7-segment numerical LED's, and then to +5 volts. There are ten of the 7-segment LED's, which are also color-coded: the 2 right-most are green, the 6 middle are red, and the 2 left-most are yellow. The numerical LED's display the word in octal (base 8) format, complementing the discrete LED's and providing easier comprehension of the data. The ranges of displays are 00_8 - 77_8 for the address and select code LED's and 000000_8 - 177777_8 for the data LED's.

MIDIM thus provides a visual display of any word transmitted on the interface, since it latches and displays the word regardless of its address. Thus, any word sent by the computer, the ADC, or MIDIM itself is displayed.

CHAPTER VI

CHROMATOGRAPHY

Chromatography is an important analytical tool for separating components in a mixture. The resolution of peaks in a chromatogram, then, is an important response for optimization.

The chromatograph used was a Varian 1200 gas chromatograph with a flame-ionization detector. Two operational parameters which directly affect resolution and which are reasonably easy to control are column temperature and carrier gas flow rate. To effect computer control, the following modifications were made to the GC:

(1) Flow rate control. A stepper motor was connected, via a slip clutch, to a standard needle valve. In position mode, the motor and valve had an allowable range of 0 - 500_{10}. Zero was chosen so as to not quite close the valve entirely; 500 was found to be the largest possible position.

(2) Temperature control. A second stepper motor was connected, via a slip clutch, to a 10-turn, 200-ohm potentiometer, which was used in place of the standard 200-ohm potentiometer, which could be rotated only 3/4 of a turn (thus not allowing sufficient resolution). The range in position mode was 0 to 960_{10}; 0 was the lower limit of the potentiometer and 960 gave approximately 180 ^{o}C (the maximum rated temperature for the first column used).

(3) Sample injection. This step also needed to be accomplished under computer control. Automated syringe-

type injectors were rejected as being too expensive. The following apparatus was constructed (Figure 31): Nitrogen was continually bubbled through the sample, picking up sample vapor. The vapor stream passed through a gas sampling valve (GSV) (Varian) and, usually, out to waste (connected to an aspirator). The GSV was actuated by a solenoid which was controlled by the computer-addressable relay. The computer could thus make an injection and control the length of time of the injection. To avoid pressure build-up during an injection, a water trap was connected to the stream. When the solenoid moved the GSV to the inject position, carrier gas was directed through the sample loop in the GSV, thereby transferring a quantity of sample vapor-laden gas into the GC.

(4) Data acquisition. The input to the ADC was linked to the GC analog output. The signal had to be sampled prior to the recorder attenuation circuitry to obtain the proper range of voltage for the ADC.

Instrumental operation. The following instrumental parameters were kept constant:

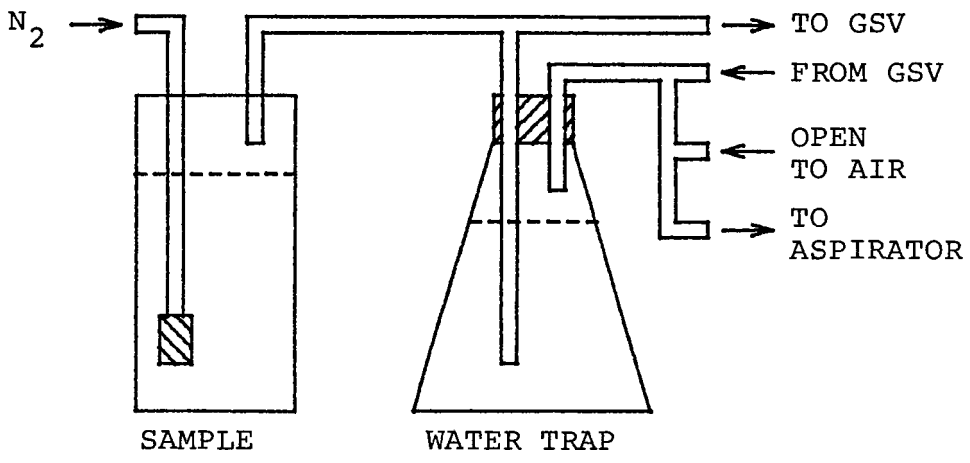
(1) Injector temperature -- set to position 3.0, corresponding to 180 ^{o}C.

(2) Detector temperature -- set to position 1.5, corresponding to 240 ^{o}C.

(3) Carrier gas (nitrogen) pressure -- set to 60 PSIG.

FIGURE 31

GC AUTOMATED SAMPLING SYSTEM


N_2
TO GSV
FROM GSV
OPEN
TO AIR
TO
ASPIRATOR
SAMPLE
WATER TRAP

(4) Flame gases (hydrogen and air) -- set to standard, Varian-recommended pressures and flow rates (44).

(5) Range control -- set to 10.

Preliminary studies. The following two preliminary studies were undertaken:

(1) Reproducibility of the carrier gas flow rate and column temperature using the stepper motors. The positioning of the valve and potentiometer were reasonably reproducible (although not linear) when moving from a lower position, but in moving to the same point from a higher position, a hysteresis was apparent. Therefore, whenever one of the motors was changed in position, it was first driven to a low value (0 for the temperature, 100_8 for the flow rate so as to always have some carrier gas flowing) and then upward to the new position.

(2) Effect of injection time and sample stream nitrogen flow rate. A higher sample stream nitrogen flow rate gave increased peak heights, but it was found that if the flow rate was fixed, the peak heights remained virtually constant. Up to a point, increasing the time which the GSV remained in the inject position increased the peak heights; it was found that a time of 10 sec gave peaks which

encompassed the ADC range.

Response. The response used for evaluating the chromatograms was the chromatographic response function (CRF) (45) (see Figure 32). The response used is:

$$\text{response} = \sum_{i=1}^{n} \log (b_i/a_i) \qquad (13)$$

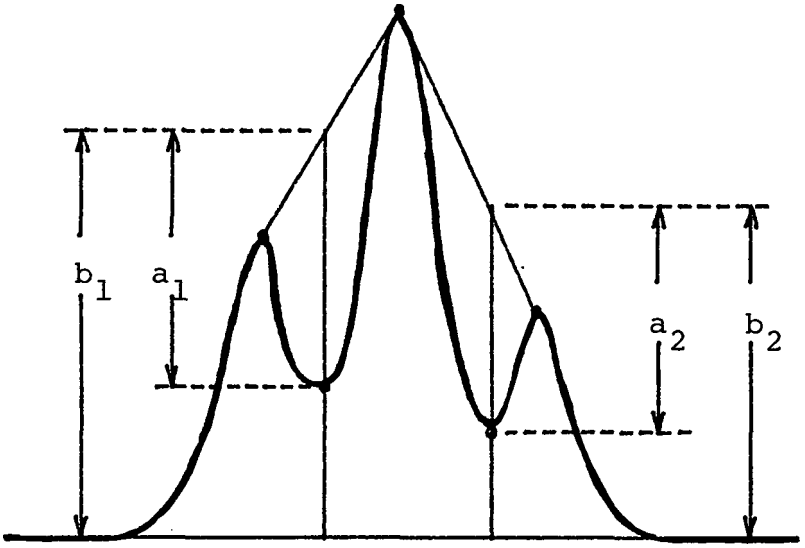
where n is equal to the number of pairs of adjacent peaks (and thus one less than the number of components) in the mixture. If complete baseline separation of all the components is achieved, a_i will equal b_i, and all the terms will be zero. As peaks merge, b_i will become less than a_i, b_i/a_i will become less than 1, and the log terms will become negative. The CRF thus varies from $-\infty$ (worst) to 0 (best).

Initial system. The first column used was graphitized carbon black coated with 2,3,4,5-tetranitrofluorenone (2,3,4,5-TeNF) (Supelco, Inc., Bellefonte, PA). The column was reported to be well-suited for the separation of aromatics (46). In early tests, this was found to be true, as even the three isomers of xylene (o, m, and p) were separable. However, an influx of noise into the interface during one of the preliminary runs caused the temperature potentiometer motor to set the column temperature to 300 ^{o}C; the resulting degradation of the column rendered it useless.

Final system. The original column (SE-30, Varian) was

FIGURE 32

CHROMATOGRAPHIC RESPONSE FUNCTION (CRF)


b1
a1
a2
b2

was re-installed and the sample mixture changed to benzene, toluene, and p-xylene.

Automated system. When running, the computer would set the temperature and flow rate corresponding to the next set of conditions (new simplex vertex or mapping point), actuate the sample injection relay, and collect data for 10 minutes. If three peaks were not found in 10 min, the retention time was deemed unacceptable and a bad response was assigned to that experiment (a boundary violation on the response). The computer plotted the data as it was acquired, and also stored the data on the mass memory disc for subsequent processing.

Simplex. A 2-factor variable-size simplex algorithm was run on the computer to seek the combination of temperature and flow rate yielding optimum separation. Table II shows the starting vertex coordinates, the step sizes, and the upper and lower bounds. Table III shows the simplex progress, with the flow rate and temperature corresponding to each vertex in motor positions. The progress is also shown in Figure 33 (boundary violations are not shown). The simplex was halted after 20 vertexes. Boundary violations in a factor were assigned a very bad response (-9.999999999 E 99, essentially negative infinity); boundary violations in response, indicated by -9 E 99, indicate that three peaks did not completely elute in 10 min or that

TABLE II

INITIAL SIMPLEX PARAMETERS

(all numbers refer to motor positions)

	Flow Rate	Temperature
Starting vertex	450	900
Step size	-350	-650
Lower bound	64	200[a]
Upper bound	500	960

[a] This setting corresponded to room temperature; control below this setting was impossible.

TABLE III

SIMPLEX PROGRESS

Simplex	Vertex	Flow Rate[a]	Temperature[a]	Response[b]
	1	450	900	-0.448
	2	112	732	-9 E 99
1	3	359	272	-9 E 99
	4	203	1360	*
2	5	320	544	0.033
	6	658	712	*
3	7	249	727	-9 E 99
	8	119	371	-9 E 99
4	9	202	503	-9 E 99
5	10	273	320	-9 E 99
	5[c]	320	544	0.042
6	11	392	361	-9 E 99
7	12	439	585	-0.021
	13	367	768	-0.158
8	14	373	666	-0.052
	5[c]	320	544	0.038
	15	255	625	-9 E 99
9	16	393	595	-0.023
	17	340	473	-9 E 99
10	18	365	618	-0.022
11	19	292	567	-0.017
	5[c]	320	544	0.019
	20	248	493	-9 E 99

* Boundary violation; response = -9.999999999 E 99

[a] In motor positions

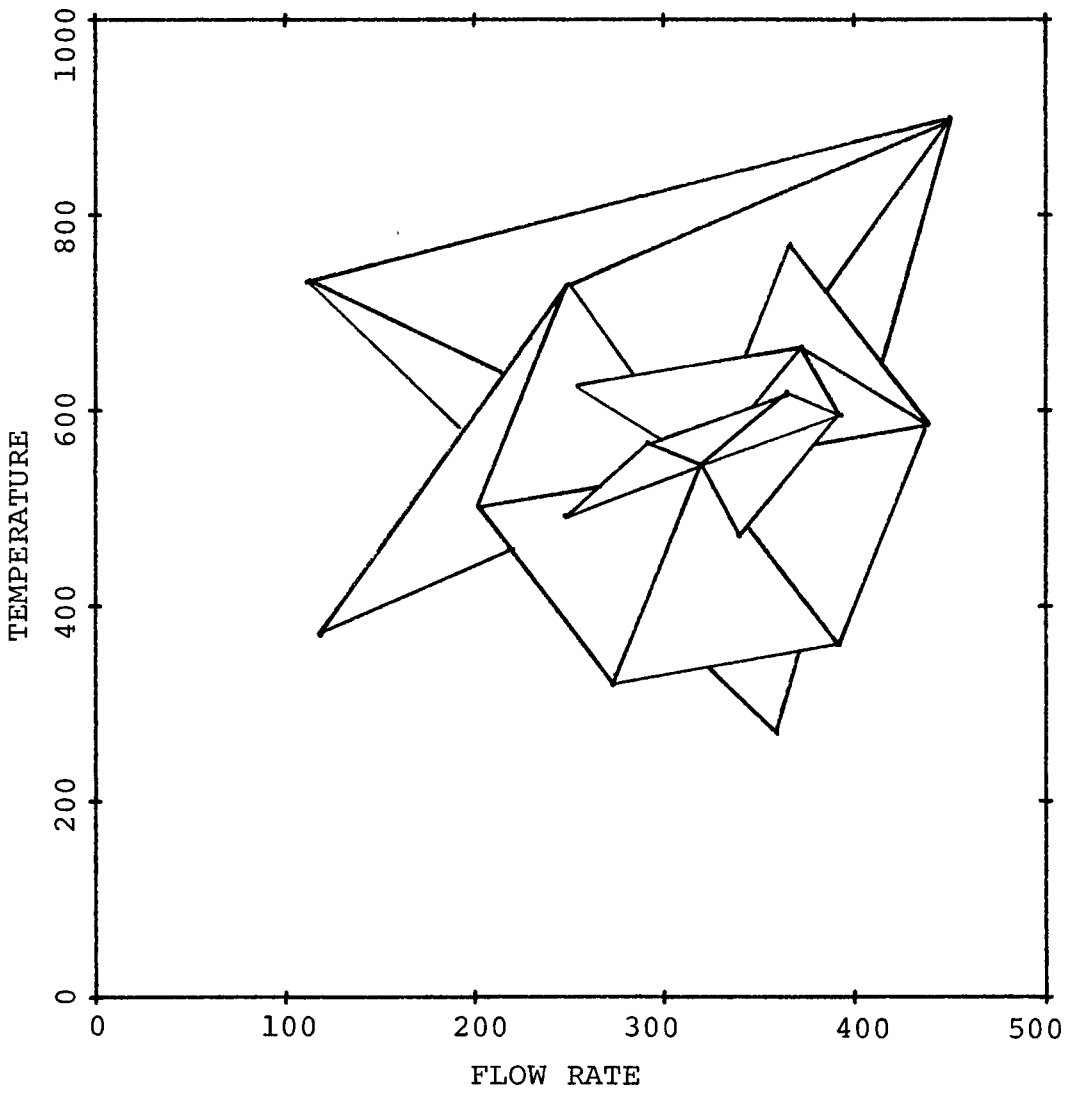
[b] E represents exponentiation (X 10 to the power)

[c] $\underline{n}$+1 re-evaluation

FIGURE 33

SIMPLEX PROGRESS

(Boundary violations are not shown)


TEMPERATURE
0
200
400
600
800
1000
0
100
200
300
400
500
FLOW RATE

the carrier gas flow was not sufficient to keep the flame lit and thus no peaks were detected. The program took the first and last 20 points of each 10-min data set and fit a straight line via least squares. This line was taken as the baseline and was subtracted from all the data points. As a consequence, due to experimental uncertainty it was possible for a valley between two peaks to be lower than the "baseline"; this is the cause of the positive value of the CRF at vertex 5. The programming responsible for controlling the GC and acquiring and analyzing the data is listed in Appendix B. Representative chromatograms from the simplex study are included: vertex 1 (Figure 34), vertex 5 (Figure 35), vertex 10 (Figure 36), and vertex 13 (Figure 37).

The best simplex vertex was number 5, corresponding to 320 motor steps in flow rate and 544 steps in temperature. A 3-level 2-factor factorial design was centered on these coordinates, with a spacing of 50 steps in each factor. The center point experiment was performed an additional 3 times to be able to assess the pure error variance; thus a total of 12 mapping study experiments were performed. Figures 38 through 49 show the chromatograms of the 12 factorial points. The first three represent high, medium, and low temperature at low flow rate; the next six represent high, medium (4 due to center point replicates), and low temperature at medium flow rate; and the final three repre-

FIGURE 34

CHROMATOGRAM FOR VERTEX 1

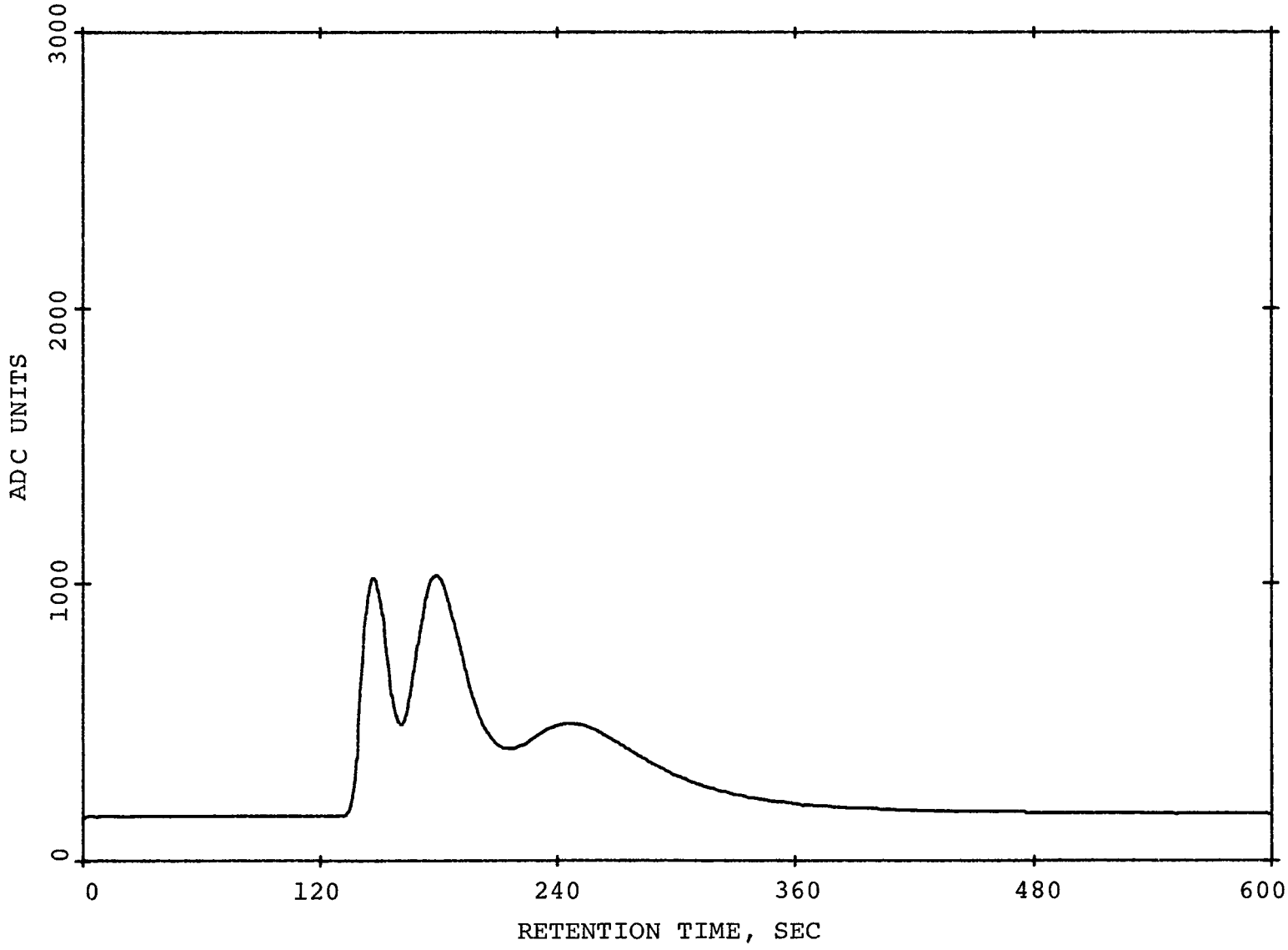

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 35

CHROMATOGRAM FOR VERTEX 5

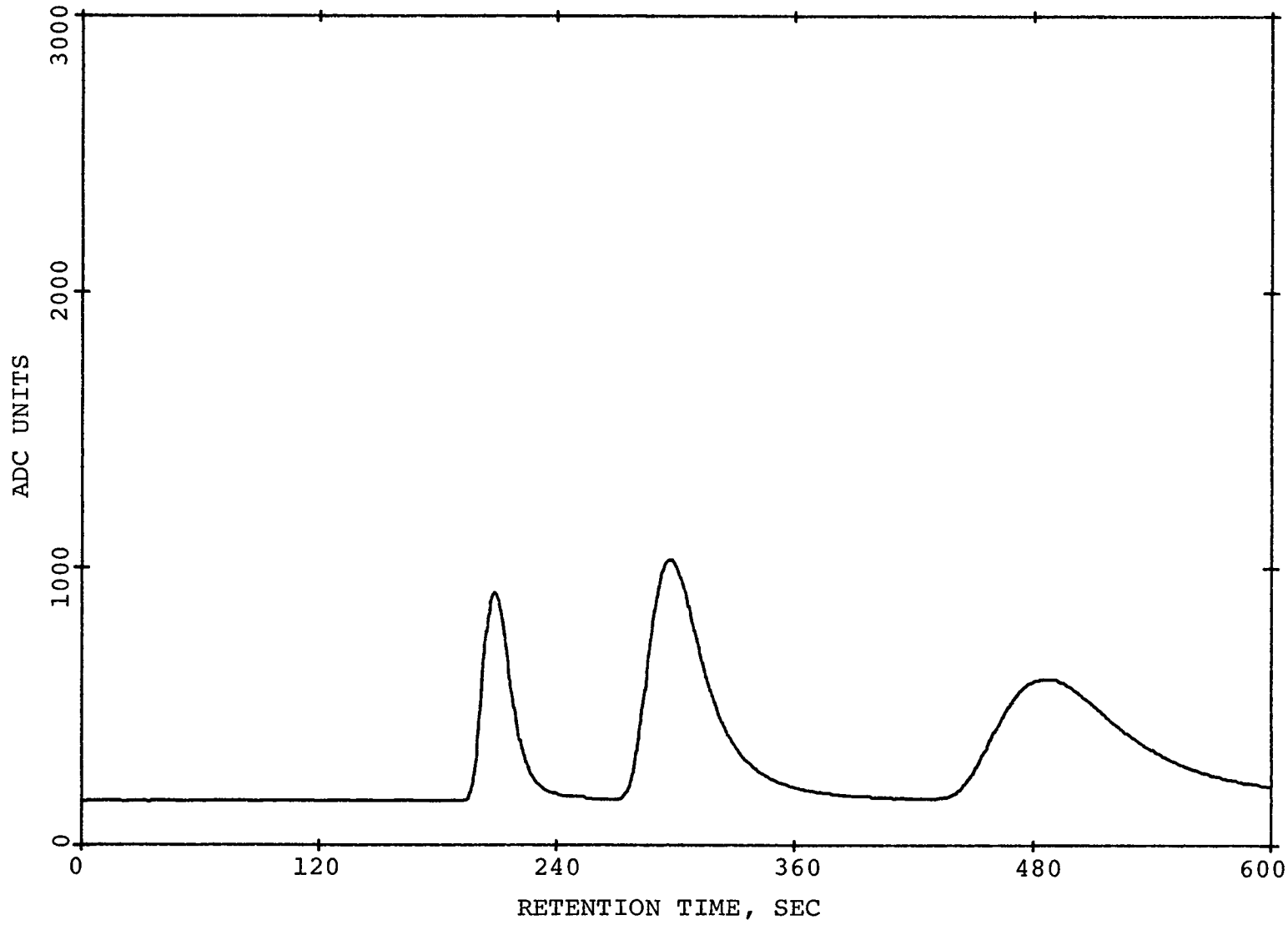

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 36

CHROMATOGRAM FOR VERTEX 10

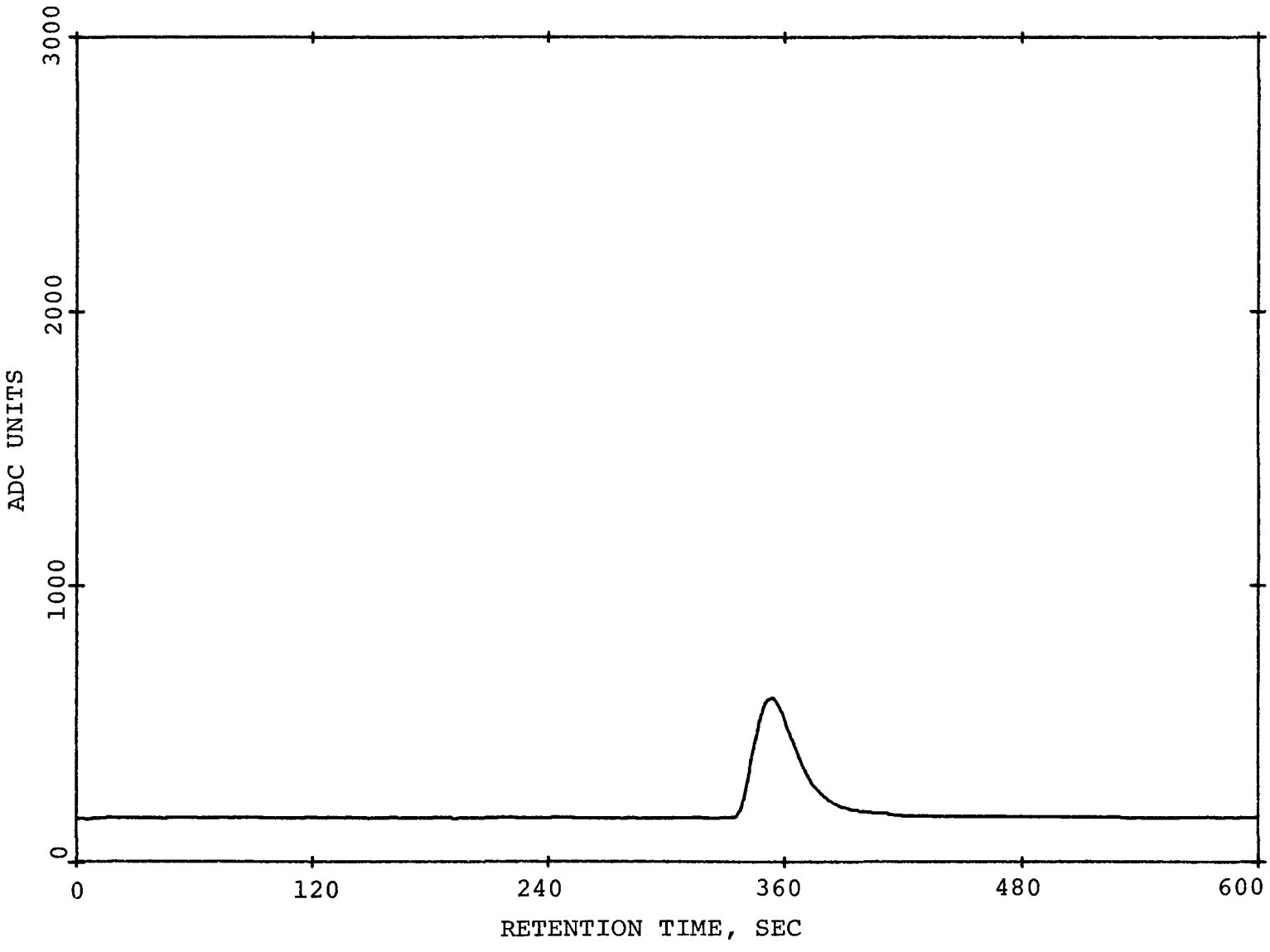

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 37

CHROMATOGRAM FOR VERTEX 13

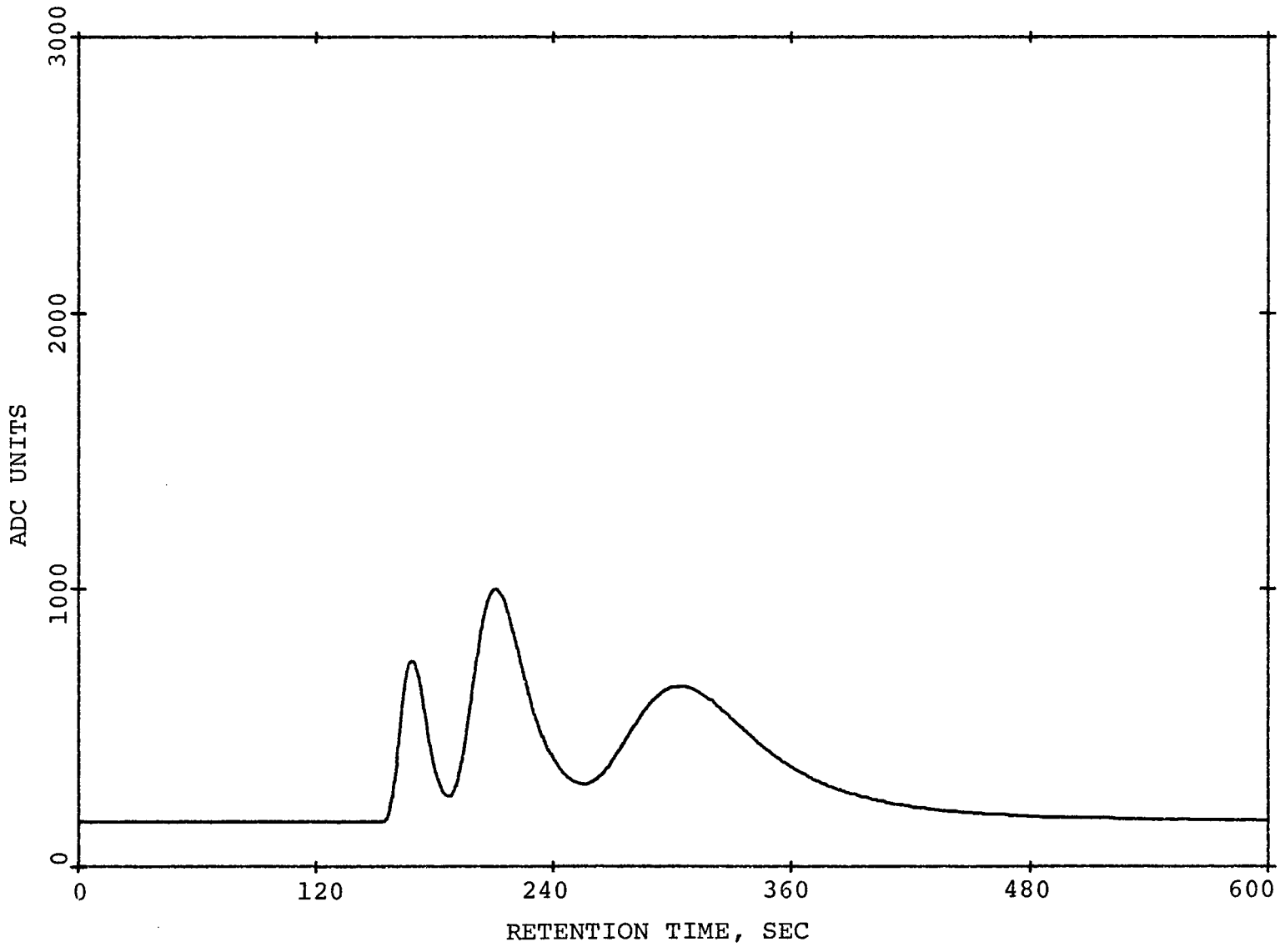

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 38

CHROMATOGRAM FOR FACTORIAL POINT 5

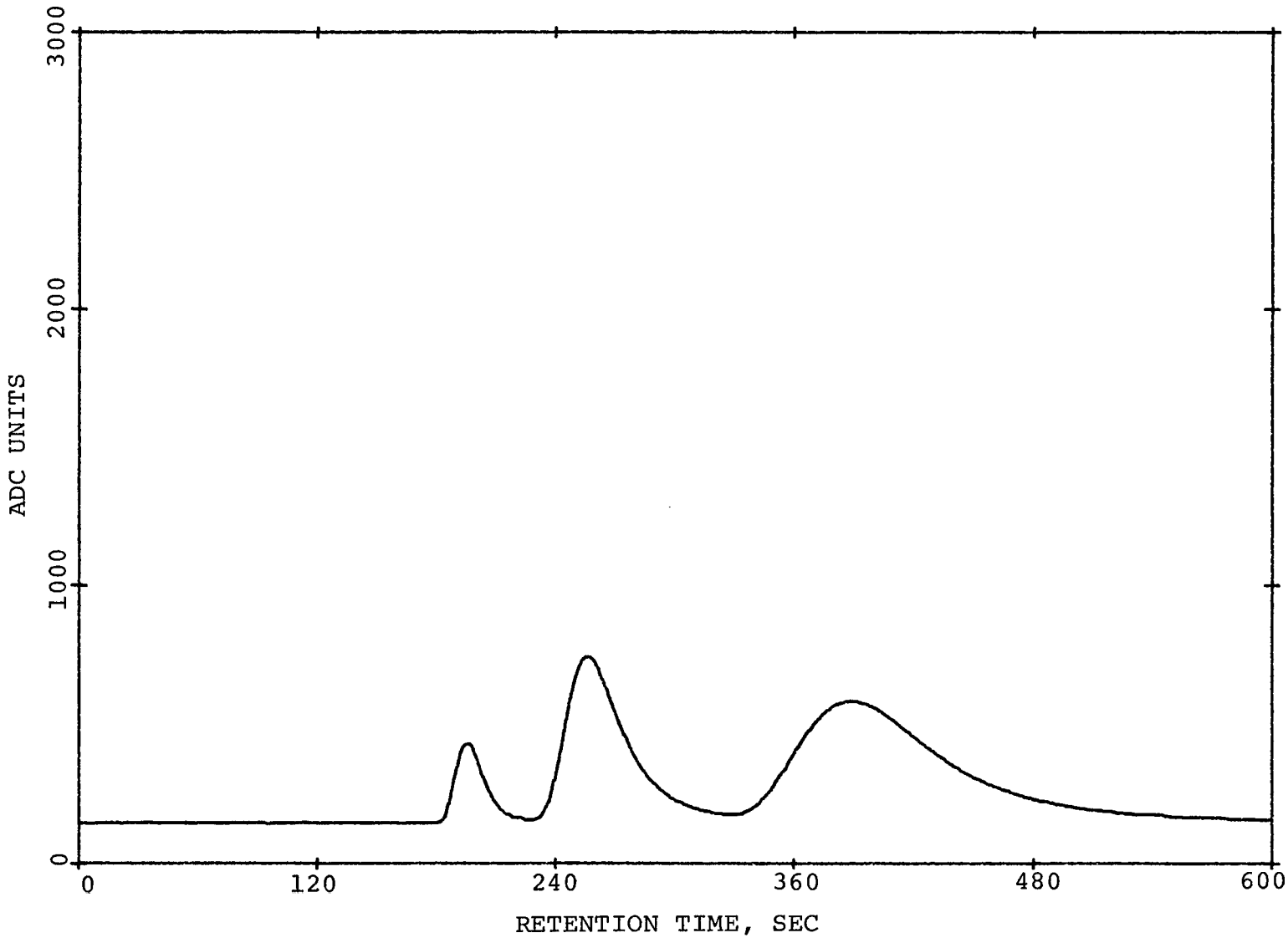

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 39

CHROMATOGRAM FOR FACTORIAL POINT 10

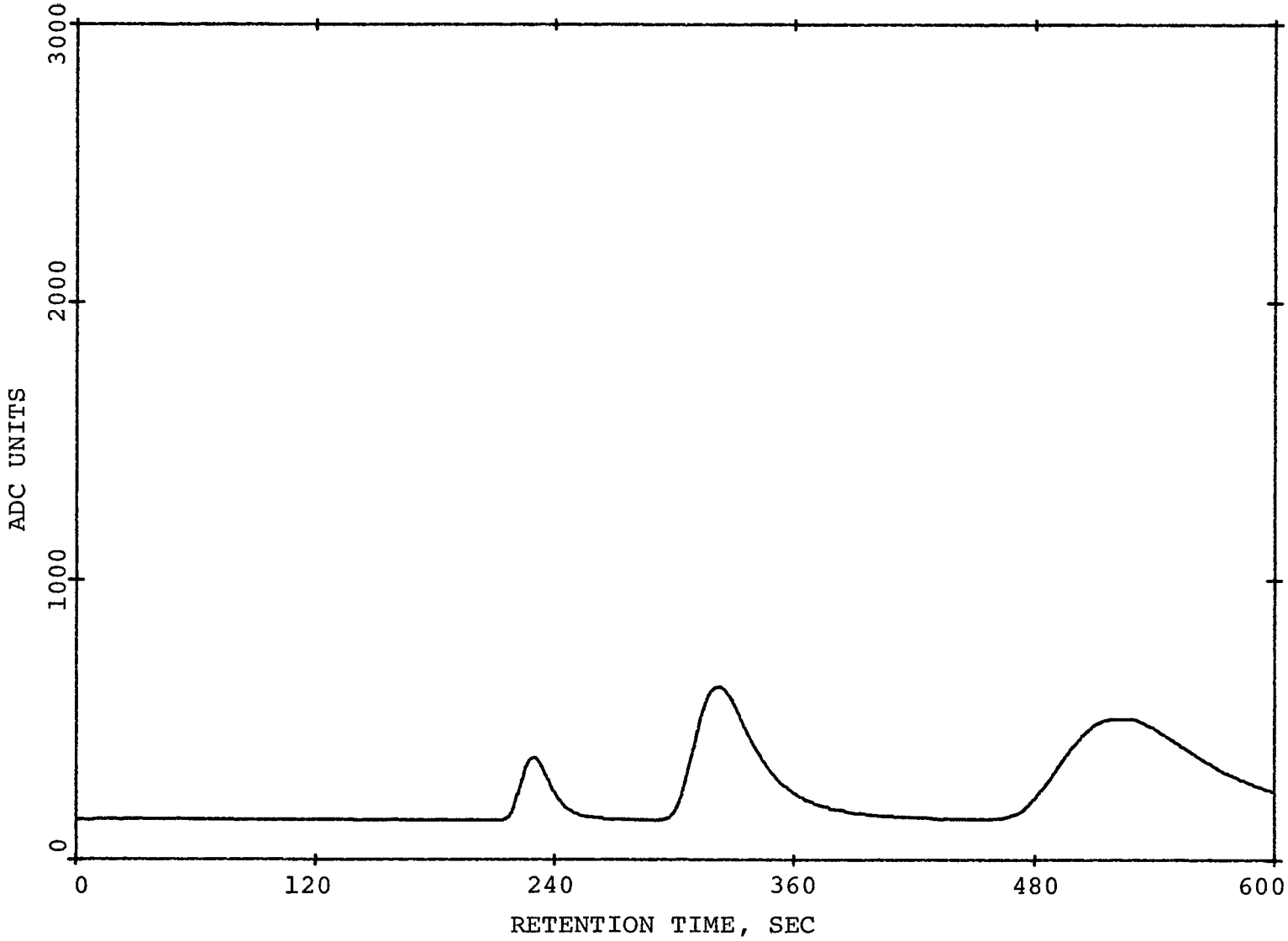

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 40

CHROMATOGRAM FOR FACTORIAL POINT 9

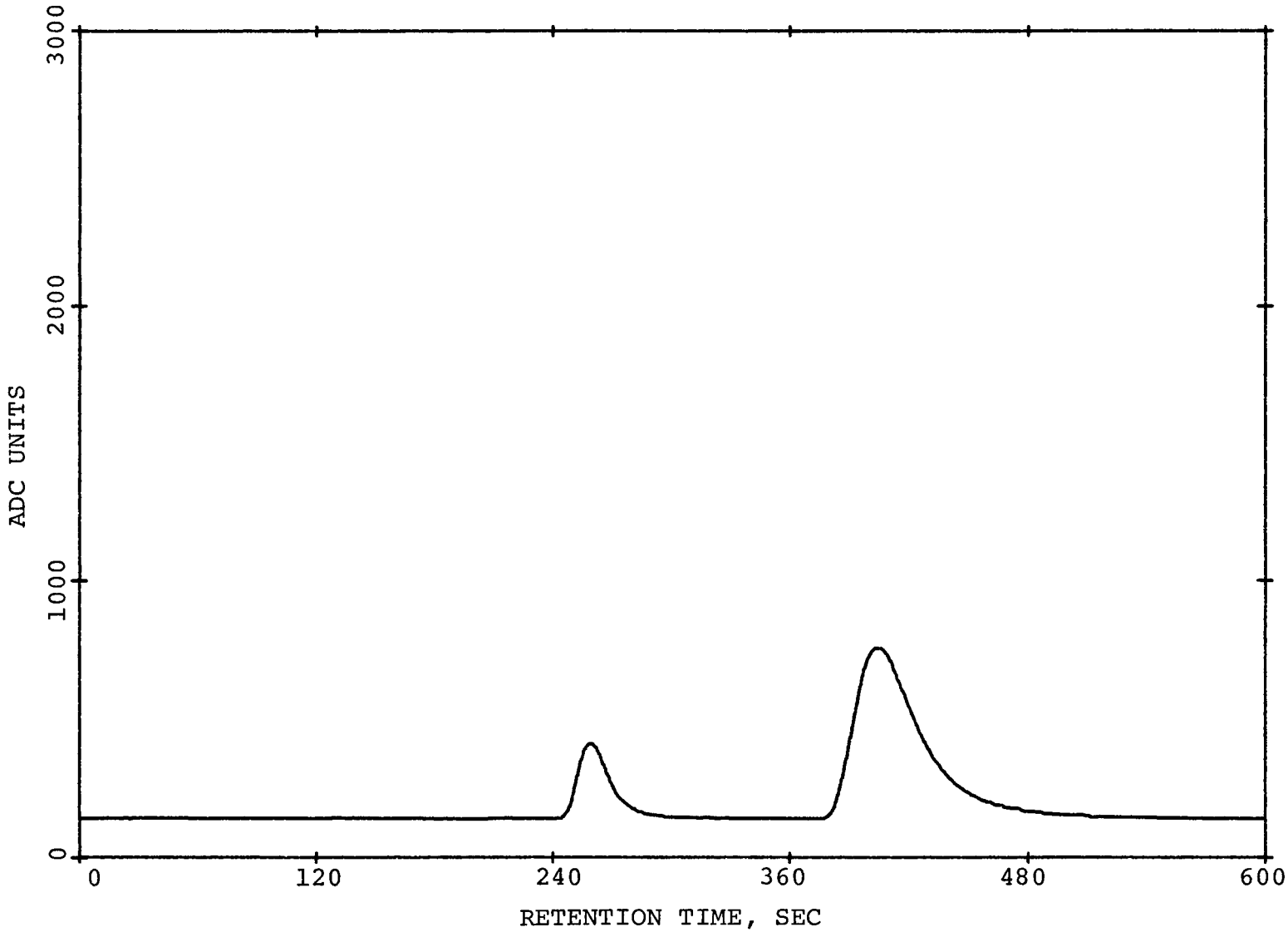

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 41

CHROMATOGRAM FOR FACTORIAL POINT 12

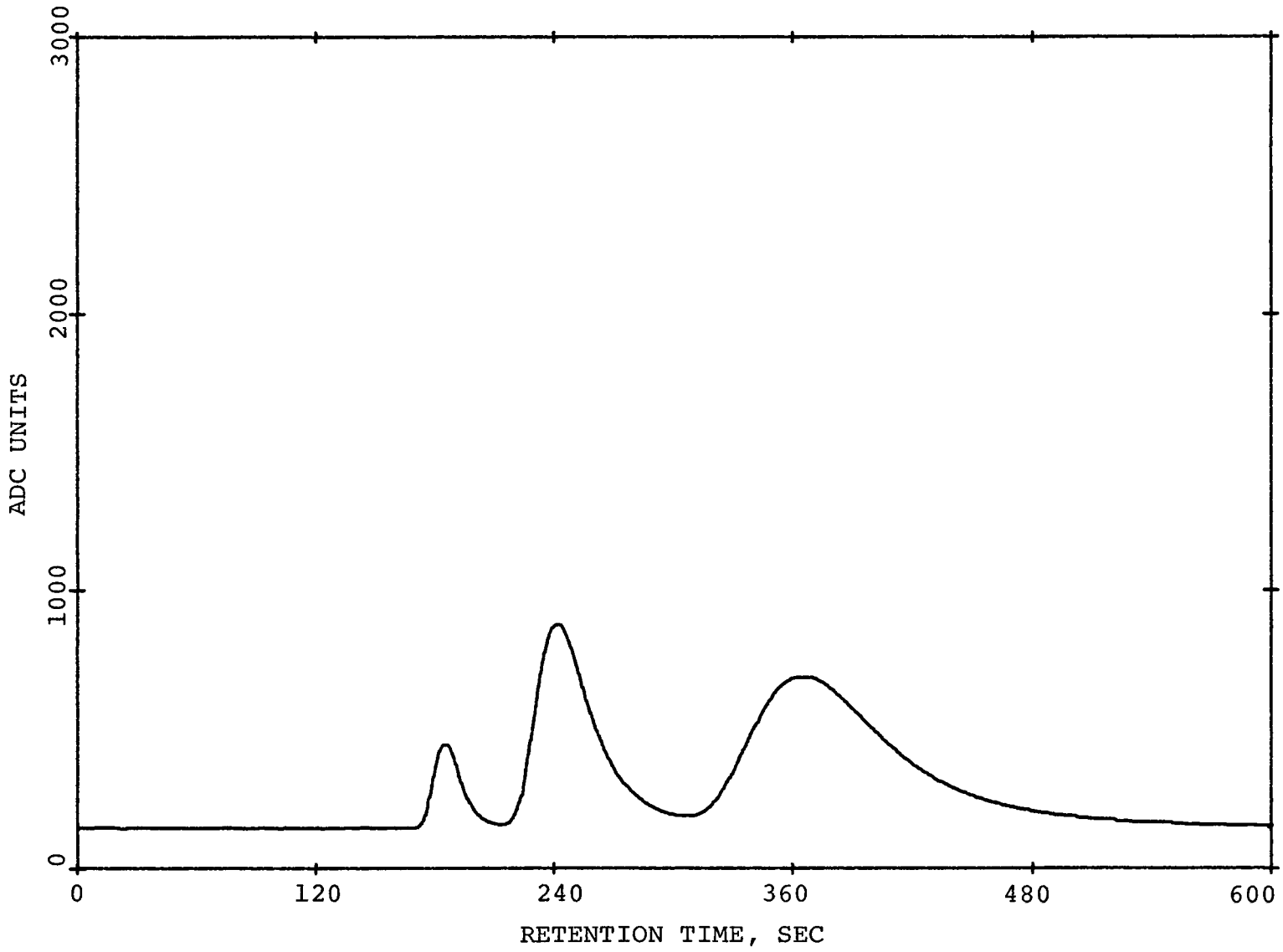

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 42

CHROMATOGRAM FOR FACTORIAL POINT 3

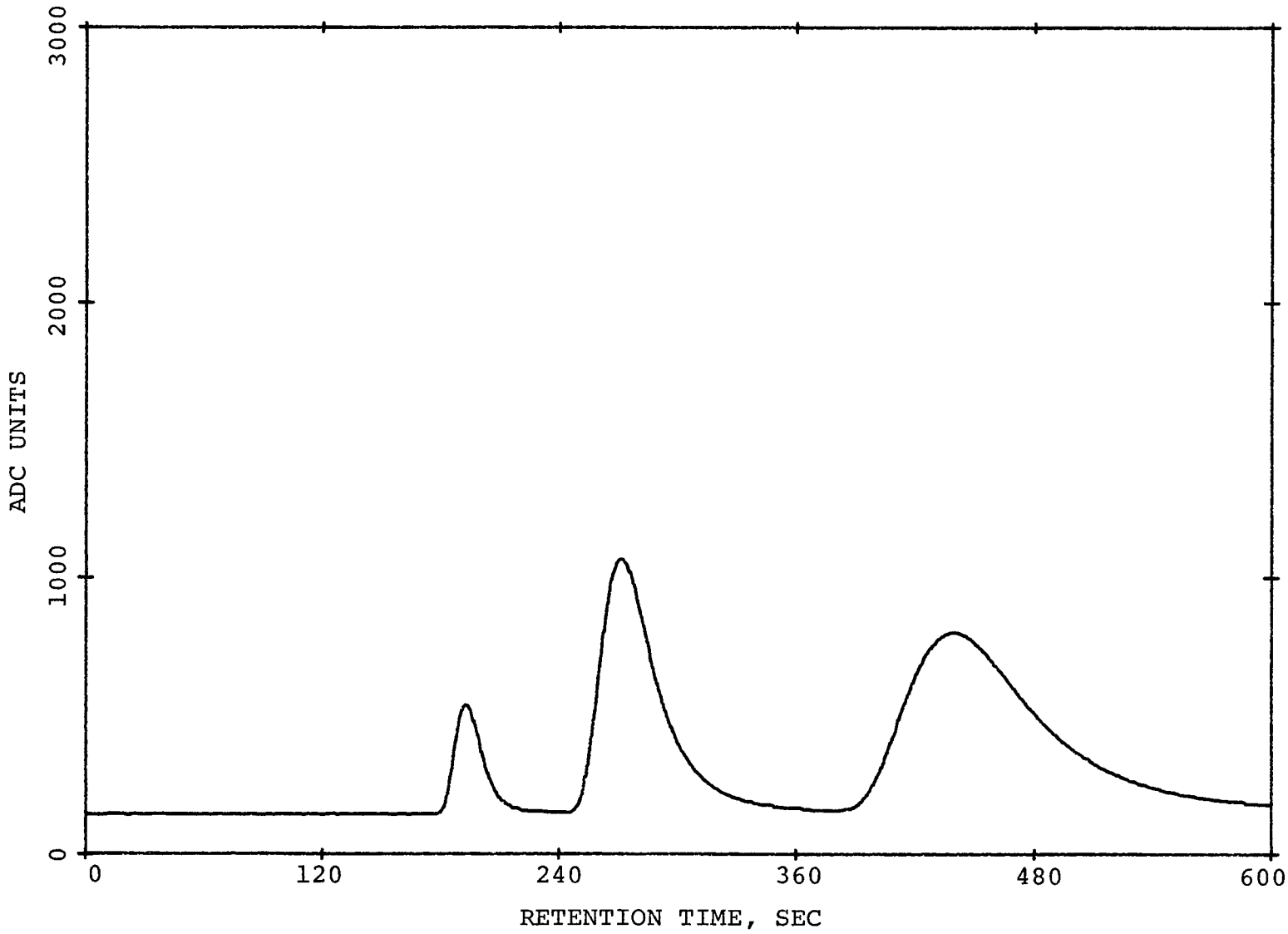

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 43

CHROMATOGRAM FOR FACTORIAL POINT 4

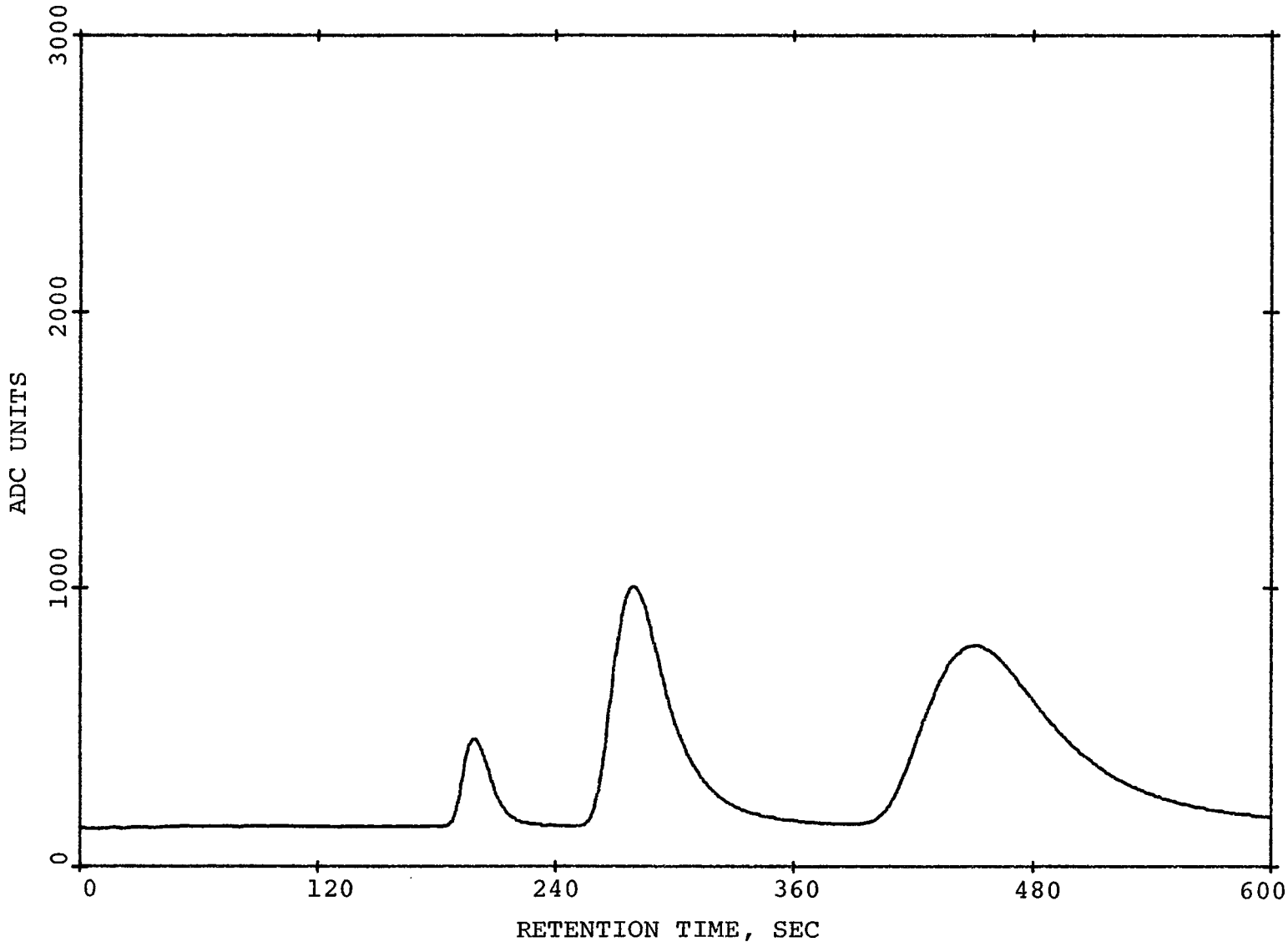

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 44

CHROMATOGRAM FOR FACTORIAL POINT 6

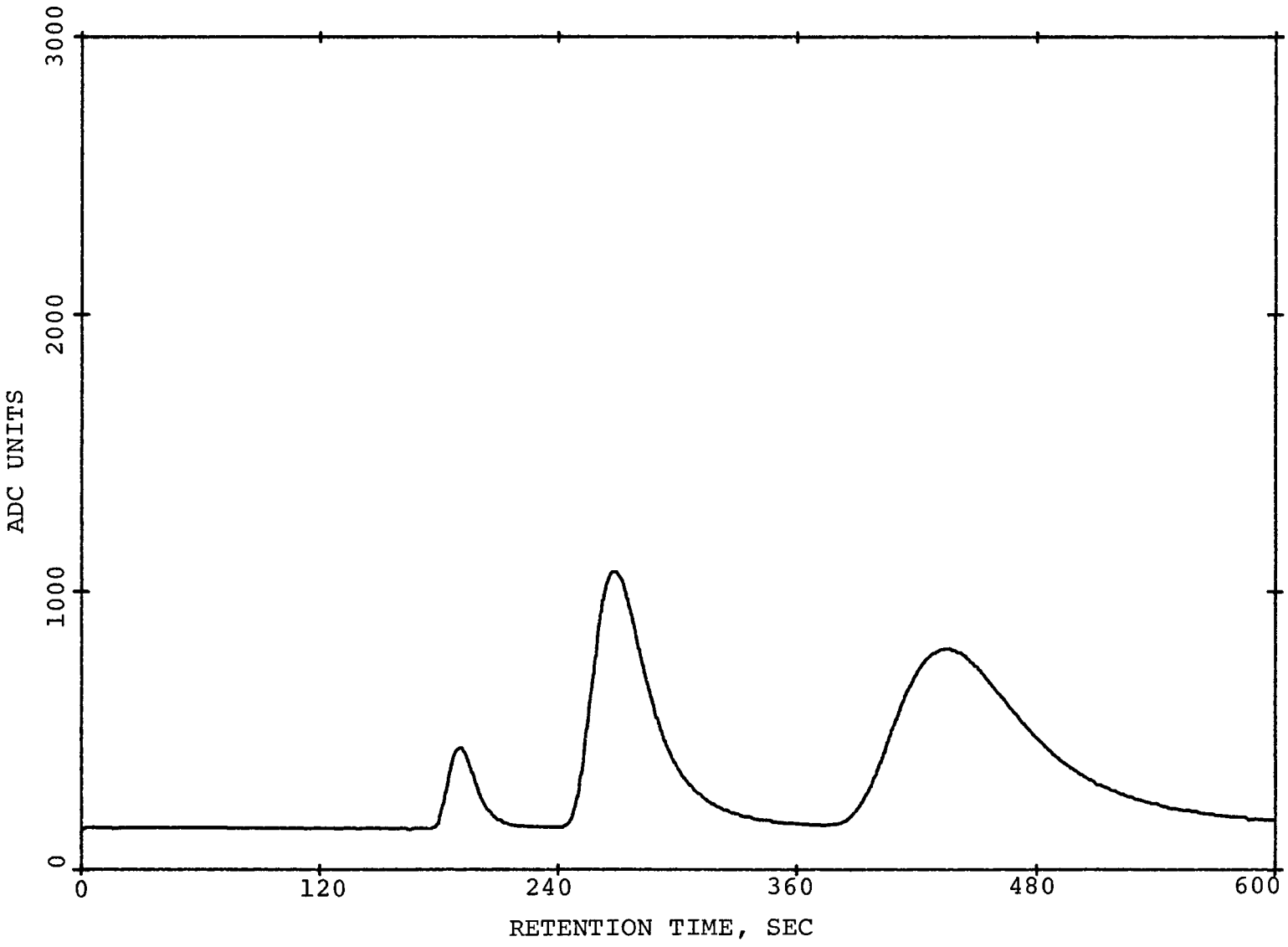

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 45

CHROMATOGRAM FOR FACTORIAL POINT 8

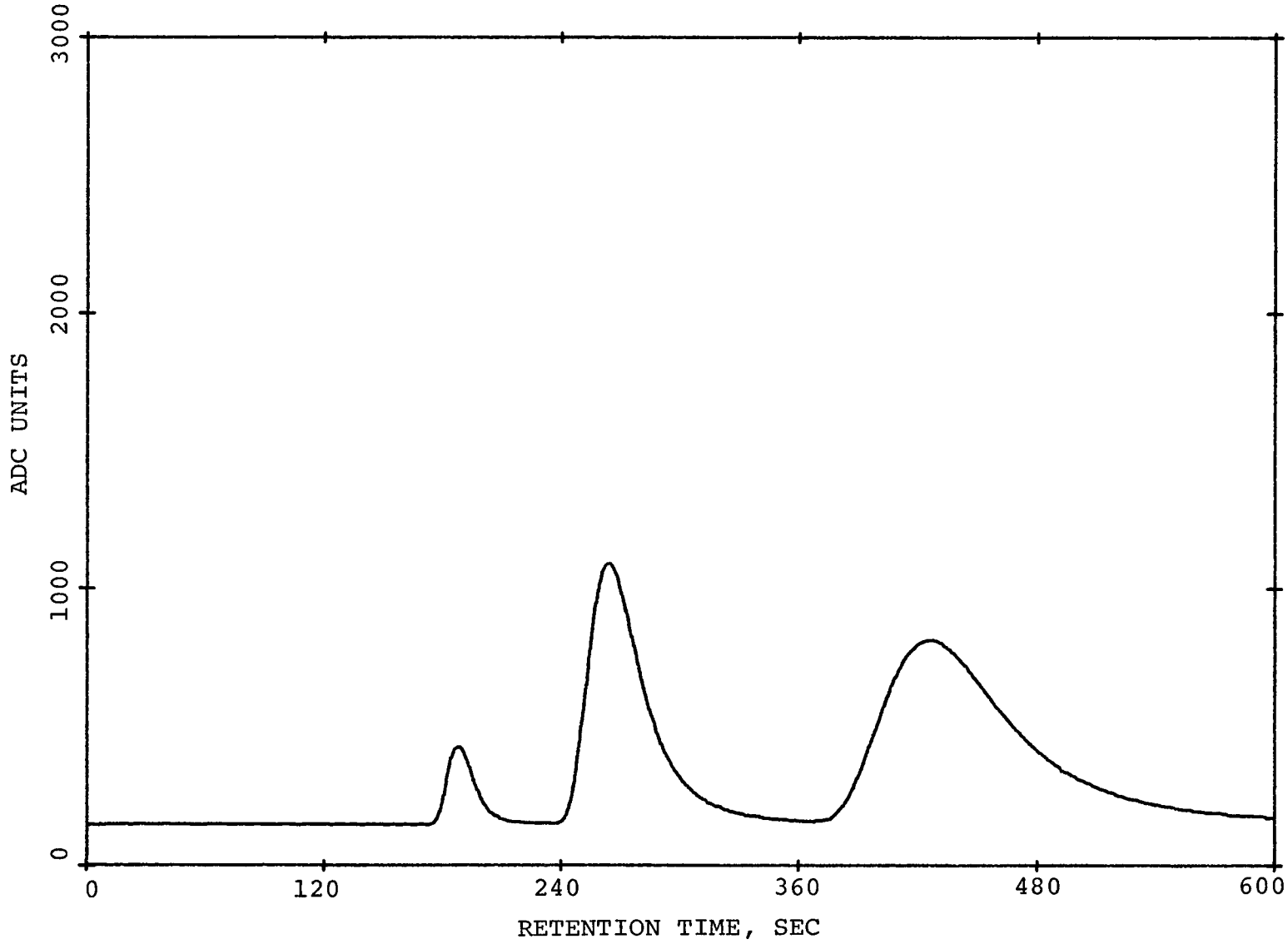

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 46

CHROMATOGRAM FOR FACTORIAL POINT 11

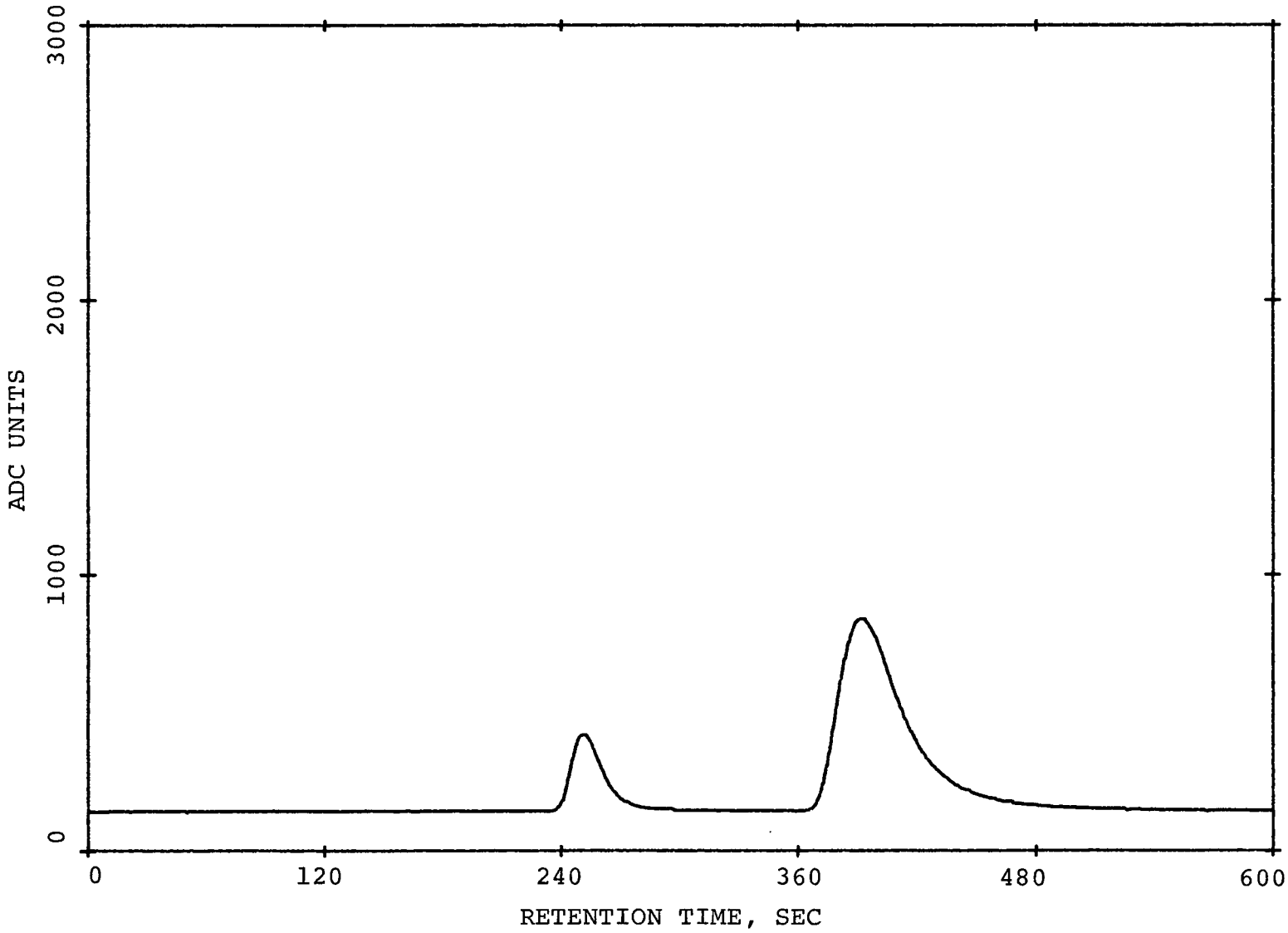

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 47

CHROMATOGRAM FOR FACTORIAL POINT 7

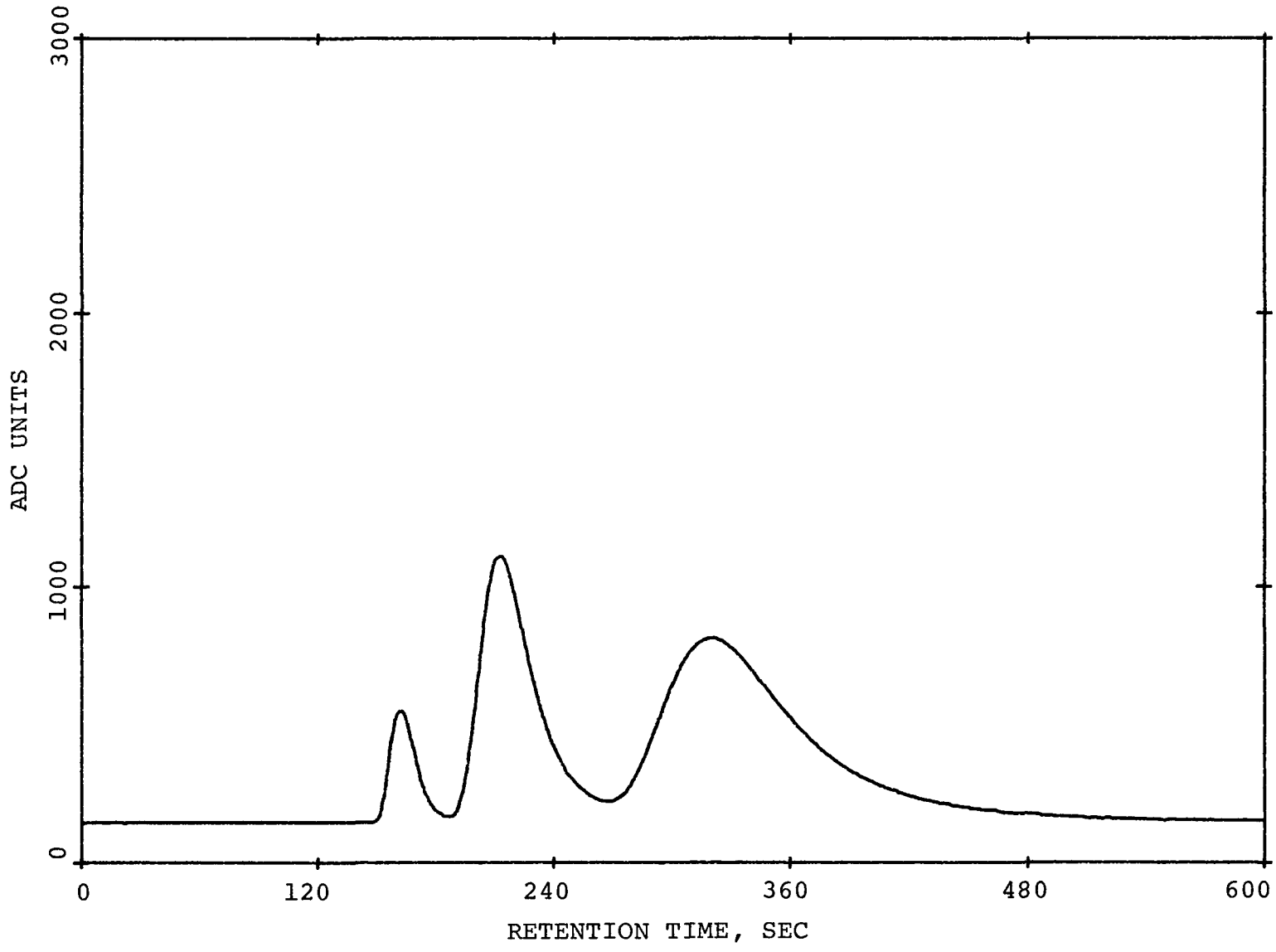

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 48

CHROMATOGRAM FOR FACTORIAL POINT 1

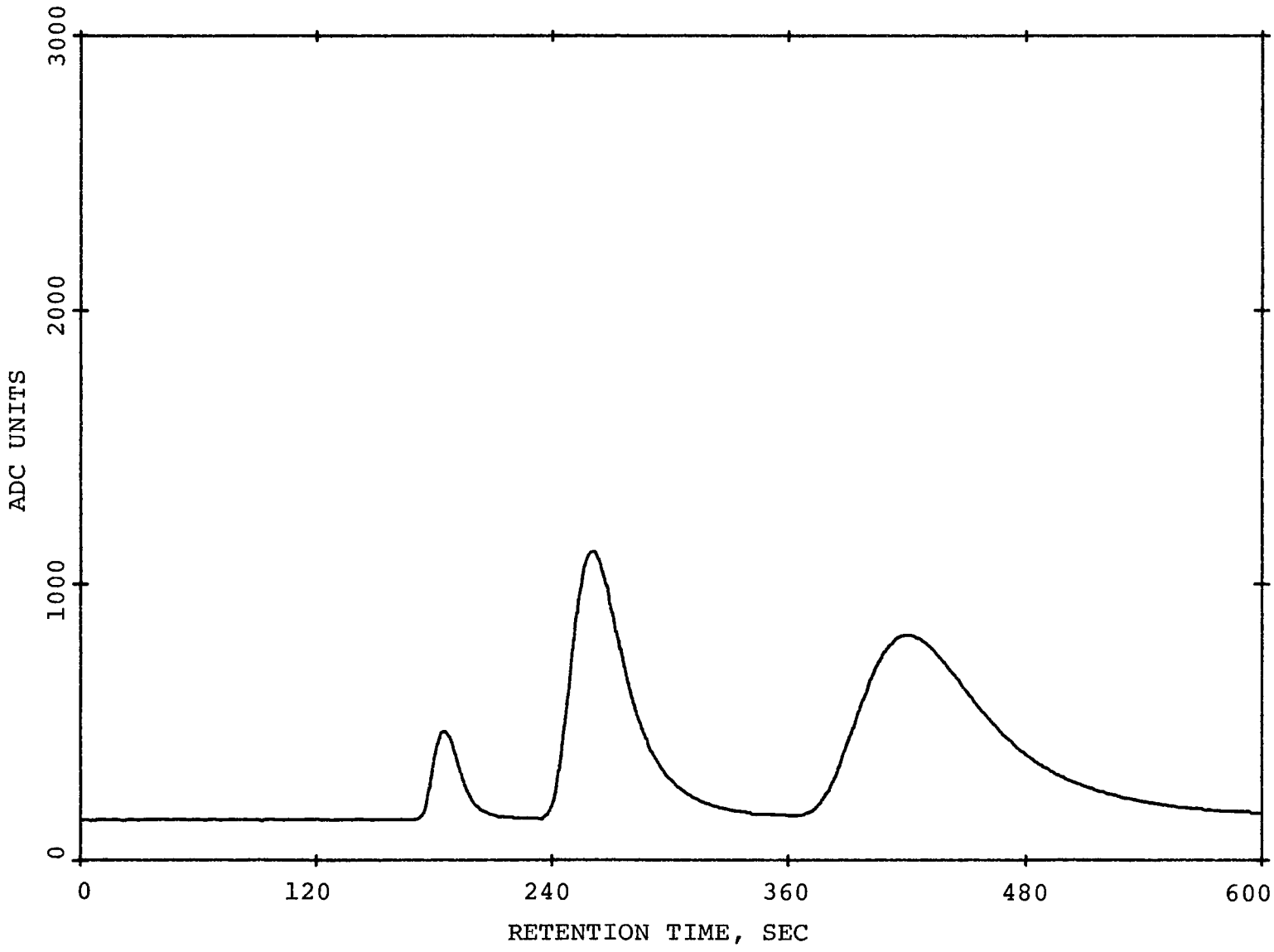
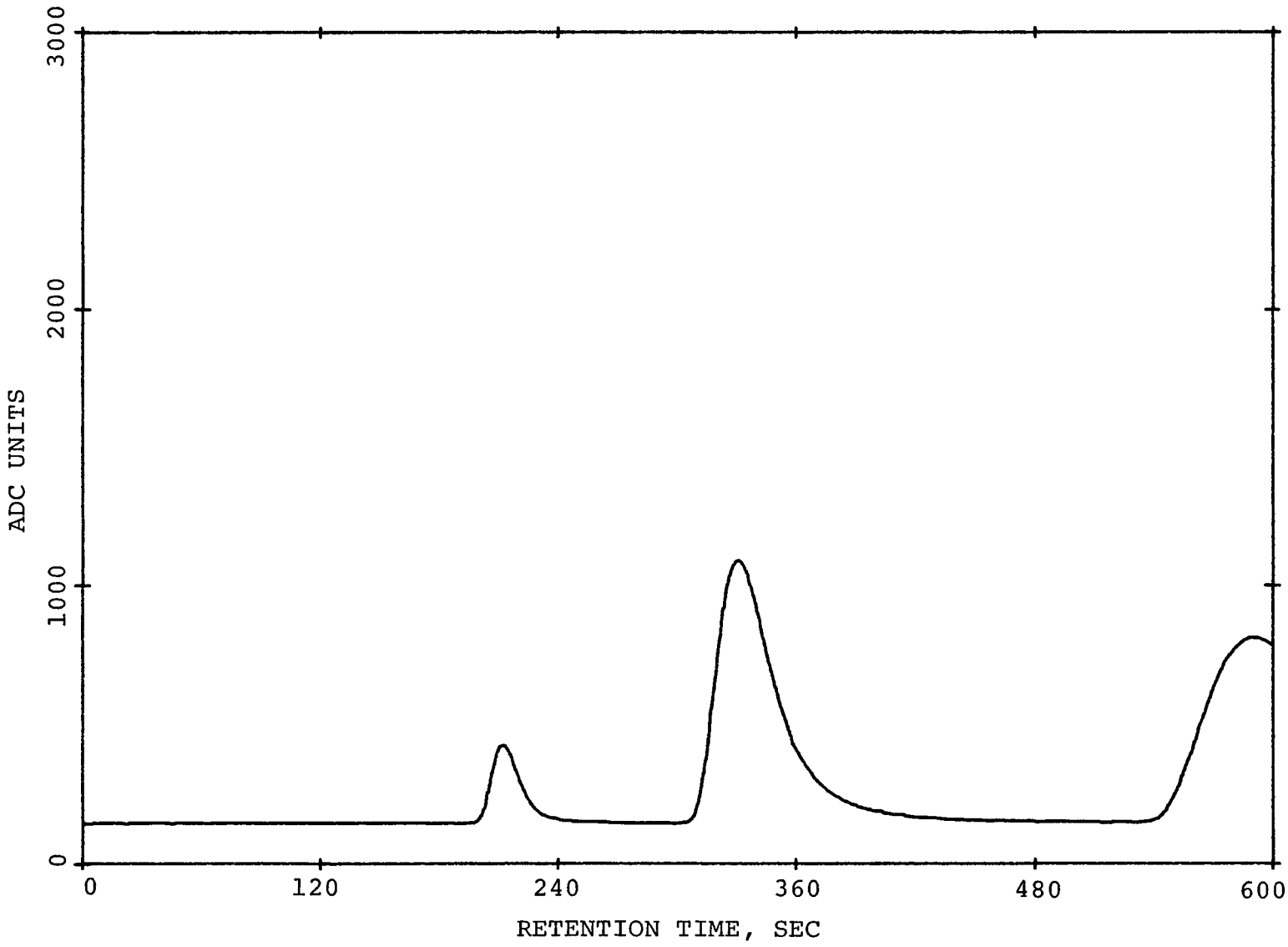

3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

FIGURE 49

CHROMATOGRAM FOR FACTORIAL POINT 2


3000
2000
1000
0
ADC UNITS
0
120
240
360
480
600
RETENTION TIME, SEC

sent high, medium, and low temperature at high flow rate. The data shows that retention time increases as flow rate is decreased and temperature is decreased; this is to be expected, inasmuch as both low temperature and low flow rate allow the sample to spend more time in the column. A larger retention time usually yields better resolution; this can also be seen in the chromatograms. The CRF is presented on the factorial design in Figure 50. A V-shaped region of the response surface gives the best resolution -- above the V, the mixture is not completely resolved; below the V, the p-xylene peak does not elute within the 10-min time restraint. Figures 51 - 53 illustrate the retention times, in seconds, of benzene, toluene, and p-xylene, respectively, on the response surface at the factorial design points. (Since data was taken at 1-sec intervals, the measurement of retention times is simplified.) In Figure 53, there is a diagonal boundary below which the retention time for p-xylene is greater than 10 minutes (600 sec).

Regression analysis of the responses from the factorial design using a second-order polynomial model provided no useful information; this was probably due to:

(1) the range of factor space covered by the design was small (thus there was no significant (at the 95% level) difference in the CRF values); and

(2) retention time, while showing a highly significant

FIGURE 50

FACTORIAL DESIGN SHOWING CRF RESPONSES

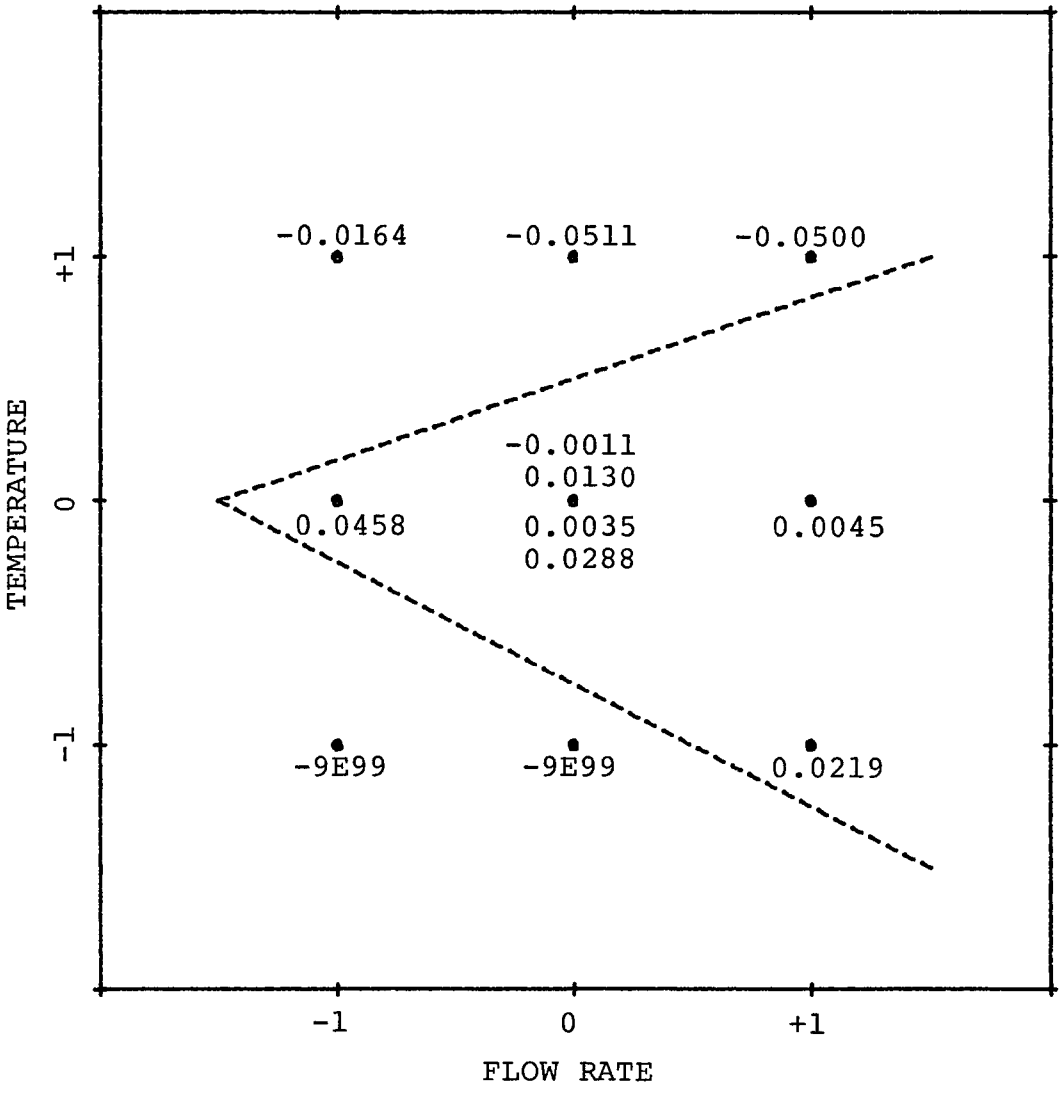

-0.0164
-0.0511
-0.0500
-0.0011
0.0130
0.0458
0.0035
0.0045
0.0288
-9E99
-9E99
0.0219
+1
0
-1
TEMPERATURE
-1
0
+1
FLOW RATE

FIGURE 51

FACTORIAL DESIGN SHOWING

RETENTION TIMES FOR BENZENE

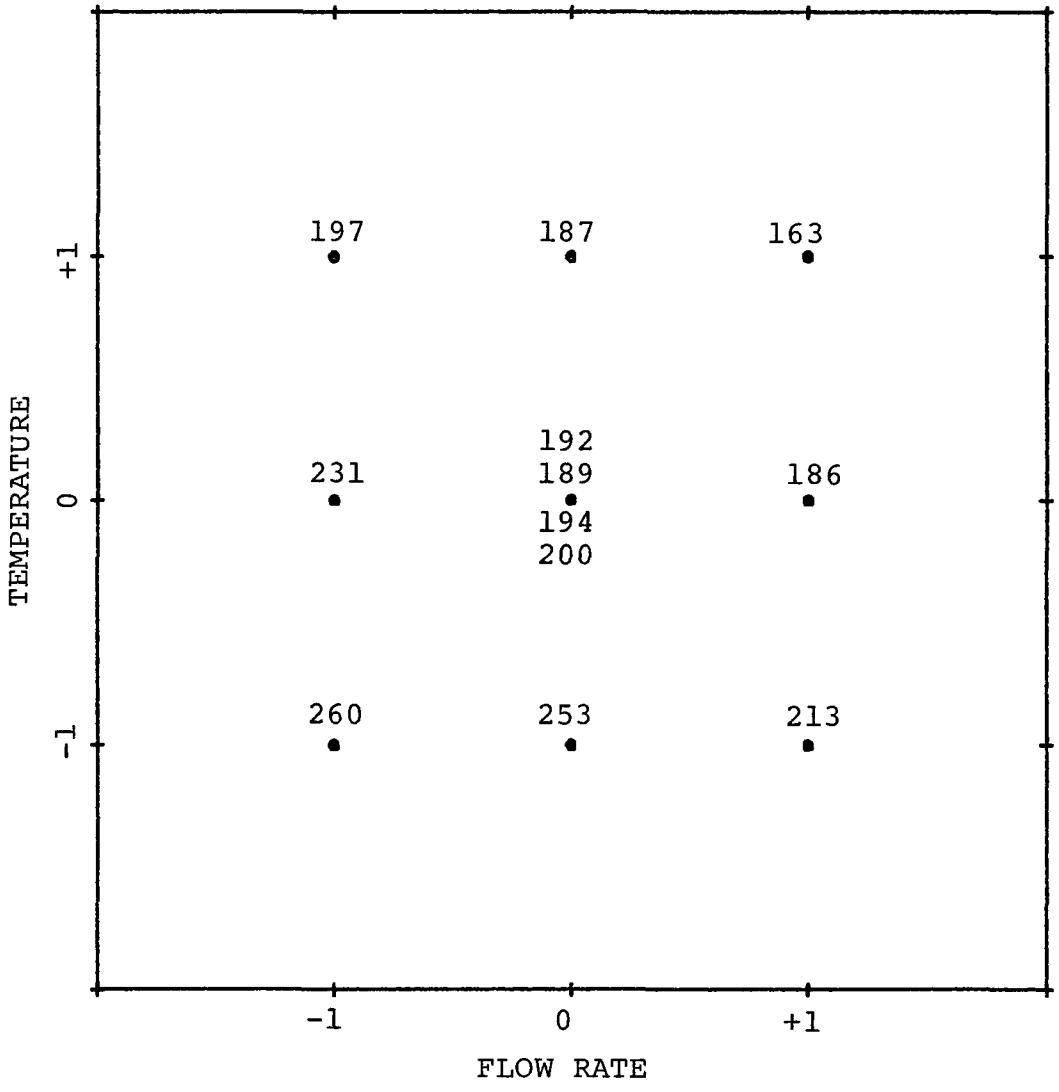

197
187
163
231
192
189
194
200
186
260
253
213
+1
0
-1
TEMPERATURE
-1
0
+1
FLOW RATE

FIGURE 52

FACTORIAL DESIGN SHOWING

RETENTION TIMES FOR TOLUENE

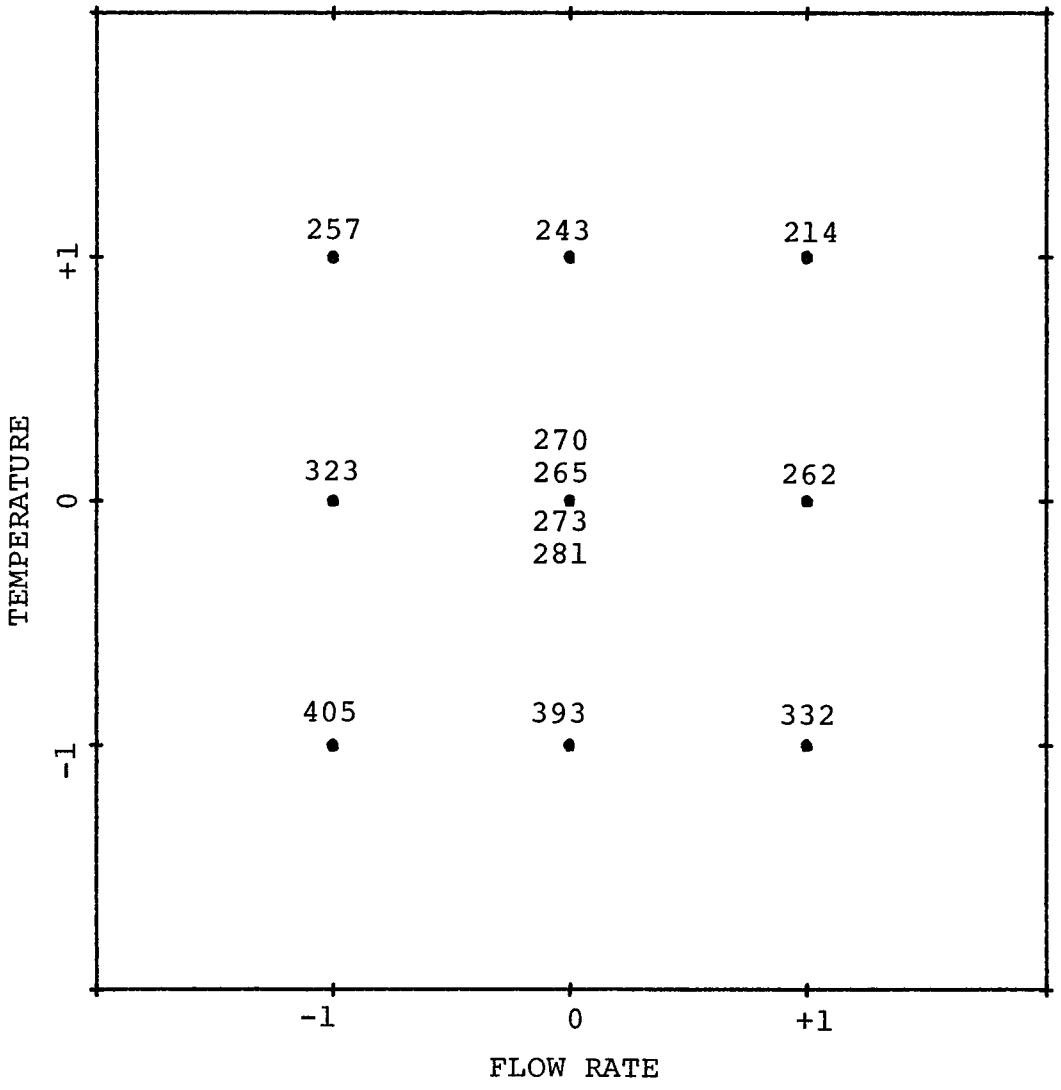
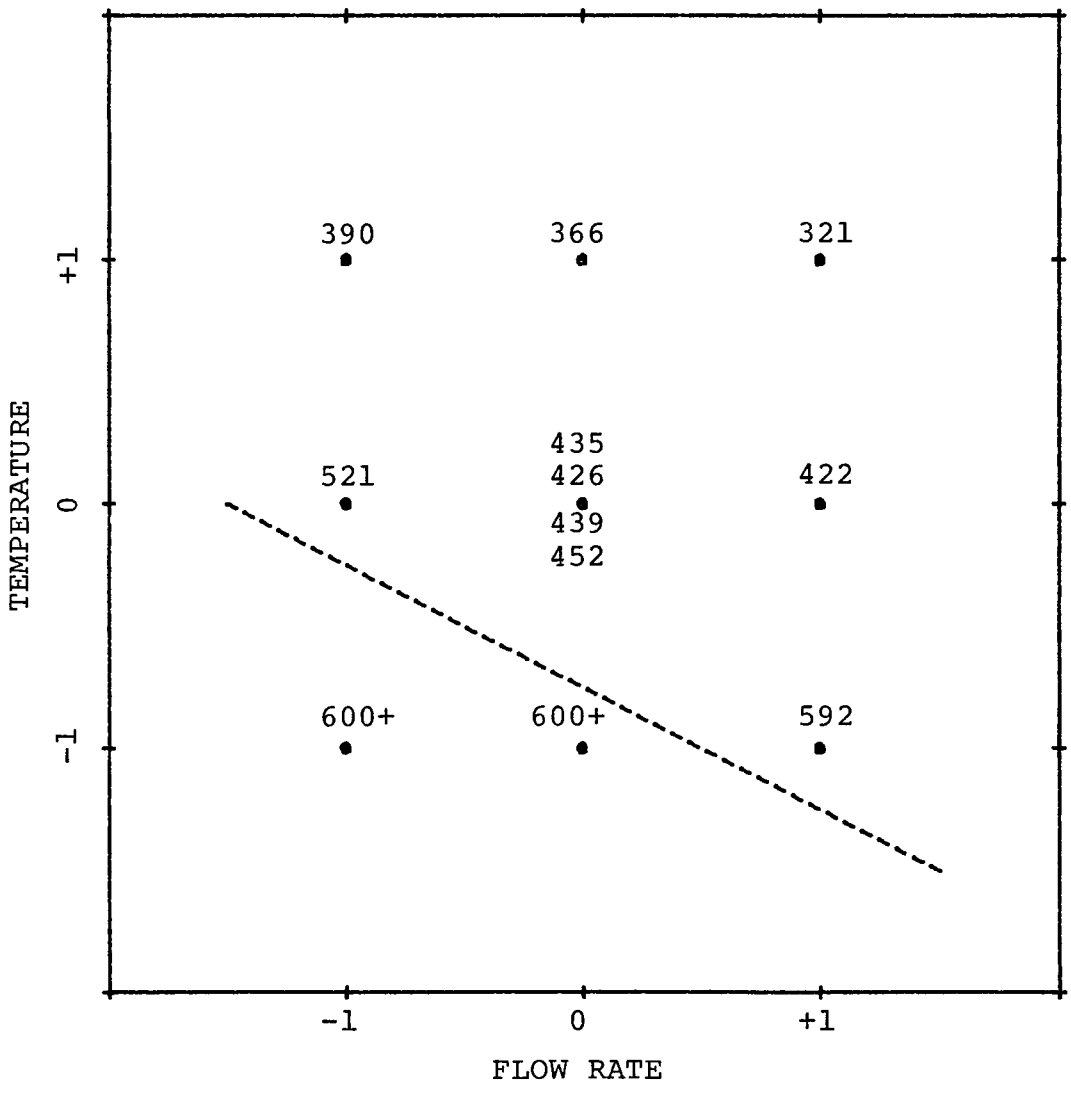

257
243
214
270
323
265
262
273
281
405
393
332
+1
0
-1
TEMPERATURE
-1
0
+1
FLOW RATE

FIGURE 53

FACTORIAL DESIGN SHOWING

RETENTION TIMES FOR p-XYLENE


390
366
321
435
521
426
422
439
452
600+
600+
592
+1
0
-1
TEMPERATURE
-1
0
+1
FLOW RATE

(99% level) dependence on temperature and flow rate, also showed a significant lack of fit for the second-order model (as might be expected, since the relationship between retention time and temperature is known to be logarithmic). Further, correction for column dead time, difficult to perform with a flame ionization detector, was not made.

Inasmuch as the relationship between motor position and flow rate and that between position and temperature had been found, in the preliminary studies, to be non-linear, the entire optimization study was performed using motor positions as the factors. After the mapping study, calibrations of these factors were carried out, using a soap-bubble flowmeter for the flow rate and the pyrometer built into the GC for the temperature. These calibrations are shown in Table IV, along with the factorial design points in order of experimentation, motor positions, and responses (CRF, and retention times of benzene, toluene, and p-xylene). The approach of calibrating only around the optimum is valid, since the actual values should be of interest only in that region of factor space.

The gas chromatography project demonstrated the feasability of achieving the following goals:

(1) An existing analytical instrument was automated (interfaced to a computer for both control and data acquisition) via stepper motors and a relay with very minimal

TABLE IV

FACTORIAL DESIGN

PARAMETERS AND RESPONSES

Point #	Flow Rate			Temperature		
	Coded	Motor	ml/min	Coded	Motor	deg C
1	+1	370	41.96	0	544	100
2	+1	370	41.96	-1	494	82
3	0	320	35.29	0	544	100
4	0	320	35.29	0	544	100
5	-1	270	28.04	+1	594	118
6	0	320	35.29	0	544	100
7	+1	370	41.96	+1	594	118
8	0	320	35.29	0	544	100
9	-1	270	28.04	-1	494	82
10	-1	270	28.04	0	544	100
11	0	320	35.29	-1	494	82
12	0	320	35.29	+1	594	118

Point #	CRF	Benzene ret. time[a]	Toluene ret. time[a]	p-Xylene ret. time[a]
1	0.0045	186	262	422
2	0.0219	213	332	592
3	-0.0011	192	270	435
4	0.0130	189	265	426
5	-0.0164	197	257	390
6	0.0035	194	273	439
7	-0.0500	163	214	321
8	0.0288	200	281	452
9	-9 E 99	260	405	600+
10	0.0458	231	323	521
11	-9 E 99	253	393	600+
12	-0.0511	187	243	366

[a] In seconds

modification to the instrument itself. For a laboratory where a number of instruments need to be interfaced once to a computer for optimization, but do not need to be continuously computer-controlled, this would be an important consideration. One general-purpose interface, without the need for multiplexing or multichannel capability, could be used.

(2) Computer-controlled optimization of an analytical method (gas chromatography) was accomplished. The computer set the experimental conditions, injected the sample, acquired the data, evaluated the response, and determined the next set of experimental conditions, all without operator intervention.

CHAPTER VII

AUTOANALYZER METHODOLOGY

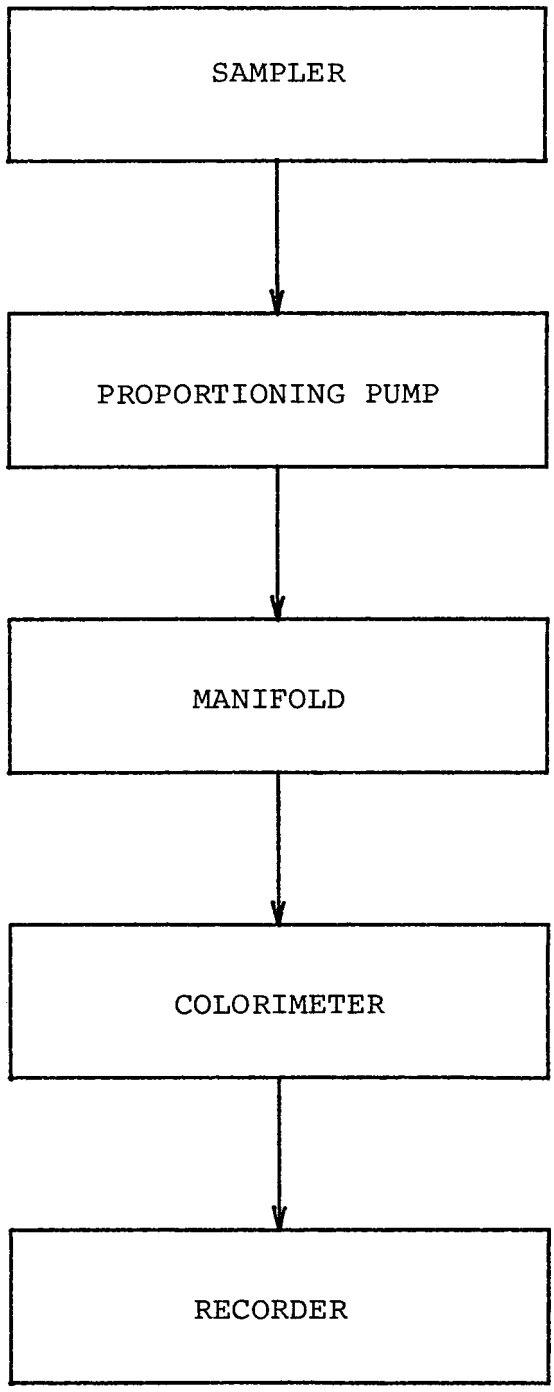
The Technicon AutoAnalyzer™ is an instrument which lends itself readily to computer control inasmuch as many of the functions of the AutoAnalyzer are already automated (47,48). A schematic of the AutoAnalyzer is shown in Figure 54. Each of the modules performs an essential function in the overall analysis:

(1) Sampler. The sampler consists of a rotary tray which holds up to 40 sample cups, a wash reservoir containing distilled water, a probe, and a timing cam. In actual use, the probe initially resides in the distilled water. A tube runs from the probe through the pump and into the manifold; distilled water is thus pumped through the sample line. When actuated by the cam, the probe rises from the reservoir, rotates, and descends into a sample cup in the tray. Sample is pumped through the sample line until the probe, again actuated by the cam, moves back into the distilled water reservoir. At this time, the sample tray rotates to bring the next sample cup into position.

(2) Proportioning pump. The pump is a fixed-speed, multichannel peristaltic pump. The sample line, reagent lines, and diluent lines (if any) pass through the pump. A line to replenish the sampler distilled water reservoir also passes through the pump. Since all the lines pass through the fixed-speed pump, the flow rate in each line depends upon the inner diameter of the tube.

FIGURE 54

SCHEMATIC OF TECHNICON AUTOANALYZER


SAMPLER
PROPORTIONING PUMP
MANIFOLD
COLORIMETER
RECORDER

(3) Manifold. The sample is introduced into the system at the manifold; the introduction is usually either into a diluent stream or a reagent stream. The sample is then segmented with air bubbles; the bubbles serve the following three purposes (49):

(a) Prevention of interactions. The sample line consists of alternating portions of sample and water. The bubbles prevent diffusion between these portions.

(b) More effective mixing. As the stream passes through coils, repeated inversion causes mixing within each air-separated segment. The short segments experience more tumbling and turbulence (and hence more effective mixing) than would an uninterrupted stream.

(c) Scrubbing of the tubes. As the bubbles pass through the tubes, they cleanse the inner walls.

The air is introduced from a line which passes through the pump; a bar actuated by the pump allows air (which is somewhat pressurized by the pumping action) to enter the stream at regular intervals and as discrete bubbles. A small amount of Brij-35^{TM}, a surfactant, is usually added to all reagents; this lowers the surface tension and allows the segmented stream to pass more freely through the tubes.

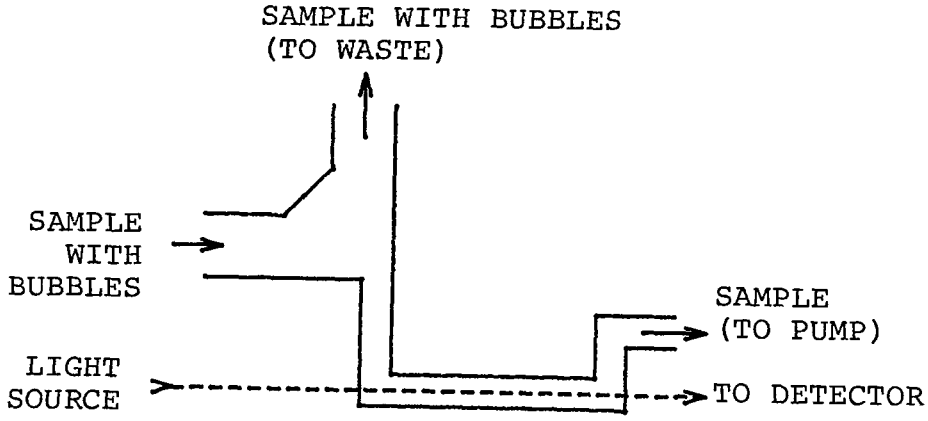
After the segmented sample stream is combined with reagents and mixed in the coils, it may be dialyzed. In dialysis, the segmented stream passes over a semi-permeable

membrane (usually cellulose acetate); a recipient segmented stream (reagent, diluent, or buffer) passes below the membrane. Small analytes (e.g., Ca^{2+}, uric acid) will pass through the membrane due to a concentration gradient existing between the two streams. Large substances (mostly proteins) cannot pass through the small membrane passages and so are kept in the sample stream. Dialysis, then, is a technique for separating substances based upon size. Since protein is frequently an interferent in the determination of analytes, dialysis provides a means of separating the analytes from the proteins; a means which is compatible with a flowing stream. After dialysis, the sample stream flows into a waste vessel; the recipient stream (now containing the analyte) may be further treated in the manifold.

(4) Colorimeter. After the sample has reacted, the colorimeter is the usual detector; here, the absorbance of the analyte is measured. The sample passes into a flowcell where a portion, without bubbles, is pulled down into the optical path (see Figure 55). This is necessary, as bubbles reaching the light path will cause highly erratic results. The portion which passes through the light path is pulled down by a tube which runs back through the proportioning pump (pull-through line); the rest of the stream (containing the bubbles) is allowed to pass out of the flowcell and into a waste vessel.

FIGURE 55

AUTOANALYZER FLOWCELL


SAMPLE WITH BUBBLES
(TO WASTE)
SAMPLE
WITH
BUBBLES
LIGHT
SOURCE
SAMPLE
(TO PUMP)
TO DETECTOR

The light is supplied by a tungsten-filament lamp; a filter between the source and the flowcell allows selection of the proper wavelength range. A second, empty flowcell, also with a filter, is used as a reference channel; the outputs of both flowcells are detected by phototubes and the sample signal is corrected for the reference signal (thus minimizing noise due to source fluctuations). The colorimeter thus produces a signal (voltage) which is proportional to the net absorbance of the sample stream.

(5) Recorder. A fixed-speed (1 in min^{-1}) strip chart recorder provides an analog record of the colorimeter output.

Automation. There are three primary areas of added automation involved with the AutoAnalyzer as used for computer-controlled optimzation studies:

(1) Sampler control. The sampler must be turned off after an experiment (simplex vertex or mapping study point) to allow calculation of a new set of conditions, to set the conditions for the next experiment, and to allow the system to equilibrate at the new set of conditions; the sampler must then be turned back on. This can be accomplished by leaving the sampler power switched on and plugging the line cord into the computer-addressable relay box (see Chapter V, p. 113). To ensure reproducibility of the sampler cam, a switch (which is actuated by the cam) was added. Both the

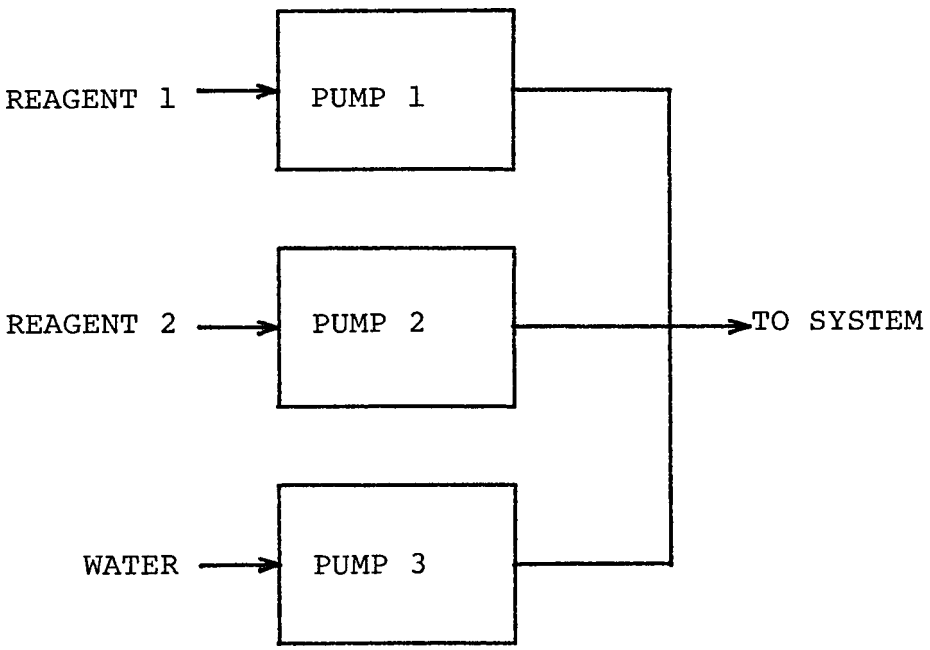
relay and this switch must be "OFF" before power to the sampler is interrupted. If the computer has turned off the relay, power to the sampler is not shut off until the cam has actuated the switch; thus, the sampler will turn off with the cam always at the same position.

(2) Reagent addition. In the standard AutoAnalyzer system, each reagent (or set of reagents) is prepared in a container and pumped via a tube through the proportioning pump. This, however, does not lend itself to easy variation of the concentration of the reagents. For a factorial-type design, where the experimental conditions for each point are pre-determined, reagents could be prepared beforehand; however, with the simplex algorithm, where the reagent conditions corresponding to a vertex are not known until that vertex has been calculated, this is impossible. While it would be possible to prepare the reagents for each vertex when that vertex has been calculated, this is hardly feasible. The other alternative, changing pump tubing for each new set of reagent conditions, is also not very feasible and, in addition, would be difficult to automate. What is needed is an automated system for varying the reagent concentrations.

The system for performing this task is shown in Figure 56. Individual computer-addressable peristaltic pumps, each controlled by a stepper motor (see Chapter V, p. 98), are

FIGURE 56

SYSTEM FOR AUTOMATED REAGENT ADDITION


REAGENT 1
PUMP 1
REAGENT 2
PUMP 2
TO SYSTEM
WATER
PUMP 3

used for all reagent lines. In addition, for each reagent or group of reagents, a water make-up line and pump are used. The concentrations of the stock reagent solutions are made relatively high; the sum of the pump speeds for each reagent or group of reagents is held constant and the speed of the make-up pump is set equal to the sum minus the speed(s) of the reagent pump(s). In this way, any concentration of the reagent is possible (up to the concentration of the stock solution), yet the flow of the reagent (or group of reagents) into the system remains constant, thus not changing the timing.

(4) Data acquisition. The computer can acquire data from the AutoAnalyzer via the ADC (see Chapter V, p. 113). The input to the instrumentation amplifier (and thus the ADC) is the colorimeter signal output to the recorder.

Preliminary study. A study (50) was undertaken to determine the long-term stability of the pump tubing. This was an important consideration, for if the tubing degraded and changed the flow rate, this would have the effect of changing the amount of reagent being added; this would, therefore, cause erroneous interpretation of the data.

Nineteen pump tubes were placed in the peristaltic pump; there were two sizes (Technicon flow ratings of 0.6 and 1.6 ml min^{-1}), three reagents (0.1 M NaOH, coded -1; water, coded 0; and 0.1 M HCl, coded +1), and nineteen

positions on the pump. The pump remained on for 327 hours (approximately 2 weeks); a total of 29 determinations of the flow rate of each tube (using a buret) were made during this period.

The flow rates for each determination were fit to the model:

$$\text{Flow Rate} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \qquad (14)$$

where x_1 is the tubing rating, x_2 is the reagent type, and x_3 is the position of the tubing in the pump. The parameter estimates are given for the last determination in Table V. Two results are evident: only the tubing size makes an important contribution to the flow rate and the actual flow rate is approximately 92% of the rated flow rate ($\beta_1 = 0.92$). No time trends were evident from examination of all the data. A second study was undertaken to evaluate the variation among the individual pumps; no significant variations were found.

TABLE V

REGRESSION RESULTS OF

FINAL FLOW RATE DETERMINATION IN

PRELIMINARY STUDY

Parameter	Estimate	Significance
β_0	0.0217	91.8 %
β_1	0.9215	100.0 %
β_2	-5.75 E-3	71.6 %
β_3	-7.19 E-5	9.2 %

CHAPTER VIII

AUTOANALYZER CALCIUM METHOD

Much of the material in this chapter was published in:

A. S. Olansky, L. R. Parker, Jr., S. L. Morgan, and S. N. Deming, Anal. Chim. Acta 95, 107 (1977).

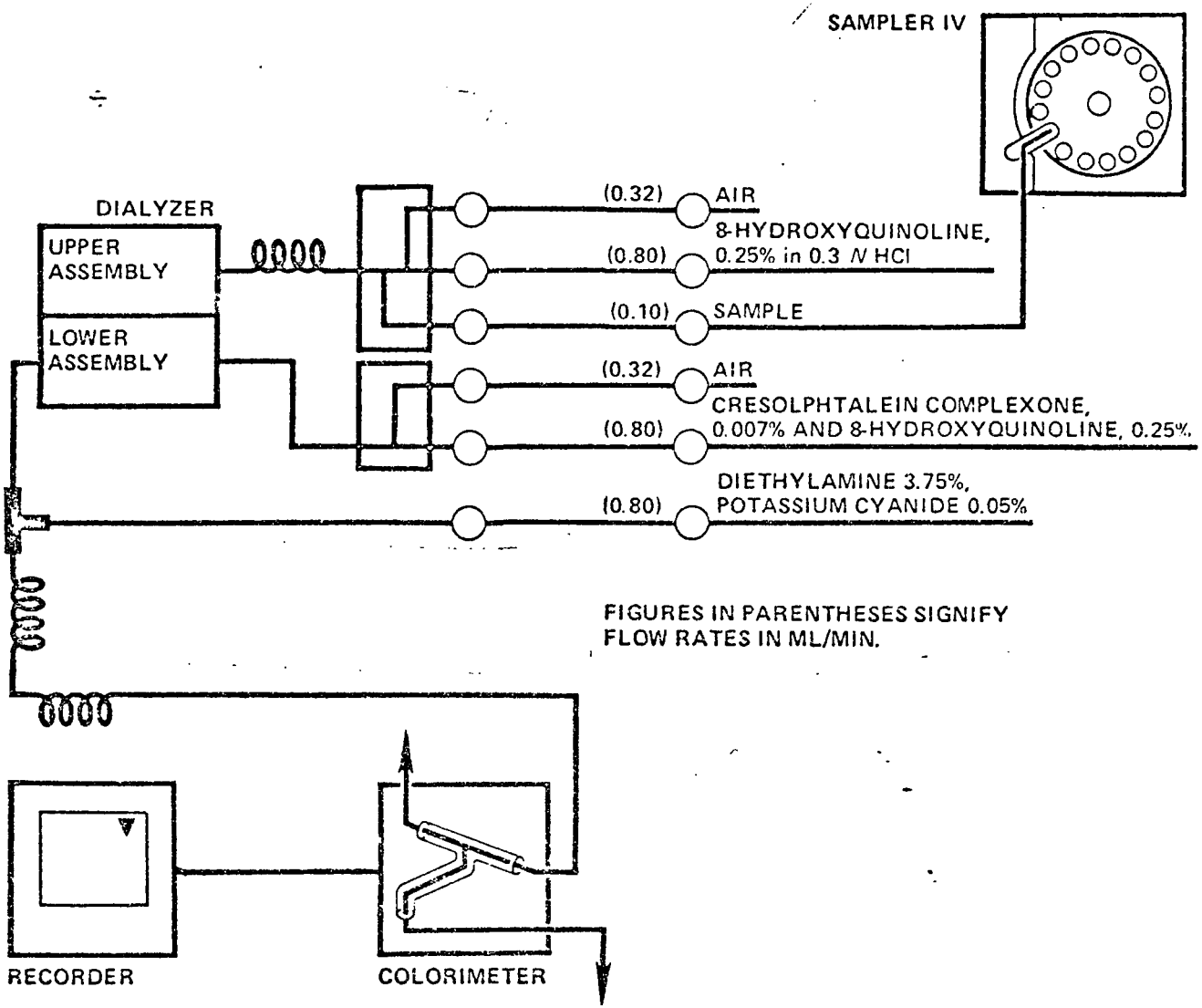
A particularly important AutoAnalyzer method is the determination of calcium in blood serum; this is clinically important as abnormal serum calcium levels can be indicative of a number of diseases (51,52). The standard AutoAnalyzer method for calcium is shown in Figure 57 (53). Samples containing calcium are pumped through the sample line, separated by segments of distilled water. (The small numbers in parentheses give the Technicon-rated flow in ml min^{-1} for the line used.) A reagent line pumps 0.25% 8-hydroxyquinoline (8HQ) in 0.3N HCl into a connector block, where the stream is air segmented. The sample stream is then added; the resulting stream passes through a mixing coil and then above a dialysis membrane. After dialysis, this stream is directed to a waste vessel.

A second stream containing 0.25% 8HQ and 0.007% cresolphthalein complexone (CPC) in 0.2N HCl is air segmented and pumped below the dialyzer membrane as a recipient stream. The calcium (along with other small species) dialyzes through the membrane. A third reagent line, 3.75% diethylamine (DEA) and 0.05% potassium cyanide (KCN), is added; the stream passes through two mixing coils and into the colorimeter flowcell. A portion of the stream (without bubbles) is pulled (by the proportioning pump) into the optical path, and the absorbance is measured and plotted on the recorder.

FIGURE 57

AUTOANALYZER METHOD FOR CALCIUM

(FROM REFERENCE 53)


SAMPLER IV
DIALYZER
UPPER ASSEMBLY
LOWER ASSEMBLY
(0.32) AIR
8-HYDROXYQUINOLINE, 0.25% in 0.3 N HCl
(0.80)
(0.10) SAMPLE
(0.32) AIR
CRESOLPHTALEIN COMPLEXONE, 0.007% AND 8-HYDROXYQUINOLINE, 0.25%.
(0.80)
DIETHYLAMINE 3.75%, POTASSIUM CYANIDE 0.05%
(0.80)
FIGURES IN PARENTHESES SIGNIFY FLOW RATES IN ML/MIN.
RECORDER
COLORIMETER

Magnesium is known to be a somewhat serious interferent (54): it dialyzes along with the calcium and will also form a colored complex which absorbs at approximately the same wavelength as the calcium complex. The 8HQ, both before and after dialysis, is added to bind the magnesium. The HCl before dialysis serves to deproteinate (free the bound ionic calcium from the proteins) the sample; the HCl after dialysis is to facilitate the dialysis process. The CPC is the color-producing reagent; a calcium-CPC complex is the species whose absorbance is measured. The calcium will bind to the CPC in alkaline solution only; thus, the DEA is added to make the solution basic. The KCN is added to the DEA solution primarily as a preservative (it was found that DEA discolors upon standing without the presence of KCN).

The responses to be evaluated (55) from this system include:

(1) Calcium sensitivity. The sensitivity to the analyte, calcium, should be maximized, as this should allow the detection of smaller amounts (i.e., lower concentrations or levels) of calcium, as well as the detection of smaller changes in the level.

(2) Magnesium sensitivity. The sensitivity to an interferent, such as magnesium, should be minimized, as otherwise erroneous results may occur. In an excess of reagents, magnesium will also form a colored complex with the CPC,

leading to an erroneously high absorbance and erroneously high values for calcium. If the reagents are not in excess, magnesium may compete with the calcium for the CPC; the resulting absorbance may then be too low, as the absorptivity of the Mg-CPC complex is reported to be about 10% of that of the Ca-CPC complex. Thus, the magnesium will cause faulty calcium analyses (54).

(3) Protein effect. It has been previously determined that the presence of protein in the donor dialysis stream causes a different dialysis rate from that in the absence of protein (56,57). If a different portion of the calcium is dialyzed, this will be interpreted as a different level of calcium in the original sample. Since the amount of protein varies, uncontrollably, from sample to sample, it is desirable to minimize the protein effect.

(4) Baseline. A lower baseline will allow a greater range of sample absorbances without overranging the colorimeter or the recorder, so a minimum baseline is desirable.

(5) Linearity. The normal procedure for this method is to run a high and a low calcium standard and construct a working curve (actually a straight line connecting the absorbances of the standards). It is desirable, therefore, that the absorbances of the levels of calcium between the standards be linear (i.e., lie on this line). Thus, the deviation of the absorbances from this line should be

minimized.

A systems view of the calcium method is given in Figure 58. The six inputs along the top represent the six reagents (b = before dialysis, a = after dialysis). The three inputs to the left are normally uncontrolled; however, they can be controlled in the optimization process. The five responses are shown on the right as outputs.

AutoAnalyzer. The sampler cam was designed to give a 1:1 ratio for sample time to wash (distilled water) time and a throughput of 40 samples hr^{-1}. The flowcell used had a 15-mm optical path; 570 nm filters were used for both the sample and reference colorimeter channels.

Modifications. As discussed previously, the reagents were added via individual, stepper-motor driven peristaltic pumps. The standard calcium method uses three reagent mixtures (but six different reagents). The addition scheme using the individual pumps is shown in Figure 59. Since the primary purpose of the KCN appeared to be to stabilize the DEA, the cyanide was not evaluated as a separate reagent. Each reagent is individually variable; the total flow for each of the reagent mixtures (and thus the total flow for the system) is kept constant by the use of three water make-up lines (one for each mixture). The stock solutions were made more concentrated than normal both to allow for dilution when mixed with the other reagents and water in the mixture

FIGURE 58

SYSTEMS THEORY VIEW OF THE

AUTOANALYZER CALCIUM METHOD

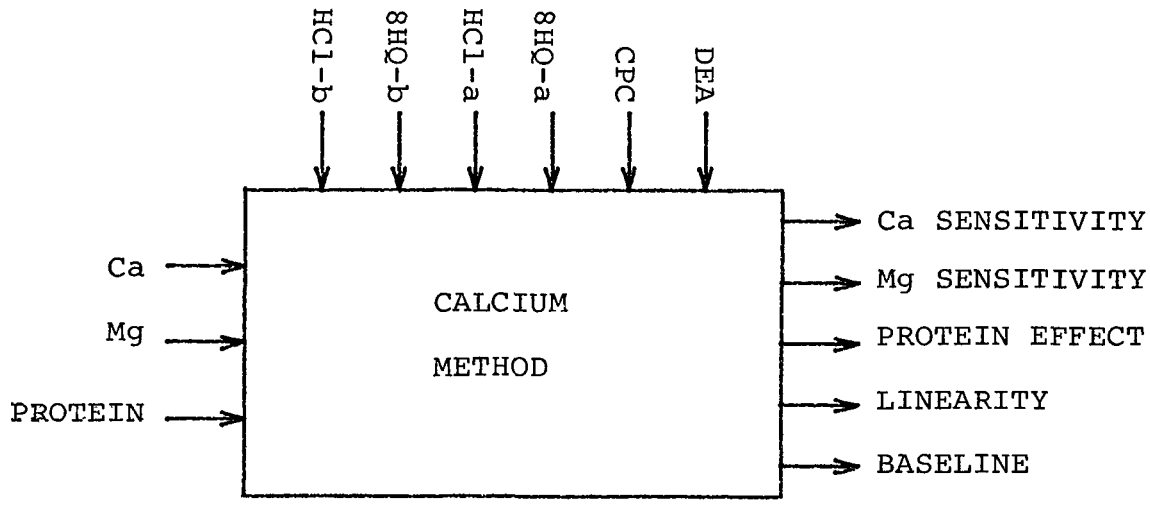
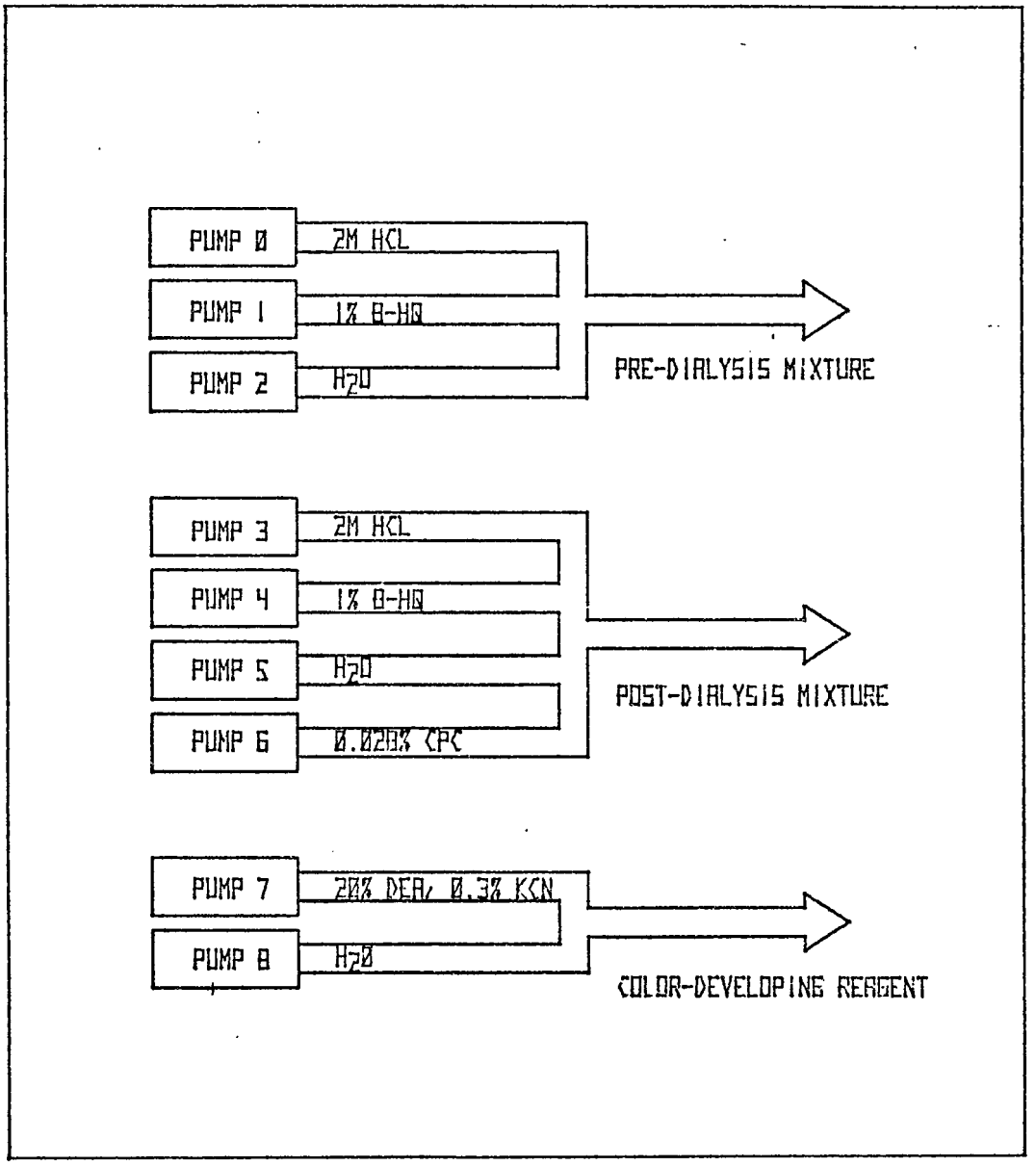

HCl-b
8HQ-b
HCl-a
8HQ-a
CPC
DEA
Ca
Mg
PROTEIN
CALCIUM
METHOD
Ca SENSITIVITY
Mg SENSITIVITY
PROTEIN EFFECT
LINEARITY
BASELINE

FIGURE 59

INDIVIDUAL REAGENT ADDITION SCHEME


PUMP 0
2M HCL
PUMP 1
1% B-HQ
PUMP 2
H2O
PRE-DIALYSIS MIXTURE
PUMP 3
2M HCL
PUMP 4
1% B-HQ
PUMP 5
H2O
PUMP 6
0.020% CPC
POST-DIALYSIS MIXTURE
PUMP 7
20% DEA, 0.3% KCN
PUMP 8
H2O
COLOR-DEVELOPING REAGENT

and to allow the simplex to investigate, if warranted, higher concentrations. The tubes used in the individual pumps were Technicon flow-rated (for the proportioning pump) at 0.23 ml min^{-1}. The air lines, sample line, and flowcell pull-through line continued to pass through the proportioning pump. Data was sampled at 1-sec intervals by the ADC.

The 8-hydroxyquinoline and cresolphthalein complexone had to be dissolved in 0.1M HCl, thereby introducing additional HCl into the system (beyond that which was introduced directly as HCl reagent). It was decided not to attempt to correct for this during the optimization, but rather to simply use the pump speeds of the six reagents investigated as the factors, with the additional HCl taken into account when the pump speeds were converted into reagent concentrations following the study.

Samples. To be able to evaluate sensitivity of calcium, sensitivity of magnesium, and effect of protein, each experiment required an evaluation of the absorbances for a number of concentrations of calcium, magnesium, and protein. A modified central composite design, randomized so as to remove the aliasing of any time trend with the concentrations, was chosen. Table VI shows the 20 samples used per experiment and the order in which they were evaluated. (The 40-

TABLE VI

SAMPLE DESIGN

AND TYPICAL PEAK ABSORBANCES

(FOR MAPPING STUDY POINT 16)

Order of evaluation	[Ca], mg/dl	[Mg], mg/dl	[BSA][a], g/dl	Absorbance, A.U.
1[b]	15.75	3.00	5.80	0.943
2	11.75	7.00	5.80	0.726
3	9.75	1.00	6.80	0.607
4	13.75	1.00	6.80	0.825
5	11.75	3.00	7.80	0.740
6	9.75	5.00	6.80	0.607
7	9.75	1.00	4.80	0.593
8	13.75	1.00	6.80	0.839
9[c]	11.75	3.00	5.80	0.718
10[d]	7.75	3.00	5.80	0.489
11	13.75	5.00	4.80	0.825
12[b]	15.75	3.00	5.80	0.959
13	11.75	3.00	3.80	0.720
14	9.75	5.00	4.80	0.645
15[d]	7.75	3.00	5.80	0.493
16	11.75	9.00	5.80	0.708
17[e]	5.75	3.00	5.80	0.366
18[e]	5.75	3.00	5.80	0.364
19	13.75	5.00	6.80	0.829
20[c]	11.75	3.00	5.80	0.726

[a] BSA = Bovine Serum Albumin; used as protein

[b,c,d,e] Replicate experiments

sample tray thus held two identical sets of 20 samples, or the equivalent of two experiments; the tray could be continuously rotated by the sampler without operator intervention.) The design was centered at 11.75 mg dl^{-1} Ca, 3.00 mg dl^{-1} Mg, and 5.80 g dl^{-1} protein. The 4 points which vary only in Ca level (Mg and protein at their center values) were replicated, thus giving an estimate of the pure experimental error. Representative absorbances (from point 16 in the subsequent mapping study) are also shown in Table VI. Each sample contained the appropriate amounts of calcium, magnesium, and protein (bovine serum albumin, BSA, was used as a representative protein), and an equal volume of Technicon Scale II serum was added to more closely simulate actual laboratory conditions. The reported average values for the serum were: calcium, 11.5 mg dl^{-1}; magnesium, 2.0 mg dl^{-1}; and protein, 7.6 g dl^{-1}. The concentrations in each sample are the average of the prepared levels and the serum levels of each constituent.

To minimize sample evaporation effects, 4-ml sample cups were used, each covered with Parafilm with a small hole melted in the top to allow entry of the sampler probe. A total of three sample trays was used, one during the simplex investigation and two during the mapping study; each tray was prepared from the same sample solutions. To promote a smoother transition from the end of one tray's use to the

beginning of the next, the sample solutions were kept unrefrigerated, even though this allowed some deterioration of the serum components in the sample.

Evaluation of response. The baseline was evaluated by taking the digitized signal 1 min before and 1 min after the sample peaks (a total of 100 points) and fitting a straight line using least squares; this value is a good estimate of the reagent blank.

The linearity was expressed as the standard deviation of residual absorbances of the samples varying only in calcium concentration (samples 1, 9, 10, 12, 15, 17, 18, and 20) about a line fit to these points.

The Ca sensitivity, Mg sensitivity, and protein effect were assessed by fitting the following model to the data:

$$\text{absorbance} = \beta_0 + \beta_1[\text{Ca}] + \beta_2[\text{Mg}] + \beta_3[\text{protein}] \qquad (15)$$

using a matrix least-squares approach (similar to Eq. 11). Then,

$$\begin{aligned} \delta(\text{absorbance})/\delta[\text{Ca}] &= \beta_1 = \text{sensitivity to Ca} \\ \delta(\text{absorbance})/\delta[\text{Mg}] &= \beta_2 = \text{sensitivity to Mg} \\ \delta(\text{absorbance})/\delta[\text{protein}] &= \beta_3 = \text{protein effect} \end{aligned} \qquad (16)$$

Simplex investigation. The variable-size simplex was used. The starting coordinates (first vertex); step sizes; and lower, upper, and summation boundaries, all in terms of pump speeds, are given in Table VII. The boundaries were chosen to be more restrictive than the actual physical bounds; this was to allow for the mapping study to be centered at the best simplex vertex.

To direct the simplex, a response must be assigned to each vertex. However, in the calcium method, a total of five responses are of interest. Three of these responses were combined to yield an overall response (also known as the objective function, or OF):

$$OF = \beta_1 - |\beta_2| - |\beta_3| \qquad (17)$$

This will tend to maximize the sensitivity to calcium and to minimize the sensitivity to magnesium and the protein effect.

In addition to the boundary violations provided for each of the factors, boundaries were placed on β_2 and β_3 as well: if $|\beta_2|$ or $|\beta_3|$ exceeded 10% of β_1, a boundary violation was considered to have occurred, and a bad response was assigned to that experiment. In addition, if the base-line absorbance exceeded the average absorbance above base-line of the two samples highest in calcium (samples 1 and 12, each with 15.75 mg dl^{-1}), then that vertex was deemed a

TABLE VII

SIMPLEX STARTING PARAMETERS

(ALL NUMBERS REFER TO PUMP SPEEDS)

	HCl-b	8HQ-b	HCl-a	8HQ-a	CPC	DEA
Start	500	500	250	250	250	1000
Step	1000	1000	500	500	500	500
Lower bound	110	110	110	110	110	110
Upper bound	2320	2320	2290	2290	2290	2360
Summation bound	2320			2290		2360

boundary violation. It was decided in advance not to use the linearity response in the simplex investigation, but to merely monitor it.

The above steps were taken in an attempt to keep the objective function as simple and straightforward as possible. It was decided in advance to terminate the simplex algorithm after 25 vertexes.

Mapping study. The mapping study consisted of a Box-Behnken design (58) -- a special type of fractional 3-level factorial design. The choice of design was to minimize the number of experiments: with 6 factors, a full 3-level factorial design would require 729 experiments; a $\frac{1}{3}$-fractional 3-level factorial design, 243 experiments; a central composite design, 77; and the Box-Behnken design, only 49. The center point experiment in the study was performed five additional times, yielding a total of 54 experiments and providing a pure error variance for comparison. Table VIII shows the coded levels (-1, 0, +1) of each factor in the mapping study and the actual concentrations.

The design was centered at the best simplex vertex. The 6 center point replicates were evenly spaced throughout the design; the other 48 points were randomized.

Comparison study. From the results of the mapping study, one level of each reagent was selected as the "optimal." These levels corresponded to the coded point

TABLE VIII

LEVELS OF FACTORS USED IN MAPPING STUDY

	-1	0	+1
HCl-b, M	0.370	0.450	0.530
8HQ-b, %	0.456	0.496	0.536
(HCl, M)[a]	0.046	0.050	0.054
HCl-a, M	0.266	0.346	0.426
8HQ-a, %	0.133	0.173	0.212
(HCl, M)[a]	0.013	0.017	0.021
CPC, %	0.0018	0.0030	0.0041
(HCl, M)[a]	0.006	0.011	0.015
DEA, %[b]	10.45	11.28	12.10

[a] Additional HCl introduced by reagent

[b] By volume; all other per cents by weight

(0,+1,+1,0,0,0). The three reagent mixtures indicated by this point were prepared and the proportioning pump used for reagent delivery. (This was to assure that the reagent concentrations were the only difference from the standard Technicon method; a different method of delivery might have affected the results.) The Technicon reagents were run twice, followed by two runs of the new reagents. A comparison of the two sets of conditions is shown in Table IX.

A new quantity of BSA (i.e, protein) had to be used for the comparison study; abnormally high values for the absorbances suggested that the protein samples contained added calcium. This was verified by atomic absorption spectroscopy; by the use of standard calcium atomic absorption solutions (Fisher), the level of calcium in the prepared BSA solution was found to be 10.37 mg dl^{-1}. The calcium values used in subsequent data analysis was corrected for this value.

Software. All computer programs used were on a magnetic disc and could be quickly loaded into the 9830A's memory. A master program determined the proper course of action: (1) begin the simplex study; (2) call the simplex algorithm at the point at which a new vertex was to be calculated; (3) terminate the simplex and call the mapping program; or (4) conclude the study.

The simplex and mapping programs calculated the next

TABLE IX

COMPARISON OF REAGENTS USED IN

THE TECHNICON METHOD AND THE MODIFIED METHOD

	Technicon Method	Modified Method
Mixture I		
8HQ, %	0.25	0.536
HCl, M	0.30	0.504
Mixture II		
8HQ, %	0.25	0.173
HCl, M	0.20	0.454
CPC, %	0.007	0.0030
Mixture III		
DEA, %[a]	3.75	11.276
KCN, %	0.05	0.169

[a] By volume; all other per cents by weight

set of pump speeds and stored these in a data file. A general data-collection routine (given in Appendix C) was then called by the simplex and mapping programs. This routine set the pump speeds, turned the sampler on and off, and collected and plotted the data. The computer then halted and allowed the experimenter to check for system malfunctions. If a malfunction was noted, the data-collection routine (remaining in memory) could be re-started after the problem was corrected.

The data was then stored. The data analysis program calculated and subtracted the reagent blank baseline from the data, extracted the peak heights, stored these in a file, and performed regression analysis on the peak heights to determine the calcium and magnesium sensitivities and the protein effect.

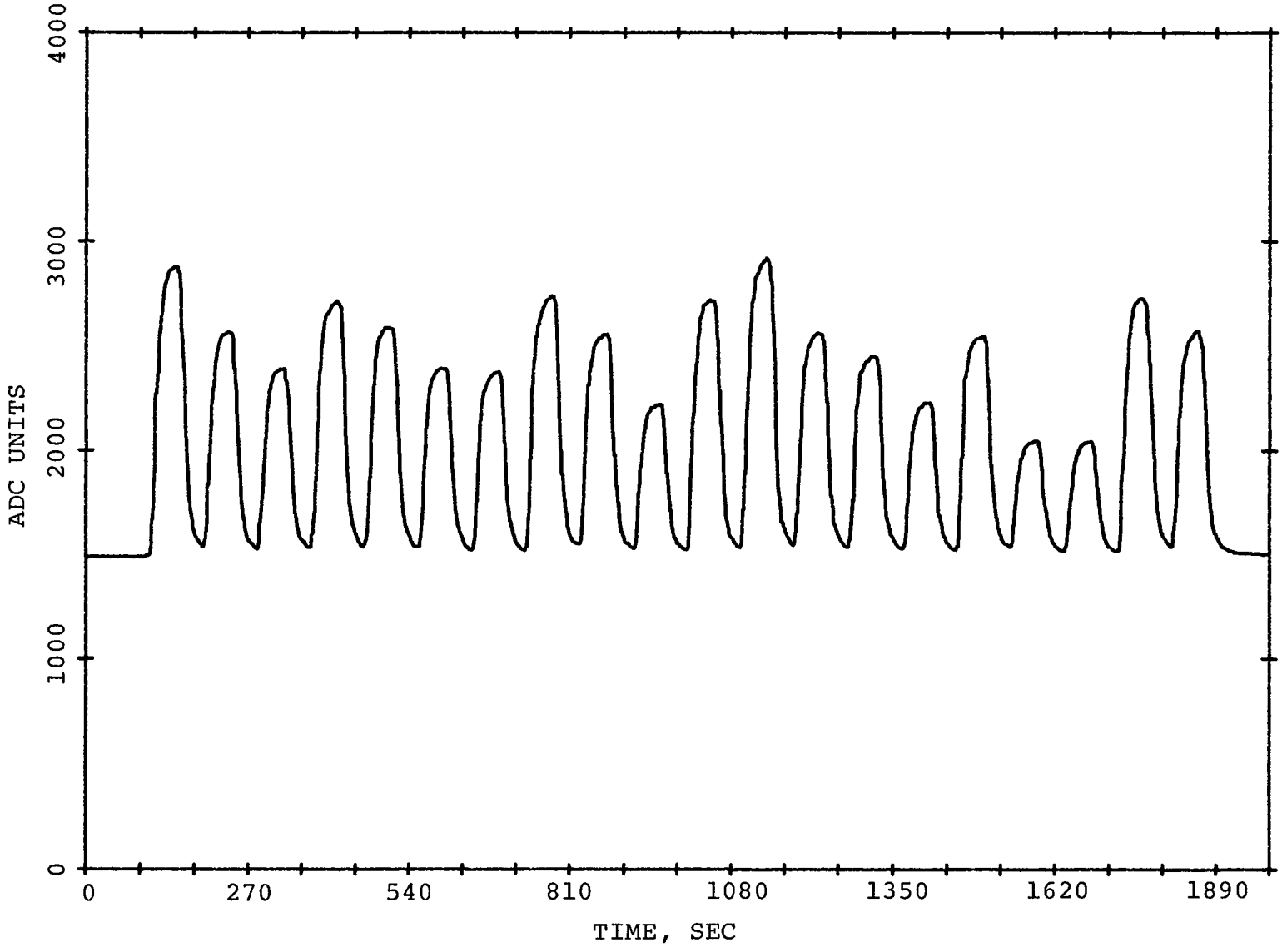
A program was called which computed the linearity of the data and combined the sensitivities and protein effect into the objective function, yielding a response for the set of experimental conditions. This response was stored and the master program again called. A representative digitized data plot (mapping study point 16) is shown in Figure 60.

Procedural note. The experimental system was constructed to be as completely automated as possible. Theoretically, the system could have been started up and then left entirely

FIGURE 60

DIGITIZED DATA PLOT

FOR MAPPING STUDY POINT 16


4000
3000
2000
1000
0
ADC UNITS
0
270
540
810
1080
1350
1620
1890
TIME, SEC

alone until the study was completed. However, the possibility of minor flow system problems (leaks around fittings, protein precipitation in the sample inlet, etc.), the need to change recorder paper and sample trays at intervals, and the desire to plot out the data continuously at the computer as it was received (each plot on a separate sheet of paper), made it convenient to place a "STOP" in the programming after the completion of each experiment (each set of 20 samples).

To minimize long-term trends and large-scale changes in the system, sample, and reagent conditions, the system was run continuously (24 hr/day) over the four-day period required to complete the study.

RESULTS

Within an experiment. Table VI contains absorbance values above baseline for each of the 20 serum samples in a representative experiment (point 16 in the mapping study, one of the center point replicates). The parameters of the model expressed by Eq. 15 were fit to the factor levels shown in Table VI and the experimentally-obtained absorbance values by means of a matrix least-squares regression program. Typical results are shown in Table X. Residual analysis showed no time trends. Parameter values from this analysis of the data were used to from the objective func-

TABLE X

REGRESSION RESULTS OF FIRST-ORDER LINEAR MODEL FOR MAPPING STUDY POINT 16

Parameter	Estimate	Calculated t	Significance
β_0	0.0313	1.367	80.9 %
β_1	0.0576	55.553	100.0 %
β_2	0.000374	0.252	19.6 %
β_3	0.001847	0.562	41.8 %

ANALYSIS OF VARIANCE

Source	SS	d.f.	Variance
Regression	0.536	3	0.1780
Residuals	0.002762	16	0.0001725
Lack of fit	0.002598	12	0.0002163
Pure error	0.0001635	4	0.0000411
Total about mean	0.536	19	

Significance of regression:

$F_{3,16}$ = 1032 (100.0%) based on residual variance

$F_{3,4}$ = 4357 (100.0%) based on pure error variance

Significance of lack of fit:

$F_{12,4}$ = 5.29 (94.0%) based on pure error variance

tion (Eq. 17).

A measure of linearity with respect to calcium level was obtained by fitting the model:

$$\text{absorbance} = \beta_0 + \beta_1[\text{Ca}] \tag{18}$$

to serum samples 17, 18, 10, 15, 9, 20, 1, and 12 (calcium replicates, Mg and protein levels constant, see Table VI) and calculating the standard deviation of points about the regression line. Figure 61 shows the regression line fit to the four pairs of replicates from mapping study point 16.

Table XI lists the results of regression analysis for mapping study point 16 fit to the full second-order linear model:

$$\begin{aligned}\text{absorbance} = {} & \beta_0 + \beta_1[\text{Ca}] + \beta_{11}[\text{Ca}]^2 + \\ & \beta_2[\text{Mg}] + \beta_{22}[\text{Mg}]^2 + \beta_3[\text{protein}] + \\ & \beta_{33}[\text{protein}]^2 + \beta_{12}[\text{Ca}][\text{Mg}] + \\ & \beta_{13}[\text{Ca}][\text{protein}] + \\ & \beta_{23}[\text{Mg}][\text{protein}]\end{aligned} \tag{19}$$

Simplex progress. The progress of the simplex is detailed in Table XII. Points 8, 10, 12, 14, and 17 (not

FIGURE 61

LINEARITY PLOT FOR CALCIUM

(MAPPING STUDY POINT 16)

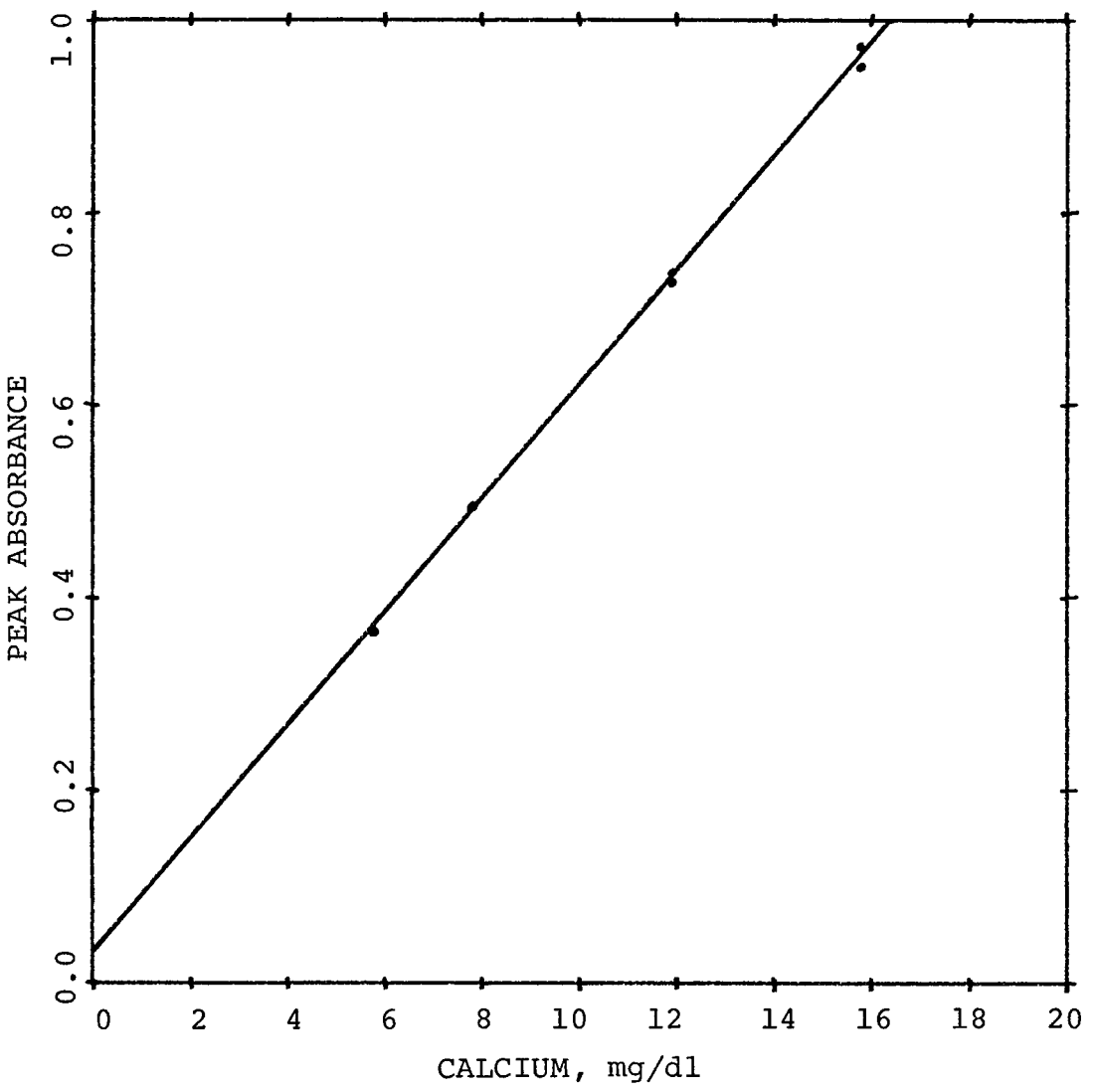

PEAK ABSORBANCE
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10
12
14
16
18
20
CALCIUM, mg/dl

TABLE XI

REGRESSION RESULTS FOR FULL SECOND-ORDER MODEL FOR MAPPING STUDY POINT 16

Parameter	Estimate	Calculated t	Significance
β_0	0.0549	0.370	28.1 %
β_1	0.576	5.048	99.1 %
β_{11}	-0.000445	1.662	86.4 %
β_2	0.0533	3.852	99.7 %
β_{22}	-0.000871	2.179	94.6 %
β_3	-0.0315	1.008	66.3 %
β_{33}	0.00121	0.582	42.7 %
β_{12}	-0.00190	2.347	95.9 %
β_{13}	0.00265	1.640	86.8 %
β_{23}	-0.00397	2.458	96.6 %

ANALYSIS OF VARIANCE

Source	SS	d.f.	Variance
Regression	0.536	9	0.0596
Residuals	0.000836	10	0.0000836
Lack of fit	0.000673	6	0.000112
Pure error	0.000163	4	0.0000409
Total about mean	0.536	19	

Significance of regression:

$F_{9,10}$ = 712 (100.0%) based on residual variance

$F_{9,4}$ = 1457 (100.0%) based on pure error variance

Significance of lack of fit:

$F_{6,4}$ = 2.74 (82.6%) based on pure error variance

TABLE XII

SIMPLEX PROGRESS

Vertex	FACTORS					
	HCl-b M	8HQ-b %	HCl-a M	8HQ-a %	CPC x 100 %	DEA[a] %
1	0.419	0.202	0.221	0.098	0.288	8.26
2	1.144	0.280	0.306	0.137	0.399	9.06
3	0.610	0.566	0.306	0.137	0.399	9.06
4	0.581	0.280	0.590	0.137	0.399	9.06
5	0.581	0.280	0.306	0.276	0.399	9.06
6	0.581	0.280	0.306	0.137	0.806	9.06
7	0.581	0.280	0.306	0.137	0.399	11.98
9	0.851	0.298	0.325	0.145	0.423	9.23
11	0.592	0.299	0.333	0.146	0.595	9.25
13	0.593	0.300	0.446	0.147	0.408	9.27
15	0.594	0.302	0.322	0.205	0.409	9.28
16	0.278	0.353	0.320	0.145	0.409	9.79
18	0.664	0.498	0.456	0.207	0.585	11.29
19	0.512	0.463	0.401	0.100	0.523	10.93
20	0.574	0.342	0.342	0.179	0.437	9.70
3[b]	0.610	0.566	0.306	0.137	0.399	9.06
21	0.506	0.479	0.241	0.170	0.534	11.09
22	0.572	0.345	0.395	0.152	0.439	9.72
7[b]	0.581	0.280	0.306	0.137	0.399	11.98
23	0.501	0.496	0.375	0.173	0.294	11.26
24	0.455	0.594	0.397	0.187	0.145	12.27
25	0.447	0.206	0.425	0.194	0.457	12.19

[a] By volume; all other per cents by weight

[b] Re-evaluated vertex (n+1 rule)

Vertex	RESPONSES[a] OF	Ca	Mg	Protein	Linearity	Baseline
1	49.73	60.68	0.67	10.29	14.14	0.455
2	42.88	49.55	1.96	4.69	9.55	0.435
3	53.98	56.23	1.27	-0.99	11.23	0.496
4	48.54	54.75	1.98	4.23	4.89	0.386
5	49.09	50.20	0.53	0.55	5.54	0.514
6	47.91	55.16	3.56	3.72	8.86	0.864
7	54.75	55.90	0.53	0.61	15.57	0.589
9	50.26	54.14	2.45	-1.42	7.40	0.512
11	52.16	56.51	1.40	2.95	6.49	0.692
13	51.74	59.24	1.84	5.66	11.33	0.475
15	50.54	54.35	0.85	2.99	10.58	0.540
16	58.05	68.52	4.57	5.87	7.08	0.603
18	54.45	56.43	0.38	1.60	7.67	0.667
19	54.65	64.28	3.13	6.51	8.31	0.665
20	54.77	57.46	1.19	1.50	10.03	0.576
3[b]	53.52	58.55	1.23	3.78	11.85	0.524
21	50.56	55.46	2.47	2.43	8.86	0.783
22	64.52	74.03	6.33	-3.18	22.71	0.576
7[b]	69.37	73.32	3.76	0.22	14.18	0.582
23	72.12	77.79	2.89	2.77	27.77	0.433
24	30.62	37.88	3.50	3.78	37.42	0.303
25	56.65	63.63	1.09	5.89	5.56	0.617

[a] All responses except baseline are X 1000

[b] Re-evaluated vertexes ($\underline{n}$+1 rule)

shown in the Table) represented conditions which would have violated the boundaries of one or more of the factors; no experiments were conducted at these points. Points 3 and 7 were re-evaluated in accordance with the n+1 rule of Spendley et al. (16); the position of these points in the Table indicates the relative time at which they were re-evaluated.

Mapping study. The mapping study was centered about the best simplex vertex, vertex 23, with levels of each factor as shown in Table VIII. Table XIII contains the results, sorted by CPC levels, with replicate center points listed at the bottom.

Because of the possibility of interaction among factors, a full second-order linear model was fitted to the mapping study data for each of the responses. The form of the model is:

$$\text{response} = \alpha_0 + \sum_{i=1}^{6} \alpha_i x_i + \sum_{i=1}^{6} \alpha_{ii} x_{ii}^2 + \sum_{i=1}^{6} \sum_{j=i+1}^{6} \alpha_{ij} x_i x_j \qquad (20)$$

where response refers to the response being fitted (OF, Ca, Mg, protein, linearity, or baseline), x_i and x_j are the factors exerting an effect (HCl-b, 8HQ-b, HCl-a, 8HQ-a,

TABLE XIII

MAPPING STUDY RESULTS

Evaluation Number	FACTORS CPC	HCl-b	8HQ-b	HCl-a	8HQ-a	DEA
3	-1	-1	0	0	-1	0
30	-1	-1	0	0	1	0
10	-1	0	-1	-1	0	0
25	-1	0	-1	0	0	-1
34	-1	0	-1	0	0	+1
37	-1	0	-1	+1	0	0
50	-1	0	+1	-1	0	0
35	-1	0	+1	0	0	-1
26	-1	0	+1	0	0	+1
21	-1	0	+1	+1	0	0
39	-1	+1	0	0	-1	0
23	-1	+1	0	0	+1	0
2	0	-1	-1	0	-1	0
44	0	-1	-1	0	+1	0
15	0	-1	0	-1	0	-1
52	0	-1	0	-1	0	+1
42	0	-1	0	+1	0	-1
13	0	-1	0	+1	0	+1
53	0	-1	+1	0	-1	0
11	0	-1	+1	0	+1	0
6	0	0	0	-1	-1	-1
45	0	0	0	-1	-1	+1
54	0	0	0	-1	+1	-1
14	0	0	0	-1	+1	+1
43	0	0	0	+1	-1	-1
18	0	0	0	+1	-1	+1
5	0	0	0	+1	+1	-1
31	0	0	0	+1	+1	+1
36	0	+1	-1	0	-1	0
20	0	+1	-1	0	+1	0
49	0	+1	0	-1	0	-1
22	0	+1	0	-1	0	+1
4	0	+1	0	+1	0	-1
46	0	+1	0	+1	0	+1
1	0	+1	+1	0	-1	0
47	0	+1	+1	0	+1	0

Evaluation Number	CPC	HCl-b	8HQ-b	HCl-a	8HQ-a	DEA
29	+1	-1	0	0	-1	0
9	+1	-1	0	0	+1	0
33	+1	0	-1	-1	0	0
38	+1	0	-1	0	0	-1
7	+1	0	-1	0	0	+1
12	+1	0	-1	+1	0	0
17	+1	0	+1	-1	0	0
27	+1	0	+1	0	0	-1
51	+1	0	+1	0	0	+1
28	+1	0	+1	+1	0	0
19	+1	+1	0	0	-1	0
41	+1	+1	0	0	+1	0
8	0	0	0	0	0	0
16	0	0	0	0	0	0
24	0	0	0	0	0	0
32	0	0	0	0	0	0
40	0	0	0	0	0	0
48	0	0	0	0	0	0

Evaluation Number	RESPONSES[a] OF	Ca	Mg	Protein	Linearity	Baseline
3	39.97	48.91	2.02	6.94	15.11	0.36
30	35.98	43.47	2.61	4.87	13.53	0.36
10	42.27	45.59	2.20	1.13	14.52	0.38
25	40.53	45.37	2.10	2.73	11.29	0.34
34	42.92	45.24	1.56	-0.75	11.12	0.36
37	42.92	45.04	0.44	1.68	8.96	0.30
50	44.52	46.05	0.89	0.63	6.88	0.34
35	43.77	45.37	0.44	1.17	8.74	0.34
26	42.45	46.05	1.80	1.82	8.52	0.36
21	42.25	44.74	1.84	0.65	7.32	0.34
39	44.15	46.96	2.51	0.30	10.96	0.32
23	40.01	41.60	0.30	-1.31	7.58	0.36
2	57.06	61.32	0.51	3.74	14.50	0.47
44	48.40	57.91	1.58	7.93	8.60	0.45
15	51.13	58.51	2.39	5.00	6.92	0.49
52	55.76	57.66	0.97	-0.93	4.75	0.49
42	51.45	59.28	1.78	6.03	8.62	0.40
13	53.94	60.37	3.09	3.34	9.30	0.47
53	59.58	61.55	0.51	1.46	7.32	0.45
11	51.64	55.92	2.59	1.68	9.51	0.49
6	52.24	57.97	1.86	3.88	7.18	0.47
45	50.79	57.87	1.01	6.09	8.09	0.49
54	52.49	54.04	-0.06	1.48	8.84	0.45
14	51.70	53.33	0.77	-0.83	8.58	0.51
43	58.03	59.48	0.97	-0.45	11.06	0.40
18	57.28	60.54	1.78	1.46	6.94	0.47
5	48.04	53.25	1.36	3.84	6.11	0.42
31	51.31	53.60	2.29	0.00	6.98	0.45
36	51.76	57.04	3.24	-2.04	8.52	0.47
20	50.91	53.54	-1.03	1.58	11.06	0.47
49	51.05	53.76	1.03	1.66	8.66	0.45
22	51.56	52.30	0.18	0.53	11.33	0.51
4	51.09	53.92	1.21	1.62	11.06	0.42
46	51.39	55.48	1.64	2.47	4.89	0.44
1	54.85	56.98	1.64	-0.49	5.28	0.44
47	50.50	52.04	-0.55	0.99	7.28	0.44

[a] All responses except baseline are X 1000

Evaluation Number	RESPONSES[a]					
	OF	Ca	Mg	Protein	Linearity	Baseline
29	58.19	63.83	2.51	3.13	5.70	0.61
9	50.99	60.84	1.46	8.39	8.88	0.59
33	53.96	58.55	1.76	2.81	6.01	0.63
38	57.64	59.56	0.42	1.52	4.93	0.55
7	52.51	60.60	2.63	5.44	10.17	0.59
12	59.20	61.77	1.70	-0.87	10.13	0.55
17	56.47	58.90	1.34	1.07	10.60	0.63
27	54.97	59.26	2.89	1.40	6.51	0.57
51	54.73	59.69	1.90	3.05	6.63	0.57
28	57.85	59.95	0.38	-1.72	8.37	0.53
19	57.30	60.76	1.90	1.58	6.88	0.59
41	52.18	55.50	0.00	3.30	5.24	0.55
8	52.10	56.94	0.67	4.17	7.18	0.45
16	55.40	57.64	0.38	1.84	6.11	0.47
24	54.47	57.04	1.68	0.87	9.08	0.47
32	56.37	56.71	-0.24	0.12	5.83	0.47
40	52.63	57.64	1.92	3.09	4.57	0.44
48	52.30	56.79	0.49	4.00	7.36	0.44

[a] All responses except baseline are X 1000

CPC, or DEA) and the α's are parameters of the model. The parameters for each of the responses are listed in Table XIV.

Comparison study. Results from the comparison study are given in Table XV.

DISCUSSION

Within an experiment. The use of a central composite design to specify levels for the three sample factors, calcium, magnesium, and protein, allowed the calculation of three of the responses which were to be investigated -- calcium sensitivity, magnesium interference, and protein interference (β_1, β_2, and β_3 in Eq. 15). The non-zero offset term observed in each experiment (β_0 in Eq. 15 and Table X, not to be confused with the absorbance baseline) is probably attributable to unknown interferences in the serum samples, and/or to the lack of a precise assay of the absolute amount of calcium in the Scale II serum. This offset does not invalidate the estimates of the other parameters.

If mapping study point 16 is taken as a representative example (Tables VI and X), replication of four of the treatment combinations allows the lack of fit of the four-parameter model (Eq. 15) to the data to be assessed; the calculated value of $F_{12,4} = 5.29$ is significant at the

TABLE XIV

PARAMETERS OF EQ. 20 RELATING

RESPONSES TO CODED FACTOR LEVELS

Parameter	RESPONSE X 1000		
	OF	Ca	Mg
α_0	53.879[a]	57.125[a]	0.817[a]
α_1	-0.305	-2.071[a]	-0.415[a]
α_{11}	-1.052	-0.008	0.291
α_2	0.564	-0.207	-0.061
α_{22}	0.750	0.071	-0.055
α_3	0.451	0.540[a]	0.172
α_{33}	0.291	-0.392	0.014
α_4	-2.375[a]	-2.427[a]	-0.380[a]
α_{44}	-0.489	-0.152	0.0008
α_5	6.844[a]	7.283[a]	0.010
α_{55}	-4.990[a]	-4.229[a]	0.546[a]
α_6	0.162	0.125	0.134
α_{66}	-0.947	-0.320	0.409
α_{12}	-0.386	0.024	-0.269
α_{13}	0.166	-0.014	0.012
α_{14}	0.833	-0.099	-0.831[a]
α_{15}	-0.993	-0.574[a]	-0.030
α_{16}	-0.789	-0.016	-0.038
α_{23}	-0.852	-0.366	0.227

[a] Significant at the 95% level

Parameter	RESPONSE X 1000		
	OF	Ca	Mg
α_{24}	-0.346	-0.461	0.386
α_{25}	-0.225	-0.229	0.083
α_{26}	0.144	0.026	-0.162
α_{34}	-2.146[a]	-0.583[a]	0.380
α_{35}	1.034	0.767[a]	-0.030
α_{36}	0.148	0.451[a]	0.360
α_{45}	-0.520	0.320	-0.164
α_{46}	0.582	-0.164	0.229
α_{56}	-0.801	0.115	0.053

[a] Significant at the 95% level

Parameter	RESPONSE X 1000 Protein	Linearity	Baseline
α_0	2.350[a]	6.683[a]	462.179[a]
α_1	-1.723[a]	-0.582	-9.379[a]
α_{11}	0.595	0.878	-1.784
α_2	-0.550	-1.118[a]	-3.200
α_{22}	-1.147	0.785	-2.255
α_3	-0.186	-0.111	-29.271[a]
α_{33}	-0.924	0.619	-0.427
α_4	0.263	-0.222	1.019
α_{44}	0.061	0.661	0.091
α_5	0.386	-1.436[a]	119.623[a]
α_{55}	0.396	1.009	3.770
α_6	0.340	0.109	18.488[a]
α_{66}	0.447	0.008	-0.473
α_{12}	1.183	-0.095	-10.232
α_{13}	-0.425	-1.292	-0.388
α_{14}	-0.146	0.099	0.305
α_{15}	0.773	0.957	-0.930
α_{16}	1.042	-0.247	1.121
α_{23}	0.045	-0.047	7.979

[a] Significant at the 95% level

Parameter	RESPONSE X 1000		
	Protein	Linearity	Baseline
α_{24}	-0.765	0.945	7.024
α_{25}	-0.289	0.955	0.247
α_{26}	0.233	-0.641	-5.839
α_{34}	1.521[a]	-0.882	2.453
α_{35}	-0.884	0.880	-15.311[a]
α_{36}	0.214	-0.617	3.471
α_{45}	1.329[a]	0.811	-11.535
α_{46}	-1.286	0.479	4.270
α_{56}	1.052	0.720	1.604

[a] Significant at the 95% level

TABLE XV

RESULTS OF COMPARISON STUDY

Method	RESPONSE X 1000		
	OF	Ca	Mg
Technicon	44.44	50.85	2.08
Technicon	45.83	51.47	1.64
average	45.14	51.16	1.86
Modified	49.39	55.36	1.27
Modified	47.69	55.66	2.65
average	48.54	55.51	1.96

Method	RESPONSE X 1000		
	Protein	Linearity	Baseline
Technicon	4.33	4.00	0.475
Technicon	4.00	2.95	0.475
average	4.17	3.48	0.475
Modified	4.71	7.81	0.398
Modified	5.34	6.84	0.407
average	5.03	7.33	0.403

94.0% level. A model containing more parameters might more closely describe absorbance as a function of calcium, magnesium, and protein. It was felt, however, that this simple model (Eq. 15) was adequate to characterize the calcium sensitivity, magnesium interference, and protein interference, and could thus serve as the basis for the objective function that was used to drive the simplex algorithm.

The magnesium and protein interferences (β_2 and β_3 in Table X) are not highly significant in these 20-sample experiments. This is probably because (a) the effects are small; (b) the ranges of magnesium and protein levels, while covering the normal serum range, were relatively small in the sample design; (c) there is a finite pure experimental uncertainty in the sample absorbances; and (d) any curvature in the effects of these factors would show up as an additional contribution to the variance of residuals in the first-order model.

Table XI presents the results from mapping study point 16 fit to the full second-order model of Eq. 19. The lack of fit of this model to the data is significant only at the 82.6% level. The calcium sensitivity (β_1) remains highly significant. In this model, the first-order effect of magnesium (β_2) is statistically significant, but the second-order (curvature) effect (β_{22}) is also significant, suggesting that for mapping study point 16, possibility (d) above

could be a major reason for the lack of statistical significance of the magnesium effect in the simple first-order model. (It is to be noted that an effect might be statistically significant, yet be so small that it makes a negligible contribution to the overall response.)

Simplex study. The simplex progress is presented in Table XII, where an overall trend toward increasing objective function values is seen. During the evaluation of point 7, the manifold sample inlet became clogged by an apparent precipitation of protein. After this and all subsequent experiments, the sample inlet was cleaned to remove any built-up obstructions. Thus, while the responses for the re-evaluation of vertex 3 are reasonably consistent with the original vertex 3 responses, the re-evaluation of vertex 7 gives much higher responses than the original vertex 7.

Calcium sensitivity is seen to increase throughout the study. There is no clear trend in either the magnesium or protein interferences, but they remain generally low; it appears that the objective function of Eq. 17 and the use of boundary violations in these responses are successful in minimizing these interferences while increasing the calsium sensitivity. The standard deviation of residuals shows some tendency to increase throughout the optimization; this might be due to increased curvature of absorbance with

respect to calcium concentration, or it might be due to increased pure experimental uncertainty.

The best value for the objective function was obtained at vertex 23; this was used as the center point of the subsequent mapping study.

Mapping study. Although the objectives of this work did not include obtaining fundamental information about the chemical system, many of the observed effects are consistent with known chemical behavior:

Calcium response: For the conditions used, increasing the amount of HCl before dialysis tended to cause the calcium sensitivity to decrease. Increasing the amount of HCl after dialysis caused the calcium sensitivity to increase. These effects can be attributed to a dialysis membrane equilibrium involving calcium and hydrogen ions: increasing the acid concentration on the recipient side and decreasing the acid concentration on the donor side favor the dialysis of H^+ ions from the recipient stream into the donor stream; this is necessary to balance the charge as a positive Ca^{2+} ion has moved from the donor stream to the recipient stream. However, too much HCl after dialysis would require considerably more DEA to make the solution basic, and at too high a concentration, the DEA attacks the pump tubes. Conversely, if the HCl before dialysis is too low, not enough Ca^{2+} will be released from the protein.

For the reasons noted earlier by Amador and Neely (59), the calcium sensitivity is relatively unaffected by the 8HQ before dialysis (8HQ will complex cations appreciably only in alkaline solution), but is decreased with increasing 8HQ after dialysis, as the alkaline 8HQ also complexes the calcium.

The increase in calcium sensitivity with increasing CPC is to be expected, since a larger amount of the color-producing reagent is present (in an equilibrium-type system, addition of CPC would shift the equilibrium to the right, forming more of the colored complex).

The effect of DEA on calcium sensitivity is negligible in the region of the design, probably because a large enough excess of DEA exists at all levels to make the solution sufficiently basic (60).

Magnesium response. The magnesium interference shows effects similar to those of calcium for HCl-b, 8HQ-b, HCl-a, and 8HQ-a. The effects of CPC and DEA on magnesium response are not clear-cut; however, from an operational point of view, the 0-level of CPC and DEA appear to give minimal interference from magnesium. (Since calcium and magnesium are quite similar, it is not surprising that conditions which favor high calcium sensitivity also favor high [undesirable] magnesium sensitivity. A compromise is thus necessary.)

Protein response. The positive protein interference

(indicating that as more protein is present, a greater fraction of the calcium is dialyzed) has been explained previously by Lott and Herman (57) as being caused by a Donnan equilibrium: protonated, positively-charged, non-dialyzable protein molecules favor the transfer of calcium through the membrane. The observed effect of HCl-b is consistent with this phenomenon.

Baseline response. The two reagents before dialysis (HCl-b and 8HQ-b) have little effect upon baseline response.

A major contribution to the baseline absorbance arises from the color of the uncomplexed CPC in alkaline solution; thus, lowering the pH would be expected to lower the baseline. The observations that increased HCl-a and decreased DEA (each contributing to a less basic solution) lower the baseline are consistent with this explanation.

Increasing the amount of CPC also causes the baseline to increase approximately linearly, as might be expected.

Comparison study. The coded mapping study levels 0, 1, 1, 0, 0, 0 (see Table VIII) were chosen as the conditions for the comparison study for the following reasons:

A low (-1) level of HCl-b produces good calcium sensitivity, but the protein interference is relatively large; a high (+1) level produces the opposite effects. A compromise may be achieved if the middle (0) level of HCl-b is used. (The objective function actually shows a slight

maximum at this level.)

The 8HQ-b was arbitrarily set at the +1 level; there are no strong arguments for setting it at any other level.

HCl-a was set at the high (+1) level primarily to reduce the baseline, even though an increase in magnesium interference was obtained.

The choice of level for 8HQ-a (0) was a compromise between increased calcium sensitivity (-1 level) and decreased magnesium interference (+1 level).

CPC trade-offs were more readily apparent: too little CPC greatly decreased the calcium sensitivity; too much CPC increased the baseline absorbance. A middle level (0) of CPC was chosen as an adequate compromise.

Finally, a DEA level of 0 was arbitrarily chosen; the OF is flat with respect to DEA in this region. Some decrease in baseline could have been achieved if less DEA had been used.

From the results presented in Table XV, it can be seen that the modified method gives higher calcium sensitivity and a lower baseline than the Technicon method. The magnesium and protein interferences are approximately the same for both methods. Although the linearity with respect to calcium is better for the Technicon method than it is for the modified method, the worst case linearity of 0.00781 absorbance standard deviation corresponds to a relative standard

deviation of only 1.4% at a calcium level of 10 mg dl^{-1}.

CONCLUSIONS

Automation of methods development and the use of recent experimental designs are two means of making the complete development of analytical chemical methods more efficient. In this work, efficient experimental designs required a total of only 80 experiments: 22 in the simplex study, 54 in the mapping study, and 4 in the comparison study. The operation of the automated system required only one analyst to prepare samples, reagents, and the flow stream before the studies began, and to detect and correct minor malfunctions (e.g., blockage of sample inlet) and carry out a few non-automated procedures (e.g., change sample trays) after the studies had begun.

The modified method is improved in two important aspects (calcium sensitivity and baseline absorbance) and is adequate in others (magnesium interference, protein interference, and linearity). Of even greater importance, however, is the comprehensive operational understanding of the system that has been easily achieved by automated methods development and efficient experimental designs.

CHAPTER IX

PHENYLEPHRINE AUTOANALYZER STUDY

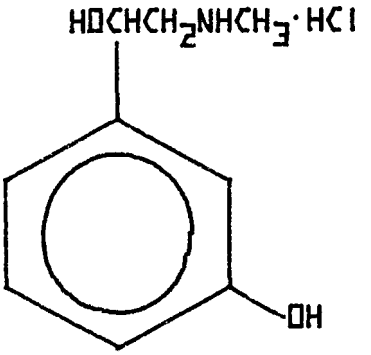
Although the Technicon AutoAnalyzer has been used primarily as a clinical chemistry instrument, it can be readily adapted for other automated analyses. One area for which the AutoAnalyzer is especially well-suited is the analysis of pharmaceuticals.

The Association of Official Analytical Chemists (AOAC) has adopted 3 procedures as "Official Methods of Analysis" for phenylephrine hydrochloride (see Figure 62) (61). Phenylephrine HCl is a sympathomimetric (decongestant) compound that is widely used in nasal sprays and sinus and cold tablets. As such, the analysis of the drug is important from a quality control standpoint, both to the manufacturing industry and to government regulatory agencies. One of the "Official" methods involves ion-pair partition chromatography; however, this method requires rather complex sample and chromatographic column preparation. The other two methods are a manual and an automated colorimetric determination.

The automated colorimetric method is designed for the Technicon AutoAnalyzer I (AAI), a predecessor of the currently used AutoAnalyzer II (AAII). The AAI is not in use very much at the present, as it suffers from several drawbacks when compared to the AAII; for example, the AAI pump could accomodate only 12 pump tubes without stacking the tubes, and the AAI colorimeter used selenium-barrier photocells,

FIGURE 62

PHENYLEPHRINE HYDROCHLORIDE


HOCHCH2NHCH3·HCl
OH

which are subject to aging and temperature effects, while the AAII colorimeter uses phototubes (51). Thus, it would be useful to adapt the AAI method for phenylephrine analysis to the AutoAnalyzer II.

The reaction employed in the analysis of phenylephrine is the Emerson reaction (62): a phenol with a H or other easily-displaced group in the para-position (to the -OH group) is oxidized in alkaline solution by potassium ferricyanide ($K_3Fe(CN)_6$) and combines with 4-aminoantipyrine to form a colored product (see Figure 63) which absorbs around 490 nm.

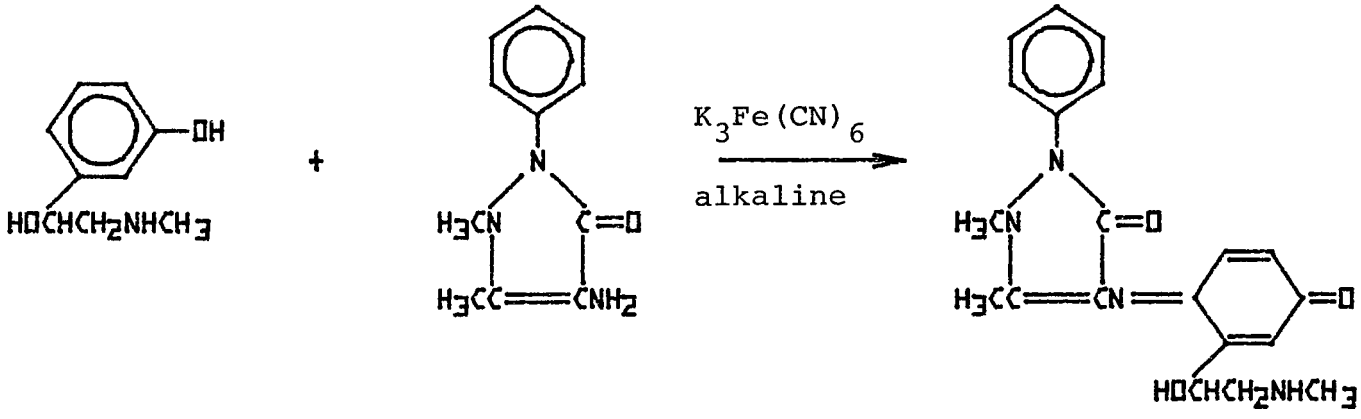
This reaction has been investigated with respect to phenylephrine. In 1958, Johnson and Savidge (63), using an NH_4Cl-NH_4OH buffer, found that the pH was important -- too low a pH gave too high a baseline and too high a pH gave too little absorbance above baseline. The authors recommended a pH between 7 and 11 for best results. They also found that an excess of $K_3Fe(CN)_6$ gave a very high baseline absorbance.

In 1961, Hiskey and Levin (64) tried a 0.1M $NaHCO_3$ buffer. No conclusions were drawn as to how the bicarbonate buffer compared with the ammonium buffer of Johnson and Savidge (63), but the authors did conclude that the amount of 4-aminoantipyrine used was important: too much of this reagent will react with the potassium ferricyanide, leaving

FIGURE 63

REACTION OF PHENYLEPHRINE WITH

4-AMINOANTIPYRINE (EMERSON REACTION)


OH
HOCHCH2NHCH3
+
N
H3CN
C=O
H3CC
CNH2
K3Fe(CN)6
alkaline
N
H3CN
C=O
H3CC
CN
=O
HOCHCH2NHCH3

an insufficient amount of $K_3Fe(CN)_6$ to oxidize the phenol.

Koshy and Mitchner in 1963 (65) found that the order of addition (potassium ferricyanide, 4-aminoantipyrine, buffer) was important. It was also found to be desirable to wait approximately 15 minutes after mixing to allow a colored imtermediate to dissipate; however, if the wait time was too long, the color of the desired product would also fade. By going to a 2% sodium borate buffer and adding the buffer first, Koshy and Mitchner were able to eliminate the interfering intermediate color. They also concluded that the concentrations of the 4-aminoantipyrine and $K_3Fe(CN)_6$ were not critical.

The system of Koshy and Mitchner was adopted by Margosis and Lane (66,67,68) as the "Official Colorimetric Method" for phenylephrine HCl and was automated for use on the Technicon AutoAnalyzer I. Two types of interferences were noted: zinc salts (although this is not a common ingredient in preparations containing phenylephrine and, in addition, could be overcome with little loss in phenylephrine sensitivity by employing EDTA in the diluent line), and other phenolic compounds (acetaminophen in particular was mentioned, as this analgesic is often found in cold and sinus tablets along with phenylephrine).

AUTOANALYZER SYSTEM

The implementation of the phenylephrine method on the AutoAnalyzer II is shown in Figure 64. The diluent line contains water with surfactant. After segmentation with air bubbles, the sample (or distilled water) is introduced. The relative flow rates affect a 1:26 dilution of the phenylephrine, which is normally in the range of 2 mg ml^{-1} (61).

After the stream is mixed in a glass coil, a resample line directs about 9% of the stream back into the pump; the remainder, including the bubbles, goes into a waste vessel. A line containing 2% sodium borate is segmented with air and the resample line added to the borate line. The potassium ferricyanide and 4-aminoantipyrine are added, with appropriate mixing, and the stream directed into the colorimeter flowcell. A portion of the stream, without bubbles, is pulled (by the pump) down into the optical path, where the absorbance of the colored product is measured.

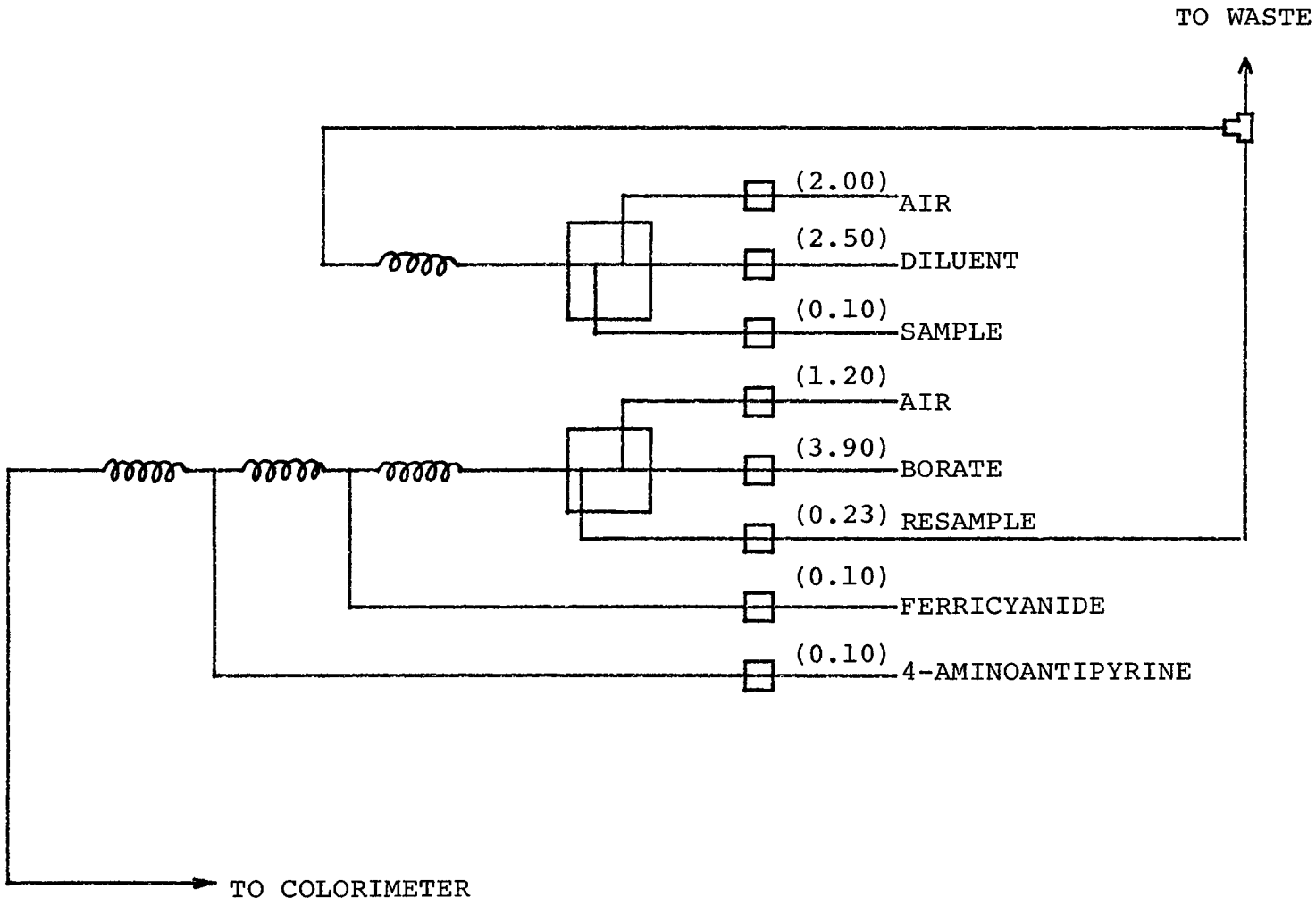
Apparatus. The 4 mixing coils were 14-turn, 2.4-mm glass coils (Technicon); the resample splitter is a standard Technicon product (part number C3). A 502-nm filter (the closest available to 490 nm) was used in the sample colorimeter channel; the reference channel was fitted with a 570-nm filter.

Preliminary studies. The following preliminary studies were undertaken:

FIGURE 64

AUTOANALYZER II PHENYLEPHRINE SYSTEM

(Numbers in parentheses refer to Technicon tubing flow-ratings)


TO WASTE
(2.00)
AIR
(2.50)
DILUENT
(0.10)
SAMPLE
(1.20)
AIR
(3.90)
BORATE
(0.23)
RESAMPLE
(0.10)
FERRICYANIDE
(0.10)
4-AMINOANTIPYRINE
TO COLORIMETER

(1) Air tubing size. Technicon AutoAnalyzer II methods normally use a 0.32 ml min^{-1} flow-rated tube for air bubble delivery (see, for example, 52); the AAI phenylephrine method (61) uses 2.00 and 2.50 ml min^{-1} tubing for the first and second air lines, respectively. Investigations showed that suitable tubing would be 2.00 and 1.20 ml min^{-1}; larger or smaller sizes gave worse bubble patterns.

During the same study, it was determined that the borate, which is introduced via a 3.90 ml min^{-1} flow-rated tube (and thus is the major contributor to the final flow rate), needed to have surfactant added to give consistent bubble patterns (the AAI method makes no mention of surfactant to any reagent other than the diluent).

(2) Flowcell pull-through line. The standard Technicon methods use 1.20 ml min^{-1} flow-rated tubing (see, for example, 52); this proved to be too large, as bubbles were pulled into the optical path. The AAI method (61) specifies 0.10 ml min^{-1} tubing; it was found that 0.80 ml min^{-1} tubing was satisfactory.

(3) Position of resample splitter. It was found that the position of the resample splitter was critical -- if it was not located as close to the pump as possible, the samples diffused to a large extent into the water between the samples and even into adjacent samples. This was due to the fact that, after the resample splitter, the stream has no segment-

ing air bubbles. (This demonstrated the need for a segmented stream in the AutoAnalyzer.)

(4) Absorbances of phenylephrine and acetaminophen. It was found that the absorptivity, a, of the acetaminophen-4-aminoantipyrine complex was approximately 8% of that of the phenylephrine-4-aminoantipyrine complex at the wavelength employed (502 nm).

Experimental system. The AutoAnalyzer was set up as shown in Figure 64. Data was taken at 1-sec intervals by the ADC and transmitted to the computer. A calibration study showed that 1 "ADC unit" corresponded to 2.755×10^{-4} absorbance units (A.U.); this conversion factor was used before subsequent data treatment. Inasmuch as a combination of factor levels yielding a reasonable response (the "Official Method") was already available, it was decided to forego the simplex search and perform a central composite mapping study centered about the recommended reagent concentrations. Because of this, the reagents were prepared in advance, thus eliminating the need for the individual pumps. The computer was still used, however: besides collecting the data, the computer turned the sampler on and off (via the relay) as needed.

Three factors were investigated: concentration of borate (recommended: 2%), concentration of potassium ferricyanide (KFC) (recommended: 4%), and concentration of

4-aminoantipyrine (AAP) (recommended: 3%). Table XVI shows the reagent design and order of evaluation. A central composite design in 3 factors requires 15 experiments; the center point experiment was performed 3 additional times for a total of 18 experiments. The 4 center point replicates were spaced uniformly throughout the design; the other 14 treatment combinations were randomized. The appropriate concentration of each reagent for each experiment was prepared in and dispensed from a separate container.

The responses investigated were the sensitivity to phenylephrine and the sensitivity to acetaminophen, the primary interferent. Table XVII shows the sample design. The levels of phenylephrine were chosen so as to bracket the concentration normally found in medication (2.0 mg ml^{-1}) (61). Originally, the same criterion was to be used in determining the levels of acetaminophen; however, acetaminophen was found to have a very low solubility; therefore, its levels were chosen so as to be comparable to those of phenylephrine. Samples 8 and 15 contained a commercial preparation for evaluation purposes.

Samples. Phenylephrine (Sigma) was prepared as a 5 mg ml^{-1} stock solution; the 18 sample solutions in Table XVII were made from dilutions of the stock solution. Acetaminophen (Sigma) was also prepared as a 5 mg ml^{-1} stock solution and diluted for the 18 samples in the Table. Both the

TABLE XVI

EXPERIMENTAL REAGENT DESIGN

Point	Borate, %	KFC, %	AAP, %
1	2.0	4.0	2.0
2	2.5	4.5	2.5
3	2.0	4.0	3.0
4	1.5	3.5	2.5
5	2.0	5.0	3.0
6	2.0	3.0	3.0
7	3.0	4.0	3.0
8	2.0	4.0	3.0
9	1.5	4.5	2.5
10	2.0	4.0	4.0
11	2.5	4.5	3.5
12	1.0	4.0	3.0
13	2.0	4.0	3.0
14	2.5	3.5	3.5
15	1.5	3.5	3.5
16	1.5	4.5	2.5
17	2.5	3.5	2.5
18	2.0	4.0	3.0

TABLE XVII

EXPERIMENTAL SAMPLE DESIGN

Sample	Phenylephrine HCl, mg/ml	Acetaminophen, mg/ml
1	2.0	0.0
2	3.0	0.0
3	1.5	1.5
4	2.5	2.5
5	0.0	0.0
6	2.0	1.0
7	2.0	3.0
8	*	*
9	1.5	2.5
10	1.5	0.0
11	2.5	1.5
12	2.0	2.0
13	2.5	0.0
14	3.0	2.0
15	*	*
16	2.0	2.0
17	3.0	2.0
18	1.0	2.0
19	1.0	2.0
20	1.0	0.0

* Contained commercial preparation (see text)

phenylephrine and acetaminophen were combined in a single sample dispensing bottle (one per sample); 2 drops of a thimerosal solution (5 g $l.^{-1}$) were added to each solution as a perservative. The commercial preparation was Neo-Synephrine (commercially-available nose drops) (Winthrop Laboratories, New York, NY), 0.25% (or 2.5 mg ml^{-1}). The other listed ingredients were methylparaben, propylparaben, and sodium bisulfite (all preservatives); the small quantities of these substances should have given rise to negligible (if any) interferences.

Reagents. Sodium borate (Fisher), potassium ferricyanide (Fisher), and 4-aminoantipyrine (Eastman) were prepared as 5% stock solutions; the 54 individual reagents (3 for each of the 18 experiments; see Table XVI) were prepared by diluting the stock solutions. Inasmuch as the KFC and AAP were reported to decompose, albeit slowly, over a period of time (69), these solutions were prepared as close as possible to the actual running of the study.

A systems view of the phenylephrine analysis is shown in Figure 65.

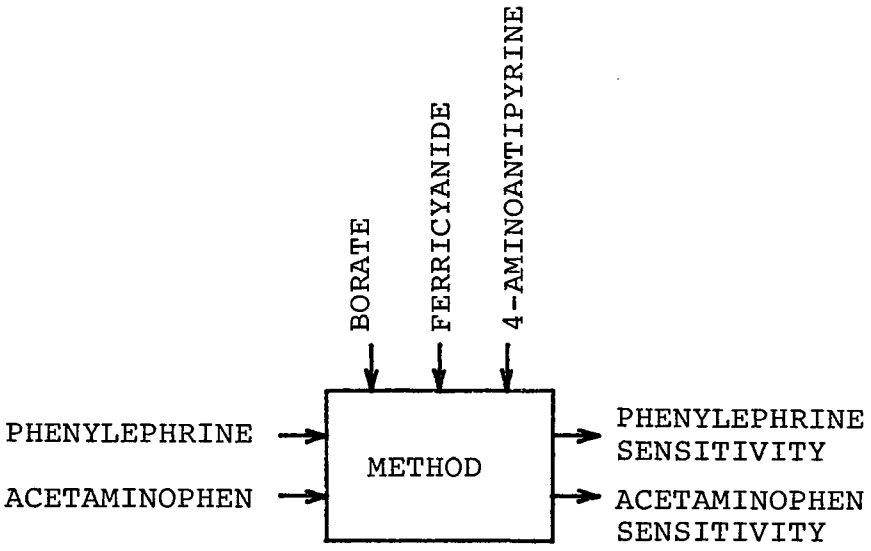
RESULTS AND DISCUSSION

The 18 experiments in the central composite design were performed in one block of time over a 12-hour period.

FIGURE 65

SYSTEMS THEORY VIEW OF THE

PHENYLEPHRINE METHOD


BORATE
FERRICYANIDE
4-AMINOANTIPYRINE
PHENYLEPHRINE
ACETAMINOPHEN
METHOD
PHENYLEPHRINE SENSITIVITY
ACETAMINOPHEN SENSITIVITY

The computer program responsible for running the experiments is listed in Appendix D. An example of the data received and plotted by the computer (that for experiment 18, the last center point replicate) is shown in Figure 66. Because the sample cups were left uncovered, some evaporation effects were discovered (evident as time trends in the data). The baseline and each of the 18 peaks were corrected for this; the 4 center point replicates were used to fit the model:

$$\text{absorbance} = \beta_0 + \beta_1(\text{time order}) \qquad (21)$$

The slopes (β_1 terms) obtained are given in Table XVIII. The peak heights, corrected for time, for each of the 20 samples in each of the 18 experiments are given in Table XIX. The baseline for each experiment was found by performing a least-squares fit on the first 25 and last 25 data points; the baseline absorbances, corrected for time, are shown in Table XX. The baseline (least-squares line) was subtracted from all data points before any data treatment.

For each of the 18 experiments, the following model was fit:

$$\text{absorbance} = \beta_1[\text{phenylephrine}] + \beta_2[\text{acetaminophen}] \qquad (22)$$

The β_0 term was omitted (forced to be zero) because this

FIGURE 66

DIGITIZED DATA PLOT FOR

POINT NUMBER 18

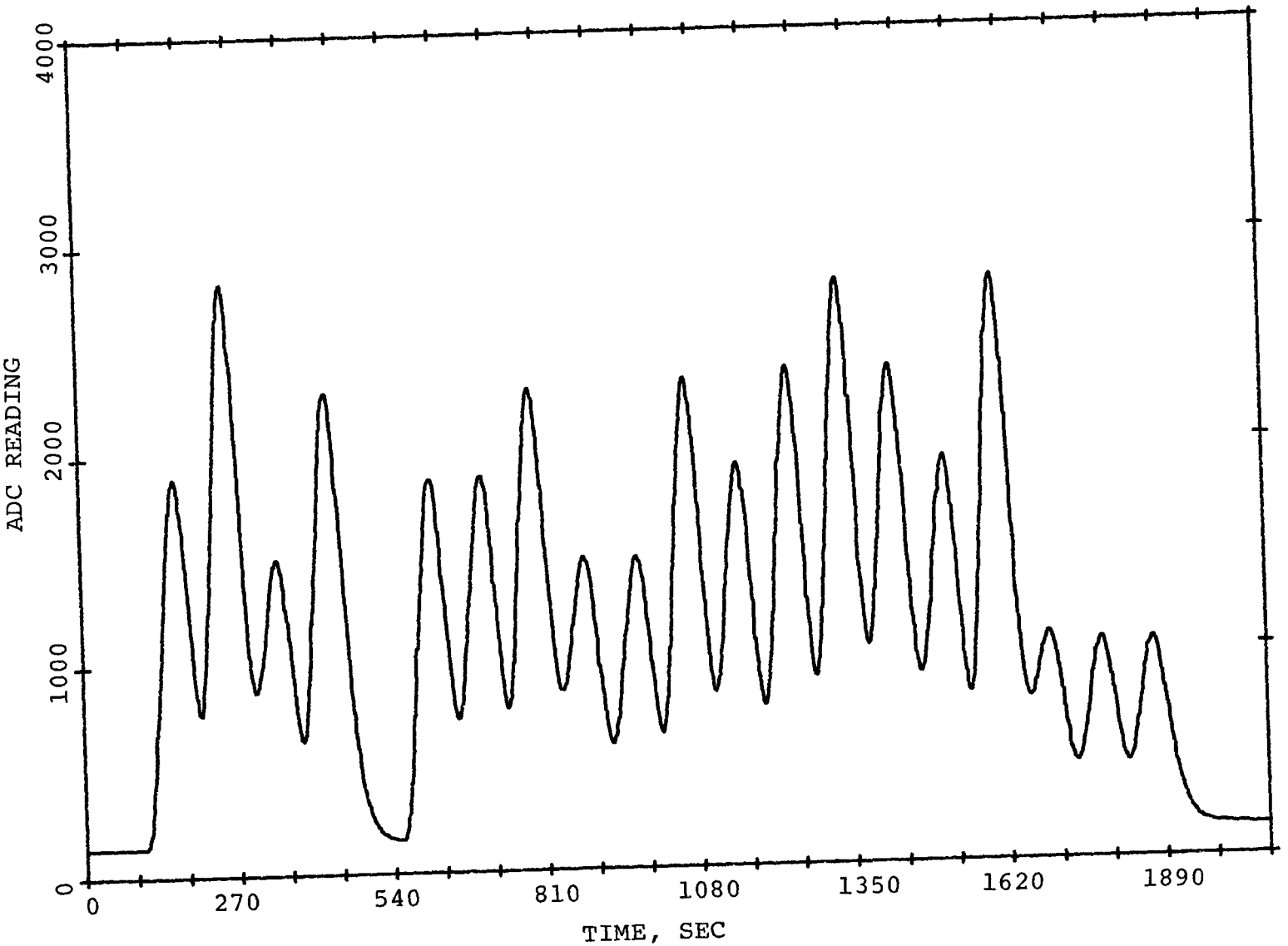

ADC READING
4000
3000
2000
1000
0
0
270
540
810
1080
1350
1620
1890
TIME, SEC

TABLE XVIII

TIME TRENDS

Sample	Slope[a], A.U.(time order)$^{-1}$
1	3.445
2	4.457
3	2.190
4	3.351
5	0.015
6	2.399
7	2.778
8	3.173
9	2.245
10	1.963
11	3.200
12	2.598
13	4.189
14	4.468
15	3.617
16	2.844
17	4.853
18	1.419
19	1.307
20	1.448
baseline	-0.824

[a] All slopes are X 1000

TABLE XIX

PEAK HEIGHTS IN ABSORBANCE UNITS

(CORRECTED FOR TIME EFFECT)

Sample Number	Experiment Number 1	2	3	4	5	6
1	0.397	0.423	0.426	0.422	0.426	0.426
2	0.632	0.663	0.666	0.664	0.662	0.671
3	0.312	0.342	0.341	0.340	0.340	0.344
4	0.521	0.539	0.540	0.542	0.540	0.548
5	0.0039	0.0045	0.0042	0.0040	0.0040	0.0040
6	0.419	0.441	0.439	0.435	0.436	0.440
7	0.422	0.437	0.437	0.437	0.436	0.438
8	0.520	0.543	0.542	0.540	0.536	0.540
9	0.322	0.332	0.332	0.331	0.331	0.330
10	0.323	0.335	0.337	0.336	0.333	0.337
11	0.529	0.551	0.550	0.549	0.546	0.547
12	0.429	0.445	0.445	0.442	0.443	0.445
13	0.527	0.541	0.542	0.549	0.540	0.544
14	0.638	0.649	0.650	0.657	0.658	0.653
15	0.531	0.544	0.551	0.549	0.549	0.547
16	0.429	0.443	0.444	0.445	0.441	0.443
17	0.630	0.642	0.645	0.649	0.646	0.644
18	0.225	0.232	0.233	0.233	0.231	0.230
19	0.217	0.225	0.226	0.224	0.224	0.224
20	0.217	0.221	0.222	0.222	0.224	0.224

Sample Number	Experiment Number 7	8	9	10	11	12
1	0.424	0.432	0.428	0.433	0.427	0.435
2	0.666	0.667	0.662	0.669	0.662	0.673
3	0.338	0.340	0.341	0.342	0.342	0.341
4	0.538	0.540	0.542	0.542	0.542	0.549
5	0.0043	0.0046	0.0044	0.0044	0.0042	0.0042
6	0.438	0.439	0.437	0.443	0.437	0.447
7	0.437	0.438	0.436	0.439	0.437	0.442
8	0.540	0.546	0.539	0.545	0.541	0.551
9	0.331	0.338	0.335	0.336	0.334	0.339
10	0.337	0.338	0.335	0.340	0.336	0.342
11	0.547	0.553	0.548	0.555	0.549	0.557
12	0.445	0.450	0.444	0.451	0.445	0.452
13	0.545	0.555	0.548	0.557	0.548	0.557
14	0.660	0.663	0.655	0.665	0.653	0.666
15	0.552	0.557	0.543	0.558	0.546	0.558
16	0.445	0.447	0.442	0.449	0.442	0.447
17	0.645	0.664	0.653	0.661	0.651	0.657
18	0.233	0.235	0.232	0.235	0.231	0.235
19	0.225	0.228	0.225	0.229	0.225	0.228
20	0.224	0.226	0.222	0.227	0.222	0.227

Sample Number	Experiment Number 13	14	15	16	17	18
1	0.430	0.429	0.431	0.426	0.429	0.427
2	0.669	0.666	0.669	0.664	0.660	0.665
3	0.344	0.338	0.344	0.339	0.341	0.339
4	0.548	0.538	0.546	0.542	0.540	0.537
5	0.0043	0.0042	0.0038	0.0047	0.0042	0.0044
6	0.443	0.441	0.445	0.443	0.440	0.438
7	0.445	0.432	0.438	0.438	0.435	0.434
8	0.546	0.543	0.548	0.541	0.543	0.541
9	0.335	0.332	0.335	0.335	0.331	0.333
10	0.339	0.338	0.340	0.338	0.336	0.337
11	0.554	0.551	0.558	0.553	0.553	0.549
12	0.449	0.445	0.450	0.451	0.445	0.446
13	0.550	0.550	0.550	0.547	0.543	0.544
14	0.653	0.660	0.654	0.661	0.648	0.654
15	0.551	0.551	0.551	0.553	0.545	0.553
16	0.445	0.443	0.445	0.445	0.440	0.445
17	0.655	0.647	0.654	0.656	0.646	0.648
18	0.232	0.233	0.232	0.235	0.230	0.234
19	0.226	0.225	0.230	0.228	0.225	0.226
20	0.225	0.224	0.225	0.224	0.223	0.223

TABLE XX

REGRESSION PARAMETERS OF EQ. 23

AND BASELINE ABSORBANCES

Experiment Number	Parameter				Baseline, A.U.[b]
	β_1	β_2[a]	β_{22}[a]	β_{12}[a]	
1	0.2092	9.817	-1.842	-1.380	0.047
2	0.2184	17.905	-3.127	-4.917	0.057
3	0.2193	15.895	-2.406	-5.008	0.053
4	0.2192	12.596	-2.130	-3.514	0.045
5	0.2182	13.617	-2.601	-3.285	0.056
6	0.2201	12.511	-1.585	-4.475	0.042
7	0.2193	14.241	-2.499	-4.032	0.047
8	0.2214	14.965	-3.099	-3.682	0.050
9	0.2192	13.496	-2.193	-3.798	0.053
10	0.2222	15.782	-3.412	-3.917	0.055
11	0.2192	14.053	-2.336	-3.985	0.054
12	0.2232	12.964	-1.920	-4.353	0.054
13	0.2212	11.978	-1.121	-4.372	0.051
14	0.2205	15.937	-3.422	-4.457	0.049
15	0.2214	17.875	-3.408	-4.968	0.051
16	0.2196	16.842	-3.674	-3.353	0.058
17	0.2189	16.262	-3.099	-4.448	0.047
18	0.2194	16.891	-3.066	-4.778	0.052

[a] Parameter X 1000

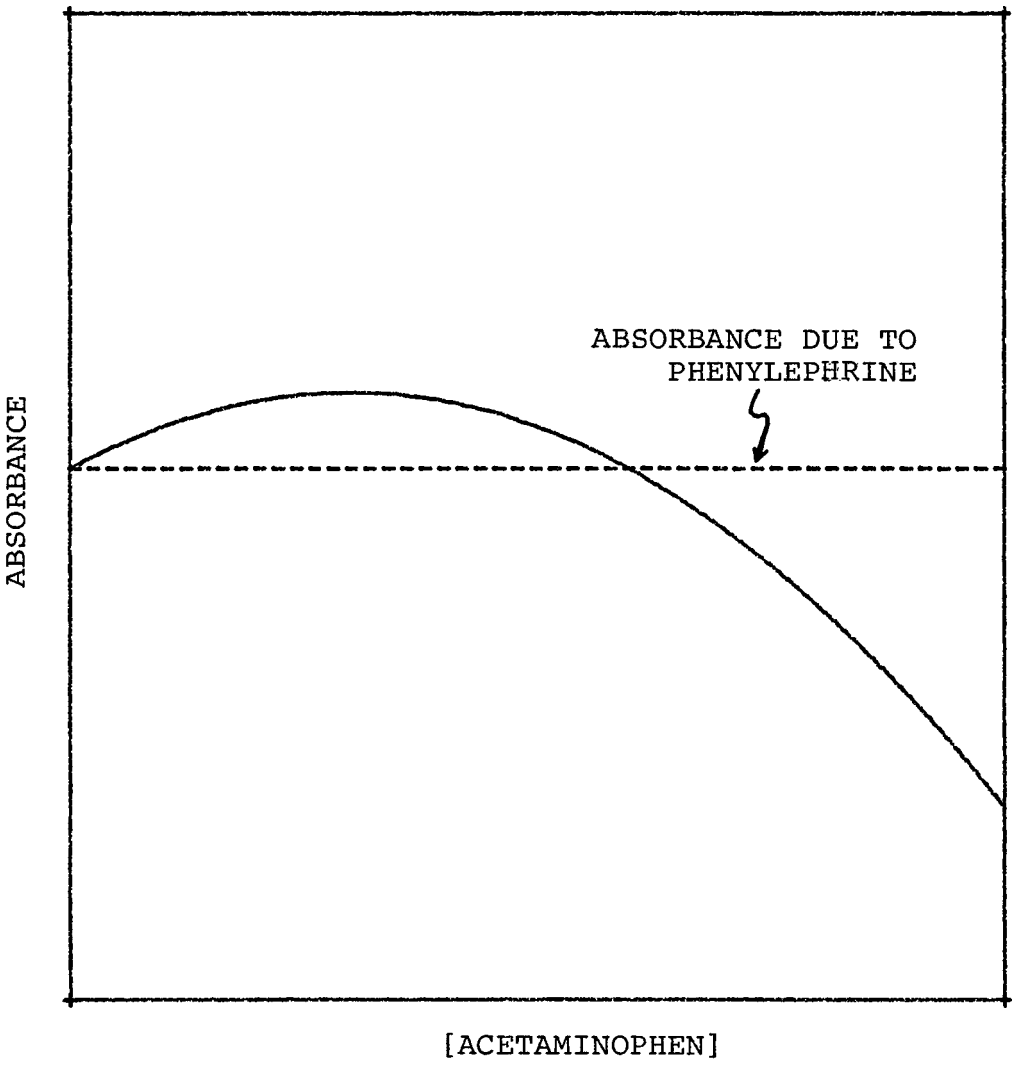
[b] Corrected for time effect

would usually be the assumption in a laboratory constructing a working curve. The lack of fit was found to be highly significant; this meant that the model was not adequate. Three other items of information provided insight into this problem: preliminary studies had found a slight positive absorbance with acetaminophen (no phenylephrine) as the sample; some of the β_2 terms in the model (eq. 22) were positive and some were negative; and other workers (66) had reported a lowering in absorbance with a phenylephrine-acetaminophen mixture as compared with a phenylephrine sample. This suggested that the true behavior of acetaminophen might be as shown in Figure 67: at low levels of acetaminophen, the additional colored complex formed by the acetaminophen makes a positive contribution to the overall absorbance; however, as the level of acetaminophen increases, the interferent competes with the phenylephrine for the reagents. The negative contribution to the total absorbance is in agreement with the preliminary study which found that the acetaminophen-AAP complex had a much lower absorptivity than did the phenylephrine-AAP complex in the spectral region used for this method. For each different level of reagents, there should be a different position of the maximum in the curve -- for higher reagent concentrations, the maximum should be shifted to higher acetaminophen levels.

In light of the apparent curvature and interaction in

FIGURE 67

POSSIBLE EFFECT OF ACETAMINOPHEN


ABSORBANCE DUE TO
PHENYLEPHRINE
ABSORBANCE
[ACETAMINOPHEN]

the acetaminophen effect, the following model was fit to each of the 18 treatment combinations:

$$\begin{aligned}\text{absorbance} = {} & \beta_1[\text{phenylephrine}] + \\ & \beta_2[\text{acetaminophen}] + \\ & \beta_{22}[\text{acetaminophen}]^2 + \\ & \beta_{12}[\text{phenylephrine}][\text{acetaminophen}]\end{aligned} \tag{23}$$

The coefficients obtained are shown in Table XX; note that the negative β_{22} coefficients correspond to the downward curvature illustrated in Figure 67.

The five parameters thus obtained (β_1, β_2, β_{22}, β_{12}, and the baseline absorbance) were fit to the following model:

$$\begin{aligned}\text{response} = {} & \alpha_0 + \alpha_1[\text{borate}] + \alpha_{11}[\text{borate}]^2 + \\ & \alpha_2[\text{KFC}] + \alpha_{22}[\text{KFC}]^2 + \alpha_3[\text{AAP}] + \\ & \alpha_{33}[\text{AAP}]^2 + \alpha_{12}[\text{borate}][\text{KFC}] + \\ & \alpha_{13}[\text{borate}][\text{AAP}] + \alpha_{23}[\text{KFC}][\text{AAP}]\end{aligned} \tag{24}$$

to assess the effects of the reagents on the responses. Table XXI lists the coefficients obtained.

The amount of phenylephrine found in the commercial

TABLE XXI

REGRESSION RESULTS USING MODEL OF EQ. 24

Coefficient[a]	Response β_1	β_2	β_{22}	β_{12}	Baseline
α_0	0.1367	-0.1185	0.0239	0.0380	-0.1049
α_1	8.1212	18.746	-5.3959	-3.9845	14.168
α_{11}	1.2134	-0.7471	-0.0284	0.1334	-0.5328
α_2	11.423	26.662	-0.6436	-5.8412	40.192
α_{22}	-0.8866	-1.2856	0.0088	0.0446	-1.9303
α_3	32.737	38.892	-4.5863	-17.592	30.015
α_{33}	-4.3366	-1.5502	-0.4457	1.6773	0.1987
α_{12}	-2.5509	-0.6294	0.7752	-0.1938	-0.7185
α_{13}	-1.5614	-4.2153	0.7374	1.3840	-3.7071
α_{23}	0.1614	-4.9149	1.3316	1.0026	-5.2730

For explanation of β coefficients, see Eq. 23

[a] All except α_0 are X 1000

preparation (samples 8 and 15) was computed using the phenylephrine sensitivity (β_1) at each experimental point; this data is tabulated in Table XXII. The average amount found, 2.514 mg ml^{-1}, is 100.6% of the reported value.

The coefficients (α's) were not found to be significant at the 95% level. This is in agreement with the work of Koshy and Mitchner (65), who reported that the concentrations of AAP and KFC were not critical.

It was decided that the amounts of borate and KFC were probably not important in this region -- instead of the center point conditions (2.0% borate and 4.0% KFC, as recommended by the "Official Method"), lower levels of these two reagents could be used (as low as 1.0% and 3.0%, respectively) without degrading the response, although the factor tolerances might need to be stricter.

The level of AAP also did not appear to be critical; however, since this was the color-producing reagent, it was decided to further investigate this factor.

Follow-up study. The borate reagents corresponding to 2.0% (center point value; reagents number 1, 3, 5, 6, 8, 10, 13, and 18) were combined into a pooled borate reagent; in a similar manner, the KFC reagents corresponding to 4.0% (center point value; reagents number 1, 3, 7, 8, 10, 12, 13, and 18) were combined into a pooled ferricyanide reagent.

The AAP reagent was varied from 0.00% to 1.50%; the

TABLE XXII

ANALYSIS OF COMMERCIAL SAMPLE

Experiment Number	Mg/ml found			% Reported Value
	Sample 8	Sample 15	Average	
1	2.508	2.559	2.534	101.4
2	2.490	2.523	2.507	100.3
3	2.523	2.569	2.546	101.8
4	2.495	2.544	2.520	100.8
5	2.487	2.550	2.519	100.8
6	2.494	2.532	2.513	100.5
7	2.493	2.561	2.527	101.1
8	2.489	2.554	2.522	100.9
9	2.481	2.515	2.498	99.9
10	2.477	2.551	2.514	100.6
11	2.489	2.527	2.508	100.3
12	2.489	2.540	2.515	100.6
13	2.473	2.517	2.495	99.8
14	2.475	2.535	2.505	100.2
15	2.490	2.529	2.510	100.4
16	2.464	2.540	2.502	100.1
17	2.489	2.525	2.507	100.3
18	2.474	2.552	2.513	100.5
average	2.488	2.540	2.514	100.6

eight experiments are shown (as columns) in Table XXIII. The 0% AAP level (experiment 5) gave no peaks. The resulting absorbance values were fit to the model given by Eq. 23; the coefficients (β's, corresponding to phenylephrine sensitivity, acetaminophen linear effect, acetaminophen curvature, and phenlyephrine-acetaminophen interaction) and the baseline absorbance are given in Table XXIV.

The five responses (4 coefficients and baseline absorbance) were fit using the following model:

$$\text{response} = \alpha_0 + \alpha_1[\text{AAP}] + \alpha_{11}[\text{AAP}]^2 \qquad (25)$$

The coefficients obtained are given in Table XXV. Canonical analysis of the regression results predicted a maximum in acetaminophen linear effect at 1.79% AAP and in phenylephrine-acetaminophen interaction at 0.95% AAP. (The maximum in acetaminophen curvature was predicted to occur at a negative concentration of AAP.) Inasmuch as the phenylephrine sensitivity is low in this region, the best working region would seem to be above 2.0%. Canonical analysis predicted a maximum in phenylephrine sensitivity at 2.02%. This is consistent with the results from the mapping study and can be explained by Figure 68. The follow-up study (covering the region from 0% to 1.5% AAP) predicted a maximum at 2.0% AAP; the mapping study (covering the region from 2.0%

TABLE XXIII

PEAK HEIGHTS IN ABSORBANCE UNITS

FOR FOLLOW-UP STUDY

(The eight experiments are shown
as columns in the order of evaluation)

Sample Number	AAP, % 0.50	1.50	1.00	0.25
1	0.175	0.404	0.332	0.099
2	0.261	0.624	0.495	0.152
3	0.140	0.322	0.260	0.080
4	0.220	0.513	0.408	0.128
5	0.0013	0.0043	0.0036	0.0009
6	0.174	0.410	0.326	0.102
7	0.177	0.417	0.333	0.107
8	0.213	0.501	0.402	0.123
9	0.135	0.319	0.258	0.081
10	0.133	0.315	0.250	0.080
11	0.218	0.543	0.414	0.135
12	0.177	0.418	0.335	0.108
13	0.217	0.524	0.419	0.132
14	0.261	0.627	0.508	0.160
15	0.214	0.515	0.409	0.129
16	0.178	0.417	0.336	0.107
17	0.263	0.628	0.503	0.160
18	0.094	0.220	0.176	0.057
19	0.094	0.212	0.171	0.055
20	0.090	0.210	0.171	0.054

Sample Number	AAP, % 0.00	1.00	0.50	0.25
1	0.000	0.344	0.194	0.101
2	0.000	0.525	0.291	0.151
3	0.000	0.272	0.155	0.081
4	0.000	0.440	0.247	0.128
5	0.0000	0.0042	0.0021	0.0010
6	0.000	0.346	0.195	0.101
7	0.000	0.355	0.197	0.104
8	0.000	0.428	0.239	0.122
9	0.000	0.271	0.154	0.080
10	0.000	0.267	0.151	0.078
11	0.000	0.455	0.245	0.132
12	0.000	0.357	0.199	0.103
13	0.000	0.440	0.249	0.126
14	0.000	0.523	0.296	0.151
15	0.000	0.437	0.243	0.124
16	0.000	0.355	0.201	0.102
17	0.000	0.529	0.298	0.155
18	0.000	0.189	0.106	0.055
19	0.000	0.180	0.103	0.053
20	0.000	0.179	0.102	0.053

TABLE XXIV

REGRESSION COEFFICIENTS IN FOLLOW-UP STUDY

(SEE EQ. 23)

Experiment Number	COEFFICIENTS[a]				Baseline, A.U.
	β_1	β_2	β_{22}	β_{12}	
1	0.0873	6.4370	-0.6853	-1.8006	0.0214
2	0.2078	12.1606	-3.5061	-1.0804	0.0299
3	0.1661	8.0743	-1.5125	-1.5083	0.0210
4	0.0514	4.2838	-1.0627	-0.0452	0.0099
5	0.0000	0.0000	0.0000	0.0000	0.0042
6	0.1752	10.4031	-1.7951	-1.8416	0.0174
7	0.0981	6.6100	-1.0529	-1.2785	0.0100
8	0.0507	3.6203	-0.6183	-0.5910	0.0047

[a] All coefficients except β_1 are X 1000

TABLE XXV

REGRESSION RESULTS OF MODEL USING EQ. 25

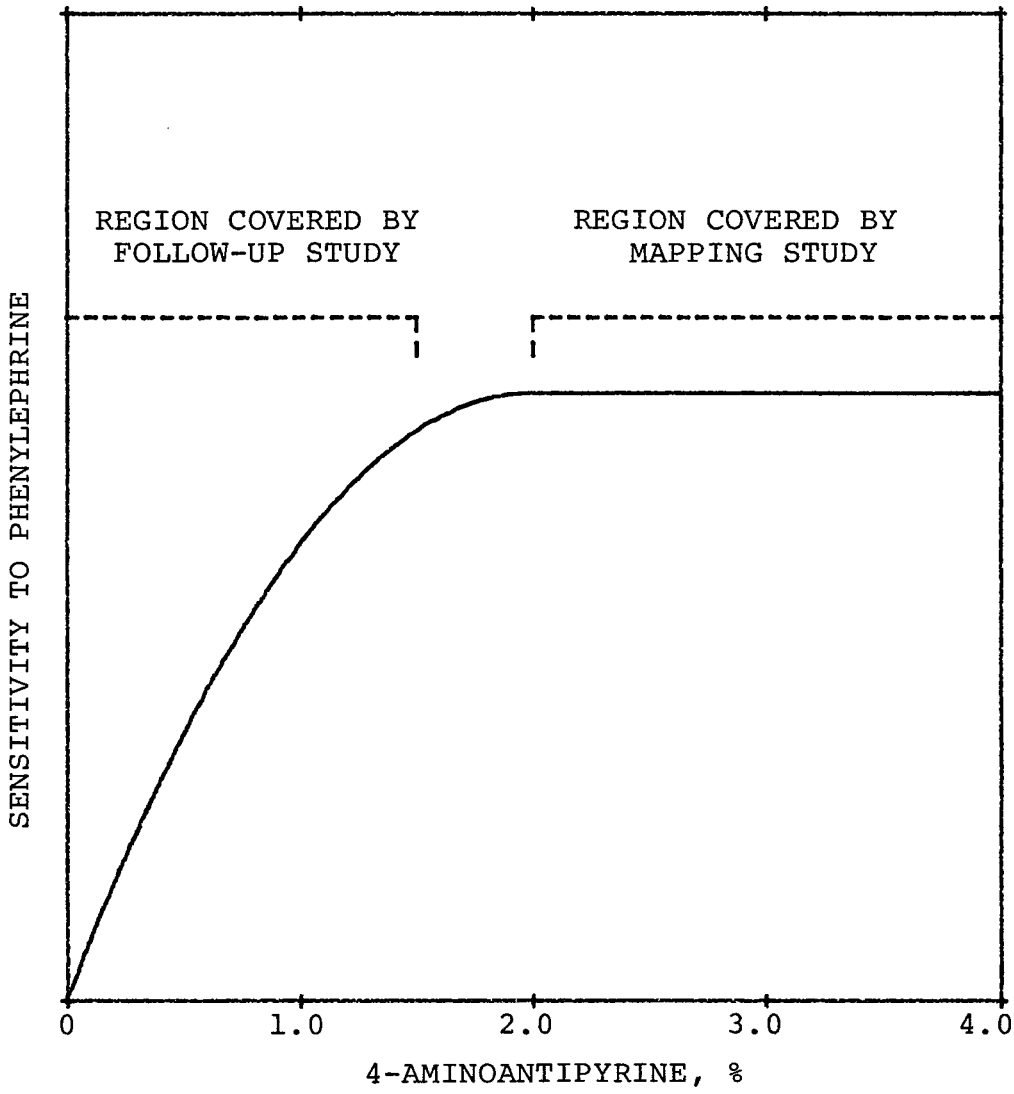
Coefficient[a]	Response β_1	β_2	β_{22}	β_{12}	Baseline
α_0	-2.0317	0.6636	-0.3720	0.2473	4.2896
α_1	224.710	12.723	-0.4573	-4.1463	17.753
α_{11}	-55.525	-3.5607	-1.0195	2.1760	-0.9554

[a] All coefficients are X 1000

For explanation of β coefficients, see Eq. 23

FIGURE 68

EFFECT OF 4-AMINOANTIPYRINE


REGION COVERED BY FOLLOW-UP STUDY
REGION COVERED BY MAPPING STUDY
SENSITIVITY TO PHENYLEPHRINE
0
1.0
2.0
3.0
4.0
4-AMINOANTIPYRINE, %

to 4.0% AAP) found essentially a flat surface. The curve shown in Figure 68 cannot be described by a simple quadratic model; however, the curve can be considered to be a combination of the two quadratic surfaces from the follow-up study and the mapping study.

Final evaluation. The AAP reagents corresponding to the center point conditions (3.0%; reagents 3, 5, 6, 7, 8, 12, 13, and 18) were combined to give a pooled AAP reagent. A commercial sinus tablet (Sinarest; Pharmacraft, Rochester, NY) was obtained. The ingredients (per tablet) were listed as: acetaminophen, 300 mg; caffeine, 30 mg; phenylephrine HCl, 5 mg; chlorpheniramine maleate, 1 mg. The tablet was crushed and placed in a test tube with 2 ml of distilled water, which should have yielded a final concentration of 2.5 mg ml^{-1} of phenylephrine. The mixture was allowed to stand for 2 days, then was centrifuged and the supernatant liquid withdrawn with a medicine dropper. Much solid residue remained, due both to the inert filler used and undissolved acetaminophen.

This solution was run in the AutoAnalyzer using the three pooled reagents; a standard of 2.5 mg ml^{-1} phenylephrine was also run. The absorbance of the mixture was found to be about 60% of that of the standard. Taking the coefficients found for a center point (Table XX, experiment 18), the amount of acetaminophen which dissolved was computed

to be 9.0 mg ml^{-1}, or 18 mg out of the original 300 mg of acetaminophen had dissolved in the 2 ml of water (this assumes that the 5 mg of acetaminophen completely dissolved). This is a reasonable value (probably the borderline of solubility, as a saturated solution could be assumed to have existed in the test tube). A test revealed that caffeine, at least in the quantity present, did not cause an interference; hence, the lowering of the absorbance by 40% was attributed to the presence of the acetaminophen.

In an earlier work, Margosis (66) reported that a 35% lowering was observed when using a phenylephrine-acetaminophen solution. However, the report was ambiguous: "a standard mixture of phenylephrine and acetaminophen, simulating the ratio" found in a commercial tablet. Three possibilities had existed as to the meaning of this:

(1) A saturated solution of acetaminophen was prepared and an appropriate amount of phenylephrine was added to simulate the ratio, but not the actual amounts. Thus, a sample containing 9 mg ml^{-1} of acetaminophen would also contain 0.15 mg ml^{-1} phenylephrine (yielding a 300:5 ratio). However, at these low levels of phenylephrine, the competition for reagents should be minimal, and there would probably be a positive contribution to the absorbance from the acetaminophen.

(2) Five mg of phenylephrine were dissolved in 2 ml of

water and the 300 mg of acetaminophen were dissolved by some means, such as acidification of the solution. However, this might be expected to change the chemistry of the method.

(3) Five mg of phenylephrine and 300 mg of acetaminophen were added to 2 ml of water and the solution (after filtration or decanting) used. This is the most likely possibility, according to the observed results. However, the actual solution analyzed does not simulate the ratio of phenylephrine to acetaminophen in the tablet, as much of the acetaminophen does not dissolve.

Conclusions. Several conclusions can be drawn from this study:

(1) It is relatively easy and straightforward to adapt an AAI method to the Technicon AutoAnalyzer II.

(2) The implementation of a method for which the AutoAnalyzer, a clinical chemistry instrument, was not really designed (i.e., pharmaceutical analysis) works quite well; in fact, the AutoAnalyzer is a sufficiently versatile instrument that many chemical processes (analyses, syntheses, etc.) can be adapted to it.

(3) The current "Official" phenylephrine method appears to be located in a good (i.e., "optimum") region of the response surface; however, all the reagents could be cut back by 1% (borate to 1.0%, potassium ferricyanide to 3.0%, and 4-aminoantipyrine to 2.0%) without degrading the response.

This would be especially attractive in the case of the 4-aminoantipyrine, inasmuch as it is expensive and somewhat limited in availability. Since potassium ferricyanide is relatively hazardous, the lower level of this reagent might also be desirable.

(4) Acetaminophen is indeed a serious interferent and the precaution in the "Official Method" (61) that the analysis should not be performed in the presence of acetaminophen seems well-founded. There are apparently only two ways to minimize this interference in the present method: (1) using more concentrated reagents (so that there would always be sufficient reagent for the phenylephrine regardless of the amount of acetaminophen present; the slight positive contribution to the absorbance due to the acetaminophen-AAP complex could probably be neglected); or (2) "swamping the system," that is, saturating the standards and samples alike with acetaminophen -- this should provide for essentially a constant (unfortunately, high) level of acetaminophen and perhaps allow construction of a phenylephrine working curve. Unfortunately, neither of these two approaches is really desirable; avoiding analyses in which acetaminophen is present, as recommended, would appear to be the best course.

CHAPTER X

CONCLUSIONS

Optimization is an important aspect of chemistry, and, in particular, of analytical chemistry. The three studies presented optimized, by use of the simplex algorithm and multivariate mapping studies, three chemical systems. Resolution within a given time constraint was improved in the chromatography project. In the investigation into the calcium analytical method, sensitivity to the analyte and the baseline absorbance were improved, while maintaining sensitivities to interferents and linearity within preselected limits. In the phenylephrine study, the existence of a stationary region in the response surface was verified and its limit in one of the reagents defined.

Just as importantly, an increased understanding of the three processes was gained. The effects of temperature and flow rate on chromatographic resolution and retention time, and the effects of the various reagents and interferences on the calcium and phenylephrine methods were investigated, and an operational (as opposed to mechanistic) understanding of these effects was gained.

These studies were carried out in a more efficient manner than is traditional: a computer controlled the instrumentation, received the data, and computed the operating conditions, utilizing a general purpose, bi-directional interface. A versatile analytical instrument, the Technicon AutoAnalyzer, was found to be a readily-adaptable

system for methods development.

Simplex optimization thus played an important role in the development (improvement and investigation) of chemical methods. Aided by automation, optimization has proven to be a valuable analytical chemistry tool.

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APPENDIX A

SUBROUTINE TO FORM
AND TRANSMIT A WORD ON THE INTERFACE

```
10 REM ############################ APPENDIX A ################################
20 REM ********************** SUBROUTINE CONVOY ***************
30 REM THIS SUBROUTINE WILL FORM AND SHIP OUT A WORD ON THE INTERFACE.
40 REM ALL DATA HAS A BINARY AND (BIAND) OPERATION PERFORMED ON IT
50 REM WITH A NUMBER REPRESENTING THE DESIRED NUMBER OF BITS SO AS TO
60 REM REMOVE ANY NOISE PRESENT IN THE OTHER BITS.
70 REM DATA IS WRITTEN OUT 8 BITS AT A TIME.  THE ROTATIONS ARE
80 REM PERFORMED BECAUSE THE 8 BITS HAVE TO BE IN THE RIGHT-MOST
90 REM PART OF THE 16-BIT COMPUTER WORD BEFORE THEY CAN BE WRITTEN.
100 REM VARIABLES USED:  A9 = ADDRESS IN OCTAL.
110 REM                  D9 = DATA IN DECIMAL.
120 REM                  C9 = SELECT CODE IN OCTAL.
130 REM                  M9 = MOTOR(1) OR OTHER DEVICE(0).
140 REM WRITING TO CHANNEL 1 ACTIVATES ONE OF THE 4 BUFFERS (CORRESPONDING
150 REM TO THE 4 BYTES OF THE WORD).
160 REM THIS LINE ACTIVATES THE RIGHT-MOST BUFFER (0).
170 REM ------------------------------------------------------------------
180 WRITE (1,*)WBYTE(0);
190 REM ------------------------------------------------------------------
200 REM THIS LINE WRITES THE ADDRESS, TAKEN AS OCTAL, TO
210 REM THE BUFFER JUST ACTIVATED (0, RIGHT-MOST).
220 REM THE ADDRESS HAS TO BE ROTATED SO AS TO BE PLACED IN THE
230 REM 6 LEFT BITS OF THE BUFFER.
240 REM ------------------------------------------------------------------
250 WRITE (2,*)WBYTE(BIAND(OCT(374),ROT(OCT(A9),14)));
260 REM ------------------------------------------------------------------
270 REM THE SECOND FROM THE LEFT BUFFER (1) IS ACTIVATED.
280 REM ------------------------------------------------------------------
290 WRITE (1,*)WBYTE(1);
300 REM ------------------------------------------------------------------
310 REM THE 8 LEAST-SIGNIFICANT BITS OF THE DARA ARE WRITTEN TO THE BUFFER
320 REM ACTIVATED (1).
330 REM ------------------------------------------------------------------
340 WRITE (2,*)WBYTE(BIAND(OCT(377),D9));
350 REM ------------------------------------------------------------------
```

```
360 REM THE SECOND FROM THE LEFT BUFFER (2) IS ACTIVATED FOR
370 REM THE 8 MOST-SIGNIFICANT BITS OF DATA.
380 REM ------------------------------------------------------------------
390 WRITE (1,*)WBYTE(2);
400 REM ------------------------------------------------------------------
410 REM THIS CHECKS TO SEE IF THE DEVICE BEING WRITTEN TO IS
420 REM A MOTOR.  IF SON, THE FREQUENCY/POSITION AND FORWARD/REVERSE BITS
430 REM NEED TO BE SET.
440 REM ------------------------------------------------------------------
450 IF M9=0 THEN 620
460 REM ------------------------------------------------------------------
470 REM THE DATA IS BIANDED SO AS TO LEAVE THE 8 MOST-SIGNIFICANT BITS.
480 REM ------------------------------------------------------------------
490 D9=BIAND(OCT(177400),D9)
500 REM ------------------------------------------------------------------
510 REM THE DATA HAS EITHER OCTAL(200) (FOR FREQUENCY, REVERSE),
520 REM OCTAL(000) (FOR FREQUENCY, FORWARD), OR OCTAL(100)
530 REM (FOR POSITION MODE) ADDED TO IT IN AN INCLUSIVE-OR OPERATION.
540 REM THIS FILLS IN THE 2 LEFTMOST BITS OF THE 16-BIT DATA.
550 REM ------------------------------------------------------------------
560 D9=INOR(ROT(D9,8),OCT(200))
570 WRITE (2,*)WBYTE(D9);
580 GOTO 660
590 REM ------------------------------------------------------------------
600 REM IF THE DEVICE ADDRESSED IS NOT A MOTOR, THE DATA IS SENT AS IS.
610 REM ------------------------------------------------------------------
620 WRITE (2,*)WBYTE(ROT(BIAND(OCT(177400),D9),8));
630 REM ------------------------------------------------------------------
640 REM THE LEFT-MOST BUFFER (3) IS ACTIVATED.
650 REM ------------------------------------------------------------------
660 WRITE (1,*)WBYTE(3);
670 REM ------------------------------------------------------------------
680 REM THE SELECT CODE IS SENT TO THE LEFT-MOST BUFFER.
690 REM ------------------------------------------------------------------
700 WRITE (2,*)WBYTE(BIAND(OCT(77),OCT(C9)));
```

```
710 REM ------------------------------------------------------------------
720 REM THIS LINE CAUSES TRANSMISSION OF THE ENTIRE 32 BITS.
730 REM ------------------------------------------------------------------
740 WRITE (1,*)WBYTE(300);
750 REM ------------------------------------------------------------------
760 REM FINALLY, THE SUBROUTINE RETURNS THE COMPUTER TO THE MAIN PROGRAM.
770 REM ------------------------------------------------------------------
780 RETURN
```

APPENDIX B

PROGRAM TO RUN GAS CHROMATOGRAPHY PROJECT

```
10 REM ######################## APPENDIX B ##########################################
20 REM ##############################################################################
30 REM PROGRAM TO CONTROL GAS CHROMATOGRAPH AND COLLECT DATA.
40 DIM DS[1,1],BS[20],C$[6],D$[6],A$[4],M[2],CS[3],XS[10,60],G[125],RS[5]
50 DIM L$[6],H[5],P[5]
60 SCALE 0,600,0,4100
70 REM FILE PUMPZ CONTAINS THE NEW MOTOR POSITIONS.
80 REM THE TWO ASTERISKS ARE FILES TO BE ASSIGNED.
90 FILES PUMPZ,RETURN,*,*
100 REM THIS DECIDES WHETHER SIMPLEX OR MAPPING IS IN PROGRESS.
110 REM THIS READS THE FIRST ELEMENT IN THE FILE PUMPZ.
120 READ #1;Z9
130 REM THIS READS THE FIRST ELEMENT IN THE FILE RETURN.
140 READ #2;R1
150 REM IF R1=4, THE MAPPING STUDY IS IN PROGRESS;
160 REM IF R1<4, THE SIMPLEX STUDY IS IN PROGRESS.
170 IF R1#4 THEN 240
180 REM THE NEXT 8 LINES FORM THE NAMES OF THE DATA FILES:
190 REM SR.XXX AND SS.XXX FOR THE SIMPLEX RAW AND BASELINE SUBTRACTED DATA;
200 REM FR.XXX AND FS.XXX FOR THE MAPPING RAW AND BASELINE SUBTRACTED DATA.
210 C$[1,3]="FR."
220 D$[1,3]="FS."
230 GOTO 260
240 C$[1,3]="SR."
250 D$[1,3]="SS."
260 DEXP Z9,A$
270 C$[4,6]=A$[2,4]
280 D$[4,6]=A$[2,4]
290 REM IF THIS EXPERIMENT IS AN N+1 RULE RE-EVALUATION,
300 REM A NEW FILE IS TO BE OPENED.
310 IF Z9<100 THEN 360
320 OPEN C$,10
330 OPEN D$,10
340 REM THE FILES ARE ASSIGNED TO FILES #3 AND #4 (THE POSITIONS
350 REM OF THE ASTERISKS IN THE FILES STATEMENT).
```

```
360 ASSIGN C$,3,W9
370 ASSIGN D$,4,W9
380 REM THE POSITIONS OF THE TWO MOTORS ARE READ FROM FILE PUMPZ.
390 READ #1;M[1],M[2]
400 REM THE EXPERIMENT NUMBER IS LABELLED ON THE PLOT.
410 PLOT 10,3500,-1
420 LABEL (*)C$
430 REM THE MOTOR POSITIONS ARE ROUNDED TO INTEGERS.
440 M[1]=INT(M[1]+0.5)
450 M[2]=INT(M[2]+0.5)
460 REM MOTOR 0 (FLOW RATE) IS SET TO 100 (OCTAL); MOTOR 1 (TEMPERATURE)
470 REM IS SET TO 0 (OCTAL).
480 A9=77
490 D9=0
500 C9=1
510 M9=1
520 GOSUB 3970
530 C9=0
540 D9=64
550 GOSUB 3970
560 REM THE CLOCK IS PROGRAMMED FOR 1 SEC INTERVALS.
570 A9=76
580 D9=60
590 C9=1
600 M9=0
610 GOSUB 3970
620 REM THERE IS A 10-SEC WAIT TO ENSURE THAT THE MOTORS
630 REM HAVE REACHED THEIR LOWER VALUES.
640 WAIT 10000
650 REM THE NEW MOTOR POSITIONS ARE PRINTED.
660 REM THE NEW MOTOR POSITIONS ARE ALSO TRANSMITTED TO THE MOTORS.
670 PRINT "MOTOR","POSITION"
680 FOR I=1 TO 2
690 PRINT I,M[I]
700 A9=77
```

```
710 C9=I-1
720 D9=M[I]
730 M9=1
740 GOSUB 3970
750 NEXT I
760 REM THERE IS A 5-MIN WAIT FOR EQUILIBRATION.
770 C8=0
780 FOR I=1 to 300
790 WRITE (3,*)WBYTE(0);
800 A9=77
810 D9=M[C8+1]
820 C9=C8
830 M9=1
840 GOSUB 3970
850 C8=(C8=0)
860 NEXT I
870 REM THIS COMMAND QUERIES THE PROGRAMMABLE CLOCK.
880 WRITE (3,*)WBYTE(0);
890 REM THE SAMPLE IS INJECTED FOR 10 SEC.
900 A9=77
910 D9=M9=0
920 C9=57
930 WRITE(3,*)WBYTE(0);
940 GOSUB 3970
950 FOR I=1 TO 10
960 WRITE(3,*)WBYTE(0);
970 A9=77
980 D9=M[C8+1]
990 M9=1
1000 C9=C8
1010 GOSUB 3970
1020 C8=(C8=0)
1030 NEXT I
1040 REM THE GAS SAMPLE VALVE IS RETURNED TO ITS NON-INJECT POSITION.
1050 A9=77
```

```
1060 D9=M9=0
1070 C9=17
1080 GOSUB 3970
1090 REM 20 POINTS ARE TAKEN FOR THE INITIAL BASELINE.
1100 FOR I=1 TO 20
1110 WRITE (3,*)WBYTE(0);
1120 REM THIS USES A READ SUBROUTINE
1130 GOSUB 3860
1140 B[I]=H6
1150 REM THE RAW DATA IS PRINTED TO FILE #3 (SR. OR FR.).
1160 PRINT #3;B[I]
1170 REM THE DATA IS PLOTTED AS IT IS READ BY THE COMPUTER.
1180 PLOT I,B[I]
1190 A9=77
1200 D9=M[C8+1]
1210 M9=1
1220 C9=C8
1230 GOSUB 3970
1240 C8=(C8=0)
1250 NEXT I
1260 PEN
1270 REM DATA IS TAKEN FOR A TOTAL OF 10 MIN.
1280 REM THE COUNTER GOES TO 580 (SEC) SINCE 20 POINTS HAVE ALREADY
1290 REM BEEN TAKEN FOR THE BASELINE.
1300 FOR I=1 TO 580
1310 REM THIS AGAIN QUERIES THE CLOCK.
1320 WRITE(3,*)WBYTE(0);
1330 REM THIS AGAIN USES THE READ DATA SUBROUTINE.
1340 GOSUB 3860
1350 D[1,1]=H6
1360 REM THE DATA IS PLOTTED.
1370 PLOT 20+I,D[1,1]
1380 REM THE DATA IS STORED ON FILE #3.
1390 PRINT #3;D[1,1]
1400 A9=77
```

```
1410 D9=M[C8+1]
1420 M9=1
1430 C9=C8
1440 GOSUB 3970
1450 C8=(C8=0)
1460 NEXT I
1470 PEN
1480 REM THIS RESETS THE POINTERS TO THE BEGINNING OF THE DATA FILES.
1490 FILES *,*
1500 REM THIS ASSIGNS SR.XXX OR FR.XXX TO FILE #1, SS.XXX OR
1510 REM FS.XXX TO FILE #2.
1520 ASSIGN C$,1,W9
1530 ASSIGN D$,2,W9
1540 REM THE 20 INITIAL BASELINE POINTS ARE READ IN FROM FILE #1.
1550 MAT READ #1;B
1560 REM THIS ROUTINE IS A LEAST-SQUARES CALCULATION.
1570 S1=S2=S3=S4=0
1580 FOR I=1 TO 20
1590 S1=S1+B[I]
1600 S2=S2+I
1610 S3=S3+B[I]*I
1620 S4=S4+I*I
1630 NEXT I
1640 REM THIS ROUTINE SKIPS TO THE LAST 20 (BASELINE) POINTS.
1650 FOR I=1 TO 560
1660 READ #1;X
1670 NEXT I
1680 REM THIS READS IN THE LAST 20 POINTS.
1690 MAT READ #1;B
1700 REM THIS IS ALSO A LEAST-SQUARES ROUTINE.
1710 FOR I=1 TO 20
1720 S1=S1+B[I]
1730 S2=S2+(I+580)
1740 S3=S3+(I+580)*(B[I])
1750 S4=S4+(I+580)*(I+580)
```

```
1760 NEXT I
1770 REM THE CALCULATION OF THE BASELINE:
1780 REM B IS THE SLOPE.
1790 B=(S3-S]*S2/40)/(S4-S2*S2/40)
1800 REM A IS THE INTERCEPT.
1810 A=S1/40-B*(S2/40)
1820 REM THE BASELINE IS SUBTRACTED FROM ALL 600 POINTS.
1830 FILES *,*
1840 REM THE POINTERS ARE AGAIN SET TO THE BEGINNING OF THE FILES.
1850 ASSIGN C$,1,W9
1860 ASSIGN D$,2,W9
1870 REM THE ENTIRE 600 POINTS ARE READ INTO THE X MATRIX.
1880 MAT READ #1;X
1890 FOR I=1 TO 10
1900 FOR J=1 TO 60
1910 X[I,J]=X[I,J]-A-B*(60*(I-1)+J)
1920 NEXT J
1930 NEXT I
1940 REM THE SUBTRACTED DATA IS WRITTEN ON FILE #2.
1950 MAT PRINT #2;X
1960 REM THIS IS THE ROUTINE THAT SEARCHES FOR 5 EXTREMA (3 MAXIMA, 2 MINIMA).
1970 FOR I=1 TO 5
1980 REM THE DATA POINTS ARE SEARCHED SERIALLY; I2 AND J2 GIVE THE LOCATION
1990 REM OF THE POINTS IN THE X MATRIX.
2000 I2=INT((I-1)/60)
2010 J2=I-60*I2
2020 REM THE R ARRAY HOLDS THE 5 POINTS CURRENTLY BEING SEARCHED.
2030 R[6-I]=X[I2+1,J2]
2040 NEXT I
2050 REM T IS THE COUNTER FOR THE DATA POINTS.
2060 T=5
2070 REM IF THE FOLLOWING CONDITION IS MET, THE FIRST MAXIMUM
2080 REM HAS BEEN FOUND.
2090 IF R[1]<R[3] AND R[2] <= R[3] AND R[4] <= R[3] and R[5]<R[3] THEN 2250
2100 REM ALL POINTS ARE SHIFTED IN THE R ARRAY
```

```
2110 R[5]=R[4]
2120 R[4]=R[3]
2130 R[3]=R[2]
2140 R[2]=R[1]
2]50 T=T+1
2160 REM IF ALL THE DATA POINTS HAVE BEEN SEARCHED, SKIP OUT OF ROUTINE.
2170 IF T>600 THEN 3450
2180 I=INT((T-1)/60)
2190 J=T-60*I
2200 REM A NEW POINT IS TAKEN INTO THE R ARRAY.
2210 R[1]=X[I+1,J]
2220 GOTO 2090
2230 REM THE FIRST PEAK HAS TO BE THIS HIGH (ADC UNITS)
2240 REM BEFORE IT IS CONSIDERED TO BE A PEAK.
2250 IF R[3]<250 THEN 2110
2260 REM THE H ARRAY CONTAINS THE VALUE OF THE EXTREMUM.
2270 REM THE P ARRAY CONTAINS THE POSITION OF THE EXTREMUM.
2280 H[1]=R[3]
2290 P[1]=T-1
2300 REM THE NEXT 5 POINTS ARE READ INTO THE R ARRAY AND THE
2310 REM SEARCH IS ON FOR THE NEXT EXTREMUM (MINIMUM).
2320 FOR I=1 TO 5
2330 I2=I+T
2340 IF I2>600 THEN 3450
2350 I3=INT((I2-1)/60)
2360 J3=I2-60*I3
2370 R[6-I]=X[I3+1,J3]
2380 NEXT I
2390 T=T+5
2400 IF R[5]>R[3] AND R[4] >= R[3] and R[2] >= R[3] and R[1]>R[3] THEN 2510
2410 R[5]=R[4]
2420 R[4]=R[3]
2430 R[3]=R[2]
2440 R[2]=R[1]
2450 T=T+1
```

```
2460 IF T>600 THEN 3450
2470 I=INT((T-1)/60)
2480 J=T-60*I
2490 R[1]=X[I+1,J]
2500 GOTO 2400
2510 H[2]=R[3]
2520 P[2]=T-1
2530 REM THE FIRST MAXIMUM AND MINIMUM HAVE BEEN FOUND.
2540 REM THE SECOND MAXIMUM IS SEARCHED FOR.
2550 FOR I=1 TO 5
2560 I2=I+T
2570 IF I2>600 THEN 3450
2580 I3=INT((I2-1)/60)
2590 J3=I2-60*I3
2600 R[6-I]=X[I3+1,J3]
2610 NEXT I
2620 T=T+5
2630 IF R[5]<R[3] AND R[4] <= R[3] AND R[2] <= R[3] AND R[1]<R[3] THEN 2740
2640 R[5]=R[4]
2650 R[4]=R[3]
2660 R[3]=R[2]
2670 R[2]=R[1]
2680 T=T+1
2690 IF T>600 THEN 3450
2700 I=INT((T-1)/60)
2710 J=T-60*I
2720 R[1]=X[I+1,J]
2730 GOTO 2630
2740 IF R[3]<100 THEN 2640
2750 H[3]=R[3]
2760 P[3]=T-1
2770 REM THE SECOND MINIMUM IS SEARCHED FOR.
2780 FOR I=1 TO 5
2790 I2=I+T
2800 IF I2>600 THEN 3450
```

```
2810 I3=INT((I2-1)/60)
2820 J3=I2-60*I3
2830 R[6-I]=X[I3+1,J3]
2840 NEXT I
2850 T=T+5
2860 IF R[5]>R[3] AND R[4] >= R[3] AND R[2] >= R[3] AND R[1]>R[3] THEN 2970
2870 R[5]=R[4]
2880 R[4]=R[3]
2890 R[3]=R[2]
2900 R[2]=R[1]
2910 T=T+1
2920 IF T>600 THEN 3450
2930 I=INT((T-1)/60)
2940 J=T-60*I
2950 R[1]=X[I+1,J]
2960 GOTO 2860
2970 H[4]=R[3]
2980 P[4]=T-1
2990 REM THE THIRD MAXIMUM IS SEARCHED FOR.
3000 FOR I=1 TO 5
3010 I2=I+T
3020 IF I2>600 THEN 3450
3030 I3=INT((I2-1)/60)
3040 J3=I2-60*I3
3050 R[6-I]=X[I3+1,J3]
3060 NEXT I
3070 T=T+5
3080 IF R[5]<R[3] AND R[4] <= R[3] AND R[2] <= R[3] AND R[1]<R[3] THEN 3190
3090 R[5]=R[4]
3100 R[4]=R[3]
3110 R[3]=R[2]
3120 R[2]=R[1]
3130 T=T+1
3140 IF T>600 THEN 3450
3150 I=INT((T-1)/60)
```

```
3160 J=T-60*I
3170 R[1]=X[I+1,J]
3180 GOTO 3080
3190 IF R[3]<100 THEN 3090
3200 H[5]=R[3]
3210 P[5]=T-1
3220 REM THIS PRINTS OUT THE 5 EXTREMA AND THEIR POSITIONS.
3230 FOR I=1 TO 5
3240 PRINT "POSITION","VALUE"
3250 PRINT P[I],H[I]
3260 NEXT I
3270 PRINT LIN2
3280 REM H(2) IS HEIGHT OF VALLEY ABOVE BASELINE.  H0 IS THE DISTANCE FROM
3290 REM THE LINE BETWEEN THE PEAKS TO THIS VALLEY.
3300 H0=(P[2]-P[1])*(H[1]-H[3])/(P[1]-P[3])+H[1]
3310 IF (H0-H[2])/H0 >= 0 THEN 3350
3320 R=0
3330 GOTO 3360
3340 REM THIS IS THE LOG ROUTINE FOR THE CRF.
3350 R=LGT((H0-H[2])/H0)
3360 H0=(P[4]-P[3])*(H[3]-H[5])/(P[3]-P[5])+H[3]
3370 IF (H0-H[4])/H0 >= 0 THEN 3410
3380 R=R+0
3390 GOTO 3420
3400 REM THIS ADDS THE SECOND LOG TERM TO THE FIRST.
3410 R=R+LGT((H0-H[4])/H0)
3420 GOTO 3510
3430 REM IF 5 EXTREMA WERE NOT FOUND IN THE 600 POINTS, A BAD RESPONSE
3440 REM IS ASSIGNED.
3450 R=-9E+99
3460 REM THE FILE RESPON CONTAINS THE RESPONSE OF THE CURRENT EXPERIMENT.
3470 REM FILES F.HOLD AND S.HOLD CONTAIN THE RESPONSES FOR ALL THE
3480 REM EXPERIMENTS IN THE MAPPING AND SIMPLEX.
3490 REM THIS ROUTINE CHECKS WHETHER SIMPLEX OR MAPPING IS IN
3500 REM PROGRESS AND FORMS THE APPROPRIATE HOLD FILE ASSIGNMENT.
```

```
3510 IF R1#4 THEN 3540
3520 L$[1,2]="F."
3530 GOTO 3550
3540 L$[1,2]="S."
3550 L$[3,6]="HOLD"
3560 FILES RESPON,*
3570 ASSIGN L$,2,W9
3580 REM THE RESPONSE IS PRINTED TO THE FILE RESPON.
3590 PRINT #1;R
3600 REM THE RESPONSE IS PRINTED.
3610 PRINT "RESPONSE = ";R
3620 REM THE HOLD FILE IS READ.
3630 MAT READ #2;G
3640 REM THE CURRENT RESPONSE IS PLACED INTO THE MATRIX.
3650 G[Z9]=R
3660 FILES *
3670 ASSIGN L$,1,W9
3680 REM THE HOLD MATRIX IS WRITTEN BACK TO THE FILE.
3690 MAT PRINT #1;G
3700 REM THE PROGRAM NOW PROMPTS THE EXPERIMENTER TO CHANGE PAPER.
3710 DISP "CHANGE PAPER"
3720 FOR I=1 TO 10
3730 BEEP
3740 WAIT 200
3750 NEXT I
3760 STOP
3770 REM THE PROGRAM NOW CALLS THE MASTER PROGRAM, "SMART."  THIS PROGRAM
3780 REM  SIMPLY DECIDES, BASED UPON THE VALUE OF THE RETURN ADDESSS IN THE
3790 REM FILE RETURN, WHETHER TO CALL THE SIMPLEX PROGRAM AT THE BEGINNING,
3800 REM AT THE POINT WHERE AN INITIAL VERTEX IS CALCULATED, AT THE POINT
3810 REM WHERE A NEW REFLECTION VERTEX IS CALCULATED, OR AT THE POINT WHERE
3820 REM AN N+1 RE-EVALUATION IS PERFORMED; OR TO CALL THE MAPPING STUDY
3830 REM PROGRAM.
3840 GET "SMART"
3850 END
```

```
3860 REM SUBROUTINE TO READ A DATA POINT.
3870 REM THIS ROUTINE WILL ADDRESS THE ADC.
3880 A9=77
3890 D9=M9=0
3900 C9=20
3910 GOSUB 3970
3920 REM THIS READS FROM THE 2 BUFFERS AND COMBINES THE 2 BYTES INTO A WORD.
3930 H6=INOR(ROT(BIAND(OCT(17),RBYTE2),8),BIAND(OCT(377),RBYTE1))
3940 RETURN
3950 REM CONVOY -- SUBROUTINE TO SHIP OUT A WORD ON THE INTERFACE.
3960 REM SEE APPENDIX 'A' FOR A DETAILED DESCRIPTION.
3970 REM ********************* SUBROUTINE CONVOY ****************
3980 WRITE (1,*)WBYTE(0);
3990 WRITE (2,*)WBYTE(BIAND(OCT(374),ROT(OCT(A9),14)));
4000 WRITE (1,*)WBYTE(1);
4010 WRITE (2,*)WBYTE(BIAND(OCT(377),D9));
4020 WRITE (1,*)WBYTE(2);
4030 IF M9=0 THEN 4080
4040 D9=BIAND(OCT(177400),D9)
4050 D9=INOR(ROT(D9,8),OCT(100))
4060 WRITE (2,*)WBYTE(D9);
4070 GOTO 4090
4080 WRITE (2,*)WBYTE(ROT(BIAND(OCT(177400),D9),8));
4090 WRITE (1,*)WBYTE(3);
4100 WRITE (2,*)WBYTE(BIAND(OCT(77),OCT(C9)));
4110 WRITE (1,*)WBYTE(OCT(300));
4120 RETURN
```

APPENDIX C

PROGRAM TO RUN AUTOANALYZER

FOR CALCIUM STUDY

```
10 REM ############################## APPENDIX C ############################
20 REM ######################################################################
30 REM PROGRAM TO RUN CALCIUM AUTOANALYZER STUDY.
40 DIM DS[1,1],ES[1,1],XS[22,90]
50 DIM P[1,9],A$[4],C$[6],D$[6],CI[19,1],II[1,19],V[1,1],ZS[1,1],NS[1,9]
60 REM EXP. NUMBER AND PUMP SPEEDS ARE IN FILE PUMPZ.
70 FILES PUMPZ
80 READ #1;Z[1,1]
90 Z9=Z[1,1]
100 SCALE 0,2000,0,4100
110 DEXP Z9,A$
120 REM THE FIRST ELEMENT IN FILE RETURN DECIDES IF THE SIMPLEX OR MAPPING
130 REM STUDY IS IN PROGRESS.
140 FILES RETURN
150 READ #1;R1
160 IF R1#4 THEN 220
170 REM THE RAW DATA WILL GO IN FILES SR.XXX OR FR.XXX;
180 REM THE BASELINE SUBTRACTED DATA IN FILES SS.XXX OR FS.XXX.
190 C$[1,3]="FR."
200 D$[1,3]="FS."
210 GOTO 240
220 C$[1,3]="SR."
230 D$[1,3]="SS."
240 C$[4,6]=A$[2,4]
250 D$[4,6]=A$[2,4]
260 REM THE ASTERISKS WILL BE ASSIGNED FILES.
270 REM THE FILES STATEMENT INTIALIZES THE FILE POINTERS.
280 FILES PUMPZ,*,*
290 ASSIGN C$,2,W9
300 ASSIGN D$,3,W9
310 PRINT C$
320 READ #1;Z[1,1]
330 REM THE 6 PUMP SPEEDS ARE READ FROM PUMPZ INTO THE P ARRAY.
340 READ #1;P[1,1],P[1,2],P[1,4],P[1,5],P[1,7],P[1,8]
350 REM THE EXP. NUMBER IS WRITTEN TO THE DATA FILE.
```

```
360 PRINT #2;Z[1,1]
370 REM THE SPEEDS OF THE 3 WATER MAKE-UP PUMPS ARE COMPUTED.
380 P[1,3]=2470-P[1,1]-P[1,2]
390 P[1,6]=2470-P[1,4]-P[1,5]-P[1,7]
400 P[1,9]=2470-P[1,8]
410 REM THE PUMP SPEEDS ARE ROUNDED TO INTEGERS.
420 FOR I=1 TO 9
430 N[1,I]=INT(P[1,I]+0.5)
440 NEXT I
450 REM THE PUMP SPEEDS ARE PRINTED OUT.
460 PRINT
470 PRINT "PUMP","SPEED"
480 FOR I=1 TO 9
490 PRINT I,N[1,I]
500 NEXT I
510 PRINT
520 REM THE PUMP SPEEDS ARE WRITTEN TO THE DATA FILE.
530 MAT PRINT #2;N
540 REM THE PUMPS ARE SET TO THE NEW SPEEDS.
550 FOR I=1 TO 9
560 A9=77
570 D9=N[1,I]
580 C9=I-1
590 M9=1
600 GOSUB 2640
610 NEXT I
620 REM A 5-MIN WAIT IS INCLUDED FOR EQUILIBRATION.
630 FOR I=1 TO 10
640 WAIT 30000
650 NEXT I
660 REM THE CLOCK IS PROGRAMMED FOR 1-SEC INTERVALS.
670 A9=76
680 D9=60
690 C9=1
700 M9=0
```

```
710 GOSUB 2640
720 REM THIS QUERIES THE PROGRAMMABLE CLOCK.
730 WRITE (3,*)WBYTE(0);
740 REM THIS IS THE DATA COLLECTION LOOP.
750 FOR L=1 TO 28
760 REM TURN ON THE SAMPLER WHEN L=3.
770 IF L#3 THEN 840
780 A9=77
790 D9=0
800 C9=47
810 M9=0
820 GOSUB 2640
830 GOTO 870
840 IF L#1 THEN 870
850 K7=1
860 REM TAKE DATA FOR 90 SEC EACH INTERATION (1 SAMPLE).
870 FOR K=1 TO 90
880 REM TURN OFF SAMPLER WHEN L=22.
890 IF L#22 OR K#70 THEN 960
900 A9=77
910 D9=0
920 C9=15
930 M9=0
940 GOSUB 2640
950 REM QUERY THE CLOCK.
960 WRITE (3,*)WBYTE(0);
970 REM ADDRESS THE ADC.
980 A9=77
990 D9=0
1000 C9=16
1010 M9=0
1020 GOSUB 2640
1030 REM READ IN THE DIGITIZED DATA.
1040 D[1,1]=INOR(ROT(BIAND(OCT(17),RBYTE2),8),BIAND(OCT(377),RBYTE1))
1050 REM IF L>=7, SAVE AND PLOT THE DATA.
```

```
1060 IF L<7 THEN 1100
1070 REM THE DATA IS WRITTEN OUT TO THE SR. OR FR. FILE.
1080 PRINT #2;D[1,1]
1090 PLOT 90*(L-7)+K,D[1,1]
1100 IF K7 <= 9 THEN 1130
1110 REM CONTINUALLY REFRESH THE PUMP SPEEDS.
1120 K7=1
1130 A9=77
1140 D9=N[1,K7]
1150 C9=K7-1
1160 M9=1
1170 GOSUB 2640
1180 K7=K7+1
1190 NEXT K
1200 NEXT L
1210 REM FINISHED WITH AN EXPERIMENT (20 PEAKS + BASELINE ON EITHER SIDE).
1220 PRINT #2;END
1230 PEN
1240 GOTO 1250
1250 REM ROUTINE TO COMPUTE AND SUBTRACT THE BASELINE.
1260 S1=S2=S3=S4=S5=0
1270 REM POINTERS MOVED TO BEGINNING OF DATA FILES.
1280 FILES PUMPZ,*,*
1290 ASSIGN C$,2,W9
1300 ASSIGN D$,3,W9
1310 REM EXP. NUMBER READ FROM DATA FILE.
1320 READ #2;Z[1,1]
1330 REM PUMP SPEEDS READ FROM DATA FILE SO THAT
1340 REM POINTER IS AT RIGHT PLACE.
1350 MAT READ #2;N
1360 REM ALL DATA POINTS READ IN.
1370 MAT READ #2;X
1380 REM FIRST 50 POINTS TAKEN AS INITIAL BASELINE.
1390 FOR I=1 TO 50
1400 S1=S1+X[1,I]
```

```
1410 S2=S2+I
1420 S3=S3+X[1,I]*I
1430 S4=S4+I*I
1440 S5=S5+X[1,I]*X[1,I]
1450 NEXT I
1460 REM LAST 50 POINTS TAKEN AS FINAL BASELINE.
1470 FOR I=41 TO 90
1480 I1=I+1890
1490 S1=S1+X[22,I]
1500 S2=S2+I1
1510 S3=S3+X[22,I]*I1
1520 S4=S4+I1*I1
1530 S5=S5+X[22,I]*X[22,I]
1540 NEXT I
1550 REM LEAST SQUARES BASELINE IS COMPUTED; B IS SLOPE.
1560 B=(S3-S1*S2/100)/(S4-S2*S2/100)
1570 REM A IS INTERCEPT OF LINE.
1580 A=S1/100-B*(S2/100)
1590 REM S6 IS STANDARD DEVIATION OF RESIDUALS.
1600 S6=SQR((S5-A*S1-B*S3)/98)
1610 REM THRESHOLD FOR DETECTING FIRST PEAK IS 3 TIMES
1620 REM THE STANDARD DEVIATION.
1630 T=3*S6
1640 REM INITIALIZE FILE POINTERS.
1650 FILES PUMPZ,*,*
1660 ASSIGN C$,2,W9
1670 ASSIGN D$,3,W9
1680 REM WRITE EXP. NUMBER AND PUMP SPEEDS TO SS. OR FS. FILE.
1690 PRINT #3;Z[1,1]
1700 MAT PRINT #3;N
1710 REM SUBTRACT BASELINE FROM ALL 1980 DATA POINTS.
1720 FOR I=1 TO 22
1730 FOR J=1 TO 90
1740 X[I,J]=X[I,J]-A-B*((I-1)*90+J)
1750 NEXT J
```

```
1760 NEXT I
1770 REM PRINT BASELINE SUBTRACTED DATA TO FILE #3.
1780 MAT PRINT #3;X
1790 PRINT #3;END
1800 REM ROUTINE TO PICK THE 20 PEAKS.
1810 REM DECIDE IF IN SIMPLEX OR MAPPING PHASE.
1820 IF R1#4 then 1860
1830 REM FP. AND SP. FILES CONTAIN THE 20 PEAK HEIGHTS FOR EACH EXP.
1840 C$[1,3]="FP."
1850 GOTO 1890
1860 C$[1,3]="SP."
1870 REM RE-INITIALIZE THE FILE POINTERS.
1880 REM FILE PEEK CONTAINS PEAK HEIGHTS FOR CURRENT EXP.
1890 FILES PUMPZ,*,*,PEEK
1900 ASSIGN C$,2,W9
1910 ASSIGN D$,3,W9
1920 REM INTERCEPT IS WRITTEN OUT AS BASELINE ABSORBANCE.
1930 PRINT #5;A
1940 PRINT #2;A
1950 REM EXP. NUMBER AND 9 PUMP SPEEDS ARE READ IN.
1960 FOR I=1 TO 10
1970 READ #3;G7
1980 NEXT I
1990 REM PEAK-PICKING ROUTINE.
2000 FOR I=1 TO 20
2010 REM W8 IS THE DATA POINT COUNTER.
2020 W8=1
2030 M=0
2040 READ #3;E[1,1]
2050 IF I#1 THEN 2170
2060 W8=W8+1
2070 REM IF NO PEAKS FOUND IN 500 POINTS, ASSUME BAD RESPONSE.
2080 IF W8>500 THEN 2350
2090 IF E[1,1]>T THEN 2120
2100 M=0
```

```
2110 GOTO 2040
2120 M=M+1
2130 REM ROUTINE MUST FIND 5 CONSECUTIVE VALUES GREATER THAN T
2140 REM ABOVE BASELINE BEFORE CONSIDERING FIRST PEAK FOUND.
2150 IF M >= 5 THEN 2170
2160 GOTO 2040
2170 R=E[1,1]
2180 REM 90-POINT (SEC) INTERVAL IS SEARCHED FOR MAXIMUM VALUE.
2190 FOR I8=1 TO 89-4*(I=1)
2200 READ #3;E[1,1]
2210 REM IF END OF DATA REACHED, SKIP OUT OF ROUTINE.
2220 IF END#3 THEN 2270
2230 IF E[1,1]<R THEN 2250
2240 R=E[1,1]
2250 NEXT I8
2260 REM PEAK HEIGHTS ARE WRITTEN TO FILES.
2270 PRINT #4;R
2280 PRINT #2;R
2290 IF R#0 THEN 2310
2300 GOTO 2350
2310 NEXT I
2320 GOTO 2390
2330 REM IF NO PEAKS FOUND, SMALL RANDOM NUMBERS ARE
2340 REM WRITTEN TO FILES.
2350 FOR I=1 TO 20
2360 PRINT #4;0.1*RND(0)
2370 PRINT #2;0.1*RND(0)
2380 NEXT I
2390 PRINT #4;END
2400 PRINT #2;END
2410 REM EXPERIMENT AND DATA ANALYSIS IS FINISHED.
2420 REM PROGRAM PROMPTS EXPERIMENTER TO CHANGE PAPER.
2430 FOR I7=1 TO 10
2440 BEEP
2450 WAIT 112
```

```
2460 NEXT I7
2470 DISP "CHANGE PAPER"
2480 STOP
2490 REM AT END OF SIMPLEX AND MIDDLE OF MAPPING STUDY,
2500 REM PROGRAM REMINDS EXPERIMENTER TO CHANGE SAMPLE TRAYS.
2510 IF (R1=4) OR(Z9#30) THEN 2540
2520 DISP "CHANGE TRAYS"
2530 STOP
2540 IF (R1#4) OR (Z9#27) THEN 2580
2550 DISP "CHANGE TRAYS"
2560 STOP
2570 REM PROGRAM CALLS REGRESSION ANALYSIS PROGRAM.
2580 REM ************* PROGRAM MASFIT
2590 GET "MASFIT"
2600 END
2610 REM ************** SUBROUTINE CONVOY **********************
2620 REM SUBROUTINE TO SHIP DATA OUT ON INTERFACE.
2630 REM SEE APPENDIX 'A' FOR COMPLETE DESCRIPTION.
2640 WRITE (1,*)WBYTE(0);
2650 WRITE (2,*)WBYTE(BIAND(OCT(374),ROT(OCT(A9),14)));
2660 WRITE (1,*)WBYTE(1);
2670 WRITE (2,*)WBYTE(BIAND(OCT(377),D9));
2680 WRITE (1,*)WBYTE(2);
2690 IF M9=0 THEN 2740
2700 D9=BIAND(OCT(177400),D9)
2710 D9=INOR(ROT(D9,8),OCT(200))
2720 WRITE (2,*)WBYTE(D9);
2730 GOTO 2750
2740 WRITE (2,*)WBYTE(ROT(BIAND(OCT(177400),D9),8));
2750 WRITE (1,*)WBYTE(3);
2760 WRITE (2,*)WBYTE(BIAND(OCT(77),C9));
2770 WRITE (1,*)WBYTE(OCT(300));
2780 RETURN
```

APPENDIX D

PROGRAMS TO RUN AUTOANALYZER

FOR PHENYLEPHRINE STUDY

```
10 REM ######################## APPENDIX D ################################
20 REM ###########################################################
30 REM PROGRAM TO RUN AUTOANALYZER FOR PHENYLEPHRINE STUDY.
40 DIM DS[1,1],ES[1,1],XS[23,90]
50 DIM A$[4],D$[6],D$[6],ZS[1,1]
60 REM LOOP FOR THE 18 EXPERIMENTS.
70 FOR Z9=1 TO 18
80 SCALE 0,2100,0,4100
90 DEXP Z9,A$
100 REM THE EXP. NUMBER IS WRITTEN ON THE PLOT.
110 PLOT 10,4000,-1
120 LABEL (*)A$
130 REM THE FILES TO BE USED ARE ASSIGNED: PR.XXX FOR RAW DATA;
140 REM PS.XXX FOR BASELINE SUBTRACTED DATA.
150 C$[1,3]="PR."
160 D$[1,3]="PS."
170 C$[4,6]=A$[2,4]
180 D$[4,6]=A$[2,4]
190 REM THE FILE POINTERS ARE INITIALIZED.
200 FILES *,*
210 ASSIGN C$,1,W9
220 ASSIGN D$,2,W9
230 PRINT C$
240 REM THE CLOCK IS PROGRAMMED FOR 1-SEC INTERVALS.
250 A9=76
260 D9=60
270 C9=1
280 M9=0
290 GOSUB 1360
300 REM THE CLOCK IS QUERIED.
310 WRITE(3,*)WBYTE(0);
320 WRITE(3,*)WBYTE(0);
330 REM THIS SETS UP THE NUMBER OF SAMPLE+BASELINE INTERVALS PER EXP.
340 L0=22
350 REM THE DATA MATRIX IS DIMENSIONED CORRECTLY.
```

```
360 REDIM X[L0+1,90]
370 REM THIS IS THE NUMBER OF ITERATIONS PER EXP.
380 FOR L=1 TO L0+5
390 REM TURN ON SAMPLER WHEN L=2.
400 IF L#2 THEN 490
410 A9=77
420 D9=0
430 C9=47
440 M9=0
450 GOSUB 1360
460 REM WAIT 25 SEC FOR TIMING CONSIDERATIONS.
470 WAIT 25000
480 REM TAKE DATA FOR 90 SEC (90 POINTS) PER SAMPLE.
490 FOR K=1 TO 90
500 REM TURN OFF SAMPLER AT THE 55TH SEC OF THE NEXT-TO-LAST SAMPLE.
510 IF L#(L0-1) OR K#55 THEN 580
520 A9=77
530 D9=0
540 C9=15
550 M9=0
560 GOSUB 1360
570 REM QUERY THE CLOCK.
580 WRITE (3,*)WBYTE(0);
590 REM ADDRESS THE ADC.
600 A9=77
610 D9=0
620 C9=16
630 M9=0
640 GOSUB 1360
650 REM READ IN DATA AND FORM A NUMBER.
660 D[1,1]=INOR(ROT(BIAND(OCT(17),RBYTE2),8),BIAND(OCT(377),RBYTE1))
670 REM IF L=5 START TO SAVE AND PLOT THE DATA.
680 IF L<5 THEN 730
690 REM PRINT OUT THE DATA TO PR. FILE.
700 PRINT #1;D[1,1]
```

```
710 REM PLOT THE DATA AS IT IS RECEIVED.
720 PLOT 90*(L-5)+K,D[1,1]
730 NEXT K
740 NEXT L
750 REM FINISHED WITH AN EXPERIMENT.
760 PRINT #1;END
770 PEN
780 REM ROUTINE FOR BASELINE CALCULATION AND SUBTRACTION.
790 S1=S2=S3=S4=S5=0
800 REM INITIALIZE FILES.
810 FILES *,*
820 ASSIGN C$,1,W9
830 ASSIGN D$,2,W9
840 REM READ IN ALL DATA POINTS FROM PR. FILE.
850 MAT READ #1;X
860 REM TAKE FIRST 25 POINTS AS INITIAL BASELINE.
870 FOR I=1 TO 25
880 S1=S1+X[1,I]
890 S2=S2+I
900 S3=S3+X[1,I]*I
910 S4=S4+I*I
920 S5=S5+X[1,I]*X[1,I]
930 NEXT I
940 REM TAKE LAST 25 POINTS AS FINAL BASELINE.
950 FOR I=66 TO 90
960 I1=I+1980
970 S1=S1+X[L0+1,I]
980 S2=S2+I1
990 S3=S3+X[23,I]*I1
1000 S4=S4+I1*I1
1010 S5=S5+X[23,I]*X[23,I]
1020 NEXT I
1030 REM CALCULATE LEAST-SQUARES BASELINE;
1040 REM B IS SLOPE.
1050 B=(S3-S1*S2/50)/(S4-S2*S2/50)
```

```
1060 REM A IS INTERCEPT AND WILL BE TAKEN AS BASELINE ABSORBANCE.
1070 A=S1/50-B*(S2/50)
1080 REM PRINT OUT BASELINE.
1090 PRINT "RUN ";Z9,"BASELINE = ";A
1100 S6=SQR((S5-A*S1-B*S3)/48)
1110 REM RE-INITIALIZE FILES.
1120 FILES *,*
1130 ASSIGN C$,1,W9
1140 ASSIGN D$,2,W9
1150 REM SUBTRACT BASELINE FROM ALL POINTS.
1160 FOR I=1 TO L0+1
1170 FOR J=1 TO 90
1180 X[I,J]=X[I,J]-A-(B*((I-1)*90+J))
1190 NEXT J
1200 NEXT I
1210 REM PRINT BASELINE SUBTRACTED DATA TO PS. FILE.
1220 MAT PRINT #2;X
1230 PRINT #2;END
1240 REM PROGRAM PROMPTS EXPERIMENTER TO CHANGE PAPER.
1250 FOR I=1 TO 30
1260 BEEP
1270 WAIT 112
1280 NEXT I
1290 DISP "CHANGE PAPER, PLEASE, SIR"
1300 STOP
1310 NEXT Z9
1320 END
1330 REM SUBROUTINE TO SHIP DATA OUT ON INTERFACE.
1340 REM SEE APPENDIX 'A' FOR COMPLETE DESCRIPTION.
1350 REM ****************** SUBROUTINE CONVOY ********************
1360 WRITE (1,*)WBYTE(0);
1370 WRITE (2,*)WBYTE(BIAND(OCT(374),ROT(OCT(A9),14)));
1380 WRITE (1,*)WBYTE(1);
1390 WRITE (2,*)WBYTE(BIAND(OCT(377),D9));
1400 WRITE (1,*)WBYTE(2);
```

```
1410 IF M9=0 THEN 1460
1420 D9=BIAND(OCT(177400),D9)
1430 D9=INOR(ROT(D9,8),OCT(200))
1440 WRITE (2,*)WBYTE(D9);
1450 GOTO 1470
1460 WRITE (2,*)WBYTE(ROT(BIAND(OCT(177400),D9),8));
1470 WRITE (1,*)WBYTE(3);
1480 WRITE (2,*)WBYTE(BIAND(OCT(77),C9));
1490 WRITE (1,*)WBYTE(OCT(300));
1500 RETURN
```

```
10 REM ############################################################
20 REM SEPARATE PROGRAM TO PICK PEAKS.
30 DIM DS[1,1],ES[1,1],XS[23,90]
40 DIM A$[4],C$[6],D$[6]
50 REM LOOP FOR THE 18 EXPERIMENTS.
60 FOR Z9=1 TO 18
70 REM ADDRESS FILES -- PP. WILL CONTAIN PEAK HEIGHTS.
80 DEXP Z9,A$
90 C$[1,3]="PP."
100 D$[1,3]="PS."
110 D$[4,6]=A$[2,4]
120 C$[4,6]=A$[2,4]
130 REM INITIALIZE FILES.
140 FILES *,*
150 ASSIGN C$,1,W9
160 ASSIGN D$,2,W9
170 GOTO 230
180 REM THESE NEXT TWO LINES TAKE THE DATA POINT NUMBER AND COMPUTE
190 REM ITS LOCATION IN THE DATA MZTRIX.
200 DEF FNA(I8)=INT((I8-1)/90)+1
210 DEF FNB(I8)=I8-(FNA(I8)-1)*90
220 REM READ IN THE SUBTRACTED DATA FROM THE PS. FILE.
230 MAT READ #2;X
240 REM LOOP FOR THE 20 SAMPLES (AND THUS 20 PEAKS).
250 FOR K=1 TO 20
260 IF K#1 THEN 440
270 M=0
280 REM I8 IS THE DATA POINT COUNTER.
290 I8=1
300 REM I AND J ARE THE MATRIX LOCATION INDICES FOR DATA POINT I8.
310 I=FNA(I8)
320 J=FNB(I8)
330 IF K#1 THEN 440
340 IF X[I,J]>50 THEN 380
350 M=0
```

```
360 I8=I8+1
370 GOTO 310
380 M=M+1
390 REM 20 DATA POINTS AT LEAST 50 ADC UNITS ABOVE BASELINE MUST
400 REM BE FOUND IN SEQUENCE BEFORE FIRST PEAK IS RECOGNIZED.
410 IF M>20 THEN 430
420 GOTO 360
430 I7=I8
440 R=X[I,J]
450 IF K#5 THEN 470
460 R=50
470 FOR I8=I7 TO I7+90-5*(K=1)
480 I=FNA(I8)
490 J=FNB(I8)
500 REM SAMPLE #5 IS DISTILLED WATER -- ROUTINE LOOKS FOR
510 REM MINIMUM IN THAT SAMPLE, MAXIMUM FOR ALL OTHERS.
520 IF K=5 THEN 560
530 IF R>X[I,J] THEN 580
540 R=X[I,J]
550 GOTO 580
560 IF R<X[I,J] THEN 580
570 R=X[I,J]
580 NEXT I8
590 REM WRITE PEAK HEIGHTS TO FILE PP.
600 PRINT #1;R
610 REM PRINT OUT PEAK HEIGHT.
620 PRINT R,
630 I7=I8-1
640 NEXT K
650 PRINT
660 NEXT Z9
670 END
```