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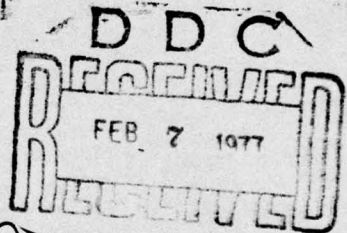
SPONTANEOUS REACTIVATION OF ACETYLCHOLINESTERASE FOLLOWING  
ORGANOPHOSPHATE INHIBITION. III. STUDIES WITH  
*p*-NITROPHENYL METHYLPHENYLPHOSPHINATE

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December 1976



DEPARTMENT OF THE ARMY  
Headquarters, Edgewood Arsenal  
Aberdeen Proving Ground, Maryland 21010



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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM	
1. REPORT NUMBER EB-TR-76113	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER 9	
4. TITLE (and Subtitle) SPONTANEOUS REACTIVATION OF ACETYLCHOLINESTERASE FOLLOWING ORGANOPHOSPHATE INHIBITION. III. STUDIES WITH <i>p</i> -NITROPHENYL METHYLPHENYLPHOSPHINATE		5. TYPE OF REPORT & PERIOD COVERED Technical Report, Started in 1974, in progress	
7. AUTHOR(s) Claire N. Lieske, Gary L. Horton James H. Clark, Donald K. Jewell John R. Lowe, Lester W. Daasch		6. PERFORMING ORG. REPORT NUMBER	8. CONTRACT OR GRANT NUMBER(s) 17 02
9. PERFORMING ORGANIZATION NAME AND ADDRESS Commander, Edgewood Arsenal Attn: SAREA-BL-REB Aberdeen Proving Ground, Maryland 21010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Task 1T161102A71A02	16
11. CONTROLLING OFFICE NAME AND ADDRESS Commander, Edgewood Arsenal Attn: SAREA-TS-R Aberdeen Proving Ground, Maryland 21010		18. REPORT DATE December 1976	11
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) 12 42 p.		19. NUMBER OF PAGES 48	
		15. SECURITY CLASS. (of this report) UNCLASSIFIED	
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE NA	
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited. Copies available from: National Technical Information Service, Springfield, Virginia 22151.			
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)			
18. SUPPLEMENTARY NOTES Life sciences basic research in support of chemical material			
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Acetylcholinesterase Spontaneous reactivation <i>p</i> -Nitrophenyl methylphenylphosphinate Kinetics			
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Inhibition constants for the phosphinylation of eel acetylcholinesterase by <i>p</i> -nitrophenyl methylphenylphosphinate are reported, as are the constants for the subsequent spontaneous reactivation reaction. The data support the rationale that phosphinates are ideally suited for studies directed at advantageously perturbing the spontaneous reactivation process.			

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## PREFACE

The work described in this report was authorized under Task 1T161102A71A02, Life Sciences Basic Research in Support of Chemical Material. Portions of the work, which is still in progress, were started in 1974. The experimental data are contained in notebooks MN 2529, MN 2772, 9321, and 9386.

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## Acknowledgments

The authors thank Mr. Joseph W. Hovanec for the synthesis of *p*-nitrophenyl methylphenylphosphinate and Mrs. Linda L. Szafranec and Mr. Harold Klapper for the NMR spectra and analysis.

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SPONTANEOUS REACTIVATION OF ACETYLCHOLINESTERASE FOLLOWING  
ORGANOPHOSPHATE INHIBITION. III. STUDIES WITH  
*p*-NITROPHENYL METHYLPHENYLPHOSPHINATE

I. INTRODUCTION.

A large number of papers in recent years have been devoted to the problems of the biochemical mechanism, toxic action, and therapy involving organophosphorus compounds which are inhibitors of cholinesterases. Regarding therapy, the development of oximes as antidotes against cholinesterase poisoning followed the observation that the very slow restoration of enzymatic activity obtained with certain phosphorylated cholinesterases was accelerated in the presence of nucleophilic agents such as choline and hydroxylamine.<sup>1,2</sup> The use of the latter nucleophiles was followed by hydroxamic acids and additional ketoximes and aldoximes. However, even the present-day oximes such as 2-PAM\* and TMB-4\*\* are of limited value in the therapy of organophosphate poisoning.<sup>3,4</sup>

Our interest in the spontaneous reactivation of phosphorylated<sup>†</sup> acetylcholinesterase (AChE, EC 3.1.1.7) is directed at understanding the basic parameters and mechanism of this phenomenon. Such knowledge is mandatory if one hopes to advantageously perturb this system as part of a therapeutic approach in organophosphate poisoning.

Our first studies in this area were carried out using a series of *para*-substituted phenyl methylphosphonochloridates.<sup>5</sup> These spontaneous reactivation studies produced the first evidence for hydrophobic interactions participating in the spontaneous dephosphonylation of inhibited AChE. We have subsequently reported the first kinetic studies<sup>6</sup> on the spontaneous reactivation of AChE inhibited with Sarin.<sup>††</sup>

In support of our continuing effort in the area of spontaneous reactivation studies, we synthesized *p*-nitrophenyl methylphenylphosphinate (NMPP). The reasons for the selection of a phosphinate in our current studies were twofold. First, in the case of phosphinate-inhibited AChE, aging should be excluded from reaction considerations since a susceptible carbon-oxygen-phosphorus bond, necessary for aging, is absent. Second, an additional positive aspect resulting from phosphinate use concerns the amino acid functional in acid-catalyzed aging. Since there is evidence that the same amino acid is involved in substrate conversion,<sup>7,8</sup> the use of phosphinates precludes an approach where the action of this amino acid may be affected. Consequently, the spontaneous reactivation process is effectively isolated and, therefore, approaches to its advantageous perturbation may be investigated.

Our work to date with NMPP supports the rationale that phosphinates are ideally suited to spontaneous reactivation studies. Of special interest is the fact that this report also documents the first spontaneous reactivation studies of AChE following inhibition by a phosphinate.

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\* Pyridinium aldoxime methochloride.

\*\* 1,1'-Trimethylene-bis(4-formylpyridinium bromide)dioxime.

† The term phosphorylation will be used to include phosphorylation, phosphonylation, and phosphinylation. [Steinberg, G. M., Lieske, C. N., Boldt, R., Goan, J. C., and Podall, H. E. Model Studies for the Reactivation of Aged Phosphonylated Acetylcholinesterase. Use of Alkylating Agents Containing Nucleophilic Groups. *J. Med. Chem.* 13, 435 (1970)].

†† Isopropyl methylphosphonofluoridate.

## II. MATERIALS AND METHODS.

### A. General.

The zwitterionic 3-(N-morpholino)propanesulfonic acid (MOPS) and N,N-bis(2-hydroxyethyl)glycine (Bicine) buffers were described by Good *et al.*<sup>9</sup> and are available commercially from several sources. MOPS was used in the pH 7.60 studies; and Bicine, in the pH 9.10 studies. Each was 0.10 M and contained 0.01 M  $Mg^{++}$ , 0.002%  $NaN_3$ , and 0.01% bovine serum albumin.

The 0.134 M phosphate buffer of pH 6.94 was prepared from 0.134 M  $K_2HPO_4$  and 0.134 M  $KH_2PO_4$  solutions. Sodium azide, 20 mg/l, was added to prevent bacterial growth. A 1:1 (v/v) dilution of this buffer with double distilled water gave a pH of 6.90 for the resulting 0.067 M phosphate buffer. This buffer was used in the pH 6.90 studies. All pH values were determined at 25.0°C using a Beckman research pH meter, Model 101900, equipped with a Markson No. 1808 Polymark combination electrode.

The eel acetylcholinesterase (AChE, EC 3.1.1.7) was purchased from Worthington Biochemical Corporation. It was obtained as a purified, salt-free powder, 4000 units/mg. Two and one-half milligrams of this powder was dissolved in 1.5 ml of a previously boiled solution (pH approximately 7.4) containing 0.225 M KCl, 0.10% gelatin, and 0.020% sodium azide to give the enzyme concentrate. The addition of 5.0  $\mu$ l of a fiftyfold dilution of the concentrate to 2.00 ml of 0.10 M MOPS buffer, pH 7.60, containing 5.0  $\mu$ l of 1.58 M phenyl acetate in acetonitrile, gives an absorbance change at 272.5 nm of *ca.* 0.60 absorbance unit/min (25.0°C). Assay concentration of the substrate equals  $4 \times 10^{-3}$  M.

### B. Preparation of *p*-Nitrophenyl Methylphenylphosphinate.

Methylphenylphosphinyl chloride (550 mg, 3.15 mmoles) dissolved in 5 ml of benzene was added in drops over a 10-minute period to a magnetically stirred solution of *p*-nitrophenol (414 mg, 2.98 mmoles) and triethylamine (290 mg, 2.87 mmoles) in 10 ml of benzene. Upon completion of the addition, the stirred reaction mixture was maintained at room temperature for several hours. It was transferred to a separatory funnel and washed progressively with small aliquots of pH 4 acetate buffer (3X), pH 7.5 MOPS buffer (3X), and finally with distilled water (2X). After the nonaqueous layer was dried over  $MgSO_4$ , the benzene was removed in vacuo, without heating, to give a viscous yellow-orange oil. The oil solidified on standing overnight. Two recrystallizations from ethyl ether gave 158 mg (19% yield) of product. The very light tan crystals melted at 85° to 86.5°C (uncorrected). The integrated NMR spectrum was in consonance with the assigned structure and showed no evidence of impurities. Anal. Calc for  $C_{13}H_{12}O_4NP$ : C, 56.33; H, 4.36; N, 5.05; P, 11.17. Found: C, 56.4; H, 4.4; N, 5.0; P, 11.2.

### C. Mass Spectrum of *p*-Nitrophenyl Methylphenylphosphinate.

The mass spectrum of NMPP was obtained on a Hitachi Model RMU6E, single-focusing, magnetic deflection mass spectrometer. The solid sample was placed near the source of the spectrometer, using the solid probe feature of the instrument. The tip of the probe was surrounded by an electrically heated copper block; its temperature controlled the vaporization of the sample. The ionizing voltage was 70 volts. A list of the relative intensities obtained with the sample heater held at 200°C appears on page 13.

#### D. Hydrolysis of *p*-Nitrophenyl Methylphenylphosphinate.

The hydrolysis of NMPP in phosphate buffer was monitored spectrophotometrically at 400 nm. The reaction was initiated by introducing, with rapid mixing, 6.0  $\mu\text{l}$  of a  $4.87 \times 10^{-2}$  M solution of NMPP in acetonitrile into an UV cell containing 3.00 ml of 0.067 M phosphate buffer of pH 6.90 at 25.0°C. The data obtained were calculated using equation 1. Linear first-order kinetics were observed for the 1.9 half lives monitored. At completion of the hydrolysis, the production of *p*-nitrophenol was stoichiometric. The results of three runs under the cited conditions gave a  $k_{\text{obsd}}$  of  $0.0219 \text{ min}^{-1} \pm 2.59\%$ , corresponding to a half life of 31.64 minutes.

The hydrolysis of NMPP in MOPS buffer was monitored spectrophotometrically at 400 nm. The reaction was initiated by introducing, with rapid mixing, 4.0  $\mu\text{l}$  of a  $5.27 \times 10^{-2}$  M solution of NMPP in acetonitrile into an UV cell containing 3.00 ml of 0.10 M MOPS buffer of pH 7.60 at 25.0°C. The data obtained were calculated using equation 1. Linear first-order kinetics were observed through the 1.97 half lives monitored. At completion of the hydrolysis, the production of *p*-nitrophenol was stoichiometric. The results of three runs under the cited conditions gave a  $k_{\text{obsd}}$  of  $0.0066 \text{ min}^{-1} \pm 1.8\%$ , corresponding to a half life of 105.0 minutes.

#### E. Inhibition of Eel Acetylcholinesterase by *p*-Nitrophenyl Methylphenylphosphinate.

The inhibition of AChE by NMPP was studied by an adaption and modification of the Hart and O'Brien<sup>10</sup> method for obtaining the inhibition constants. The kinetic experiments were initiated by mixing reactants by an Aminco-Morrow stopped-flow apparatus which was attached to a Beckman DUR monochromator. A data acquisition, storage, and retrieval system (DASAR, American Instrument Company) was used for visual examination and acquisition of the data. A high-speed paper-tape punch, interfaced with the DASAR, was employed to produce a permanent record of experimental results on paper tape. The permanent data record on paper tape was transferred to the Univac 1108 computer files. Program and data files, as described by Horton *et al.*,<sup>11</sup> were called and manipulated by use of a remote terminal keyboard.

The reactant solutions were prepared at twice the desired final concentration after mixing in the stopped-flow instrument. The AChE solution in 0.134 M phosphate buffer was prepared by diluting 10.5  $\mu\text{l}$  of enzyme concentrate (section II, A) with 30 ml of the buffer. Final buffer concentration was 0.067 M after mixing and the pH was 6.90. Just prior to use, solutions of (1) the substrate, *p*-nitrophenyl acetate, and (2) the inhibitor, NMPP, plus substrate were prepared in 4% ethanol/96% distilled water (v/v). This, of course, resulted in a 2% ethanol solution in each case following mixing in the stopped-flow apparatus.

The *p*-nitrophenyl acetate solution was used to determine control velocities, and the *p*-nitrophenyl acetate plus NMPP solution was used for the inhibition runs. The concentrations of substrate were identical in the control velocity runs and the inhibition runs. In all determinations, the final substrate concentration was  $1.00 \times 10^{-3}$  M; and, in the inhibition determinations, the final concentration of NMPP was  $2.67 \times 10^{-6}$  M. The temperature of the reaction cell in the stopped-flow apparatus was controlled at 25°C by circulating water from a constant temperature bath. Residual enzymatic activity and background hydrolysis of the substrate after complete inhibition were determined and were incorporated in the calculations of the inhibition constants resulting from five determinations (see page 14).

#### F. Spontaneous Reactivation of Methylphenylphosphinylated Eel Acetylcholinesterase.

A detailed description of the technique involved in a spontaneous reactivation study of methylphenylphosphinylated AChE at pH 7.60 is given below. Other kinetic investigations at pH 7.60, as well as those at pH 9.10, were handled similarly.

An enzyme stock solution was prepared by adding 10.0  $\mu$ l of enzyme concentrate (section II, A) to 1.5 ml of 0.10 M MOPS buffer of pH 7.60. For a control, 0.500 ml of this stock solution was added to 49.50 ml of the buffer. Phenyl acetate assays of this 1 to 100 dilution showed an activity of 0.566 absorbance unit/min at 272.5 nm, allowance being made for the nonenzymatic hydrolysis of phenyl acetate in the buffer medium (0.002 absorbance unit/min).

The remaining 1.0 ml of enzyme stock solution was inhibited with 10.0  $\mu$ l of  $4.87 \times 10^{-2}$  M NMPP dissolved in acetonitrile. Three minutes later, 0.500 ml of the inhibited enzyme solution was added via a Chromatronix calibrated sample loop to a 1/2-inch by 6-3/4-inch column bed of Ultrogel AcA44 (4% acrylamide and 4% agarose) and eluted with the buffer at a flow rate of 0.50 ml/min. The zero time for reactivation was taken as the time when the 0.500-ml sample of inhibited AChE had passed into the gel bed of the column. The first 17.00 ml was collected and diluted to 50.0 ml with buffer. During the course of this elution, another aliquot of the inhibited enzyme was checked for residual activity with none being detected. Previous tests established that all the AChE and no inhibitor was present in the 17.00-ml fraction collected from the column.

The 50.0-ml fraction was incubated at 25.0°C. At appropriate intervals, 1.00-ml aliquots of this solution were assayed. Activity was calculated from the phenyl acetate assays, the reaction being monitored in this manner for *ca.* 100 hours. Corrections were made for the loss of activity observed in the control during this time period. The results of this spontaneous reactivation experiment gave  $k_{\text{obs}} = 0.02120 \text{ hr}^{-1}$  with a 100.1% return of activity (figure 1). A summary of our spontaneous reactivation experiments is presented on page 15.

#### G. Calculation of the Observed Spontaneous Reactivation Rate of Methylphenylphosphinylated Eel Acetylcholinesterase.

A modification of the computer program originally developed by Hayo and Wilcoxson<sup>1,2</sup> was used in these calculations. The FORTRAN program (EXPFIT) fits the reactivation data to the exponential rate equation

$$V = V_{\infty} [1 - e^{-k_{\text{obs}}(t-t_0)}] \quad (1)$$

where  $V$  = velocity function (e.g., zero-order rates, absorbances, and % reactivation),  $V_{\infty}$  = value of velocity function at infinite time (limiting value),  $k_{\text{obs}}$  = observed first-order rate constant, and  $t_0$  = the time value when the velocity function is equal to zero (abscissa intercept of graphical treatment). An iterative least-squares procedure is used to fit ( $V$ ,  $t$ ) data, and the best values for  $V_{\infty}$ ,  $k_{\text{obs}}$ , and  $t_0$  are calculated. The validity of equation 1 has been previously reported.<sup>5</sup> Calculation of first-order kinetic constants when the experimental end point is not available, using the Hayo and Wilcoxson method, offers a distinct advantage over the more common procedure of Guggenheim.<sup>1,3</sup> The former method does not require that the kinetic parameters be spaced at constant time intervals. A complete documentation of program EXPFIT is presented in appendix A.

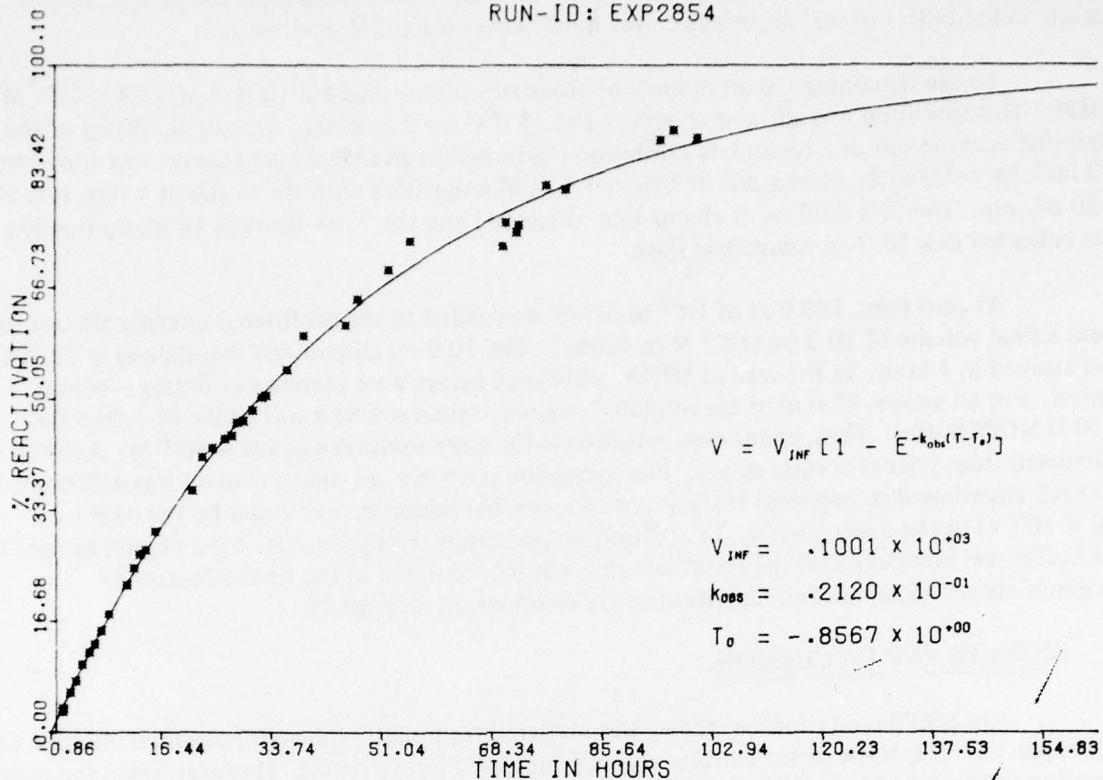


Figure 1. Spontaneous Reactivation of Methylphenylphosphinylated Eel Acetylcholinesterase (pH 7.60, 0.10 M MOPS buffer, 25.0°C)

In addition to calculating the kinetic parameters, program EXPFIT also creates a separate plotting parameter file to provide the capability of examining the results graphically. This plotting file can be subsequently read by program EXPFITPLOT which provides the required machine control commands to operate the off-line CALCOMP plotter. Appendix B contains a complete documentation of program EXPFITPLOT.

H. Induced Reactivation of Methylphenylphosphinylated Eel Acetylcholinesterase.

A detailed description of the technique involved in our induced reactivation studies of NMPP-inhibited AChE is given below. All experiments were carried out at pH 7.60 in 0.10 M MOPS buffer (25.0°C). The oximes used were 2-PAM, TMB-4, and MINA.\* Each oxime study was treated in the same manner.

An enzyme stock solution was prepared by adding 10.0  $\mu$ l of enzyme concentrate (section II, A) to 1.5 ml of 0.10 M MOPS buffer. A control was prepared by adding 0.500 ml of this stock solution to 49.50 ml of the buffer. Phenyl acetate assays of this 1 to 100 dilution showed

\* Monoisnitrosoacetone.

an activity of 0.577 absorbance unit/min (272.5 nm). The nonenzymatic hydrolysis of phenyl acetate in the buffer (0.002 absorbance unit/min) was corrected in each assay.

To the remaining 1.0 ml of enzyme stock solution was added 10.0  $\mu$ l of  $4.87 \times 10^{-2}$  M NMPP. The inhibition was allowed to proceed at 25.0°C for 3 minutes. Exactly 0.500 ml of the inhibited enzyme solution (complete inhibition confirmed in an independent assay) was added to a 1/2-inch by 6-3/4-inch column bed of Ultrogel AcA44 and eluted with the buffer at a flow rate of 0.50 ml/min. The first 6.00 ml of eluent were discarded and the 7.00- through 16.90-ml fraction was collected in a 10.0-ml volumetric flask.

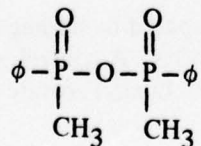
At zero time, 100.0  $\mu$ l of  $10^{-2}$  M oxime was added to the gel-filtered enzyme aliquot to yield a final volume of 10.0 ml ( $10^{-4}$  M in oxime). The 10.0-ml aliquot was maintained at 25.0°C and assayed in 1 hour. In the case of MINA, additional assays were carried out during a 4-hour period. For all assays, 95.0  $\mu$ l of the inhibited enzyme/oxime solution was added to 1.90 ml of 0.10 M MOPS buffer. Thus, oxime concentration in the assay solution was  $5.0 \times 10^{-6}$  M. Activity was calculated from phenyl acetate assays. For correction purposes, an oxime control was determined for each experiment to ascertain the amount of reversible inhibition exhibited by the oxime at  $5.0 \times 10^{-6}$  M in the assay aliquot. In addition, oxime-catalyzed hydrolysis of the phenyl acetate in the buffer was measured and the resulting value was incorporated in the final calculations. The results of these oxime-induced reactivations are summarized on page 16.

### III. RESULTS AND DISCUSSION.

The NMPP used in these studies was prepared from methylphenylphosphinyl chloride and *p*-nitrophenol. The NMR of the starting chloride indicated purity >98%. However, since the mass spectrum of the chloride indicated small quantities of a variety of compounds (figure 2), we characterized the NMPP obtained by melting point, elemental analysis, NMR, and mass spectroscopy (section II, B and C).

The mass spectrum of NMPP is given in table 1. The relative intensities were found to be dependent on the conditions for heating the sample, especially the temperature of the probe at the time the sample was introduced. The intensities given in table 1 were obtained with the sample heater held at 200°C, a temperature which induced the solid to volatilize quickly. Table 1 also lists the observed metastable peaks. Metastable peaks are very broad signals which can appear at nonintegral mass values, whose position is determined according to the formula  $m = (m_2)^2/m_1$ , where  $m_1$  is the parent ion and  $m_2$  the daughter ion of the fragmentation involved. The origin of all the strong peaks in the spectrum can be rationalized by cleavages in which no complicated rearrangements are required. In this regard, it is interesting to note that there is usually considerable rearrangement of hydrogen atoms in the fragmentation of the organic phosphates and phosphonates which is absent in this phosphinate.

The impurity peak at  $m/e$  294 may be the pyro compound



which was one of the impurities detected in the mass spectrum of the methylphenylphosphinyl chloride used to prepare NMPP (figure 2). The fact that all strong peaks can be accounted

for without involving the presence of impurities, coupled with the stoichiometry of *p*-nitrophenol production in the hydrolysis studies, permits a purity >99.5% to be assigned to the NMPP used in these studies.

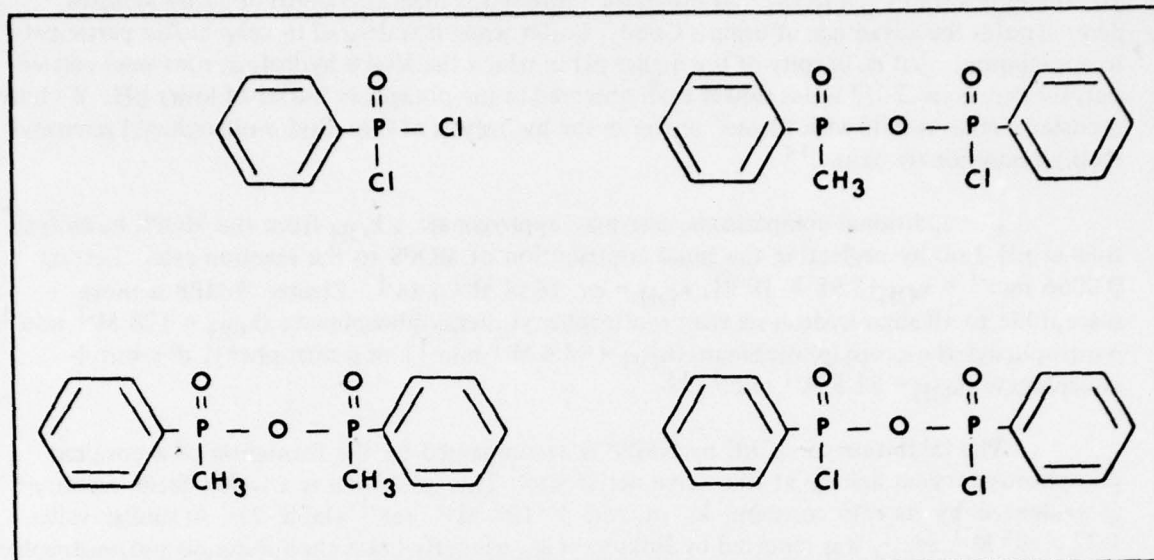


Figure 2. Structural Formulas of Trace Impurities in Methylphenylphosphinyl Chloride

Table 1. Mass Spectrum of *p*-Nitrophenyl Methylphenylphosphinate

m/e	Intensity	m/e	Intensity	Metastables
294	1	141	3	275.2
279	5	140	10	264.9
278	5	139	100	249.0
277	24	121	3	219.2
276	60	109	6	206.0
268	0.5	107	0.5	191.8
262	2	95	2	134.8
261	2	93	0.5	105.3
247	1	92	1	85.5
235	0.2	91	8	69.3
231	1	79	0.5	59.8
230	3	78	2	46.9
222	1	77	24	42.8
221	0.2	76	2	
218	0.4	75	1.5	
217	0.4	65	4	
216	1	64	1	
215	2.5	63	2	
201	0.2	51	3	
199	1	50	2	
185	0.2	47	2	
156	1	39	3	

To additionally characterize NMPP and, as a prelude to our enzymatic studies with this compound, its hydrolysis was examined spectrophotometrically at pH 6.90 in 0.067 M phosphate buffer and at pH 7.60 in 0.10 M MOPS buffer. The half life in the MOPS buffer (pH 7.60) of 105.0 minutes compared to the half life in the phosphate buffer (pH 6.90) of 31.64 minutes demonstrates the advantage of using a Good<sup>9</sup> buffer when it is desired to keep buffer participation to a minimum: that is, in spite of the higher pH in which the MOPS hydrolysis runs were carried out, the rate is *ca.* 3-1/2 times slower than observed in the phosphate buffer of lower pH. We have reported similar results with Bicine<sup>9</sup> buffer in the hydrolysis of O-methyl *p*-nitrophenyl phenacyl methylphosphonate oxime.<sup>14</sup>

For additional comparisons, one may approximate a  $k_{OH}$  from the MOPS hydrolysis runs at pH 7.60 by neglecting the small contribution of MOPS to the reaction rate. Letting  $0.0066 \text{ min}^{-1} = k_{OH}[3.98 \times 10^{-6}]$ ,  $k_{OH} = \text{ca. } 1658 \text{ M}^{-1} \text{ min}^{-1}$ . Clearly NMPP is more susceptible to alkaline hydrolysis than *p*-nitrophenyl diethylphosphinate ( $k_{OH} = 128 \text{ M}^{-1} \text{ min}^{-1}$ ), *p*-nitrophenyl *di-n*-propylphosphinate ( $k_{OH} = 93.4 \text{ M}^{-1} \text{ min}^{-1}$ ), or *p*-nitrophenyl *di-n*-butylphosphinate ( $k_{OH} = 81.5 \text{ M}^{-1} \text{ min}^{-1}$ ).<sup>15</sup>

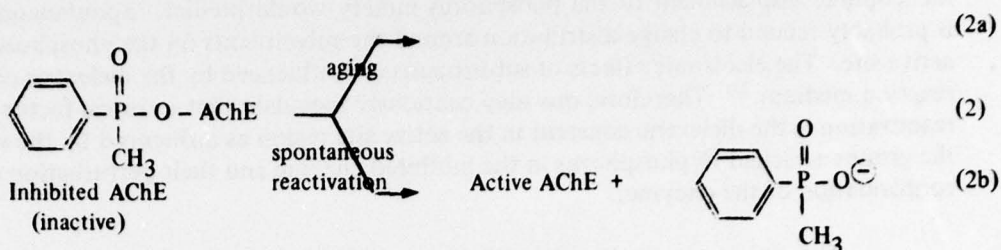
The inhibition of AChE by NMPP is accompanied by the formation of a covalent phosphorus-enzyme linkage at the serine active site. This inhibition is a rather facile reaction\* as evidenced by its rate constant,  $k_i$ , of  $2.95 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$  (table 2). A similar value,  $1.32 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ , was reported by Fukuto *et al.*, using fly-brain cholinesterase and *p*-nitrophenyl ethylphosphinate.<sup>16</sup>

Table 2. Constants for the Inhibition of Eel AChE by NMPP. Substrate, *p*-nitrophenyl acetate; pH 6.90, 0.067 M phosphate buffer; 25°C

Run No.	$K_d \times 10^6$ M	$k_2$ $\text{sec}^{-1}$	$k_i \times 10^{-4}$ $\text{M}^{-1} \text{ sec}^{-1}$
1	7.74	0.230	2.97
2	8.29	0.258	3.11
3	6.22	0.175	2.82
4	8.74	0.268	3.06
5	7.15	0.198	2.78
Mean	7.63	0.226	2.95
Standard deviation	0.99	0.039	0.15

\* Under identical conditions and based upon  $k_i$ , it is approximately 15 times more reactive than paraoxon and 1.6 times more reactive than amiton. G. L. Horton, J. R. Lowe, and C. N. Lieske, unpublished results.

Spontaneous reactivation of NMPP-inhibited AChE involves displacement of the phosphinyl group with concomitant return of enzymatic activity (equation 2b).



Its counterpart has been termed aging (equation 2a). With phosphates and phosphonates this pathway has been shown to involve loss of an alkoxy group from the phosphorus moiety.<sup>7,17,18</sup>

In the case of phosphinylated AChE one should be able to dismiss aging from reaction considerations (see INTRODUCTION). Our work to date with NMPP supports this rationale. A summary of our spontaneous reactivation results with AChE inhibited with NMPP is shown in table 3. They clearly reflect 100% spontaneous reactivation at both pH 7.60 and pH 9.10, with  $t_{1/2} = 32.2$  hours at the lower pH and  $t_{1/2} = 9.6$  hours at the higher pH. By way of contrast, the  $t_{1/2}$  for the spontaneous reactivation of phenyl methylphosphonylated AChE under the same conditions at pH 7.60 is 42.5 hours, with 83% return of activity.<sup>5</sup> The less than 100% return of activity reflects operation of the parallel first-order aging reaction. The trend of the spontaneous reactivation rates for the above-mentioned compounds is in the same direction as those frequently found for hydrolysis rates of the analogous esters; i.e., phosphinates > phosphonates > phosphates. However, the magnitude of the difference between these spontaneous reactivation rates is not as great as one might expect. For example, *p*-nitrophenyl diethylphosphinate is 14.6 times more susceptible to alkaline hydrolysis than ethyl *p*-nitrophenyl ethylphosphonate. Likewise, ethyl *p*-nitrophenyl ethylphosphonate hydrolyzes 39 times faster in pH 8.3 buffer than the corresponding phosphate, diethyl *p*-nitrophenyl phosphate.<sup>19</sup> These trends for hydrolysis rates also prevail upon replacement of the ethyl groups in the above-cited phosphinate and phosphate compounds by *n*-butyl groups; that is, at 25.0°C, the  $k_{OH}$  for the hydrolysis of *p*-nitrophenyl *di-n*-butylphosphinate is 81.5 M<sup>-1</sup> min<sup>-1</sup>, whereas the  $k_{OH}$  for *di-n*-butyl phosphate is 0.456 M<sup>-1</sup> min<sup>-1</sup>. This corresponds to the phosphinate being 179 times more susceptible to alkaline hydrolysis than the analogous phosphate.

Table 3. Spontaneous Reactivation of Methylphenylphosphinylated Eel Acetylcholinesterase, 25.0°C

pH	Replicates	$k_{obs}$	$t_{1/2}$	Percent reactivation <sup>a</sup>
		hr <sup>-1</sup>	hr	
7.60 <sup>b</sup>	4	0.0215 ± 0.0003	32.2	98.9 ± 2.3
9.10 <sup>c</sup>	2	0.072 ± 0.004	9.6	103.4 ± 4.0

<sup>a</sup> Calculated using equation 1.

<sup>b</sup> MOPS buffer.

<sup>c</sup> Bicine buffer.

The statements just noted strongly suggest that the spontaneous reactivation of organophosphorus-inhibited AChE is far more complex than either general base catalysis or nucleophilic displacement of the phosphorus moiety would predict. Spontaneous reactivation is probably related to charge distribution around the substituents on the phosphorus atom in the active site. The electronic effects of substituents are influenced by the dielectric constant of the reaction medium.<sup>20</sup> Therefore, one may cautiously speculate that a critical factor in spontaneous reactivation is the dielectric constant in the active site region as influenced by the steric factors of the groups attached to phosphorus in the inhibited enzyme and their perturbation on the conformation of the enzyme.

Our oxime reactivation experiments confirmed the stoichiometry obtained in our spontaneous reactivation studies and they support the absence of aging in NMPP-inhibited enzyme. Both TMB-4 and 2-PAM completely reactivated methylphenylphosphinylated enzyme in 1 hour. As expected, the same concentration of MINA was found to be less efficacious;<sup>21</sup> that is, MINA has been reported to be only about 1/75th as effective as 2-PAM in the reactivation of diethylphosphoryl AChE (human erythrocyte). The structural formulas of these oximes and the results of these investigations are shown in table 4.

Table 4. Oxime-Induced Reactivation of NMPP-Inhibited Eel AChE,  
[Oxime] =  $1 \times 10^{-4}$  M, pH 7.60, 0.10 M MOPS Buffer, 25.0°C

OXIME	STRUCTURAL FORMULA	REPLICATES	% REACTIVATION IN 1.0 HOUR
2-PAM		2	96.4 + 0.2
TMB-4		2	99.8 + 0.8
MINA		2	28.5 + 0.4

Since induced reactivation by MINA was incomplete at the end of 1 hour, the reaction was monitored through a 4-hour period in two experiments. The apparent second-order reactivation rate constant,  $k_{2(\text{app})}$ , was calculated using equation 3

$$k_{2(\text{app})} = \frac{k_{\text{obsd}}}{[\text{OXIME}]} \quad (3)$$

where  $k_{\text{obsd}}$  is the observed first-order reactivation in the presence of an oxime concentration. The value obtained was  $48.2 \text{ M}^{-1} \text{ min}^{-1} \pm 0.51\%$ , no distinction being made between protonated and unprotonated forms of the oxime. Likewise, cognizance is taken that the reaction is not truly second order but that it involves an intermediate complex of the oxime with the inhibited enzyme.

Thus, although  $k_{2(\text{app})}$  values give only a rough measure of the reactivating effectiveness of a compound, it frequently suffices for qualitative screening purposes.<sup>22</sup> As such, it is interesting to note that the MINA-induced reactivation of eel AChE inhibited with NMPP is more effective than MINA reactivation of human erythrocyte cholinesterase inhibited with Sarin ( $21.5 \text{ M}^{-1} \text{ min}^{-1}$ ) or tetraethyl pyrophosphate ( $7.2 \text{ M}^{-1} \text{ min}^{-1}$ ).<sup>23</sup>

In conclusion, this report documents (1) methylphenylphosphinylated eel AChE as a model system suitable for use in examining the effects of various perturbing agents on the spontaneous reactivation process and (2) the first spontaneous reactivation data on a phosphinate-inhibited cholinesterase. We are currently in the process of examining the effects of various compounds on the spontaneous reactivation of methylphenylphosphinylated eel AChE. In addition, several additional phosphinate esters are being synthesized, which, based upon linear free energy relationships,<sup>24</sup> will display reactivating characteristics different from those found in this study. The use of such compounds should allow greater flexibility in spontaneous reactivation studies of inhibited AChE and ultimately contribute to the therapy of organophosphate poisoning.

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## APPENDIX A

### DOCUMENTATION FOR PROGRAM EXPFIT

#### A.1. Macrodocumentation of Program EXPFIT

- a. Title: MEDICA\*BIOCHEM.EXPFIT
- b. Programmer: CPT John R. Lowe, Biomedical Laboratory (SAREA-BL-RE), 671-3836 or 2626.
- c. Machine and Language: UNIVAC 1108, FORTRAN V.
- d. Purpose: This program, a modification of one written by W.L. Wilcoxson,<sup>12</sup> will calculate by an iterative least squares method the observed first-order rate constant and the limiting velocity for the spontaneous reactivation of inhibited enzyme using equation 1 and also will generate a computer graphics datafile for subsequent off-line automated plotting. This program will accommodate up to 200 sets of velocity versus time data for each rate constant to be calculated. A flowchart for this program is given in section A.2. A copy of the program is listed in section A.3.

#### e. Input Parameters:

IDRUN	experiment identification
ID2	continuation of IDRUN
ID3	continuation of IDRUN and ID2
IBLANK	dummy variable to accept a value in the datafile which is used in an alternate computer program
IB2	continuation of IBLANK
IB3	continuation of IBLANK and IB2
TUNIT	units of time (hours, minutes, or seconds)
VUNIT	velocity function (% reactivation, absorbance, % activity, or velocity)
VU2	continuation of VUNIT
VU3	continuation of VUNIT and VU2
TIME(I)	time elapsed at which velocity function was measured
RATE(I)	velocity function measured at time <sub>i</sub>

#### f. Input Format:

Line/Card No.	Parameter	Columns	Format Specification
1	IDRUN	1-4	A4
1	ID2	5-8	A4
1	ID3	9-12	A4

Line/Card No.	Parameter	Columns	Format Specification
2	TUNIT	1-6	A6
3	VUNIT	1-6	A6
3	VU2	7-12	A6
3	VU3	13-18	A6
4	TIME(1)	1-8	F8.3
4	RATE(1)	9-17	F9.4
5	TIME(2)	1-8	F8.3
5	RATE(2)	9-17	F9.4
.	.	.	.
.	.	.	.
.	.	.	.
N+4	TIME(N+1)=0.0	1-8	F8.3
N+4	RATE(N+1)=0.0	9-17	F9.4
N+5	IDRUN='NONE'	1-4	A4

g. Output Parameters: In addition to each of the parameters listed in section A.1.e, the following parameters are printed out in one of two output files, a hard-copy analytical summary (unit 6) and a plotting data-file (unit 16).

DELTA	predetermined small number used to terminate iterations based on evaluating percentage change between successive estimations of $k_{obs}$ , $V_0$ , and $V_\infty$
HALF	calculated half life of the reaction
IT	iteration number
ITMAX	maximum number of iterations allowed
M	calculated plotter control digit for plotting program
N	number of sets of time versus velocity data
NN	number of plotting points generated for plotting datafile
OBSK	calculated observed rate constant
QHALF	number of half lives over which the data were collected
SAS	analytic variance for the fitted curve
SS	experimental variance for the fitted curve
SOD	sum of differences between experimental and calculated velocities
TCALC	time calculated by fractionation of total experimental time interval, used for generating plotting data
TIMO	abscissa intercept, time when velocity = 0



Line/Card No.	Parameter	Columns	Format Specification
N+216	TCALC	1-8	F8.3
N+216	VCOMP	9-17	F9.4
.	.	.	.
.	.	.	.
N+414	TCALC	1-8	F8.3
N+414	VCOMP	9-17	F9.4
N+415	M	1-3	I3
N+416	N	1-3	I3
N+417	TIME(1)	1-8	F8.3
N+417	RATE(1)	9-17	F9.4
.	.	.	.
.	.	.	.
2N+416	TIME(N)	1-8	F8.3
2N+416	RATE(N)	9-17	F9.4
2N+417	M	1-3	I3
2N+418	IDRUN='NONE'	1-4	A4

h. Operating Instructions:

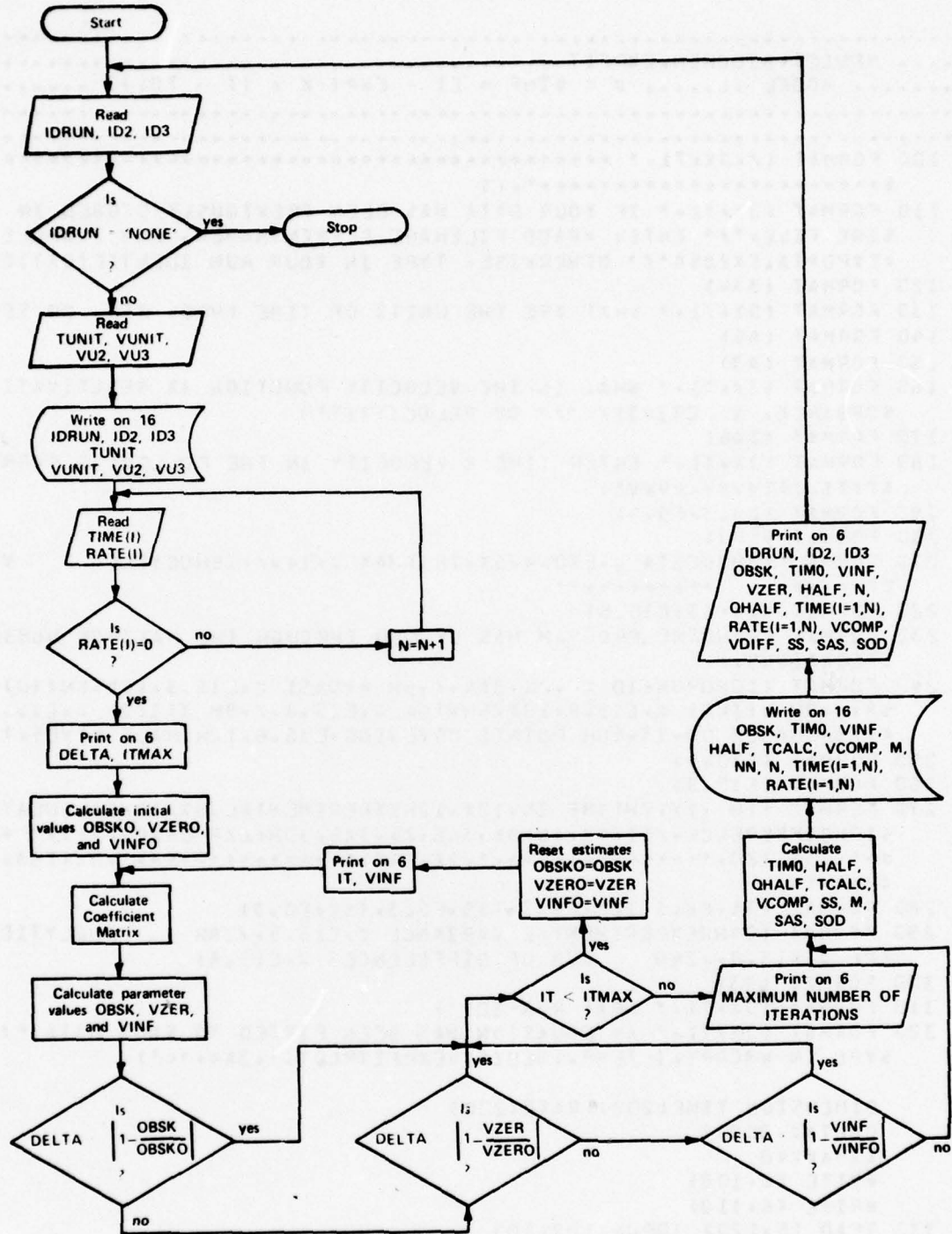
(1) For batch operations, follow the instructions listed in sections 3, 7, 16, and 17 of the Edgewood Arsenal UNIVAC 1108 User's Guide.

(2) For demand operations, the required UNIVAC 1108 system interactions are described in the following section. All of our data reduction is performed in the demand mode. The telecommunication port to the computer is initialized by calling the appropriate phone number for the baud rate being used and by typing in the site identification number. After initialization, the demand runstream is begun with an Executive 8 control statement (master space RUN). This control statement contains the run identifier, account number, project identifier, and time/page estimates. Experimental data are then entered into an element of an on-line datafile (MEDICA\*EXPDATA.) in the format listed in section A.1.f by using the ELT processor for program file maintenance. Proofreading and subsequent corrections to this data element are made by using the editing processor (@ED,U FILE.ELEMENT). After proper format has been established for the data element, the data reduction is begun by typing in the following set of system control commands.

```
@ERS
@ASG,T TEMP.,F2
@USE 16. TEMP.
@FOR,N MEDICA*BIOCHEM.EXPFIT,TPF$.
@MAP,N
IN TPF$.
@XQT
```

In actual practice, these statements have been stored as a canned runstream in the file MEDICA\*RUNEXPFIT. Therefore, in order to start the data processing by the FORTRAN curvefitting program, we type in @ADD MEDICA\*RUNEXPFIT. Now the only requirement to complete the data reduction is to answer the questions which are printed out at the teletype. A sample of the output is shown in section A.4.

A.2. Flowchart for Program EXPFIT



A.3. FORTRAN Statements for Program EXPFIT

```

C.....C
C.... MEDICA*BIOCHEM.EXPFIT .....C
C..... MODEL ..... V = VINP * (1 - EXP(-K * (T - T0))) .....C
C.....C
C.....C
100 FORMAT (/JX,T1,' .....
      S.....')
110 FORMAT (JX,T1,' IF YOUR DATA HAS BEEN PREVIOUSLY STORED IN AN ON-L
      LINE FILE.*/' ENTER #ADD FILENAME.ELEMENTNAME#. FOR EXAMPLE: #ADD
      #EXPDATA.EX2854*/' OTHERWISE, TYPE IN YOUR RUN IDENTIFICATION.')
```

120 FORMAT (3A4)

130 FORMAT (JX,T1,' WHAT ARE THE UNITS OF TIME (HRS, MIN, OR SEC)?')

140 FORMAT (A6)

150 FORMAT (A3)

160 FORMAT (JX,T1,' WHAT IS THE VELOCITY FUNCTION (% REACTIVATION, ABS
 SOPBANCE, % ACTIVITY,\*/' OR VELOCITY)?')

170 FORMAT (3A6)

180 FORMAT (JX,T1,' ENTER TIME & VELOCITY IN THE FOLLOWING FORMAT:\*/'
 STTTT.TTTVVVV.VVVV')

190 FORMAT (F8.3,F9.4)

200 FORMAT (1HJ)

210 FORMAT (8HDELTA =,E10.4,5X,7HITMAX =,I4,/,18HOCYCLE V(INF),/
 S' +++++ +++++')

220 FORMAT (1X,I3,E15.5)

230 FORMAT (65H)THE PROGRAM HAS CYCLED THROUGH THE MAXIMUM NUMBER OF I
 TERATIONS.)

240 FORMAT (10H)RUN-ID = ,2X,3A4,/,9H K(OBS) =,E15.8,10X,6HT(D) =,E15.
 8,/,9H V(INF) =,E15.8,10X,6HV(O) =,E15.8,/,9H T(1/2) =,E15.8,1X,A6
 #,/,9H)BASED ON,I3,16H POINTS COVERING,E15.6,12H HALF LIVES.)

250 FORMAT (E13.4)

260 FORMAT (E15.8)

270 FORMAT (1H ,1X,7HTIME IN,10X,12H)EXPERIMENTAL,8X,10H)CALCULATED,8X,
 510H)DIFFERENCE,/,T1,4X,A6,9X,3A6,2X,3A6,10H)EXP-CALC,/,T1,' ++++++
 #',17X,T2,' ++++++',7X,T40,' ++++++',5X,T58,' ++++++
 3++++')

280 FORMAT (T1,F8.3,T20,F8.3,T39,F8.3,T57,F8.3)

290 FORMAT (24H)EXPERIMENTAL VARIANCE =,E15.8,/24H ANALYTIC VARIAN
 SCE =,E15.8,/24H SUM OF DIFFERENCES =,E15.8)

300 FORMAT (I3)

310 FORMAT (JX,T1,' NEXT RUN-ID?')

320 FORMAT (JX,T1,' AN EQUATION HAS BEEN FITTED TO YOUR DATA.\*/' NOW T
 YPE IN #COPY,I TEMP.,MEDICA\*EXPFIT#PLOT.,',3A4,'#')

C

```

      DIMENSION TIME(200),RATE(200)
      DELTA=.00001
      ITMAX=40
      WRITE (6,100)
      WRITE (6,110)
      330 READ (5,120) IDRUN,ID2,ID3
      WRITE (16,120) IDRUN,ID2,ID3
```

```

340 IF (IDRUN.EQ.'NONE') GO TO 540
ID4=IDRUN
ID5=ID2
ID6= ID3
N=0
WRITE (6,130)
READ (5,140) TUNIT
WRITE (16,150) TUNIT
WRITE (6,160)
READ (5,170) VUNIT,VU2,VU3
WRITE (16,170) VUNIT
WRITE (6,180)
DO 350 I=1,200
READ (5,190) TIME(I),RATE(I)
IF (RATE(I).EQ.0.) GO TO 360
N=N+1
350 CONTINUE
360 WRITE (6,200)
WRITE (6,210) DELTA,ITMAX
VZERO=RATE(1)
TEST=.5*(RATE(N)+VZERO)
DO 370 I=3,N
IF (RATE(I).GT.TEST) GO TO 380
370 CONTINUE
OBSKO=1./TIME(N)
GO TO 390
380 OBSKO=1./TIME(I)
390 VINFO=RATE(N)
IT=0
400 C11=0.0
C12=0.0
C13=0.0
C1R=0.0
C22=0.0
C23=0.0
C2R=0.0
C33=0.0
C3R=0.0
CRR=0.0
DO 430 I=1,N
EXPON=-OBSKO*TIME(I)
IF (EXPON.GT.-49.) GO TO 410
U=1.0
V=0.0
W=0.0
GO TO 420
410 V=EXP(EXPON)
U=1.-V
W=TIME(I)*V
420 TEST=RATE(I)

```

```

C11=C11+U*U
C12=C12+U*V
C13=C13+U*W
C1R=C1R+U*TEST
C22=C22+V*V
C23=C23+V*W
C2R=C2R+V*TEST
C33=C33+W*W
C3R=C3R+W*TEST
CRR=CRR+TEST*TEST
430 CONTINUE
A=C11*C33-C13*C13
B=C12*C33-C13*C23
C=C22*C33-C23*C23
E=C1R*C33-C3R*C13
F=C2R*C33-C3R*C23
VINP=(E+C-F*B)/(A+C-B*B)
VZER=(F-VINP*B)/C
Q=(C3R-VINP*C13-VZER*C23)/C33
OBSK=OBSK0+Q/(VINP0-VZERO)
IT=IT+1
OKTEST=1.-OBSK/OBSK0
OBSK0=OBSK
VTEST=1.-VZER/VZERO
VZERO=VZER
VITEST=1.-VINP/VINP0
VINP0=VINP
IF (DELTA.LE.ABS(OKTEST)) GO TO 440
IF (DELTA.LE.ABS(VTEST)) GO TO 440
IF (DELTA.LE.ABS(VITEST)) GO TO 440
GO TO 450
440 WRITE (6,220) IT,VINP
IF (IT.LT.ITMAX) GO TO 430
WRITE (6,230)
450 TIMD=(ALOG(1.-VZER/VINP))/OBSK
HALF=.69315/OBSK
QHALF=TIME(N)/HALF
WRITE (6,240) IDRUN, ID2, ID3, OBSK, TIMD, VINP, VZER, HALF, TUNIT, N, QHALF
WRITE (6,250)
WRITE (16,250) OBSK
WRITE (16,250) TIMD
WRITE (16,260) TIMD
WRITE (16,250) VINP
WRITE (16,250) VZER
WRITE (16,260) HALF
WRITE (6,270) TUNIT, VUNIT, VU2, VU3, VUNIT, VU2, VU3
ENM3=FLOAT(N-3)
SAS=(CRR-VINP*C1R-VZER*C2R-Q*C3R)/ENM3
SOS=0.0
SOD=0.0

```

```

DO 470 I=1,N
EXPON=-ORSK*TIME(I)
VCOMP=VINP
IF (EXPON.LE.-49.) GO TO 460
VCOMP=VCOMP-(VINP-VZER)*EXP(EXPON)
460 VDIFF=RATE(I)-VCOMP
SOS=SOS+VDIFF*VDIFF
SOD=SOD+VDIFF
WRITE (6,280) TIME(I),RATE(I),VCOMP,VDIFF
470 CONTINUE
SS=SOS/ENM3
WRITE (6,290) SS,SAS,SOD
TIMN=TIME(N)
TINF=5.*HALF
480 NN=200
WRITE (16,300) NN
VCOMP=0.0
WRITE (16,190) TIM0,VCOMP
DO 490 I=1,199
TCALC=TIM0+FLOAT(I)*(TIMN-TIM0)/199.
VCOMP=VINP*(1.-EXP(-ORSK*(TCALC-TIM0)))
WRITE (16,190) TCALC,VCOMP
490 CONTINUE
M=0
WRITE (16,300) M
WRITE (16,300) N
DO 500 J=1,N
WRITE (16,190) TIME(J),RATE(J)
500 CONTINUE
M=-N/30
IF (M) 520,510,510
510 M=-1
520 WRITE (16,300) M
IF (TIMN.EQ.TINF) GO TO 530
TIMN=TINF
GO TO 480
530 WRITE (6,100)
WRITE (6,310)
READ (5,120) IBLANK,IBL2,IBL3
IF (IBLANK.EQ.' ') GO TO 330
IDRUN=IBLANK
ID2=IBL2
ID3=IBL3
GO TO 340
540 WRITE (6,100)
WRITE (6,320) ID4,ID5,IDE
WRITE (6,100)
STOP
END

```

A.4. Sample of Teletype Interactive Output for Program EXPFIT

a. The following is a sample of what is seen during a demand operation at the teletype terminal. The first input is the site identification. The system responds with the Edgewood Arsenal identification line. The operator then enters his run control command. The system responds with the date and time and then indicates that it is ready for input with a ">" sign in column one. Each time a ">" sign is printed, the operator enters his control commands or data.

```
#SITE-ID
*EDGEWOOD ARSENAL 1108 SYSTEM VER. 31-244 UPD D(RSI)*
@RUN JFLOWE,COST-CODE,PROJECT-ID,30,1000
DATE: 062176      TIME: 165422
>@ADD MEDICA*RUNEXPFIT.
RURPUR: 0026-06/21-16:54
READY
READY
FOR 30E3-06/21/76-16:54:44 (0.)
END FOR
MAP28R1 RL71-3 06/21/76 16:54:52
END MAP
*****

IF YOUR DATA HAS BEEN PREVIOUSLY STORED IN AN ON-LINE FILE,
ENTER "@ADD FILENAME.ELEMENTNAME". FOR EXAMPLE: @ADD EXPDATA.EX2854
OTHERWISE, TYPE IN YOUR RUN IDENTIFICATION.
>@ADD EXPDATA.EX2854
WHAT ARE THE UNITS OF TIME (HRS, MIN, OR SEC)?
WHAT IS THE VELOCITY FUNCTION (% REACTIVATION, ABSORBANCE, % ACTIVITY,
OR VELOCITY)?
ENTER TIME & VELOCITY IN THE FOLLOWING FORMAT:
TTTT.TTTUUUU.UUUUU

DELTA = .1000+04      ITMAX = 40

CYCLE      U(INF).
+++++      ++++++
  1      .99804+02
  2      .99985+02
  3      .10007+03
  4      .10007+03
  5      .10007+03

RUN-ID = EXP2854
K(OBS) = .21199663+01      T(0) = -.85671768+00
U(INF) = .10007400+03      U(0) = .18011509+01
T(1/2) = .32696274+02 HRS

BASED ON 39 POINTS COVERING .308139+01 HALF LIVES.
```

TIME IN HRS *****	EXPERIMENTAL % REACTIVATION *****	CALCULATED % REACTIVATION *****	DIFFERENCE (EXP-CALC) *****
1.000	3.050	3.863	-.813
1.080	3.560	4.026	-.466
2.160	5.940	6.200	-.260
3.000	7.640	7.857	-.217
4.050	10.180	9.887	.293
5.160	12.050	11.984	.066
6.000	13.240	13.539	-.299
7.000	15.280	15.354	-.074
8.250	17.820	17.570	.250
11.050	22.100	22.325	-.225
12.160	24.650	24.133	.517
13.080	26.700	25.600	1.100
14.050	27.380	27.115	.265
15.500	30.100	29.324	.776
21.500	36.340	37.774	-1.434
23.000	41.330	39.724	1.606
24.580	42.680	41.712	.968
26.500	44.130	44.040	.090
27.660	44.550	45.401	-.851
28.500	46.540	46.366	.174
29.500	46.710	47.493	-.783
31.000	48.350	49.139	-.789
32.360	50.340	50.586	-.246
33.060	50.560	51.315	-.755
36.330	54.450	54.581	-.131
38.910	59.470	57.002	2.468
45.500	61.170	62.618	-1.448
47.420	65.120	64.112	1.008
52.250	69.480	67.612	1.868
55.660	73.820	69.876	3.944
70.160	73.030	77.868	-4.838
70.610	76.540	78.078	-1.538
72.330	75.090	78.866	-3.776
72.660	76.180	79.014	-2.834
76.950	82.110	80.845	1.265
80.000	81.480	82.049	-.569
94.920	88.960	86.936	2.024
97.000	90.370	87.503	2.867
100.750	89.260	88.464	.796

EXPERIMENTAL VARIANCE = .28565553+01  
ANALYTIC VARIANCE = .28565209+01  
SUM OF DIFFERENCES = .33676624-04

\*\*\*\*\*  
NEXT RUN-ID?  
NONE

\*\*\*\*\*  
AN EQUATION HAS BEEN FITTED TO YOUR DATA.  
NOW TYPE IN "@COPY,I TEMP.,MEDICA\*EXPFITPLOT.EXP2854"

\*\*\*\*\*  
>@COPY,I TEMP.,MEDICA\*EXPFITPLOT.EXP2854  
FURPUR 0026-06/21-16:56

b. In the previous example, the data were entered by using an on-line datafile. If data are to be entered during program operation manually, the following is a sample of the portion of the runstream which is different from that shown in A.4.a. Instead of responding to the first statement with @ADD EXPDATA.EX2854, the operator would type in the run identification, EXP2854. The other questions are then answered in turn as the ">" sign appears.

```
>EXP2854
WHAT ARE THE UNITS OF TIME (HRS, MIN, OR SEC)?
>HRS
WHAT IS THE VELOCITY FUNCTION (% REACTIVATION, ABSORBANCE, % ACTIVITY,
OR VELOCITY)?
>% REACTIVATION
ENTER TIME & VELOCITY IN THE FOLLOWING FORMAT:
TTTT.TTTUUUU.UUUU
> 1.00 3.05
> 1.08 3.56
> 2.16 5.94
> 3.00 7.64
> 4.05 10.18
> 5.16 12.05
> 6.00 13.24
> 7.00 15.28
> 8.25 17.82
> 11.05 22.10
> 12.16 24.65
> 13.08 26.70
> 14.05 27.38
> 15.50 30.10
> 21.50 36.34
> 23.00 41.33
> 24.58 42.68
> 26.50 44.13
> 27.66 44.55
> 28.50 46.54
> 29.50 46.71
> 31.00 48.35
> 32.36 50.34
> 33.06 50.56
> 36.33 54.45
> 38.91 59.47
> 45.50 61.17
> 47.42 65.12
> 52.25 69.48
> 55.66 73.82
> 70.16 73.03
> 70.61 76.54
> 72.33 75.09
> 72.66 76.18
> 76.95 82.11
> 80.00 81.48
> 94.92 88.96
> 97.00 90.37
> 100.75 89.26
> 0.0 0.0
```

## APPENDIX B

### DOCUMENTATION FOR PROGRAM EXPFITPLOT

#### B.1. Macrodocumentation for Program EXPFITPLOT

a. Title: MEDICA\*BIOCHEM.EXPFITPLOT

b. Programmer: CPT John R. Lowe, Biomedical Laboratory (SAREA-BL-RE), 671-2626/3836.

c. Machine and Language: UNIVAC 1108, FORTRAN V.

d. Purpose: This program will provide the required system control commands to plot the calculated least-squares-fitted exponential curve from program EXPFIT. The off-line CALCOMP plotter at the main computer site and the library plotting subroutines for this plotter are used in this program. Up to 200 sets of abscissa-ordinate data can be accommodated to draw smooth curves for the fitted exponential equation. A copy of the flowchart for this program is shown in section B.2. A copy of the program is listed in section B.3.

e. Input Parameters:

HALF	calculated half life of the reaction
IDRUN	experiment identification
ID2	continuation of IDRUN
M	plotter control digit used to selectively choose the number of points to draw on the plot
N	number of sets of TIME versus RATE data
OBSK	observed rate constant in alphanumeric format
OBSKA	continuation of OBSK
OBSKB	continuation of OBSK and OBSKA
RATE(I)	velocity function measured at time <sub>i</sub>
TIME(I)	time elapsed at which velocity function was measured
TIMO	abscissa intercept, time when velocity = 0, in alphanumeric format
TIMOA	continuation of TIMO
TIMOB	continuation of TIMO and TIMOA
TIMON	TIMO in numeric format

TUNIT units of time (hours, minutes, or seconds)

VINF calculated maximum velocity at infinite time in alphanumeric format

VINFA continuation of VINF

VINFB continuation of VINF and VINFA

VINFN VINF in numeric format

f. Input Format:

Line/Card No.	Parameter	Columns	Format
1	IDRUN	1-6	A6
1	ID2	7-12	A6
2	TUNIT	1-3	A3
3	VUNIT	1-6	A6
4	OBSK	1-3	A3
4	OBSKA	4-7	A4
4	OBSKB	8-11	A4
5	TIMO	1-3	A3
5	TIMOA	4-7	A4
5	TIMOB	8-11	A4
6	TIMON	1-15	E15.8
7	VINF	1-3	A3
7	VINFA	4-7	A4
7	VINFB	8-11	A4
8	VINFN	1-10	E10.4
9	HALF	1-15	E15.8
10	N=200	1-3	I3
11	TIME(1)	1-8	F8.3
11	RATE(1)	9-17	F9.4
12	TIME(2)	1-8	F8.3
12	RATE(2)	9-17	F9.4
.	.	.	.
.	.	.	.
.	.	.	.
210	TIME(N)	1-8	F8.3
210	RATE(N)	9-17	F9.4
211	M	1-3	I3
212	N	1-3	I3
.	.	.	.
.	.	.	.
.	.	.	.
N+212	TIME(N)	1-8	F8.3
N+212	RATE(N)	9-17	F9.4
N+213	M	1-3	I3
N+214	N=200	1-3	I3
N+215	TIME(1)	1-8	F8.3
N+215	RATE(1)	9-17	F9.4
.	.	.	.
.	.	.	.
.	.	.	.

Line/Card No.	Parameter	Columns	Format Specification
N+414	TIME(N)	1-8	F8.3
N+414	RATE(N)	9-17	F9.4
N+415	M	1-3	I3
N+416	N	1-3	I3
N+417	TIME(1)	1-8	F8.3
N+417	RATE(1)	9-17	F9.4
.	.	.	.
.	.	.	.
.	.	.	.
2N+416	TIME(N)	1-8	F8.3
2N+416	RATE(N)	9-17	F9.4
2N+417	M	1-3	I3
2N+418	IDRUN='NONE'	1-6	A6

g. Output Parameters: All output from this program is through the library subroutines available on-line to provide CALCOMP plotter control. In addition to the parameters listed in B.1.e, the following parameters are also printed.

ASLEN            length in inches of the asymptote line at VINFN  
 ASYMP           length in inches to the ordinate value of VINFN  
 ZERO            length in inches of time = 0 on the abscissa axis  
 PLOTID          character used to identify the two graphs described

h. Output Format: Section B.4. contains the graphical CALCOMP output which results from the operation of this program. For each fitted exponential equation two plots are drawn. The first has an axis scale based on the range of time values from TIMON to TIME(N) and the second has an axis scale based on the range in velocity values from zero to VINFN and the range of time values from TIMON to five times the calculated half life for the reaction. The sample graphs show the differences in graphical output.

i. Operating Instructions:

(1) For batch operations, follow the instructions listed in the UNIVAC 1108 User's Guide.

(2) For demand operations the required UNIVAC 1108 system interactions include site and runstream initialization as described in appendix A, section A.1.i.(2). To initiate plotting, a canned runstream located in MEDICA\*PLOTEXPFIT is entered at the teletype keyboard. This file contains the following:

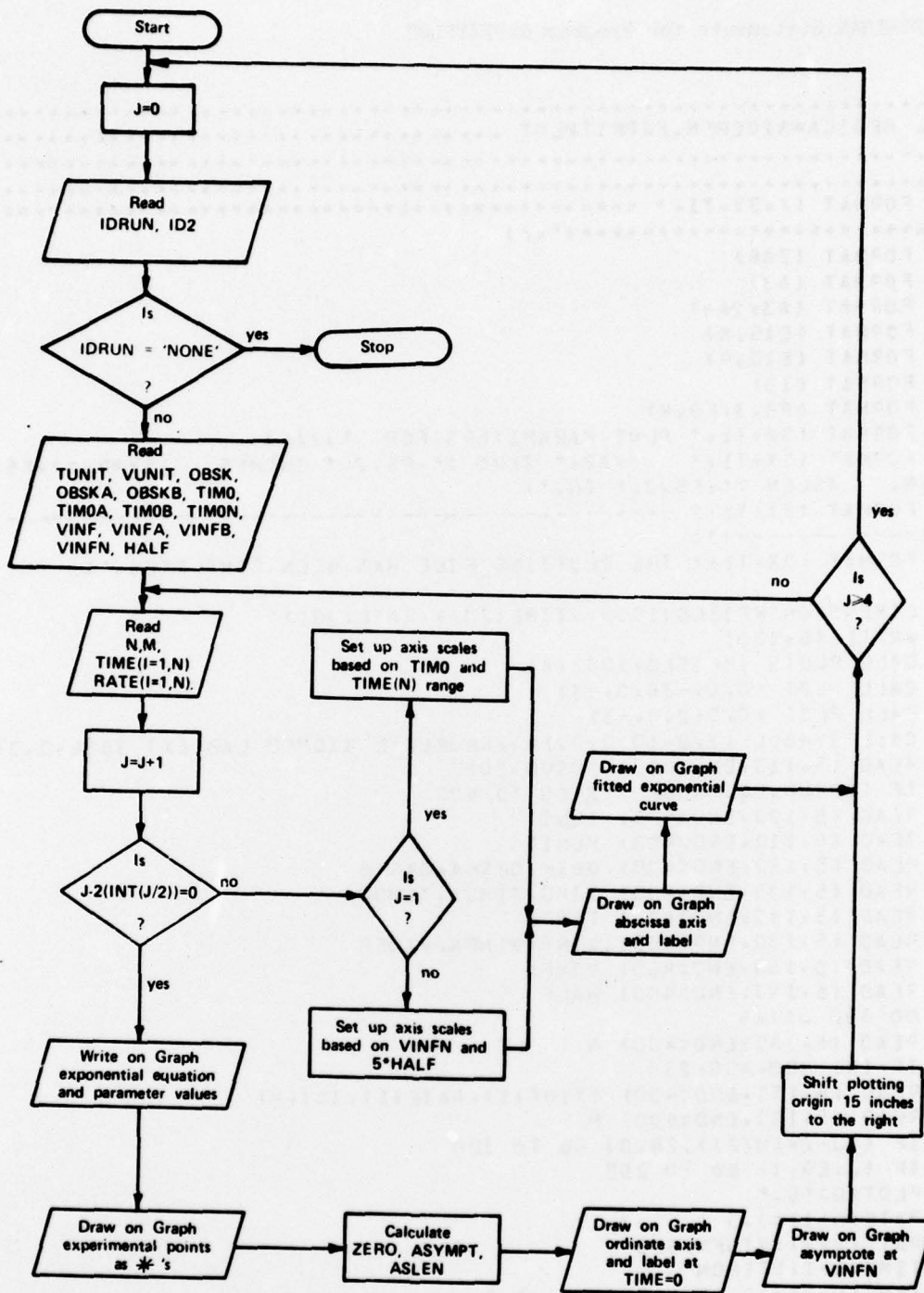
```
@ERS
@ASG,T TEMP.,F2
@USE 16.,TEMP.
@FOR,N MEDICA*BIOCHEM.PLOTTEREXPFT,TPF$.
@MAP,N
IN TPF$.
@XQT
```

As in the use of program EXPFIT, the only requirement to have the plots made is to type in one statement which for plotting is "@ADD MEDICA\*PLOTEXPFIT." and then answer the questions printed out at the teletype terminal. This runstream compiles and runs the FORTRAN program PLOTTEREXPFT which is a small control runstream generator. A copy of the program PLOTTEREXPFT is listed in section B.5. The system control commands which are generated by PLOTTEREXPFT are:

```
@ERS
@ASG,T 8.,F2
@FOR,N MEDICA*BIOCHEM.EXPFITPLOT,TPF$.
@MAP,N
IN TPF$.
@XQT
@ADD MEDICA*EXPFITPLOT.EXP2854
@COPY 8.,MEDICA*INHIBPLOT.
@MSG,W PLOT,JRLOWE,X3836
@PLOT,N MEDICA*INHIBPLOT.
```

A sample of the teletype interactive output during demand operation of the plotting program is shown in section B.6.

B.2. Flowchart for Program EXPFITPLOT.



B.3. FORTRAN Statements for Program EXPFITPLOT

```

C.....C
C.....MEDICA*BIOCHEM.EXPFITPLOT.....C
C.....C
C.....C
100 FORMAT (/,OX,T1,' *****')
      S('*****',/)
110 FORMAT (2A6)
120 FORMAT (A3)
130 FORMAT (A3,2A4)
140 FORMAT (E15.8)
150 FORMAT (E10.4)
160 FORMAT (I3)
170 FORMAT (F8.3,F9.4)
180 FORMAT (JX,T1,' PLOT PARAMETERS FOR ',2A6)
190 FORMAT (JX,T1,' ',A2,' ZERO ='F5.2,' INCHES ASYMP ='F5.2,' I
      SN. ASLEN ='F5.2,' IN.')
200 FORMAT (JX,T1,' -----')
      S('-----')
210 FORMAT (JX,T1,' THE PLOTTING FILE HAS BEEN COMPLETED.')
C
      DIMENSION KFIELD(1000),TIME(202),RATE(202)
      WRITE (6,100)
      CALL PLOTS (KFIELD,1000,8)
      CALL PLOT (0.0,-36.0,-3)
      CALL PLOT (0.0,2.0,-3)
      CALL SYMBOL (1.0,10.0,0.14,26HJRWLOWE BIOMED LAB EXT 3836,0.0,26)
220 READ (5,110,END=400) IDRUN,ID2
      IF (IDRUN.EQ.'NONE ') GO TO 400
      READ (5,120,END=400) TUNIT
      READ (5,110,END=400) VUNIT
      READ (5,130,END=400) OBSK,OBSKA,OBSKB
      READ (5,130,END=400) TIMD,TIMDA,TIMOB
      READ (5,140,END=400) TIMDN
      READ (5,130,END=400) VINP,VINFA,VINFB
      READ (5,150,END=400) VINFN
      READ (5,140,END=400) HALF
      DO 390 J=1,4
      READ (5,160,END=400) N
      IF (N) 400,400,230
230 READ (5,170,END=400) (TIME(I),RATE(I),I=1,N)
      READ (5,160,END=400) M
      IF ((J-2*(J/2)).EQ.0) GO TO 300
240 IF (J.EQ.1) GO TO 250
      PLOTID='B.'
      RATE(N+1)=0.0
      RATE(N+2)=VINFN/6.0
      TIME(N+1)=TIMDN
      TIME(N+2)=(5.*HALF-TIMDN)/9.5
      GO TO 260
250 CALL SCALE (TIME,9.5,N,+1)
      CALL SCALE (RATE,6.0,N,+1)

```

```

PLOTID='A.'
260 IF (TUNIT.NE.'SEC') GO TO 270
    CALL AXIS (0.0,0.0,15HTIME IN SECONDS,-15,9.5,0.0,TIME(N+1),
    $TIME(N+2))
    GO TO 290
270 IF (TUNIT.NE.'MIN') GO TO 280
    CALL AXIS (0.0,0.0,15HTIME IN MINUTES,-15,9.5,0.0,TIME(N+1),
    $TIME(N+2))
    GO TO 290
280 CALL AXIS (0.0,0.0,15H TIME IN HOURS,-15,9.5,0.0,TIME(N+1),
    $TIME(N+2))
290 CALL SYMBOL (3.3,8.6,0.21,16MEXPONENTIAL PLOT,0.0,16)
    GO TO 310
300 CALL SYMBOL (6.0,2.5,0.14,5HV = V,0.0,5)
    CALL SYMBOL (999.,2.46,0.07,4HINF ,0.0,4)
    CALL SYMBOL (999.,2.5,0.14,6HCL - E,0.0,6)
    CALL SYMBOL (999.,2.6,0.08,1H=,0.0,1)
    CALL SYMBOL (999.,999.,0.053,1HK,0.0,1)
    CALL PLOT (7.9,2.6,3)
    CALL PLOT (7.9,2.69,2)
    CALL PLOT (7.9,2.6,2)
    CALL SYMBOL (999.,2.58,0.04,3H08S,0.0,3)
    CALL SYMBOL (999.,2.6,0.08,4H(T-T,0.0,4)
    CALL SYMBOL (999.,2.58,0.04,1HQ,0.0,1)
    CALL SYMBOL (999.,2.6,0.08,1H),0.0,1)
    CALL SYMBOL (999.,2.5,0.14,1H),0.0,1)
    CALL SYMBOL (6.0,1.6,0.14,1HV,0.0,1)
    CALL SYMBOL (999.,1.56,0.07,4HINF ,0.0,4)
    CALL SYMBOL (999.,1.6,0.14,1H=,0.0,1)
    CALL SYMBOL (999.,999.,0.14,VINF,0.0,3)
    CALL SYMBOL (999.,999.,0.14,VINFA,0.0,4)
    CALL SYMBOL (999.,1.62,0.10,3H X ,0.0,3)
    CALL SYMBOL (999.,1.6,0.14,2H10,0.0,2)
    CALL SYMBOL (999.,1.7,0.08,VINF8,0.0,4)
    CALL SYMBOL (6.0,1.2,0.09,1HK,0.0,1)
    CALL PLOT (6.0,1.2,3)
    CALL PLOT (6.0,1.35,2)
    CALL PLOT (6.0,1.2,2)
    CALL SYMBOL (6.09,1.18,0.07,4H08S ,0.0,4)
    CALL SYMBOL (6.42,1.2,0.14,1H=,0.0,1)
    CALL SYMBOL (999.,999.,0.14,08SK,0.0,3)
    CALL SYMBOL (999.,999.,0.14,08SKA,0.0,4)
    CALL SYMBOL (999.,1.22,0.10,3H X ,0.0,3)
    CALL SYMBOL (999.,1.2,0.14,2H10,0.0,2)
    CALL SYMBOL (999.,1.3,0.08,08SK8,0.0,4)
    CALL SYMBOL (6.0,0.8,0.14,1HT,0.0,1)
    CALL SYMBOL (999.,0.78,0.07,4H0 ,0.0,4)
    CALL SYMBOL (999.,0.8,0.14,1H=,0.0,1)
    CALL SYMBOL (999.,999.,0.14,TIMQ,0.0,3)
    CALL SYMBOL (999.,999.,0.14,TIMQA,0.0,4)
    CALL SYMBOL (999.,0.82,0.10,3H X ,0.0,3)

```

```

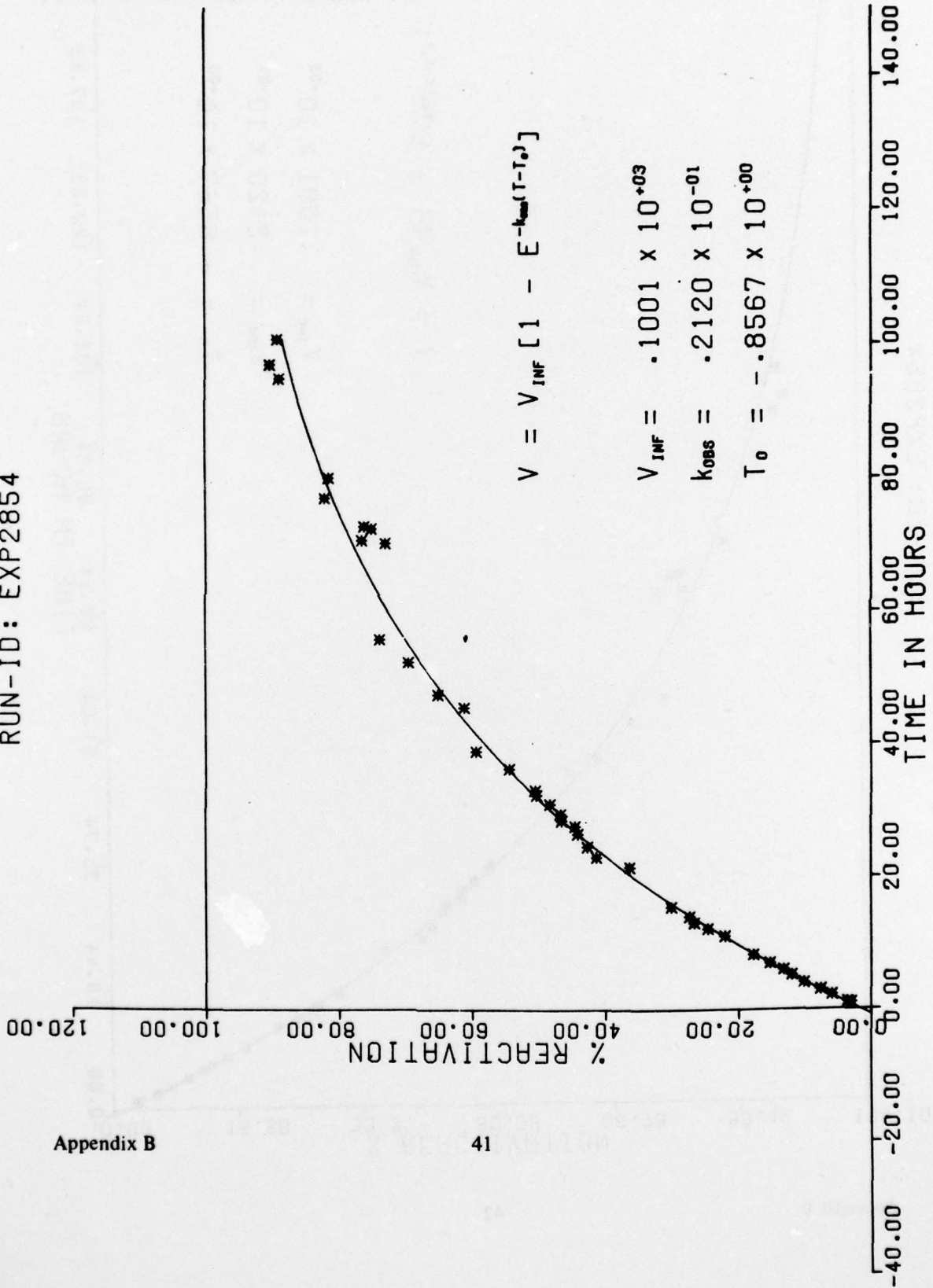
CALL SYMBOL (999.,0.8,0.14,2H10,0.0,2)
CALL SYMBOL (999.,0.9,0.08,TIM08,0.0,4)
TIME(N+1)=TB
TIME(N+2)=TS
RATE(N+1)=RB
RATE(N+2)=RS
CALL LINE (TIME,RATE,N,1,M,11)
GO TO 320
310 CALL LINE (TIME,RATE,N,1,M,0)
CALL SYMBOL (4.0,6.4,0.14,8HRUN-ID:,0.0,8)
CALL SYMBOL (5.1,6.4,0.14,IDRUN,0.0,6)
CALL SYMBOL (999.,999.,0.14,IO2,0.0,6)
CALL PLOT (0.0,0.0,3)
TB=TIME(N+1)
TS=TIME(N+2)
RB=RATE(N+1)
RS=RATE(N+2)
GO TO 390
320 ZERO=-TIME(N+1)/TIME(N+2)
CALL PLOT (ZERO,0.0,-3)
IF (VUNIT.NE.'% REAC') GO TO 330
CALL AXIS (0.0,0.0,14HZ REACTIVATION,14,6.0,90.0,RATE(N+1),
$RATE(N+2))
GO TO 360
330 IF (VUNIT.NE.'ABSORB') GO TO 340
CALL AXIS (0.0,0.0,10HABSORBANCE,10,6.0,90.0,RATE(N+1),
$RATE(N+2))
GO TO 360
340 IF (VUNIT.NE.'% ACTI') GO TO 350
CALL AXIS (0.0,0.0,10HZ ACTIVITY,10,6.0,90.0,RATE(N+1),
$RATE(N+2))
GO TO 360
350 CALL AXIS (0.0,0.0,8HVELOCITY,8,6.0,90.0,RATE(N+1),
$RATE(N+2))
360 ASYMP=VINFN/RATE(N+2)
ASLEN=TB/TS+9.5
IF (ASYMP.GT.6.01) GO TO 370
CALL PLOT (0.0,ASYMP,3)
CALL PLOT (ASLEN,ASYMP,2)
370 CALL PLOT (15.0,0.0,-3)
IF (J.EQ.4) GO TO 380
WRITE (6,180) IDRUN,IO2
380 WRITE (6,190) PLOTID,ZERO,ASYMP,ASLEN
390 CONTINUE
WRITE (6,200)
GO TO 220
400 CALL PLOT (15.0,0.0,999)
WRITE (6,210)
WRITE (6,100)
STOP
END

```

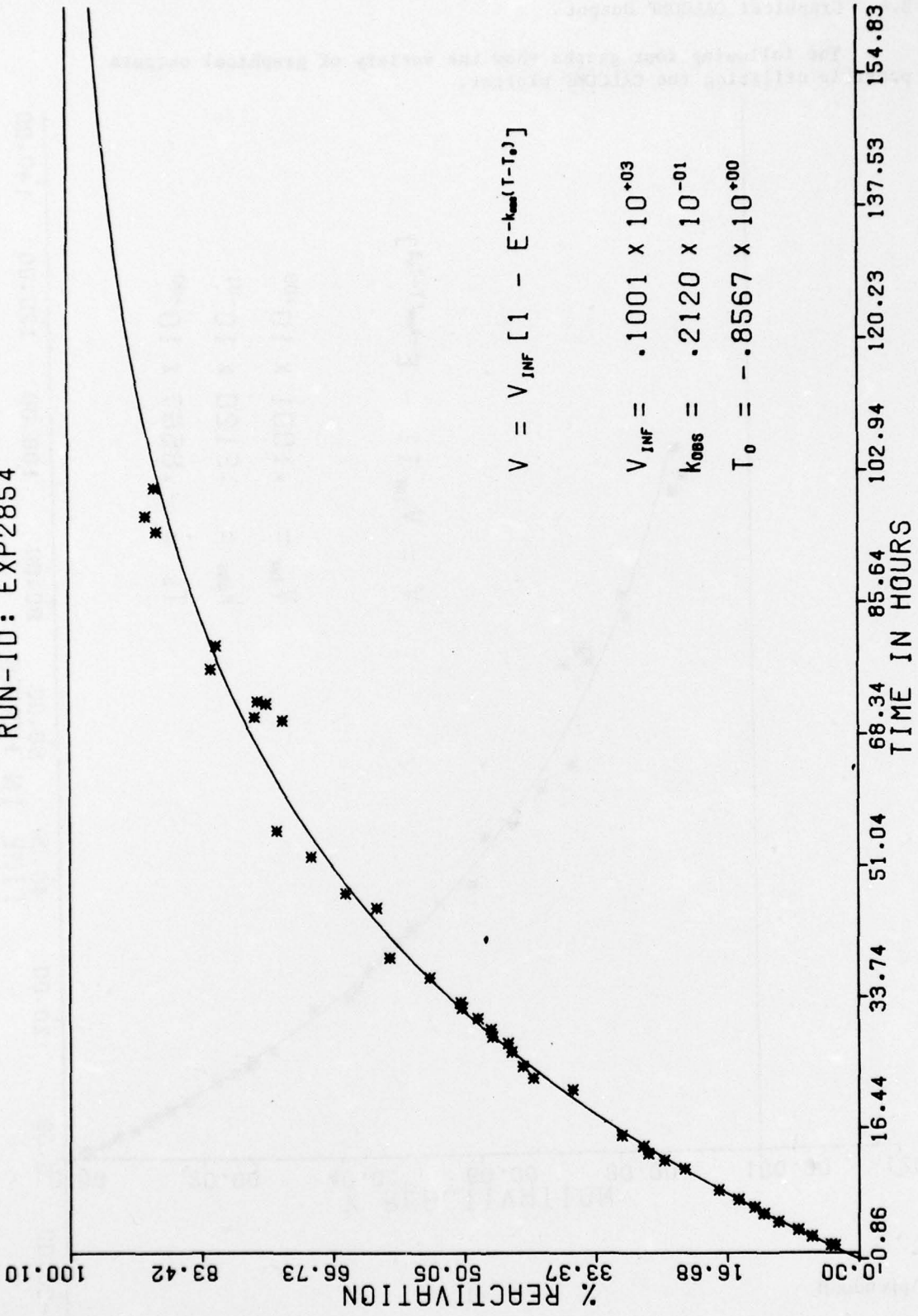
B.4. Graphical CALCOMP Output

The following four graphs show the variety of graphical outputs possible utilizing the CALCOMP plotter.

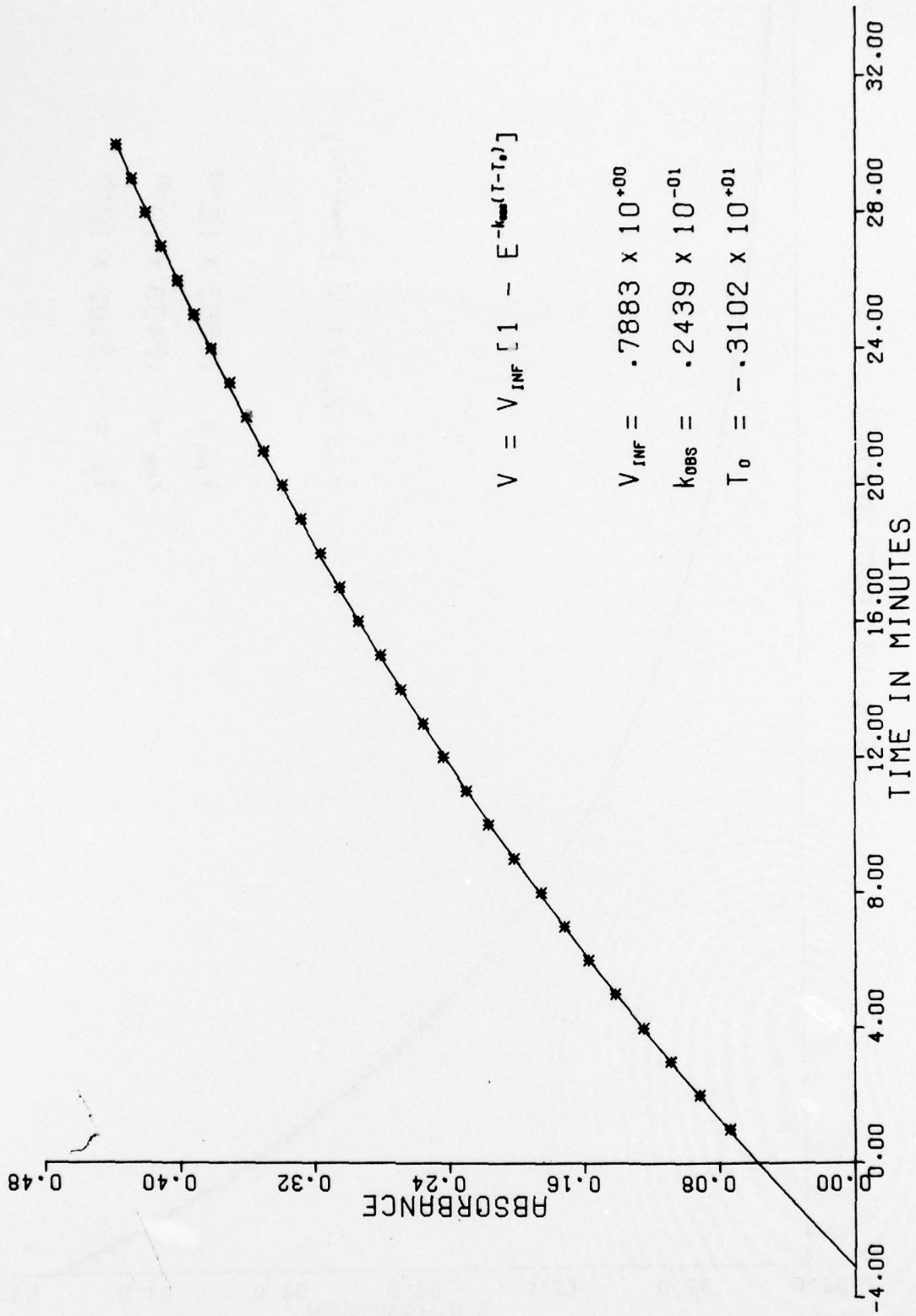
RUN-ID: EXP2854



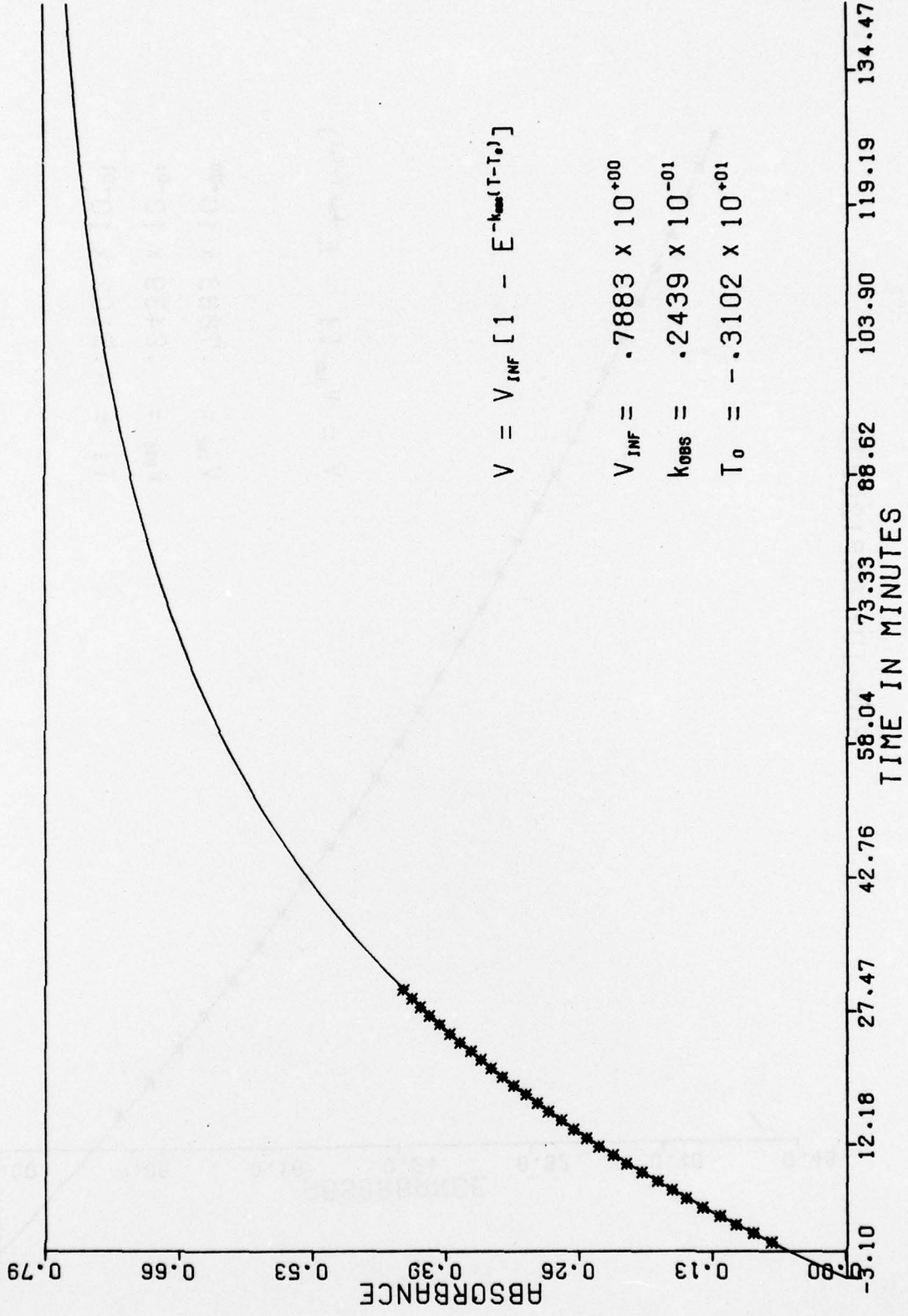
RUN-ID: EXP2854



RUN-ID: EXP120376



RUN-ID: EXP120376



B.5. FORTRAN Statements for Program PLOTTEREXPFT

```

C.....C
C.... MEDICA*BIOCHEM.PLOTTEREXPFT .....C
C.....C
C.....C
100 FORMAT (/,IX,T1,' .....
3.....,/)
110 FORMAT (IX,T1,' ENTER YOUR NAME USING SIX CHARACTERS OR LESS.')
120 FORMAT (A6)
130 FORMAT (IX,T1,' WHAT IS THE DATA-ID?')
140 FORMAT (IX,T1,' @ERS')
150 FORMAT (IX,T1,' @ASG.T 8.,F2')
160 FORMAT (IX,T1,' @FOR.N MEDICA*BIOCHEM.E XPFITPLOT.TPFS.')
170 FORMAT (IX,T1,' @MAP.N')
180 FORMAT (IX,T1,' IN TPFS.')
190 FORMAT (IX,T1,' LIB MISD*PLOT.')
200 FORMAT (IX,T1,' NEXT DATA-ID?')
210 FORMAT (3A4)
220 FORMAT (IX,T1,' @XGT')
230 FORMAT (IX,T1,' @ADD MEDICA*EXPFITPLOT.',3A4)
240 FORMAT (IX,T1,' @COPY 8.,MEDICA*INHIBPLOT.')
250 FORMAT (IX,T1,' @MSG.W PLOT.',A6,',X3836')
260 FORMAT (IX,T1,' @PLOT.N MEDICA*INHIBPLOT.')
270 FORMAT (IX,T1,' -----')
280 FORMAT (IX,T1,' NOW TYPE IN #@ADD TEMP.#')
WRITE (6,100)
WRITE (6,110)
READ (5,120) NAME
WRITE (6,130)
WRITE (16,140)
WRITE (16,150)
WRITE (16,160)
WRITE (16,170)
WRITE (16,180)
WRITE (16,190)
GO TO 300
290 WRITE (6,200)
300 READ (5,210) IDRUN,ID2,ID3
IF (IDRUN.EQ.'NONE') GO TO 310
WRITE (16,220)
WRITE (16,230) IDRUN,ID2,ID3
WRITE (16,240)
WRITE (16,250) NAME
WRITE (16,260)
WRITE (6,270)
GO TO 290
310 WRITE (6,100)
WRITE (6,280)
WRITE (6,100)
WRITE (16,140)
STOP
END

```

B.6. Sample of Teletype Interactive Output for Program EXPFITPLOT

```
#SITE-ID
*EDGEWOOD ARSENAL 1108 SYSTEM VER. 31-244 UPD D(RSI)*
@PUN JALONE, COST-CODE, PROJECT-ID, 30, 1000
DATE: 062176      TIME: 170818
>@ADD MEDICA*PLOTEXPFIT.
FURPUR 0026-06/21-17:08
READY
READY
FOR S0E3-06/21/76-17:08:46 (0.)
END FOR
MAP28R1 RL71-3 06/21/76 17:08:50
END MAP
```

\*\*\*\*\*

```
ENTER YOUR NAME USING SIX CHARACTERS OR LESS.
>JALONE
WHAT IS THE DATA-ID?
>EXP2854
```

```
-----
NEXT DATA-ID?
NONE
```

\*\*\*\*\*

```
HOW TYPE IN "@ADD TEMP."
```

\*\*\*\*\*

```
>@ADD TEMP.
FURPUR 0026-06/21-17:10
READY
FOR S0E3-06/21/76-17:10:39 (0.)
END FOR
MAP28R1 RL71-3 06/21/76 17:10:50
END MAP
```

\*\*\*\*\*

```
PLOT PARAMETERS FOR EXP2854
A. ZERO = 2.00 INCHES  ASYMP = 5.00 IN.  ASLEN = 7.50 IN.
B. ZERO = 1.05 INCHES  ASYMP = 6.00 IN.  ASLEN = 9.45 IN.
```

```
-----
THE PLOTTING FILE HAS BEEN COMPLETED.
```

\*\*\*\*\*

```
FURPUR 0026-06/21-17:11
      1 BLOCK COPIED
```

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