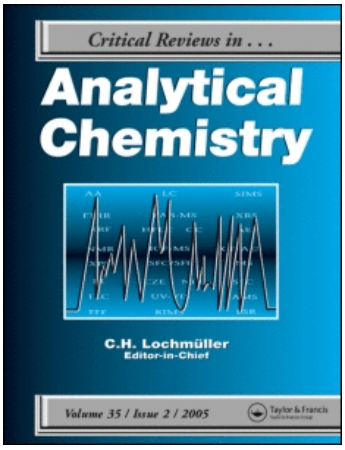

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REACTION-RATE METHODS OF CHEMICAL ANALYSIS

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I. INTRODUCTION

We are now witnessing the birth of a new generation of elegant, completely automated and computer-controlled chemical instrumentation. It seems certain that these new systems will add a new perspective to analytical chemistry and laboratory operation. But what has this to do with a discussion of reaction-rate (or kinetic) methods of analysis? Just this – the rather recent breakthroughs in instrumentation make it just as easy to obtain sensitive quantitative chemical results using reaction-rate methods as with conventional stoichiometric (equilibrium or end-point) methods. By eliminating the barriers imposed by difficult laboratory rate techniques it now becomes very worthwhile to consider the inherent advantages of reaction-rate methods.

So first let us consider what the inherent advantages of reaction-rate methods might be and then also note some of their possible limitations compared to endpoint or equilibrium methods.

A. Advantages of Reaction-Rate Methods

With rate methods it is often possible to measure, immediately after mixing the reactants, the rate of change of some parameter P of the reactant whose concentration is to be determined, or another reactant or product of the reaction, and not wait for the reaction to go to completion (equilibrium). This saving in time may or may not be significant, depending on the specific reaction, but there are good examples[1-8] of obtaining quantitative rate results in seconds for selective reactions that would have required many minutes or hours to go to completion. This is especially true for many of the highly selective enzymatic reactions.

Because it is possible to obtain quantitative rate data shortly after the reagents are mixed, the measurement may be completed before interfering side reactions start. This can be a distinct advantage in providing higher accuracy for some determinations.

One of the most important characteristics of the reaction-rate method is that it involves a relative measurement. The absolute value of the parameter (absorbance, cell potential, fluorescence, etc.) chosen to monitor the reaction does not have to be measured accurately. It is only necessary to measure the parameter's rate of change with time accurately. Hence, even for very rapid reactions, the reaction-rate method can offer freedom from those interferences which contribute to the absolute value of the parameter (turbidity, dirty cells, junction potentials, other fluorescing materials, etc.), but which do not enter the chemical reaction and do not contribute to the rate of change of the parameter with time.

Then, of course, there are those applications where it is the rate per se, rather than the absolute concentration of a specific species, which is the important quantity to be determined. Most noteworthy in this category is the determination of *enzyme activity*.

Another possible advantage of kinetic methods is that they sometimes provide a means of determining the concentration of two or three consti-

tuents of closely related chemical properties without physical separation. As a rule of thumb, the successful development of differential rate methods requires that the first order rate constants of the individual components differ by at least a factor of ten. For example, silicate and phosphate in mixtures have been determined by a differential rate procedure based on the formation reactions of the heteropolymolybdate and the reduced heteropolyblues.[9]

B. Limitations of Reaction-Rate Methods

There are some limitations in the general application of reaction-rate methods. The most important is that imposed by the reaction rate itself. The half time of the reaction must be greater than the mixing time of the instrumental system available. Considering the other extreme, very slow reactions with half times greater than a few hours are not practical for routine analysis. Also, the accuracy and precision of the measurement depend upon good reproducibility (although not necessarily good accuracy) for all experimental conditions such as temperature, pH, ionic strength, size and shape of reaction vessels, etc.

C. Type of Presentation

Specific applications of rate methods will be only briefly summarized in this presentation, primarily in the form of tables. More details of specific methods are not presented because several detailed books and reviews have already been published on existing procedures.[10-15] Also, the authors are convinced that the general acceptance of rate methods for most chemical laboratories will be greatly dependent on the automated instrumentation now beginning to appear on the market and still under development. Much of this instrumentation information needs to be reviewed. Therefore, this presentation will be largely devoted to a consideration of the basic measurement concepts and specific instrumentation systems for rate methods. We have also attempted to organize the material so as to provide some insights into future trends and possibilities for reaction-rate methods of analysis. The new instrumentation could reduce the time required for mechanistic rate studies by an order of magnitude or more. This, in turn, could lead to the rather rapid development of many reliable reaction-rate methods.

II. GENERAL CONSIDERATIONS IN ENCODING CHEMICAL REACTION–RATE INFORMATION

A. Determination of Glucose

A few practical examples of reaction-rate methods are presented here to illustrate the general considerations involved in the encoding of rate information. The first example is the quantitative determination of glucose using the well-known selective oxidation of glucose in the presence of the enzyme glucose oxidase, as illustrated by Equation 1.

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic Acid} + H_2O_2 \qquad (1)$$

1. Encoding Indirectly via a Coupling Reaction

In our own laboratory we first became heavily committed to the development of rate methods more than a dozen years ago by devising an automated rate measurement system for the determination of glucose in blood serum.[16] For expediency we first chose to determine the rate of change of glucose by indirectly following the rate of formation of H_2O_2. Since the H_2O_2 did not have a physical parameter that could be easily and directly measured, the relatively fast coupling reaction of H_2O_2 with an organic dye was used. This was conventional practice at the time for the end-point methods because the colored reaction product, as illustrated in Equation 2, provided high sensitivity by photometric measurements.

$$\text{Organic dye A} + H_2O_2 \xrightarrow[\text{enzyme}]{\text{Peroxidase}} \text{colored product B} \qquad (2)$$

Unfortunately, the peroxidase enzyme that catalyzed the coupling fast reaction was rather unstable and expensive and the automated 2-point, reciprocal time, rate-measuring system was at first relatively crude. But from this example it became apparent that sensitive and precise quantitative measurements could be realized with the aforementioned advantages over end-point methods if 1. suitable reactions could be selected and controlled, 2. sensitive devices for converting (transducing or encoding) the concentration change for one of the reaction species as a measurable electrical signal could be developed, and 3. reliable electronic systems for rate measurement could be developed.

The glucose reaction then became a test system for demonstrating new encoding systems and rate measurement systems. By using a different fast coupling reaction, as illustrated in Equation 3, it was possible to determine the rate of change of H_2O_2

$$H_2O_2 + 3I^- \xrightarrow[\text{Catalyst}]{\text{Molybdate}} I_3^- + 2OH^- \qquad (3)$$

by following the I_3^- using potentiometric,[17] spectrophotometric,[18] and amperometric[19] encoding (transducer) systems, and thus eliminate the troublesome peroxidase enzyme.

The rate of formation of gluconic acid also could be used to obtain the initial rate information. Therefore, a very sensitive digital pH-stat system[20] was devised wherein the pH was held constant by adding small increments of NaOH during the glucose reaction. The number of increments of base added during a short fixed period of time proved to be directly proportional to the glucose concentration. However, this system is not as sensitive as the methods based on the H_2O_2 coupling reactions.

2. Encoding Directly via the Primary Reaction

The obvious desire to eliminate any type of secondary or coupling reaction also led to the investigation of direct methods for following the rate of change of O_2 (Equation 1). Voltammetric encoding systems that could measure O_2 directly[21-23] were developed, even though the method is not as sensitive as the indirect H_2O_2 color methods. The conversion of glucose or gluconic acid rate information to a directly measured physical parameter has not been reported.

B. Determination of Phosphate

1. Encoding Indirectly via a Secondary Reaction

The determination of phosphate by a rate method also illustrates similar considerations as those for the glucose determination. First, it was found[24] that the classical molybdenum blue procedure could be developed into a sensitive rate method, involving the primary reaction of phosphate with Mo(VI) to form 12-molybdophosphoric acid (12-MPA) and its subsequent reduction to form the heteropolyphosphomolybdenum blue, PMB, as shown in Equations 4 and 5.

$$H_3PO_4 + 6Mo(VI)_t \rightarrow 12\text{-MPA} + 9H^+ \qquad (4)$$

$$12\text{-MPA} + n\text{Red} \rightarrow \text{PMB} + n\text{Ox} \qquad (5)$$

In this case, the measured reaction rate depends on the nature of the reductant, the order of adding reagents, as well as the acidity, etc.[25]

2. Encoding Directly via the Primary Reaction

It was again obvious that it would be advantageous to eliminate the reduction reaction (Equation 5) and convert one of the species in the primary reaction to a physical parameter that could be readily measured. Fortunately, the 12-MPA has a relatively high molar absorptivity at readily available wavelengths. However, for the first time in our development of quantitative reaction rate procedures there was a confrontation with a reaction whose half life was so short that it was necessary to make rate measurements in milliseconds rather than seconds. A rather long development program was thus required to develop the new automated equipment that would provide very rapid mixing and readout of rate information. After the automated system was developed it was then possible to determine phosphate directly[26] utilizing the primary reaction shown in Equation 4. It is feasible to make as many as 3,000 determinations of phosphate concentration per hour with this type of system.

C. General Conclusions

It can be seen from these examples for glucose and phosphate that a sequence of chemical reactions is often used to 1. obtain a chemical species that is related to the rate information and is readily converted to a sensitive measurable signal, or 2. obtain a readily measured chemical species whose rate of formation is slow enough to be measured by available equipment. Although the methods utilizing multiple sequential reactions can be dependable under carefully controlled conditions, almost inevitably they will be less reliable than methods using measurements on one primary reaction.

Much research effort has been invested in providing more sensitive and selective encoding systems and high-speed automated rate-measuring instruments, and a relatively wide choice of methods is now available. The method of choice for encoding the rate information from either the primary or coupling reactions depends, of course, on several factors, including sensitivity, freedom from interferences, simplicity, and dependability.

III. ANALYTICAL KINETIC METHODS

Reaction rate techniques have been used in the development of analytical methods for inorganic, organic, and biological compounds in a variety of complex samples. The number of kinetic methods developed has increased considerably in the last few years. Selected kinetic procedures reported in the literature primarily after 1968 are summarized in Tables 1 to 3. The procedures for the determination of the concentration of inorganic ions given in Table 1 are categorized according to the type of reaction utilized. A significant portion of the methods cited involve the catalytic effect of the analyte on the oxidation of an organic compound. The rate procedures for organic compounds are tabulated according to functional groups in Table 2. The large number of methods cited in Table 3 attests to the popularity of enzymatic methods of analysis. This is explained by the high degree of specificity and sensitivity for substrates such reactions provide, and by the importance of measuring enzyme activity.

A rather novel application of reaction-rate methods is the use of a kinetically controlled reaction as the endpoint indicator reaction for a titration. Both catalysts and inhibitors have been used as indicator systems and the general scheme for catalytic endpoint detection is given below:

$$\text{Titrant} + \text{Sample} \rightarrow \text{Products}$$

$$\text{Indicator Reaction Excess of titrant (catalyst)} \xrightarrow{} \text{Products}$$

After the first reaction is stoichiometrically completed, the indicator reaction proceeds at a rate which is dependent upon the excess of titrant added. The endpoint can be determined from a plot of reaction rate vs. volume of titrant added. The break in the reaction rate – titrant volume curve indicates the endpoint. Kinetic titrations have been used for the determination of metallic ions,[265-268] ligands,[269] and organic acids.[270]

IV. INSTRUMENTATION SYSTEMS FOR REACTION–RATE METHODS

The early work on automating reaction-rate methods primarily provided improved rate measurement devices and sensitive data domain transducers for converting the chemical rate information to measurable electrical signals. Today the attention is being focused on complete systems that start with the raw samples and reagents and end with a formated printed readout in the desired quantitative units. A complete system for obtaining quantitative data is illustrated in Figure 1. By preliminary treatments (dissolution, dilution, filtration, ion exchange, etc.), the sample and reagent solutions are prepared as required for the specific procedures. Predetermined volumes of sample and reagents are then introduced, mixed, and transported to a vessel which serves as the reaction cell. The chemical reaction is monitored by a suitable transducer which converts the information about a specific species inherent in the reaction to a measurable signal in the electrical domain. Frequently several interdomain conversions are required to obtain data in the prefered form (usually digital readout of concentration). Readout can be visually displayed on a digital counter or a printout. When desired, continuous monitoring of the reaction is accomplished using a servo recorder or storage oscilloscope for visual display of the rate curve. Information obtained by continuous monitoring of the entire measurement process can be used to provide feedback control of the process in an experimenter-specified manner using an appropriate control system.

V. SAMPLE AND REAGENT HANDLING

The weakest links in instrumentation systems in the recent past have been the sample and reagent preparation and the aliquoting and mixing systems. Therefore, the new developments in these areas will be discussed in some detail. In fact, it becomes apparent that the greatest differences in completely automated reaction-rate instruments will probably be in the methods of sample and reagent preparation, and in aliquoting and mixing.

These operations not only are tedious, repetitious, and time demanding when done manually, but also are subject to human error and bias. It is not unusual that the sample handling procedures require much more time than the rate measurement itself. This is not a problem exclusively associated with the development of reaction-rate methods; rather, it is an important consideration in the development of all analytical methods.

TABLE 1

Determination of the Concentration of Inorganic Ions by Kinetic Methods

Type of reaction	Reagents	Analyte ion	Sensitivity	Interferences	Ref.
A. Catalytic effect on oxidation of an organic compound					
1. Oxidation of an organic dye by H_2O_2					
	H_2O_2-Indigo carmine	Cr(VI)	6×10^{-3} μg/ml	Fe(III), Cu(II)	27
	H_2O_2-*o*-diansidine	Cr(VI)	2×10^{-3} μg/ml		28
	H_2O_2-Pyrocatecol violet	Co(II)	1 ng/ml		29
	H_2O_2-Bordeaux S	Co(II)	ng/ml Range		30
	H_2O_2-9-phenyl-3,4,7 dihydroxy-fluorone	Co(II)	ng/ml Range		31
	H_2O_2-Tiron	Co(II)	0.6-10 ng/ml		32
	H_2O_2-3,4 diamino-phenol	Cu(II)	0.2 μg/ml		33
	H_2O_2-Congo Red	Cu(II)	10^{-3} μg/ml		34
	H_2O_2-Acid chrome dark blue	Fe(III)	2×10^{-3} μg/ml		35
	H_2O_2-Pyrocatechol violet	I^-	10-100 ng/ml		36
	H_2O_2-Alizarin S	Mn(II)	0.3-56 ng/ml		37
	H_2O_2-Pyrocatechol violet	Mn(II)	2×10^{-2} μg/ml		38
	H_2O_2-Rubeanic acid	Mo(VI)	2×10^{-3} μg		39
2. Oxidation of an organic dye by $K_2S_2O_8$	$K_2S_2O_8$-*p*-nitrodiazo-aminobenzoate	Ag(I)	5×10^{-3} μg/ml	CN^-,SCN^-,I^- Br^-	40
	$K_2S_2O_8$-pyrocatechol	Pb(II)	10 ng/ml	Ag(I),Cd(II), Co(II),Cu(II), Mn(II),Ni(II)	41
	$K_2S_2O_8$-3,4-dihydroxy-azobenzene	Pb(II)	10 ng/ml		42
	$K_2S_2O_8$-stilbaze				
3. Oxidation of an organic compound	H_2O_2-hydroquinone and pyridine	Cu(II)	10^{-3} μg/ml		43
	H_2O_2-Lucigenin	Ag(I)	3 ng/ml		44
		Bi(III)	10 ng/ml		
		Co(II)	0.5 ng/ml		
		Cr(III)	20 ng/ml		
		Cu(II)	20 ng/ml		
		Mn(II)	50 ng/ml		
		Ni(II)	200 ng/ml		
		Pb(II)	10 ng/ml		
	$KBrO_3$-methurin	Cr(VI)	3×10^{-3} μg/ml		45
	$KBrO_3$-5-amino-N-phenylanthranilic acid	V(V)	2×10^{-2} μg/ml		46

Table 1 (continued)

Type of reaction	Reagents	Analyte ion	Sensitivity	Interferences	Ref.
	$KBrO_3$-I° aromatic amines	V(V)	10^{-6} *M*		47
	O_2-Furfuraldehyde	Cu(II)	10^{-6} *M*		48
	O_2-L-ascorbic acid	CN^-	10^{-7} *M*		49
	O_2-Methyl orange	Fe(III)	$2x10^{-2}$ μg/ml		50
	IO_4^--Tris(1,10-phenanthroline)-Iron(II)	Ru(III)	10^{-9} *M*		51
B. Catalytic effect on an inorganic redox reaction					
1. Oxidation of I^-	BrO_3^--I^-	V(V)	$1-3x10^{-2}$ μg/ml		52
	H_2O_2-I^-	Cr(VI)	0.6 μg		53
	H_2O_2-I^-	Mo(VI)	$5x10^{-2}$ μg/ml		54
	H_2O_2-I^-	Mo(VI)	0.25 μg		55
	H_2O_2-I^-	W(VI)	0.5 μg		56
	MoO_3^--I^-	Ge(IV)	1 μg/ml		57
	MoO_4^{2-}-I^-	PO_4^{3-}	$5x10^{-3}$ μg/ml		58
2. Reduction of Ce(IV)	Ce(IV)-As(III)	Ag(I)-Hg(II)	10^{-8} *M*		59
		I^-	10^{-8} *M*		
		Os(VIII)	$3x10^{-10}$ *M*		
	Ce(IV)-As(III)	Os(VIII)	10^{-9}-10^{-11} *M*		60
	Ce(IV)-As(III)	Os(VIII)	160 ng/ml		61
	Ce(IV)-Hg_2(II)	Au(III)	$0.5\text{-}4.5x10^{-7}$ *M*		62
	Ce(IV)-Hg_2(II)	Ir	10^{-1} μg/ml		63
	Ce(IV)-Tl(I)	Tl(I)			64
3. Other redox reactions	Ag(I)-Fe(II)	S^-	10^{-4} μg/ml		65
	$NaNO_2$-$Fe(SCN)^{+2}$	I^-	$2x10^{-3}$ μg/ml		66
	MnO_4^--acetodiphosphoric acid	Ni(II)	0.25 μg/ml	Ag, Co, Cu	67
	SeO^{2-}-Sn(II)	Mo(VI)	$1x10^{-3}$ μg/ml		68
C. Reaction involving complexation					
1. Formation of heteropoly acid	Molybdate, Phosphate, ascorbic acid	Bi(III)	40 ng/ml		69
	Mo(VI), ascorbic acid	PO_4^{3-}	0.38 μg/ml		25, 71,72,76
	Mo(VI), HNO_3	PO_4^{3-}	0.1 μg/ml P		26
2. Ligand substitution reactions	Methyleneglycol bis (2 amino ethyl-ether)N,N,N,N' tetroacetic acid + 4-(2 pyridylazo)-resorcinol	Co(II)-Ni(II) Co(II)-Fe(II) Cu(II)-Pb(II) Ni(II)-Fe(II) Zn(II)-Cd(II)	10^{-6} *M* levels		73
	Me-CyDTA and acid or exchange metal	Lanthanides Transition metals			6
		Group II metals	10^{-6} *M*		
		Group III metals			

Table 1 (continued)

Type of reaction	Reagents	Analyte ion	Sensitivity	Interferences	Ref.
	Me-CyDTA and Pb(II)	Mixtures of Mg(II),Ca(II) Sr(II),Ba(II)			7
3. Catalytic effect on coordination reactions		Cu(II)	10^{-6}-10^{-8} *M*	Ag(I),Hg(II), Fe(III) Masked with $S_2O_3^{2-}$,CN^-, and Chloral-hydrate	74
	Pentacyanocobaltate(II)	O_2	±0.003 μg/ml		75
	$ZrOCl_2$-Xylenol Orange	F-	5×10^{-3} μg/ml		76
	Zr-Methylthymol Blue	SO_4^{2-}	0.1 μg/ml		77
D. Inhibitory effect on redox reaction	Me-CN complex, cyanohydrin o-dinitrobenzene	Ag(I)	0.2 μg/ml		78,79
		Cd(II)	1 μg/ml		
		Co(II)	0.06 μg/ml		
		Cu(II)	0.003 μg/ml		
		Ni(II)	1 μg/ml		
		Zn(II)	1 μg/ml		
	Ce(IV)-As(III)	Ag(I)	10^{-8} *M*		59
		Hg(II)	10^{-8} *M*		

TABLE 2

Determination of the Concentration of Organic Compounds by Kinetic Methods

Functional group	Analyte	Type of reaction or method	Sensitivity	Ref.
A. Alcohols		Alkaline hydrolysis of alcohols' 3,5 dinitro-benzoate esters	0.02 mg	80
B. Glycols and polyols	Glycol mixtures	Difference in rate for cleavage of glycols with Pb(IV)	1:1000 ratio	81
	Polyols	Reaction with phenyl-isocyanate		82
C. Phenolic Compounds	Phenols	Reaction with *N*-(benzene sulfonyl) quinonimine	1μg/ml	83
	Phenols	Controlled bromination	1 μg/ml	84
	α-naphtol and β-naphtol	Reaction with KBr and $KBrO_3$	1 μg/ml	85
D. Carbonyls	Binary ketone mixtures	Reaction with hydroxyl-amine hydrochloride	10^{-5} moles	87
E. Amino acids and proteins	Mixtures of tryptophan and tyrosine	Time-resolved phosphorimetry		88
	R.N.A.	Cu(II) catalyzed orcinol reaction	2 μg/ml	89
F. Amines and amides	Ethanol amides	Differential saponification rates		90
	o-phenylendiamine	Amperometric titration using catalytic electrode reaction for endpoint determination	$10^{-6}M$	91
F. Amines and amides	Thiourea, N-methyl urea *N*-ethyl urea *N*-phenyl urea *N,N'*-diphenyl urea *N*-alkyl urea	Induction of reaction $2\,NaN_3+I_3^- \rightarrow 3I^-+3N_2$	2μg	92
	Creatinine	Reaction with alkaline picrate solution	10 μg/ml	93
	2-amino ethanethiol ethylene diamine	Reaction with Cu(II) which acts as catalyst for redox reactions	$1x10^{-5}$ *M* $2x10^{-6}$ *M*	94
G. CN-containing organic compounds	CN-containing organic compounds	Hydrolysis. The CN^- formed catalyses reduction of *o*-dinitrobenzene	0.006-10 μg/ml	78
H. Organophosphorous compounds	Organophosphorous esters	Organophosphorous esters react with an excess of hydroxamic acid The concentration of nonreacted acid is determined by measuring the rate of splitting of an acylating agent by the hydroxamic acid.	0.5 μg/ml	95

TABLE 3

Enzymatic Analyses

Analyte class	Analyte	Reagent (enzyme substrate)	Method	Sensitivity	Ref.
A. Substrates					
1. Alcohols and esters	Ethanol	Alcohol oxidase	Fluorometric	0.1 μg/ml	96,98,100
		Alcohol dehydrogenase	Fluorometric	0.1 μg/ml	99,97
	Acetylcholine	Acetylcholine esterase	Paper strip		102
	Xanthine and hypoxanthine	Xanthine oxidase and uricase	Spectrophotometric	2 μg	103,106,105
2. Amines and amino acids	Asparagine	*E. coli* L-asparaginase	Colorimetric	0.05 μmol	106
	L-amino Acid	L-amino acid Oxidase	Enzyme electrode		107
	Creatinine	Creatinine	Colorimetric	μg/ml	108,109
	Urea	Urease	Enzyme immobilized on NH_4^+-ion electrode	6 μg/ml	110-112
	Urea	Urease, glutamate dehydrogenase α-ketoglutamate			113,114
	Urea	Urease	Colorimetric	0.1 μmol	115
	Amines and amino acids (Asparagine, glutaminase, urea, etc.)	Asparaginase glutaminase, ureases, etc.	Electrochemical-cation electrode	0.5 μg/ml	116
3. Carbohydrates	Glucose	Glucose oxidase peroxidase	Fluorometric	0.03 μg/ml	117,118

TABLE 3 (continued)

Enzymatic Analyses

Analyte class	Analyte	Reagent (enzyme substrate)	Method	Sensitivity	Ref.
		Glucose oxidase, peroxidase and dye	Spectrophotometric	1 μg/ml	119,133
	Glucose	Hexokinase-Glucose-6-phosphate dehydrogenase system	Fluorometric or spectrophotometric	0.05 μg/ml	134-139,86
	Glucose (in biological fluids)	Glucose oxidase	Polarographic (O_2 sensor)	0.5 μg	21,22,142
	Galactose	Galactose oxidase, peroxidase, Dye	Spectrophotometric	0.9 μg	143
		D-Galactose dehydrogenase	Spectrophotometric	0.10 μmol	144
	Lactose	β-galactosidase	Spectrophotometric	0.1 μmol	145
4. Organic Acids	Citrate	Malate dehydrogenase and NADH	Fluorometric	10^{-12} mol	146
	Free and conjugated 3-hydroxyanthranilic acid	3-hydroxyanthranilate-oxygenoxyreductase	Spectrophotometric	0.45 μg/ml	147
	Galacturonic acid	Isomerase and Dehydrogenase	Spectrophotometric	5 μg	148
	Lactate	Lactate dehydrogenase	Enzyme electrode	0.5 m mol	149
	Pyruvic acid	Lactic dehydrogenase	Spectrophotometric	10 μmol	150
	Uric acid	Uricase	Spectrophotometric (Technicon Autoanalyzer)	0.4 μmol/ml	117,152,153

TABLE 3 (continued)

5. Aldehydes and Ketones	Aldehydes	Carbonic Anhydrase	Hydration		154,155
	D-glyceraldehyde and dihydroxyacetone	Glycerol kinase and Triokinase	Spectrophotometric	0.02 μmol/ml	156
6. Inorganic Substances	Ammonia	Oxoglutarate glutamic dehydrogenase-NADH	Fluorometric	4×10^{-11} *M*	157
	Inorganic pyrophosphate	UDP-glucose pyrophosphorylase, Pyroglucomutase, Glucose-6-phosphate dehydrogenase	Fluorometric	2×10^{-12} *M*	158
B. Enzymes					
1. Deaminases	Asparaginase Glutaminase Urease	Asparagine glutamine urea	Electrochemical-cationic electrodes	1.0×10^{-3} I. U.	116
2. Dehydrogenases	Dehydrogenases-general	Resazurin, NAD or NADP	Fluorometric	10^{-4} I. U./ml	159,160,161
	Hydroxybutyric dehydrogenase	α-hydroxybutyrate, NAD^+ and dye	Colorimetric – NADH formed reduces dichloroindophenol		162
	Glucose-6-phosphate dehydrogenase	NADP	Fluorometric-follow formation of NADPH	10^{-2} I.U./ml	163,164
	Lactate dehydrogenase	Lactate and NAD^+ or pyruvate and NADH	Colorimetric-follow NADH		165,168
	Lactate dehydrogenase		Fluorometric	10^{-2} I.U./ml	169

TABLE 3 (continued)

Analyte class	Analyte	Reagent (enzyme substrate)	Method	Sensitivity	Ref.
3. Esterases	Amylase	Cibachron Blue F3GA-amylose	Colorimetric	50 I.U./ml	170,171
	Amylase	Starch-^{125}I-^{127}I.	Radiochemical		172
	Cellulose	Fluorescein dibutyrate, α- and β-Naphthyl acetates, Indoxyl acetate, Resorufin butyrate	Fluorometric	1.0×10^{-4} I.U.	173
	Cholinesterase	Comparison of Acetyl thiocholine, Butyrlthiocholine, *o*-nitrophenylbutyrate	Colorimetric	7 I.U./ml	174,175
		Acetylcholine and Bromthymol blue	Colorimetric – indicator papers		176,177
		Acetate, propionate, and butyrate esters of *N*-methylindoxyl, umbelliferone and 4-methyl umbelliferone compared	Fluorometric	5×10^{-5} I.U./ml	178

TABLE 3 (continued)

Lipase	4-methyl umbelliferone heptanoate	Fluorometric	2×10^{-5} I. U.	179
	N-methylindoxyl myristate	Fluorometric	2×10^{-4} I.U./ml	180,181
Aminopeptidase	*l*-leucyl-β-naphthylamide	Spectrophotomteric		182
Phosphatase	*p*-nitrophenylphosphate	Spectrophotometric		183
Alkaline phosphatase	*p*-nitrophenylphosphate	Colorimetric	4.5×10^{-2} I.U./ml	167,188,189
Alkaline and acid phosphatase	Umbelliferone phosphate	Fluorometric	10^{-6} I.U. Alk. Phosphatase, 10^{-5} I.U. Acid phosphatase	190
	Naphthol AS-BI AS-MX and AS-IR phosphates	Fluorometric	5×10^{-4} I.U. Alk. Phosphatase 2×10^{-6} I.U. Acid phosphatase	191
Glucose-6-phosphatase		Radiometric		192,193
Sulphatases	Fluorescein sulphate β-naphthyl-sulphate Umbelliferone sulphate N-methyl indoxyl sulphate	Fluorometric	10^{-6} μg/ml	180,194
Urease	Urea	Colorimetric	25 μg	195

TABLE 3 (continued)

Enzymatic Analyses

Analyte class	Analyte	Reagent (enzyme substrate)	Method	Sensitivity	Ref.
4. Kinases, transmitases and transferases	Creatine kinase	Coupled Hexokinase-glucose-6-phosphate dehydrogenase	Fluorometric		196,197
	Creatine phosphokinase	Phosphocreatine, Glucose, Hexokinase	Fluorometric or spectrophotometric		162,167 198,199
	S.G.O.T.	Oxaloacetic acid coupled with fast pancreau	Colorimetric		200
	S.G.O.T.	Oxaloacetic acid coupled with NADH catalyzed reaction	Colorimetric-oxaloacetic acid oxidizes NADH in presence MDH to nonadsorbing NAD^+	8×10^{-3} I.U./ml	201
	S.G.O.T.	Oxaloacetic acid	Colorimetric		167,202
	S.G.P.T.		Colorimetric		167
5. Oxidases	All oxidative enzymes	Homovanillic acid	Fluorometric-follow formation of 2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic acid	10^{-5} I.U.	203
	All oxidative enzymes	*p*-hydroxyphenyl acetic acid	Fluorometric	10^{-5} I.U.	117
	Alcohol oxidase	Alcohols-*p*-hydroxyphenyl acetic acid and peroxidase	Fluorometric	2.0×10^{-4} I.U.	100

TABLE 3 (continued)

	Amine oxidases	Amines, *p*-hydroxyphenyl acetic acid	Fluorometric	μg range	140,141 204,205 206
	Amino acid oxidases	Homovanillic acid	Fluorometric	9.0×10^{-5} I.U.	207
	Galactose oxidase	Galactose	Electrochemical-0_2 electrode	0.5 μg	208,209
	Peroxidase	Leuco 2,3',6-trichloroindophenol	Spectrophotometric		210
6. Reductases	Azoreductase	1,2 Dimethyl-4-(*p*-carboxyphenolazone)-5-hydroxybenzene	Colorimetric		211
C. Activators and coenzymes					
1. Cation activators	Ba^{2+}	Alkaline phosphatase reactivated	Spectrophotometric	14 μg	212
	Ca^{2+} and Zn^{2+}	Apo enzyme of calf intestinal alkaline phosphatase	Spectrophotometric	1 μg Ca 6 ng Zn	213
2. Anion activators	CN^-,I^-,S^{2-}	Invertase	Polarimetric-based on anion decrease of inhibitory effect of Ag(I) and Hg(II)	$10^{-8}M$ Ca^{2+} $10^{-7}M$ S^{2-} 0.1 μg I^-	214
Coenzymes	Cyclic 3',5'-AMP (in mammalian tissues	Phophodiesterase, myokinase and pyruvate kinase	Fluorometric	$0.5 \times 10^{-8}M$	215
	Cyclic AMP (in fat cells)		Radiometric		96
	ATP	Luciferase reaction	Fluorometric	10^{-14} mol	216-219

TABLE 3 (continued)

Enzymatic Analyses

Analyte class	Analyte	Reagent (enzyme substrate)	Method	Sensitivity	Ref.
	FMN (in photobacteria)	Luciferase and Dodecylaldehyde	Photometric	10^{-4} μg/ml	220
	NADPH	NADPH Specific enzyme	Fluorometric or colorimetric	0.3 n mole	221
D. Inhibitors					
1. Pesticides	DDT	Pancrease	Fluorometric	10 μg/ml	222
	Lindane	Lipase		0.8 μg/ml	
	Aldrin			0.1μg/ml	
	Heptaclor			0.1 μ/ml	
	Sevin			0.1 μg/ml	
2. Metalic Ions	Hg(II)	Glucose oxidase	Colorimetric	0.1 μg/ml	223
		Invertase	Polarimetric	$2 \times 10^{-8} M$	224
		Urease	pH-Stat method	$2 \times 10^{-7} M$	225
		Alcohol-dehydrogenase	Fluorometric	2 ng	226
	Zn(II)	Alkaline phosphatase	Fluorometric or spectrophotometric	0.6 μg	227
	Be(II)	Phosphatase	Spectrophotometric	18 ng	227
	Ag(I)	Alcohol-dehydrogenase	Fluorometric	1 ng	226
	Ag(I)	Urease	pH-Stat method	2×10^{-8}	225
	Ag(I)	Invertase	Polarimetric	2×10^{-7}	224
	Cu(II),Cd(II) Co(II),Ni(II) Mn(II),Pb(II)	Urease	pH Stat method	2×10^{-6}	225

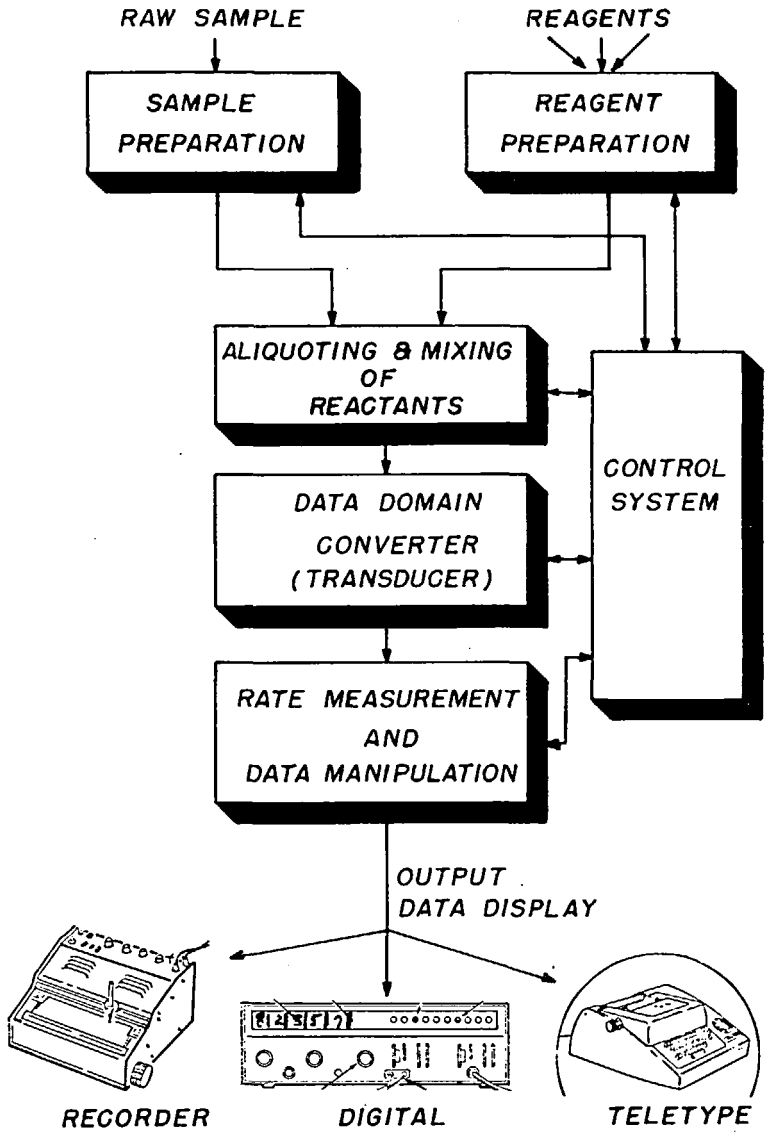

FIGURE 1. Block diagram of a complete instrumentation system for reaction-rate methods.

A. Sample Preparation

In many cases the raw sample is not in suitable form for direct aliquoting and mixing with reagents. The sample preparation system must provide treatment operations which include dissolution in the case of solid samples, dilution, pH adjustment, extraction, removal of suspended particles, and removal of interfering constituents associated with some complex matrices.

B. Reagent Preparation

The reagent preparation system, in addition to simply preparing reagents of the required concentration and pH which can be individually dispensed, can be used to dispense and mix appropriate volumes of each reagent to form a single *composite* reagent for the analysis. It is desirable to have this operation automated since such *composites* frequently have limited stability.

C. Aliquoting and Mixing of Reactants

Considerable research, design, and engineering activities are currently being directed to automation of aliquoting and mixing of reactants. A variety of dispensing pipets and valving systems that can be manipulated by a suitable control center have been developed. A number of ap-

proaches have been taken to incorporate such devices into aliquoting and mixing systems. Most of these systems can also be used to implement some of the operations required in reagent and sample preparation. However, further developments are necessary for implementing operations such as digestion and separations which are not easily automated with the devices currently available.

The first sample and reagent handling approach taken was merely that of manually pipeting sample and reagents into a mixing chamber which frequently served as the reaction vessel and observation cell. For slow reactions, it was not unusual to mix the reactants externally in a suitable vessel and then manually transfer the contents to the observation cell. Semiautomated instrumentation which employs manual sample and reagent handling is still commercially available. For example, the Digicon® system (Sherwood Medical Industries, Inc., St. Louis, Missouri) provides automatic measurement of initial rates of chemical reactions and digital printout of concentration but requires that the sampling process be executed manually. Sample and reagents are manually pipeted into the reaction chamber where they are mixed by a magnetic stirring bar. The reactant mixture is pulled by vacuum into a cuvette where the kinetic measurement is made. A manually activated pump and vacuum system then flushes out the old solution and rinses the cuvette with water.

1. Continuous Flow Methods

One of the first approaches taken to automated sample and reagent processing was associated with the development of continuous flow methods. The Technicon Autoanalyzer®, shown schematically in Figure 2, was the first widely accepted system which automated sample and reagent handling. Samples and reagents, segmented by air bubbles, are aspirated by means of a proportioning pump which compresses the plastic tubing carrying them. This is the heart of the system, in that it determines and maintains temporal and volumetric relationships throughout the modular system. If necessary, dialysis is performed on stream to

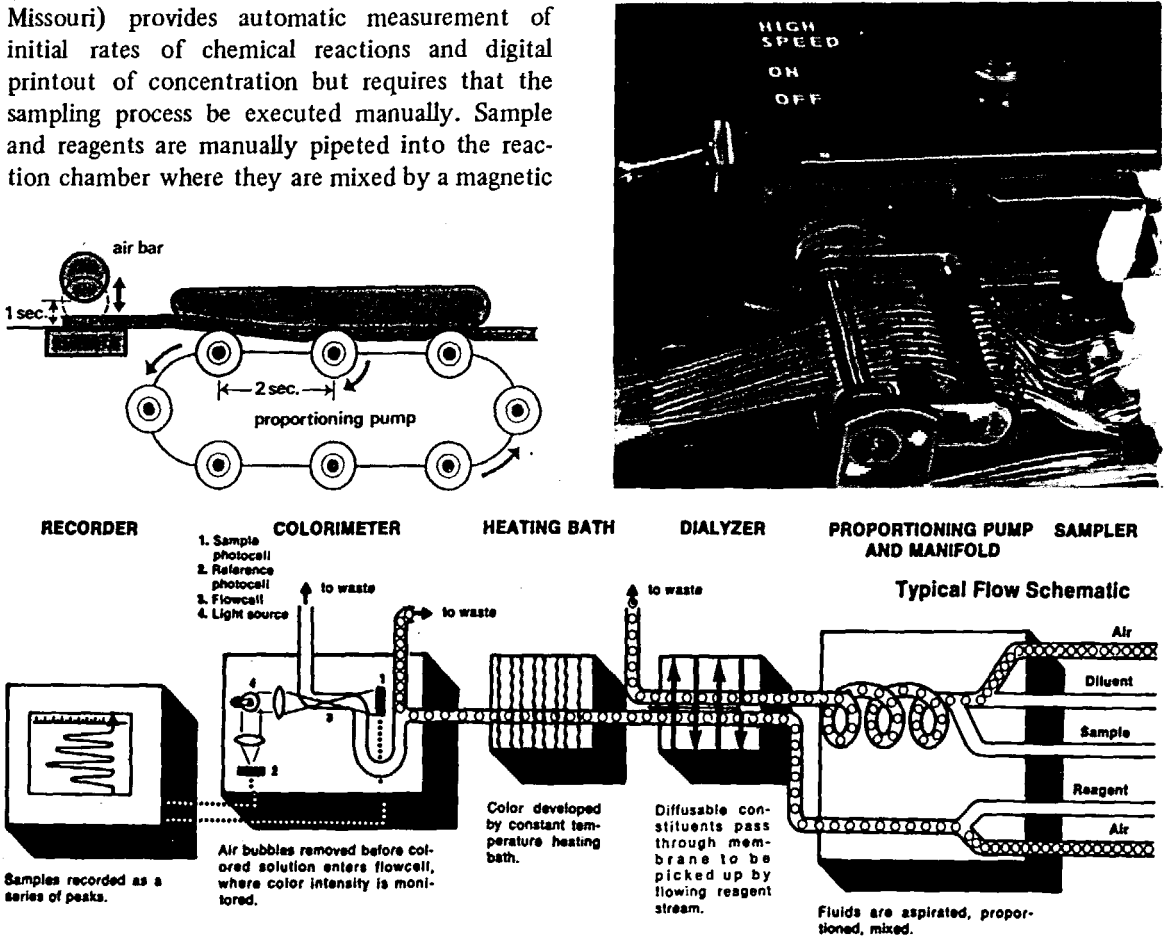

FIGURE 2. Upper left and right – peristaltic proportioning pump. Bottom – schematic of basic AutoAnalyzer modular system. (Courtesy of Technicon® Instruments Corp., Ardsley, New York.)

remove some interfering constituents (suspensions, proteins, etc.), and the sample stream is combined with the color developing reagent stream. Heating and incubation are accomplished by passing the stream through a constant temperature heating bath prior to entry into the flow cell of the colorimeter.

While such continuous flow systems have enabled laboratories to handle a rapidly expanding workload, they suffer from several disadvantages which are inherent in any continuous flow technique. Carry-over effects between samples with widely differing values can occur, and such systems require large volumes of sample and reagents, which is equivalent to a loss in sensitivity compared with an analogous discrete procedure. Generally, meticulous maintenance is required to insure proper operation of all of the mechanical parts to avoid errors due to clogging and leakage in the dialyzer and heating coils. The use of caustic reagents requires frequent replacement of tubing to insure that the proper volumes of reactants are aspirated into the system.

2. Discrete Sample Handling

a. Basic Approach

An alternate instrumental approach incorporates methods which allow for discrete volumes of sample and reagents to be measured and mixed in a reaction vessel. Such an approach requires the use of one or more automatic pipets for the introduction of samples and reagents.

One of the first automated discrete sample and reagent handling systems, developed by Malmstadt and Pardue,[5] used an automatic washout pipet to aliquot the sample and two injection pipets, one for flushing the sample from the washout pipet and diluting, and one for aliquoting composite reagent. In this system, deproteinizing reagents are manually added to the sample, the sample mixed, and the solution immediately drawn by vacuum into the measuring pipet through a glass-fiber filter which retains the precipitate. When the calibrated tip is filled with filtrate, the 3-way stopcock is turned to connect the tip with the sample injection pipet. The filter is knocked off and the tip of the washout pipet is moved over the sample cell. A fixed volume of water is automatically drawn into the injection pipet and dispensed, thus flushing the sample from the stem of the washout pipet into the sample compartment. An aliquot of the composite reagent is automatically delivered to the sample compartment by a second injection pipet. The reactants are mixed by means of a magnetic stirring bar in the sample compartment. Upon completion of the potentiometric rate measurement, the reaction mixture is removed by an aspiration tube.

b. Commercial Pipeting Station

Among the newer commercial sample and reagent handling instrumentation is a modular automated pipeting station (Micromedic Systems, Inc., Philadelphia, Pa.) which can dilute sample or dispense two reagents through a common delivery tip (Figure 3). The pipeting unit consists of two

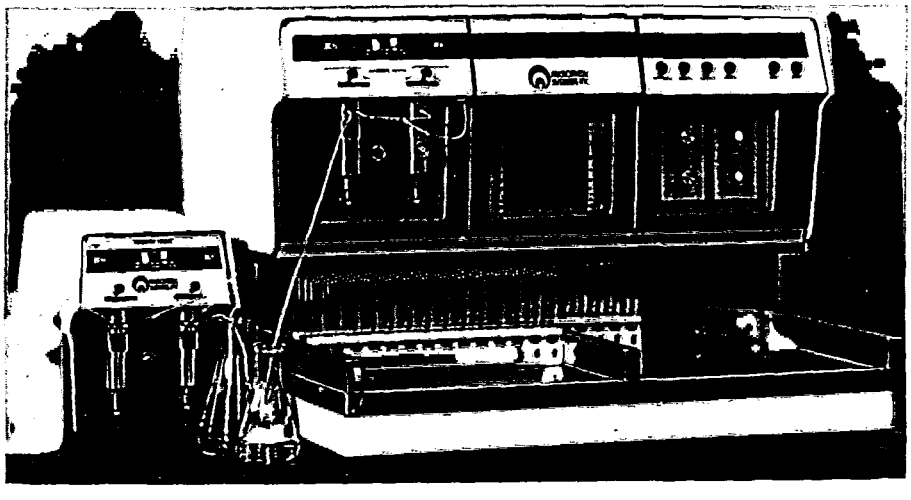

FIGURE 3. The Micromedics Pipeting Station (Courtesy of Micromedic Systems Inc., Philadelphia, Pennsylvania).

positive displacement pumps which provide liquid measurement with a relative standard deviation of less than 0.5% for consecutive delivery and with an accuracy to within 2% of the volume index settings. Each pump can be set independently to deliver from 0 to 100% of full stroke volume by varying the length of the piston stroke. Carry-over is reduced by specially fabricated tips and automatic wiping of the outside of the pipet tip with a reel-fed disposable absorbent paper. The liquids are dispensed into test tubes placed on a moving rack. The module can be used in conjunction with instruments which have an aspirating sample-feed mechanism.

c. Single Digital Pipet Operated Sequentially

A system that employs a single digital pipet has been developed by Eggert, et al.[228] as a part of a computer controlled measurement system designed to perform kinetic experiments. A block diagram of the hardware used for ELLA (Experimental LINC Laboratory Analytical System), excluding the computer I/O devices, is shown in Figure 4. The reactants are placed on the sample tray in the order of desired addition to the system. For example, for an enzymatic experiment the order is buffer, enzyme, buffer, substrate, buffer. The initial volume of enzyme to be aliquoted and incubation time required prior to rate measurement are specified via a teletype by the experimenter in response to the system's programmed request. The thermostated digital pipet sequentially draws reactants through a dip tube from the turntable and into a holding coil which is also thermostated in a heating bath. The volumes of solutions to be picked up and delivered are determined by the number of clock pulses received from the accumulator buffer of the computer (DEC PDP-12). These impulses activate a stepping motor which allows delivery of 5 $\mu\ell$ from the pipet per pulse. The reactants are expelled from the pipeting system into a thermostated mixing chamber which contains a magnetic stirring bar. A sequence timer controls the operation of valves which sample the reaction mixture, drain, and water wash the mixing chamber. When the sample valve is opened, the reaction mixture is transferred by vacuum into the flow cell where the spectrophotometric measurement is made. The flow cell is drained by vacuum and rinsed by allowing the next reactant mixture to be drawn through the cell for a 10 second time interval.

D. Human Mimic Systems

A sample and reagent handling system that consists of a number of modules to mimic stan-

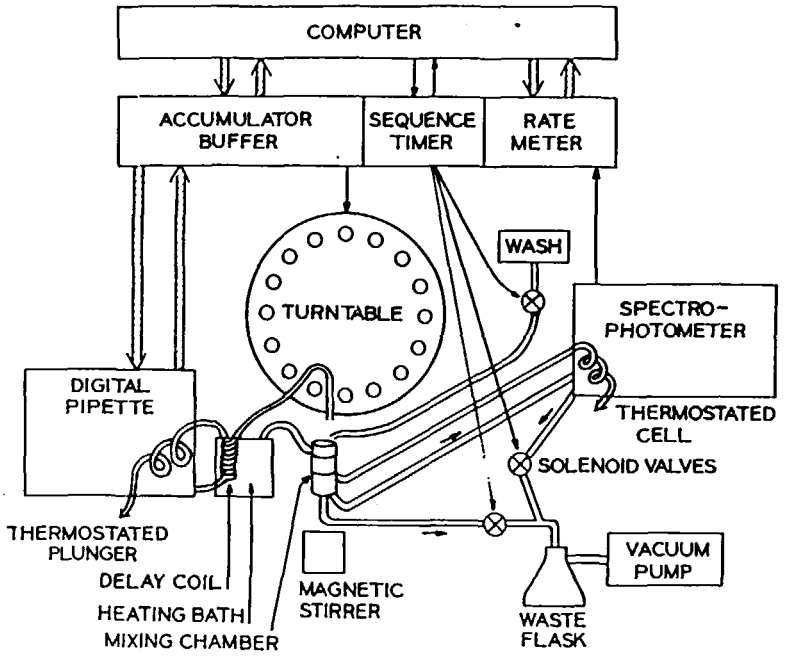

FIGURE 4. Block diagram of hardware used in ELLA. (From Reference 228 courtesy of the American Chemical Society, Washington, D. C.)

dard human operations in processing samples is an obvious approach. A reaction train is set up through which samples pass at predetermined intervals. The robot train includes stations where either discrete volumes of reagents are added from automatic pipets or where processes such as heating, filtration, and dialysis are performed. The Diclan 240 Digital Clinical Analyzer (Honeywell®, Denver, Colorado) is an example of such a system. Interlocking plastic holders containing disposable sample tubes form the sample chain (Figure 5a) which enters a sampling module (Figure 5b). Here the sample chain joins a second chain, the analysis chain, where an aliquot of sample is automatically transferred to the analysis chain. A pipet dips into a tube in the sample chain and a transfer syringe, which is manually preset, extracts the required volume of sample through the pipet. Reagent syringes are simultaneously loaded with a presc volume of required reagents from supply bottles.

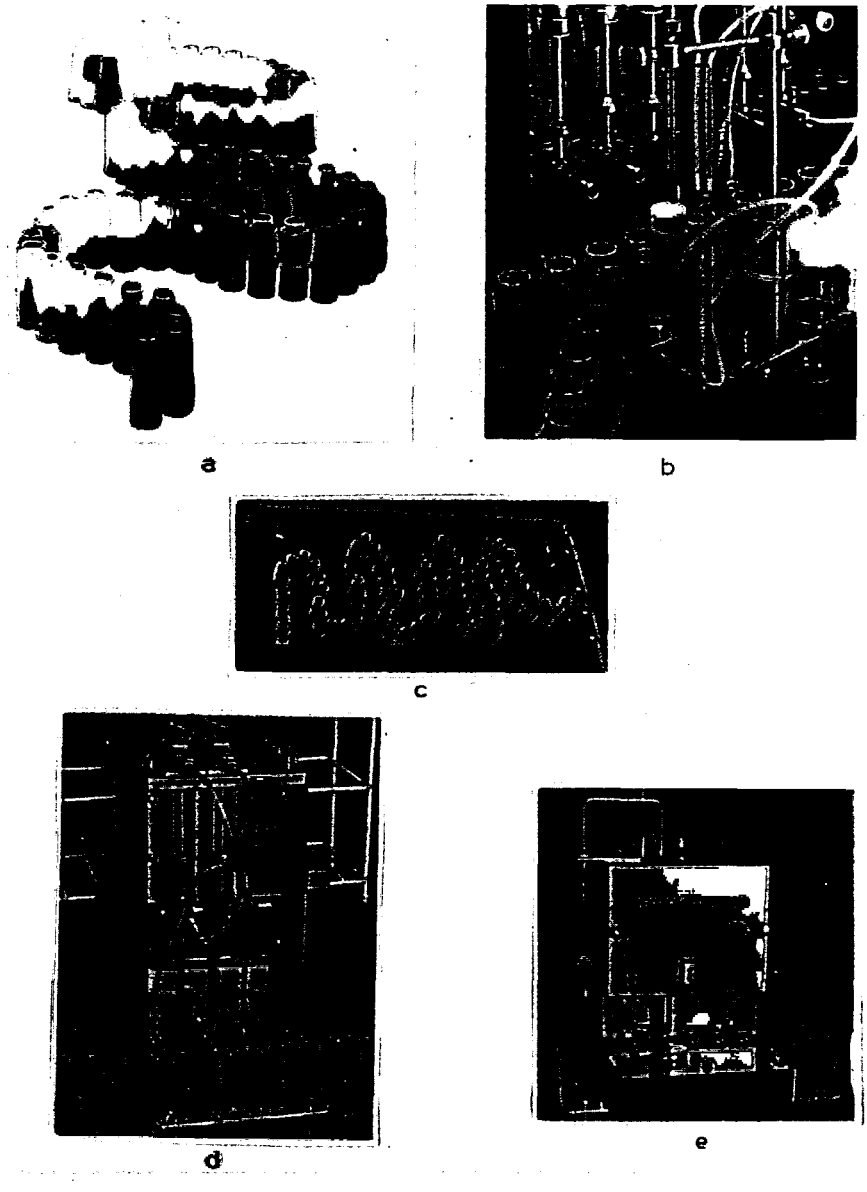

FIGURE 5. Modular components of the Diclan 240 Digital Clinical Analyzer. a) Formation of sample train; b) automatic aliquoting of sample; c) incubation of sample train; d) automatic aliquoting of reagents; and e) automatic transfer to observation cell and rate measurement. (Courtesy of Honeywell®, Inc., Denver, Colorado.)

The pipet then moves over the corresponding tube in the analysis chain. The transfer syringe discharges the sample through the pipet into the tube. One reagent syringe discharges its reagent through the tip of the transfer syringe, through the pipet, and into the tube. The remaining reagent syringes discharge any additional reagents required at this time directly into the analysis tube. The transfer pipet is designed to accomodate the addition of sample and one reagent so that a flushing action cleanses the interior of the pipet, thereby minimizing sample carry-over. The pipet is enclosed in a collar which rinses and removes any residue from its exterior before the next sample is aspirated. A Teflon® coated stirrer blends the reaction mixture before the analysis chain proceeds to the water bath module (Figure 5c) for any necessary incubation period at a selected temperature. For tests which require post incubation addition of reagents, the analysis chain passes through a reagent module (Figure 5d) where up to three additional reagents are dispensed from syringes and mixed. The analysis chain proceeds to the photometer module (Figure 5e) where the reaction mixture is drawn into a cuvette and the spectrophotometric rate measurement is made.

E. Automated System With Prepackaged Reagents

A novel method for automation of sample and reagent handling which minimizes the problem of carry-over and reduces reagent contamination has been developed by DuPont (E. I. du Pont de Nemours and Co., Wilmington, Delaware). The Automatic Clinical Analyzer (aca) system is built around disposable plastic reagent packs such as the one shown in Figure 6. A rigid header contains sealed ports through which sample and diluent are injected. For determinations requiring elimination of interfering components, the header includes a gel filtration or an ion exchange column. Reagents are stored in the pack in seven compartments individually enclosed by temporary seals. The pack also serves as the reaction chamber and observation cell.

The sample is placed either manually or by automatic sampler into a plastic cup to which a patient identification card is clipped. The cup assembly is placed in the input tray of the analyzer and the test packs required for the desired analyses are then loaded in any order. The sample cup and test packs are moved automatically into the filling station shown in Figure 7. Here the sample moves

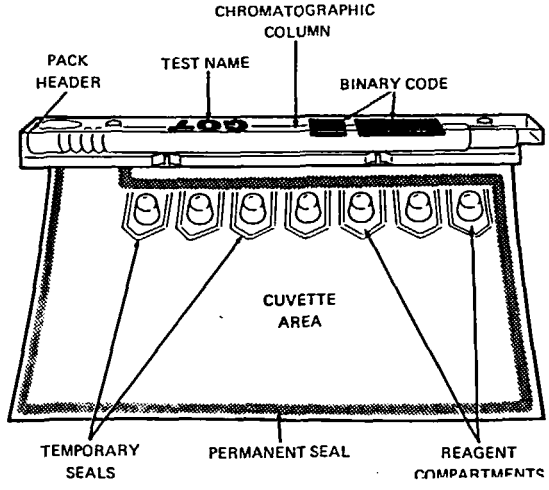

FIGURE 6. Du Pont Automatic Clinical Analyzer analytical pack. (Courtesy of E. I. du Pont de Nemours and Co., Wilmington, Delaware.)

to one side and the test packs are decoded by the built-in read-only-memory computer. The binary code on the pack header provides instructions which specify sample volume, type of diluent, elution volume if a chromatographic column is used, and processing cycle. The filling needle moves over the drain at B and flushes the injection system with selected diluent. The pump of the fluid metering unit reverses and fills the system with the specified quantity of diluent. In the next operation the needle moves over the sample cup at A and withdraws the specified volume of sample. If a chromatographic column is used, it is this point at which the sample and elution diluent are injected into the column (at C). If this is not the case, the needle injects sample and diluent into the pack at D. After filling the pack, the needle returns to its position over the drain (B) and the fluid system is flushed twice with distilled water. The time required for the filling sequence is 36 seconds for noncolumn packs and 72 seconds for packs containing columns.

The fluid metering system consists of a series of reagent reservoirs which are connected to a piston pump through a series of precision micro valves as shown in Figure 8. The pump is driven by a stepping motor. The volume of liquid drawn and delivered by the pump is proportional to the number of impulses applied to the stepping motor by the built-in computer. The operation of the micro valves is also computer controlled.

At the conclusion of the filling cycle each pack

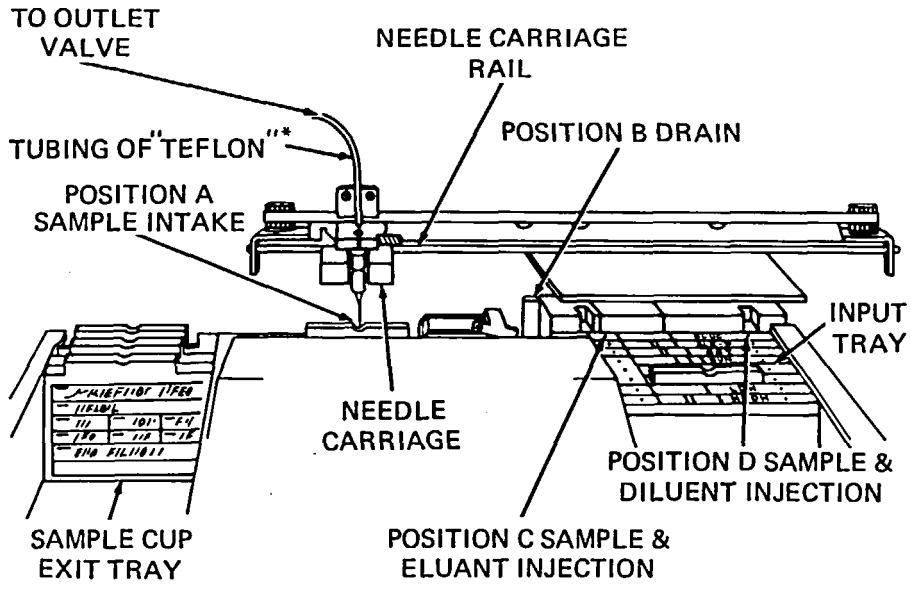

FIGURE 7. Du Pont Automatic Clinical Analyzer filling station. (Courtesy of E. I. du Pont de Nemours and Co., Wilmington, Delaware.)

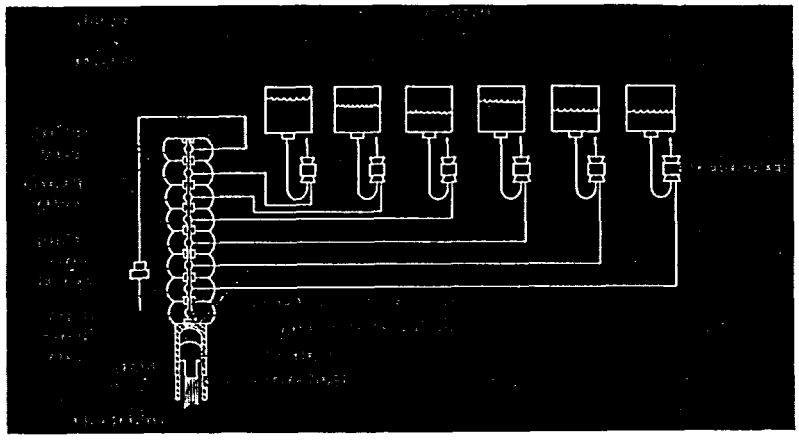

FIGURE 8. Du Pont Automatic Clinical Analyzer fluid metering system. (Courtesy of E. I. du Pont de Nemours and Co., Wilmington, Delaware.)

is automatically moved into the main processing section of the analyzer, allowing the filling station to process the next pack. The pack proceeds through the chemical processing section on a transport chain. The pack is heated to 37° C by contact with two pairs of metal heating plates at two successive heating stations. The temperature of the pack is maintained at 37° C by a circulating air bath throughout the analyzer. The pack then proceeds to the first breaker-mixer station shown in Figure 9. Here it is subjected to pressure between a back plate and platen which ruptures the first four reagent compartments. The reagents which are released are mixed with the main reaction fluid by an oscillating action of the platens for 25 seconds. The pack is then indexed through five delay stations providing a three minute time interval for incubation of the reactant mixture. At the second breaker-mixer station the remaining reagents are released from their sealed compartments and mixed with the contents of the pack. Upon completion of this cycle the pack advances to the photometer station.

F. Parallel vs. Sequential Mixing of Samples and Reagents

In the sample and reagent handling approaches described above the reactants are aliquoted and

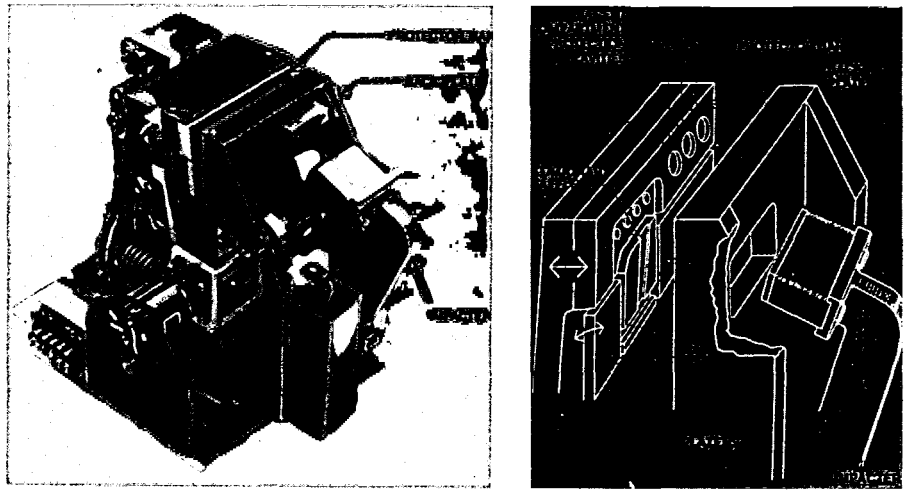

FIGURE 9. Du Pont Automatic Clinical Analyzer breaker-mixer. (Courtesy of E. I. du Pont de Nemours and Co., Wilmington, Delaware.)

dispensed into the mixing chamber and the process repeats sequentially for a series of samples. In most cases the reagents are directly expelled from a pipet into the mixing chamber where the mixing operation is immediately carried out. An alternate approach can be taken by using a time separation of these two operations, and then the simultaneous (parallel) mixing of reactants for a series of samples. All reactants are premeasured and introduced into reactant wells. The mixing operation takes place at a later time, and is illustrated by the simple techniques of Yatsimirskii.[229] Reactants were pipeted into reactant wells of the reaction vessel shown in Figure 10. Mixing of reagents and initiation of the reaction were then accomplished by inverting and shaking the vessel. This approach is also applied in biological assays requiring measurement of gas evolution or uptake using a Warburg constant-volume respirometer.

This principle of mixing is also applied in the unique GeMSAEC analyzer developed by N. G. Anderson and co-workers at Oak Ridge National Laboratory, Oak Ridge, Tennessee.[230,274] The latest system[274] allows parallel mixing of reactants for up to 42 samples. Centrifugal force is used to transfer reactants into the mixing chamber which also serves as the observation cell.

Samples and reagents are manually or automatically pipeted into individual holding compartments of the transfer disk as shown in Figure 11. The transfer disk is then manually locked into place in the rotor which also insures proper

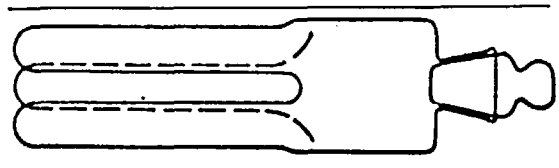

FIGURE 10. Reaction-mixture vessel with three compartments for inserting and mixing original solutions. (From Reference 229 courtesy of Pergamon Press, New York, New York.)

indexing of the samples (Figure 11). The holding compartments are arranged in two or more concentric circles in the transfer disk, with each circle containing the same number of compartments. The compartments are aligned radially and are angularly arranged so that samples and reagents are dumped through a transfer cavity into the corresponding reaction chamber when the rotor reaches a sufficient speed (Figure 11), typically 400 rpm.

To prevent lateral splashing of the solutions in the transfer disk, the rotor is gradually accelerated and then during a breaking interval, a pulsed vacuum is applied which draws a stream of small air bubbles through each cuvette. The resultant turbulence produces complete mixing of the reactants. The air bubbles are removed by rapid acceleration followed by deceleration to the normal operating speed of about 500 rpm. In present designs this entire process requires only a few seconds. After the spectrophotometric rate or stoichiometric measurements are completed, the samples are discarded through drainage siphons located in each cell by application of an air stream

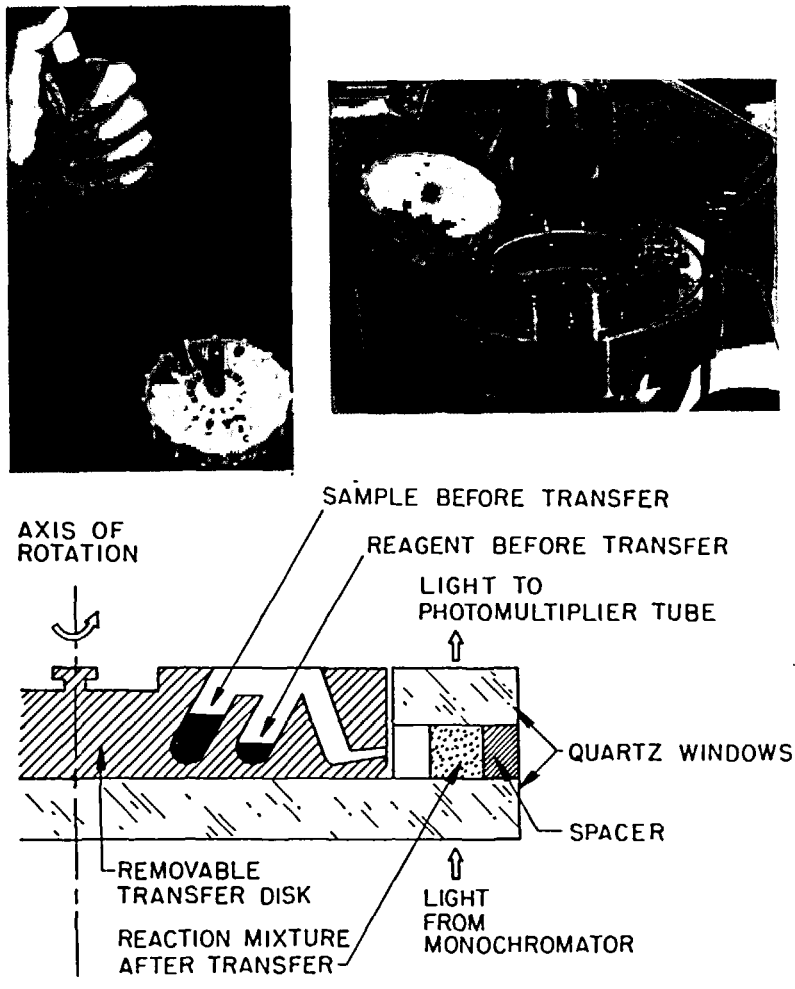

FIGURE 11. The GeMSAEC Fast Analyzer. Upper left – loading of transfer disk. Upper right – transfer disk and centrifugal analyzer section. Bottom – cross section of transfer disk and centrifugal analyzer section. (From Hatcher, D. and Anderson, N., *Am. J. Clin. Pathol.*, 52, 645, 1969. Courtesy of J. B. Lippincott Co., Philadelphia, Pennsylvania and from Coleman, R. et. al., *Am. Lab.*, 3, 26, 1971. Courtesy of International Scientific Communications, Inc., Green Farms, Connecticut.)

to the center cavity of the rotor which extends radially to each cell. The rotor is then stopped and wash water is added from a wash bottle to both the sample and reagent compartments of the transfer disk. The rotor operational sequence is repeated, transferring the wash water to the cuvettes which are drained as described above.

To increase sample through-put rate, research is being conducted to provide automatic loading of sample and reagent and cleaning and drying of the rotor between runs. To extend the utility of the analyzer to more complex methodology, two additional research objectives reported[230] are incorporation of ion exchange and gel filtration columns into the system and provision for post transfer self-decanting of supernatant from a precipitate.

G. Stopped-Flow Methods

The sample and reagent handling systems presented above are only suitable for utilizing relatively slow reaction-rate systems. As pointed out earlier, there are advantages in selecting a fast reaction for analytical purposes. The limitation in making rate measurements on a fast reaction is that the time taken for mixing and observation must be shorter than the halflife of the reaction. Today, observations can be made in the

milliseconds by using fast-responding detector-readout systems. The real limitation becomes the time required for the physical mixing of reactants and the initiation of the reaction. To minimize this time, mechanical systems are used which drive reactants rapidly enough to promote turbulent flow through the system and thereby insure rapid mixing and uniformity of solution composition in the observation cell.

While a number of flow methods have been used for studying fast reactions, the stopped-flow method has been most widely applied for analytical purposes. This technique consists of rapidly mixing reactants by forcing the solutions through a mixing chamber and into an observation cell. The flow of solution is abruptly stopped, creating a back pressure which completes the mixing, and the rate measurement is rapidly made. The more sudden the stopping, the faster the reaction that can be observed. To insure that complete mixing has occurred, the observation cell is placed a short distance from the mixing chamber.

The efficiency of the stopped-flow system is dependent upon the flow velocity, mixer design, and the distance between the mixer and observation cell. To provide efficient mixing, the flow through the system must be turbulent. The critical velocity of flow which produces turbulence can be calculated from Reynold's equation:

$$u_c = \frac{N_R \eta}{\rho d} \quad (6)$$

where

u_c = critical velocity in cm/sec
N_R = Reynold's number (about 2000 for short tubes with streamline entry).
η = viscosity of the liquid in poises
ρ = density in g/ml
d = diameter of the flow tube in cm

Thus high flow rates must be used to obtain a high degree of turbulence and rapid, efficient mixing. However, very high flow rates can cause cavitation as a result of unequal hydrostatic pressure of the streaming fluid in different portions of the flow tube. While cavitation is a turbulent condition, it does not represent the ideal case. Rather, it introduces an error in the kinetic measurement, particularly when spectrophotometric rate measurements are made. Therefore a compromise is made in selecting the flow rate. The rate must be high enough to produce turbulent flow, yet low enough to prevent cavitation. Flow rates ranging from 1 m/sec to 30 m/sec have been reported.

The efficiency of the mixing of reactants is highly dependent upon mixer design. High mixing efficiencies have been obtained using tangentially offset jets which promote a rotary motion to the fluid. When the jets are placed in these positions, greater turbulence results. Mixing efficiency is also dependent upon bore size; the smaller the bore, the higher the jet velocity. The total area of the jets and observation tube should be made equal to avoid excess pressure drop in the mixer. Mixing performance is improved by increasing the number of jets. To maintain the same relative area, the diameter of the jets must be made smaller as the number of jets is increased. Multiple mixers are used for mixing several solutions. Ruby[231] described a system which used three mixers in cascade to mix four solutions in sequence. A chemical stop could be made at any point along the flow path, using one, two, or all of the mixers.

A measure of the mixing efficiency is the dead time which is the time interval from initial contact of two solutions in the mixing chamber to the observation measurement in the cell. It is desirable to provide as small a dead time as possible. This is accomplished by either improving the mixer design or, for a given mixer, increasing the flow velocity. Frequently mixing is not completed inside the mixing chamber, therefore the observation point is placed a short distance from the outlet of the mixer. This distance is given by

$$d_m = t_m \cdot v \quad (7)$$

where

d_m = distance from mixing chamber outlet to observation point in cm
t_m = dead time in sec
v = flow velocity in cm/sec

The sample delivery unit, mixing chamber, observation cell, and stopping device of a stopped-flow system used successfully in our laboratories are shown in Figure 12.[26] The sample introduction system is based on a high precision rapid injection and automatic refill pipet.[232] Upon activation of the system, the stopcock motor rotates the double 3-way stopcock clockwise 90° causing the refill tubes to be opened. The syringe drive motor then immediately rotates the cam counterclockwise one half cycle allowing sample and reagents to be drawn into the spring-loaded gas-tight syringes. The cam is set so

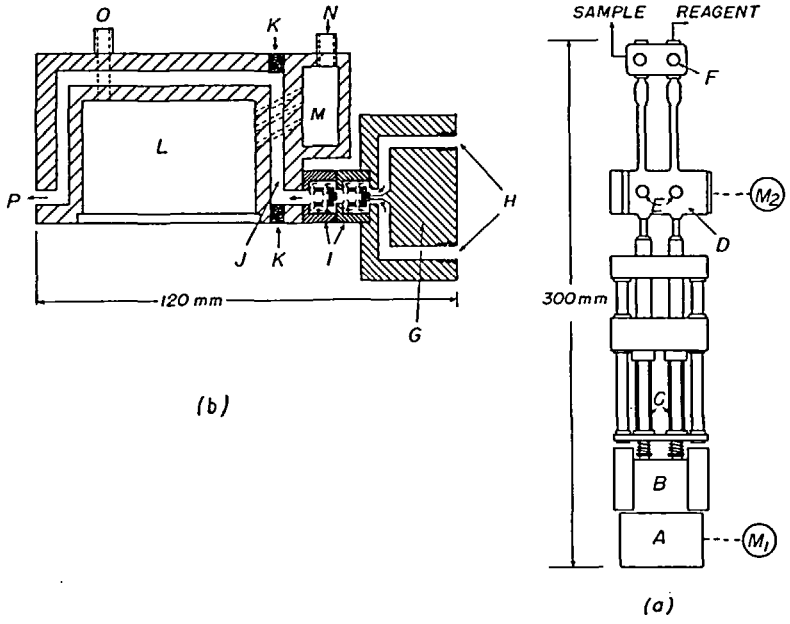

FIGURE 12. Stopped-flow aliquoting and mixing system. a) Reagent delivery unit. A. Teflon cam, B. sliding brass bar, C. gas-tight syringes with stainless steel springs, D. Teflon double 3-way stopcock, E. outlet to delivery block, F. 3-way Hamilton valves, M_1 and M_2. Synchronous motors. (From Javier, A., Ph.D. Thesis, University of Illinois, Urbana, Illinois, 1969.) b) Vertical section of delivery block, mixers, and observation chamber. G. delivery block, H. reagent and sample inlets, I. tangential mixers, J. observation cell, K. quartz windows, L. and M. thermostating jacket, N. thermostating water inlet, O. thermostating water outlet, P. Reaction mixture outlet (to check valve). (From Javier, A., Ph.D. Thesis, University of Illinois, Urbana, Illinois, 1969.)

that the stroke fills each 2 ml syringe with 0.25 ml of fresh solution. The drive motor then rotates the cam clockwise one half cycle which rapidly drives 0.25 ml of each solution out of the syringes. The solutions are forced into the mixing chamber and on to the observation cell. The flow of solution through the observation cell creates a pressure build-up which is sufficient to open a check valve connected to the drainage port of the cell. At the end of the reactant introduction drive cycle, the check valve closes rapidly which abruptly arrests the flow of solution in the observation cell. At this point the rate of change in absorbance is rapidly measured.

Samples are placed on a motor-driven thermostated turntable which can be programmed to operate in synchronization with the stopped-flow system. The reagent is picked up from a large reservoir which is also thermostated. Use of a 72 rpm motor to drive the syringes produces a linear flow velocity of 1 m/sec. A two-stage mixing chamber based on the design of Gibson and Milnes[233] provides a 20 m/sec mixing time. The mixing time is defined as the time required for complete replacement of solution in the observation cell and attainment of an equilibrium in absorbance. The cylindrical observation cell is located 1 cm from the end of the mixer.

H. Evaluation of Available Sample and Reagent Handling Systems

Most of the sample handling systems presented have been designed in conjunction with the particular approach desired for monitoring the reaction and manipulating the resultant rate data. Often the system was designed for a few specific applications. Unfortunately, as mentioned before, not all possible operations on the sample prior to measurement have been incorporated into present sample preparation systems. The ideal system would minimize the turn-around time required in preparing the measurement system for determination of a different analyte, and would be flexible enough to allow modifications which may be

required as future methodology is developed. The volume of sample and reagents required would be minimized and carry-over between successive samples eliminated.

Considering commercially available approaches, two provide automation of some sample preparation steps. The continuous flow systems (Technicon) use a semipermeable membrane to dialyze macromolecules and suspensions from the stream. The system which uses disposable reagent packs (DuPont aca) incorporates a small chromatographic column for removal of some interfering species. As to dilution of reagents, most approaches provide a fixed dilution of sample or reagents. The DuPont system does automatically vary the volume of diluent from one test method to another according to a computer interpreted code on the test pack. A further step toward automation is provided by ELLA. This system automatically varies the amount of diluent or reactant from run to run for a given test based on computer evaluation of the chemical data obtained.

The turn-around time inherent in the present approaches varies considerably. The continuous flow systems require manual changing of peristaltic pump mantel, reconnecting of tubing, and purging of the flow system with new reactants. The entire process can require as long as 15 to 20 minutes. The disposable pack system has a short turn-around time since all required reagents are prepackaged and the computer controls the sample handling process by interpretation of the binary code on the test pack. For the other systems, the turn-around time results from the need to manually reset the pipets to dispense a new volume and/or flush with the new reagents. New systems will undoubtedly provide for computer control of pipets. On the other hand, the DuPont system does not provide flexibility in incorporating independent methods or development of new methodology. One possible disadvantage with aca is that one must rely on the manufacturer for prepackaged reagents (including quality control) and methodology. The GeMSAEC analyzers hold considerable promise because of the parallel mixing and monitoring of many samples. It also appears that prepackaged discs could eventually be provided to greatly reduce reagent handling problems.

Some degree of flexibility is provided in the continuous flow approach since mantels can be specifically made for new methods. The approaches which mimic manual pipeting and manipulation of sample are readily adapted to new methodology.

The volume of samples required is an important consideration, particularly in biological assays. The reagent volume must also be considered since it influences the cost of the analyses and, more importantly, the time involved in executing the sample handling operations. Discrete sample handling approaches inherently minimize the volume of reactants required, while continuous flow methods generally require larger volumes to maintain a flowing stream throughout the entire system. As mentioned earlier, continuous flow systems are more susceptible to carry-over effects. For the discrete sampling systems, the degree of carry-over depends upon the design and how efficiently the sample syringe or pipet is flushed.

VI. DATA DOMAIN CONVERSION (TRANSDUCERS)

A. General Considerations

In principle, any suitable means of monitoring the concentration of a chemical species can be used in reaction rate methods. Most frequently, spectrochemical and electrochemical methods are used for monitoring; consequently, the transducers fall into these two general categories. In selecting an appropriate transducer, the type of reaction utilized, the nature of species monitored, and the rate of the reaction must be considered. The transducer must be sufficiently sensitive to provide detection of very small changes in measured reaction parameter and must have a response time which is short relative to the reaction half time to prevent distortion of the resultant rate curve.

B. Spectrochemical Transducers

Photomultiplier tubes and photodiodes are used almost exclusively for measurements involving ultraviolet and visible absorbed or fluorescent radiation. Both transducers have a response time in the nanosecond range allowing very fast reactions to be followed. For a particular type of chemical system and spectrophotometric method, the transducer which provides the higher signal-to-noise ratio is chosen. The photomultiplier tube exhibits better characteristics than photodiodes

when low light levels are measured and the photocathodic current is below 10^{-9} A.[234] For measurement of higher light intensities, the photodiode may be advantageous. This difference results from the fact that with a photomultiplier tube the process of internal amplification results in amplification of only shot noise with amplification of the signal. However, a photodiode requires an external high-gain amplifier which results in amplification of both shot noise and Johnson noise with amplification of the signal.

Photomultipliers are recommended for measurements requiring narrow absorption bandwidths as well as for fluorescence and phosphorescence spectrometry. The photodiode might be used to advantage in molecular absorption spectrometry where large bandwidths allow the use of a filter or a monochromator with large slitwidths.[235]

C. Electrochemical Transducers

Electrochemical methods allow the determination of rates of reactions with half times as low as 10^{-4} sec. The electrochemical transducers most frequently used include platinum electrodes, dropping mercury electrodes, ion-selective electrodes, and enzyme electrodes.

1. Platinum Electrodes

Platinum electrodes are used for both amperometric and potentiometric measurement systems. An amperometric method for the determination of glucose by a continuous flow technique has been described by Bladel and Olsen.[236] A pair of tubular platinum electrodes was used in the flow system. The current passing through the electrodes was on the order of nanoamperes, and the product of the coupled reaction (potassium ferricyanide) could be determined at concentration levels as low as 10^{-5} *M*.

2. Dropping Mercury Electrode

Dropping mercury electrodes have been used for amperometric and polarographic measurements. As in the case of platinum electrodes, the response time of the electrode system is limited by the rate of diffusion of the monitored species to the electrode. The dropping mercury electrode can be used for reactions where $(k_f/K)^{1/2}$ is greater than 5 sec^{-1}, where k_f is the forward rate constant and K is the equilibrium constant for the reaction monitored.[237] Osterlind[238] has described the use of a dropping mercury electrode for the determination of oxygen in vivo in blood. The bloodstream is flowed past the electrode which is held at a fixed potential sufficient for oxygen reduction. The measured current is proportional to oxygen concentration.

3. Ion-Selective Electrodes

Ion-selective membrane electrodes are presently available for more than 20 ions including the alkali metals, alkaline earths, and heavy metals. These electrodes exhibit a sensitivity which is often below 10 ng/ml. The three classes of specific-ion electrodes are designated glass electrodes, liquid-liquid membrane electrodes, and solid-state membrane electrodes. The electrode response time varies according to both the type of electrode and the conditions under which it is used. For example, a calcium ion-selective liquid membrane electrode requires 10 seconds to reach a constant potential if the calcium concentration of the solution increases by 30%. In the presence of an equivalent magnesium concentration, the response time is increased five-fold.[239] Specific-ion electrodes can be used to advantage for relatively slow reactions with half times on the order of minutes. Rechnitz and co-workers have reported several reaction-rate methods using ion-selective electrodes. Species studies include HF,[240] AlF^{2+} and FeF^{2+},[241] Cu^{2+} complexes,[242] Ag^{+}, Ni^{2+},[243] and tetraphenylborate.[244]

4. Enzyme Electrodes

Enzyme electrodes are transducers which combine electrochemical procedures with immobilized enzyme activity. Such electrodes consist of an enzyme-active membrane layered on an ion-selective electrode. The enzyme-catalyzed reaction generates a product for which the selective electrode is sensitive. For example, an ammonium electrode can be used in conjunction with several enzymes (urease, glutaminase, asparaginase, etc.) which catalyze the decomposition of urea and amino acids (glutamine, asparagine, etc.). Such an electrode can be used to determine substrate concentration as well as enzyme activity. In most cases the sensitivity obtained for substrate determination is on the order of 1 to 10 μg/ml while sensitivity for enzyme determination is about 0.001 I.U.[245]

VII. RATE MEASUREMENT AND DATA MANIPULATION

A. General Considerations

The initial rate of a reaction can be related to the concentration of the reactants by the general kinetic law given in Equation 8[246]:

$$R_i = k(T) \prod [A_i]_o^{n_i} \quad (8)$$

where R_i is the initial reaction rate in mole·l^{-1}·sec^{-1}

k(T) is the rate constant which is a function of temperature, T

$[A_i]_o$ is the initial concentration of reactants in mole·l^{-1}

n_i is the reaction order for reactant i

For mechanistic investigations, one starts from a known concentration $[A_i]$ for all reactants and determines the remaining parameters in Equation 8. Once the mechanism has been elucidated, or optimum conditions for a reaction have been determined, a kinetic method can be developed. For analytical purposes the initial reaction rate is measured which, according to Equation 8, is proportional to concentration. However, since the initial rate, R_i, depends on the concentration of all reactants, the concentration of i-1 reactants must be kept in sufficient excess so that their initial concentrations do not change appreciably during the measurement of initial rate. These factors remain constants and can be incorporated into a new rate constant k', yielding Equation 9:

$$R_i = k'(T) [A_1]_o^{n_1} \quad (9)$$

By keeping the temperature constant so k' (T) does not change, the reaction rate depends only on the concentration of the sought-for-species, $[A_1]_o$.

The most advantageous situation is that of a first order reaction (n = 1), where there exists a linear dependence between reaction rate and concentration. However, for reactions of higher order (n = 2 or 3), reaction-rate methods still have analytical utility in that the initial rate is a known function of the initial concentration. Even for these reaction orders a linear dependence can be obtained by adjusting the experimental conditions (concentration of reagents, measurement interval, etc.) so that pseudo-first order kinetics are observed.

B. Methods of Reaction Rate Computation

1. Derivative Method

Procedures for measurement of initial reaction rate can be classified as single-point and two-point methods. In the single-point method, or derivative method, the derivative of the transduced signal is measured directly. The measurement is made at time t = 0 (immediately after mixing) or at a specified time after the reaction has been initiated yet before the concentrations of reactants have changed appreciably. The proportionality between the derivative of the rate curve and concentration of rate-determining species is valid for both linear and nonlinear response curves. In the latter case, the measurement must always be made at the same time after initiation of the reaction to insure that the proportionality factor remains constant.

The two-point methods can be subdivided as a function of the parameter which is held constant during the measurement – transduced signal or time. These subcategories are referred to as variable-time and fixed-time methods. In the two-point methods the ratio between a finite variation of selected parameter, ΔP, and a finite interval of time, Δt, is measured. This ratio, ΔP/Δt, from an analytical point of view, is just as useful as the derivative dP/dt.

2. Variable Time Method

In applying the variable time method, the time interval, Δt, required for a predetermined fixed change in monitored parameter, ΔP, is measured as shown in Figure 13. The initial rate is expressed as ΔP/Δt and Equation 9 can be rewritten as:

$$\frac{\Delta P}{\Delta t} = k'[A_o]_1^{n_1} \quad (10)$$

Since ΔP is held constant, this value may be incorporated into a new rate constant, k'', yielding:

$$\frac{1}{\Delta t} = k''[A_o]_1^{n_1} \quad (11)$$

Hence, the reciprocal of the measured time interval is directly proportional to the concentration of the sought-for species. This is graphically displayed in Figure 13. As the concentration of analyte increases ($C_2 > C_1$), the slope of the initial rate curve increases ($\alpha'' > \alpha'$), and the measured time interval decreases ($\Delta t_{C_2} < \Delta t_{C_1}$).

The primary operations required in using the variable time method are measurement of the time interval, conversion to the reciprocal value, and

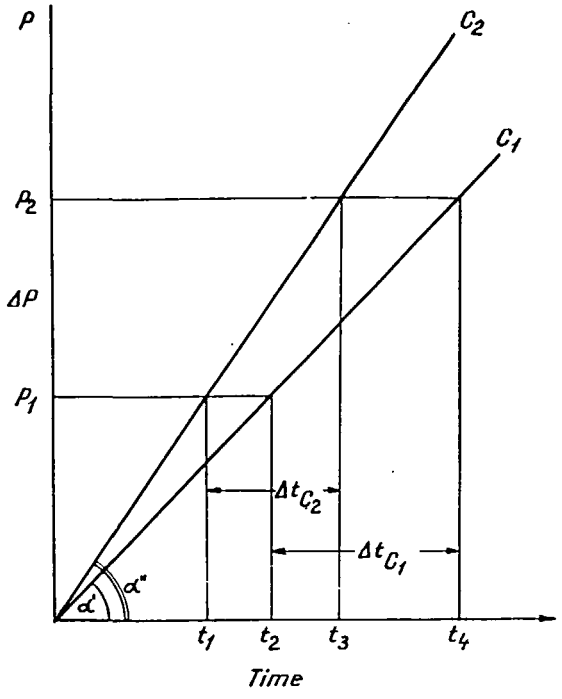

FIGURE 13. Principle of variable-time method for reaction-rate measurement. (From Cordos, E., *Chim. Anal.*, 1, 11, 1971.) Courtésy of Centrul de Documentare al Industriei Chimice, Bucharest, Romania.)

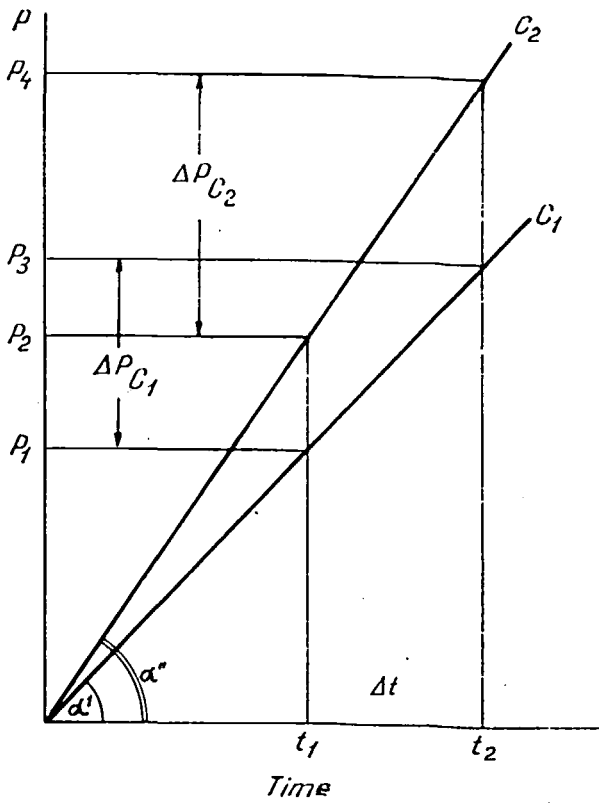

FIGURE 14. Principle of fixed-time method for reaction-rate measurement. (From Cordos, E., *Chim. Anal.*, 1, 11, 1971. Courtesy of Centrul de Documentare al Industriei Chimice, Bucharest, Romania.)

computation of concentration from Equation 11 or extrapolation of concentration from a working curve.

3. Fixed-Time Method

In using the fixed-time method, the change in transduced signal, ΔP, is measured over a preselected time interval, Δt, as shown in Figure 14. Referring to Equation 10 above, the constant, Δt, can be included in a new rate constant, k''', giving:

$$\Delta P = K''' [A_o]_1^{n_1} \quad (12)$$

Thus, a direct proportionality exists between the measured value, ΔP, and analyte concentration. Again, this is graphically displayed in Figure 14. As the concentration of analyte increases ($C_2 > C_1$), the slope of the rate curve increases ($\alpha'' > \alpha'$) and the measured value increases ($\Delta P_{C_2} > \Delta P_{C_1}$). In applying this technique, the measured value, ΔP, is not theoretically dependent upon the absolute value of the transduced signal.

4. Integration Method

Integration techniques may be considered a special case within fixed-time methods. Rather than making the measurement between two points, two portions of the rate curve are integrated and the resultant areas are subtracted as shown in Figure 15. Referring to Figure 15a, if the integration is made over two equal time increments, Δt, which are sequential, the difference, ΔA, between the resultant areas A_1 and A_2 is the parallelogram ABCD. The area of the parallelogram is given by:

$$\Delta A = (\Delta t)a \quad (13)$$

The slope is defined as:

$$S = \text{Tan } \alpha = \frac{a}{\Delta t} \quad (14)$$

Substitution of Equation 13 into Equation 14 provides an expression relating slope to the difference in area:

$$S = \frac{\Delta A}{(\Delta t)^2} \quad (15)$$

Since Δt is a constant, the slope is directly

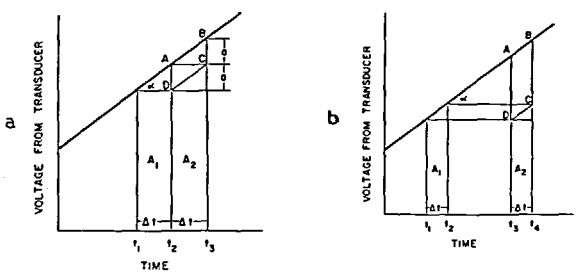

FIGURE 15. Principle of integration method for reaction-rate measurement. a) Expanded section of typical rate curve illustrating integration and subtraction of two sequential areas A_1 and A_2. b) Expanded section of typical rate curve illustrating integration and subtraction of 2 areas A_1 and A_2 separated by a measurement delay interval $t_3 - t_2$. (From Reference 258 courtesy of the American Chemical Society, Washington, D. C.)

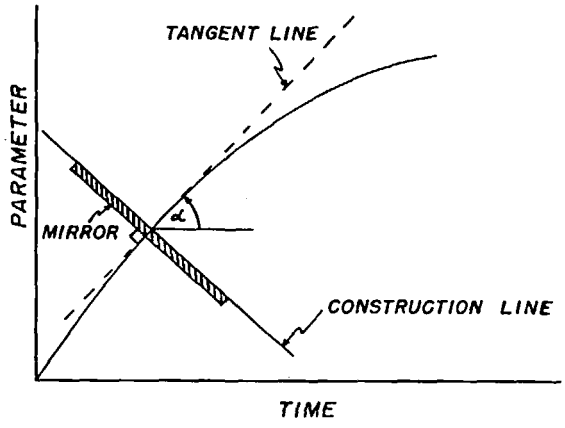

FIGURE 16. Manual reaction rate measurement by construction of tangent using a mirror.

proportional to the measured difference ΔA. In Figure 15b the two time increments are not consecutive but are separated by a time interval $t_3 - t_2$. In this case Equation 15 can be rewritten as:

$$S = \frac{\Delta A}{(t_3 - t_2)\,\Delta t + (\Delta t)^2} \qquad (16)$$

Since $(t_3 - t_2)$ is also kept constant, the slope remains directly proportional to the measured area. The total time for the measurement is $2\Delta t + (t_3 - t_2)$. For the best signal-to-noise ratio, it is preferable to work with $(t_3 - t_2) = 0$ and Δt made as long as possible for a specific reaction.

C. Manual Data Manipulation

The operations required in the methods described above can be carried out using either a manual, semiautomated, or completely automated measurement system. Manual methods require that the initial rate curve be plotted either point by point or continuously with a suitable recorder. The reaction rate is then determined from the recorded rate curve and the concentration of sought-for species is either calculated or extrapolated from an analytical curve.

The measurement of the initial rate from a recorded curve by the derivative method requires that the slope be determined at a point near the initiation of the reaction. The tangent can be constructed by merely using a ruler, or a better approximation may be obtained by using a mirror or total reflection prism as illustrated in Figure 16. The mirror is placed perpendicular to the plane of the chart paper and on the point of the curve where the tangent is to be drawn. The mirror is then rotated about its vertical axis until the curve and its image are linearly aligned, and a construction line is drawn along the plane of the mirror. A line drawn perpendicular to this construction line is the tangent. This technique is applicable when the rate curve is accompanied by a relatively low noise level.

Manual determination of analyte concentration by the two-point methods as illustrated in Figure 13 and 14 is facilitated by use of templates which are precalibrated in desired concentration units. The template is placed on the linear portion of the recorded rate curve and is oriented so that its axes are parallel to the axes of the plot. The point at which the rate curve intersects the graduated concentration scale of the template determines the concentration. Experimental conditions for the analysis must be rigidly controlled to insure that the conditions existent during template preparation are maintained.

Manual determination of initial rate is subject to serious personal bias. A high percentage of the 10% error admitted for many kinetic methods [247] is a result of determination of the rate from recorded curves. The precision and accuracy of rate measurements can often be improved by an order of magnitude by automating the operations required for data manipulation and readout. A number of hardware approaches have been taken for automating the rate measurement procedures described above.

D. Semiautomatic and Automatic Data Manipulation with Hardware Systems

1. Derivative

The experimental system for rate measurements using the derivative method is relatively simple. An operational amplifier (OA) is wired as a differentiator (Figure 17), and the voltage from a suitable transducer is applied to the input. The relationship between the input voltage (e_{in}) and the output voltage (e_o) is given as:

$$e_o = - RC \frac{de_{in}}{dt} \qquad (17)$$

The derivative method best characterizes the kinetics of a given reaction since it provides the instantaneous value of the tangent at one point on the rate curve. Paradoxically, this is a factor which limits the method since, at the required sensitivity, the rate curves are accompanied by noise. If an expanded portion of a typical rate curve shown in Figure 18 is examined, the value of the tangent is observed to change dramatically from point-to-point. For example, at point A the value is zero, at B it is approximately 2, at C it is again zero, etc. Thus, the differentiator will behave as a simple amplifier which amplifies not only the signal to be measured but the noise as well.

To diminish the influence of noise on the measurement, a number of different experimental approaches have been taken. The simplest approach is to decrease the frequency response of the differentiator by placing a small capacitor, C_f, parallel with the feedback resistor, R_f, and a small resistor, R_i, in series with the input capacitor, C_i, as shown in Figure 19. The feedback capacitor, C_f, acts as a filter for the high frequency component of the signal.

An alternate approach was taken by Pardue and

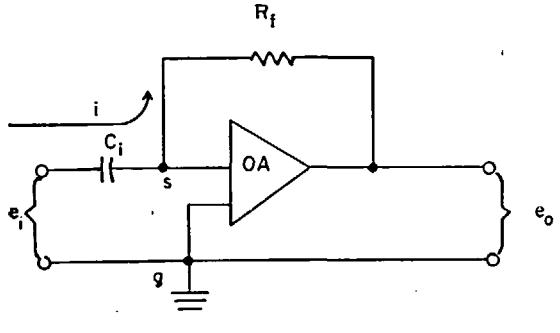

FIGURE 17. Classical operational amplifier differentiator. (From Reference 251 courtesy of the American Chemical Society, Washington, D. C.)

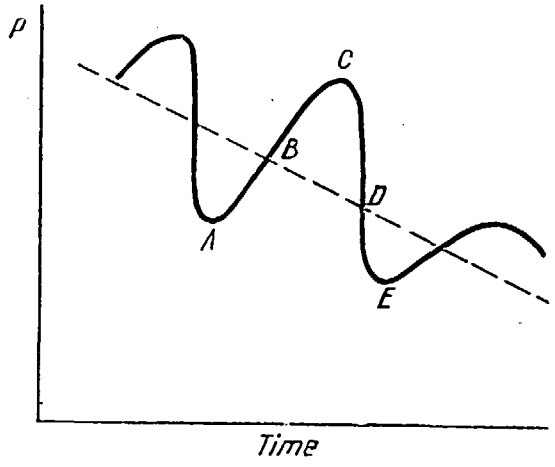

FIGURE 18. Expanded portion of a typical reaction rate curve. (From Cordos, E., *Chim. Anal.*, 1, 11, 1971. Courtesy of Centrul de Documentare al Industriei Chimice, Bucharest, Romania.)

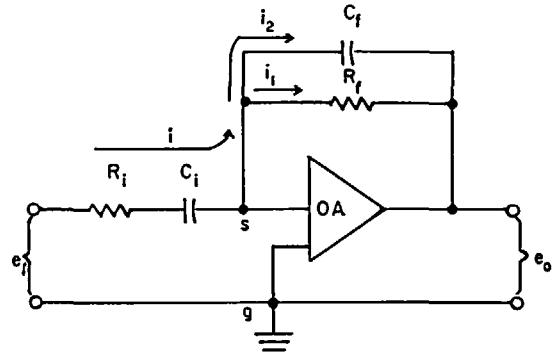

FIGURE 19. Operational amplifier differentiator with decreased frequency response. (From Reference 251 courtesy of the American Chemical Society, Washington, D. C.)

Dahl and Malmstadt and Crouch,[248-251] who described several different systems for rate measurement which were based on the principle of slope matching. In this technique an analog signal is generated whose slope is maintained equal to the slope of the transduced signal. The reference slope is generated by an integrator, whose output is given by:

$$e_{out} = - \frac{1}{RC} \int_o^t e_{in}\, dt \qquad (18)$$

Integration and rearrangement of Equation 18 yields the following expression for slope of the artificial curve:

$$\frac{\Delta e_{out}}{\Delta t} = - \frac{e_{in}}{RC} \qquad (19)$$

Thus, the input voltage of the integrator is a

measure of the initial slope of the rate curve. The principle of the slope matching method is illustrated in Figure 20, where e_s represents the slope generating source which is in series with e_u, the output voltage from the transducer. The voltage e_u changes as the reaction proceeds while e_s is varied by mechanical linkage or by an electronic device. The potential difference e_{sg} at any time is equal to the difference between the potential drop across R_2 and the voltage e_s:

$$e_{sg} = iR_2 - e_s \tag{20}$$

and since

$$i = \frac{e_u + e_s}{R_1 + R_2} \tag{21}$$

substitution into Equation 20 gives the expression:

$$e_{sg} = \frac{e_u R_2 - e_s R_1}{R_1 + R_2} \tag{22}$$

If R_1 and R_2 are equal, for a given time t_1, Equation 22 may be rewritten as:

$$(e_{sg})_t = \frac{e_u - e_s}{2} \tag{23}$$

The potential difference at some later time, $t + \Delta t$, is given by:

$$(e_{sg})_{t+\Delta t} = \frac{(e_u)_t - (e_s)_t + (\Delta e_u)_{\Delta t} - (\Delta e_s)_{\Delta t}}{2} \tag{24}$$

To maintain e_{sg} constant, the changes in e_u and e_s should be kept identical so that:

$$\frac{de_u}{dt} = -\frac{de_s}{dt} \tag{25}$$

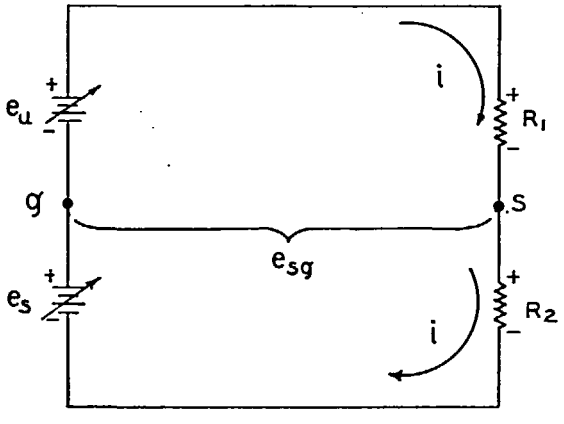

FIGURE 20. Basic comparison circuit for rate measurements. (From Reference 251 courtesy of the American Chemical Society, Washington, D. C.)

Hence, a measure of de_u/dt is given as the voltage required to generate the artificial slope de_s/dt.

A schematic for an all-electronic rate meter designed by Crouch and Malmstadt [251] which uses this principle is shown in Figure 21. The transducer voltage is represented by e_u while e_s is generated by the two operational amplifiers OA1 and OA2. The series combination of the integrator (OA1) and inverter (OA2) serves as the feedback element of the control amplifier (OA3) which maintains point S at vertical ground. Recalling Equation 19, the rate of change of the potential established in the feedback loop is given by:

$$\frac{de_s}{dt} = \frac{e_r}{R_s C_s} \tag{26}$$

Any voltage change at point S is amplified and applied to the input of OAl. The integrated voltage at the output of OAl is inverted at OA2 and applied to the summing point (S) of OA3. The output voltage of OA3, which serves as the input voltage to the slope generating circuit, is measured with a suitable device and is given by:

$$e_r = R_s C_s \frac{de_u}{dt} \tag{27}$$

Hence, the output of the control amplifier is directly proportional to de_u/dt. Typical recordings of rate curves and measured slopes for two glucose concentrations are shown in Figure 22. Noise fluctuations in the input signal to the rate meter can often be quite severe and necessitate the use of slow response times. The reason is that the fundamental principle applied in making the derivative measurement point-by-point has not changed.

2. *Variable-Time*

The primary difficulty in automating rate measurements using the variable-time approach is

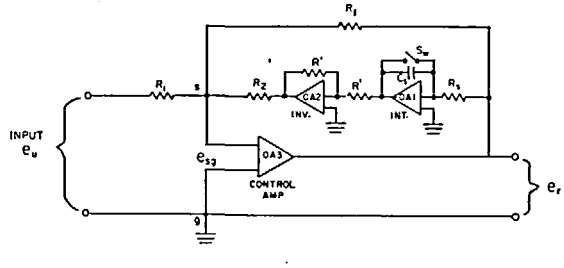

FIGURE 21., Basic circuit of the all-electronic comparison ratemeter. (From Reference 251 courtesy of the American Chemical Society, Washington, D. C.)

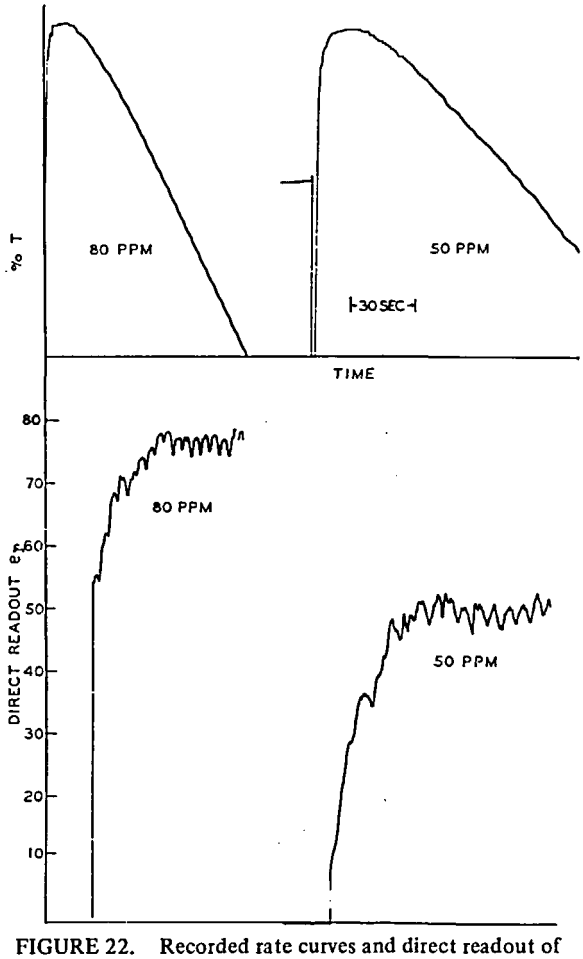

FIGURE 22. Recorded rate curves and direct readout of slopes for glucose concentrations of 50 and 80 ppm. (From Reference 251 courtesy of the American Chemical Society, Washington, D. C.)

providing for the computation of reciprocal times. Variable-time rate meters must also include a device which is capable of starting and stopping a timer at two predetermined transducer signal levels. The first automated reciprocal-time measurement system developed by Pardue et al.,[252] is shown in Figure 23. When the transduced signal becomes equal to the first preset value, switch S_1 opens, allowing the bias voltage E_b to be applied in opposition to the transduced signal. Simultaneously, switch S_2 closes, actuating a synchronous motor mechanically linked to the shaft of a potentiometer which serves as the input resistance to the inverting operational amplifier. When the transducer voltage has increased by the amount E_b, the control system closes S_1 and opens S_2, deactivating the motor. During the time interval, Δt, the input resistance is increased from 0 to some value, R_{in}, given by:

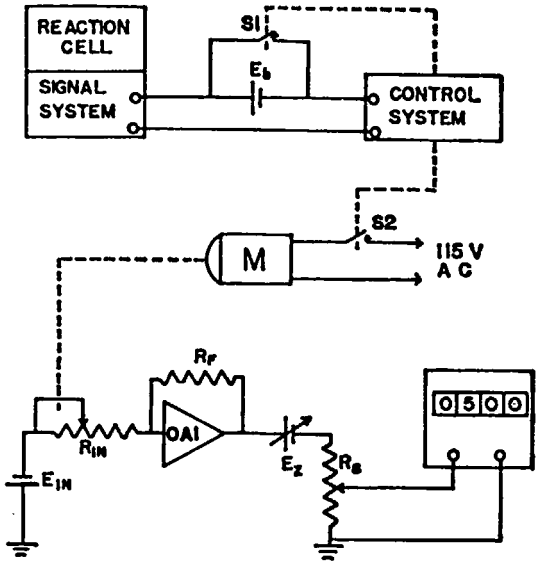

FIGURE 23. Automated reciprocal-time measurement system. (From Reference 252 courtesy of the American Chemical Society, Washington, D. C.)

$$R_{in} = k\,\Delta t \tag{28}$$

Thus, the output voltage of the inverting OA is given by:

$$e_{out} = -e_{in}\frac{R_f}{R_{in}} = -e_{in}\frac{R_f}{(k\Delta t)} \tag{29}$$

The constants $-e_{in}$, R_f, and k can be incorporated into a new constant, k', resulting in the expression:

$$e_{out} = k'\left(\frac{1}{\Delta t}\right) \tag{30}$$

The amplifier output is divided by potentiometer R_s to allow direct readout in desired concentration units at the digital voltmeter. A variable source E_Z provides compensation for nonzero intercept working curves.

An all-electronic variable time rate meter has been developed by Stehl et al.,[253] which measures the interval Δt, computes log Δt, and subsequently differentiates the expression to provide a readout voltage directly proportional to $1/\Delta t$. A similar approach has been taken by James and Pardue[254] for computing reciprocal time. The basic computation circuit illustrated in Figure 24 uses four operational amplifiers which perform a series of operations sequentially. The first OA (OA4) integrates the signal from a voltage interval detector providing an output for a time interval, Δt, which is given by:

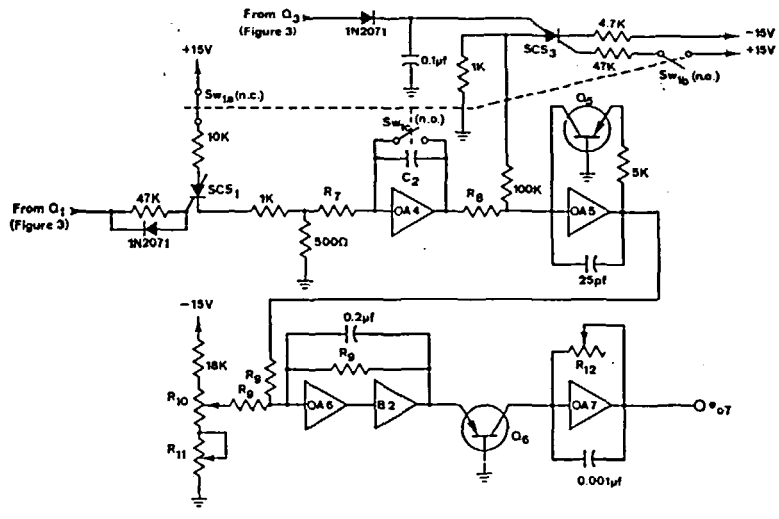

FIGURE 24. Basic circuit for an all-electronic reciprocal-time computer. (From Reference 254 courtesy of the American Chemical Society, Washington, D. C.)

$$e_{04} = -\frac{e_{in}}{R_7C_2}\Delta t \qquad (31)$$

This output is applied to the input of a log amplifier (OA5) whose output is given by:

$$e_{05} = E_o \text{ Log } \frac{e_{04}}{R_8I_1} \qquad (32)$$

where E_o and I_1 are characteristics of the log element, Q_5. OA6 is wired in a summing configuration allowing the addition of a reference voltage, e_{ref}, to the log output:

$$e_{06} = -\left(e_{ref} + E_o \text{ Log } \frac{e_{04}}{R_8I_1}\right) \qquad (33)$$

The addition of a reference voltage is necessary to insure that the output of OA6 is maintained within the exponential range of transistor Q_6. The combination of Q_6 and OA7 provides computation of the exponential of the output of OA6. The output of OA7 is given by:

$$e_{07} = -R_{12}I_2 10^{e_{06}/E_o} \qquad (34)$$

where I_2 is a characteristic of the transistor, Q_6. Successive substitutions from Equations 33 and 31 into Equation 34 yields the final equation:

$$e_{07} = -\left[\frac{R_7R_8R_{12}C_2I_1I_2}{e_{in}} 10^{-e_{ref}/E_o}\right]\frac{1}{\Delta t} \qquad (35)$$

Since all factors within the brackets are constants, the output of the computation circuit is directly proportional to the reciprocal of the measurement time interval. The rate meter has been evaluated using a colorimetric procedure[255] for the determination of alkaline phosphatase activity. The results, shown in Table 4, indicate there is no significant difference between data obtained from recorded rate curves and from the rate meter.

A digital reciprocal time computer which eliminates the use of logarithmic elements and is capable of determining synthetic slopes ranging from 5 mV/sec to 20 V/sec has been reported by Crouch.[256] A block diagram of the measurement system is shown in Figure 25. The transduced signal, applied to a voltage interval sensor, starts and stops a time interval-to-voltage converter when two preset voltage levels, V_1 and V_2, are reached. Hence, the time required for the reaction to proceed over the selected concentration range is converted to a voltage V. A voltage-to-frequency converter provides an output signal with a frequency which is proportional to the input voltage V. A digital period meter is used to measure the period of this signal which is the reciprocal of frequency. Thus, the output of the rate meter is directly proportional to the reciprocal of the time interval.

3. Fixed-Time

The fixed-time approach to measurement of initial rates requires that the difference in the transduced signal level be measured at times t_1 and t_2. Since the measurement of the two signal levels is not simultaneous, the value obtained at time t_1 must be held or stored until the value at time t_2 is

TABLE 4.

Comparison of Computer Output with Recorder Data for the Determination of Alkaline Phosphatase in Reconstituted Blood Serum.[a]

Activity (mV)		Rel std dev (%)		
Computer	Recorder	Computer	Recorder	Rel diff (%)
113	109	0.63	0.92	3.67
87.7	87.9	0.74	0.65	0.28
64.7	64.8	0.96	0.93	0.15
43.5	43.7	0.71	1.00	0.46
21.6	21.6	1.43	1.18	0.00
11.0	11.0	1.11	0.64	0.00

[a]From Reference 254 courtesy of the American Chemical Society, Washington, D. C.

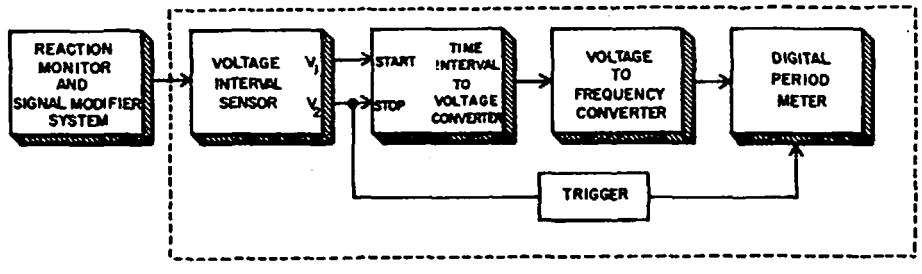

FIGURE 25. Block diagram of digital reciprocal-time reaction-rate measurement system. (From Reference 256 courtesy of the American Chemical Society, Washington, D. C.)

determined. Consequently, rate meters employing this approach must include a "memory" device. Since in the past such memory devices were not readily available, an alternate approach was taken. This technique was applied primarily to rate measurements on a continuously flowing stream. A block diagram for the method developed by Blaedel and Hicks[257] is shown in Figure 26. The reagents and samples are mixed and transported downstream at a constant flow rate through two observation cells. The signals provided by the transducers monitoring the two cells correspond to two different phases of the reaction separated by a constant time interval t_2-t_1. The transducer signals are fed to a sensitive differential recording filter photometer whose output is directly proportional to the initial rate. The primary disadvantage of this measurement system is that the measurement is made on a continuously flowing stream. This requires large volumes of reagent and sample and makes the approach somewhat limited from the analytical viewpoint.

4. Integration

The integration approach, illustrated in principle in Figure 15, uses integrators which have a "memory." The first portion of the integrated curve is always taken as a reference for the measurement regardless of the absolute value of the input signal. A block diagram of a measurement system using the integration technique is shown in Figure 27.[258] Sample and reagents are introduced in a reaction cell and the reaction is monitored by a suitable transducer. Proper input voltages for the integration and subtraction circuits are obtained from a signal modifier and are directed by relays to appropriate sections of the circuit where the integration (over two equal periods of time) and subsequent subtraction operations are performed.

The logic circuit, which acts as a control system by actuating the relay drivers, can work continuously to provide sequential measurements or can provide for a single measurement. Triggering can be manual or automatic by synchronization

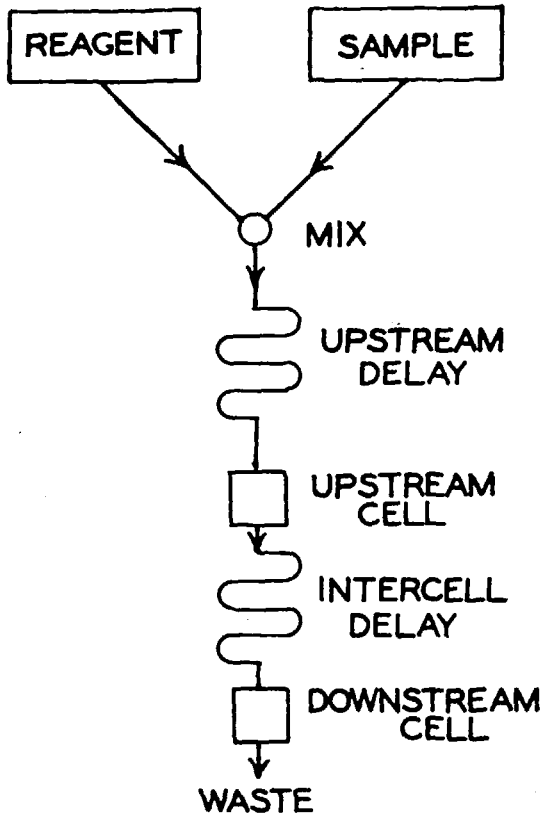

FIGURE 26. Outline of continuous measurement of reaction rates by a fixed-time method. (From Reference 257 courtesy of the American Chemical Society, Washington, D. C.)

with the sample introduction system. Upon receipt of a suitable trigger pulse, the logic system controls the sequencing of delay time, the length of the integration periods, the subtraction, and the readout. The analog circuit used for the integration and subtraction operations, shown in Figure 28a, uses two operational amplifiers (OA's) which are connected to one another and to the signal modifier by a relay network. During the first integration period, the signal is directed through relays 1 and 2 to the integrator (OA2). If the signal modifier output is as illustrated in Figure 28b, then line BC represents the signal applied to the integrator which charges capacitor C causing the output of OA2 to rise to a voltage V_1 as shown in Figure 28c. During the second integration period, the signal from the modifier is first directed to the gain-of-one inverter (OA1) by relay 1 and then to the integrator by relay 3. The voltage represented by line DE is applied to the integrator input which discharges capacitor C, and the integrator output decreases in magnitude by the amount V_2. The voltage difference ΔV, which is read out at the end of the measurement period, is proportional to the difference in area, ΔA, and is therefore proportional to the initial slope according to Equation 15.

The rate meter is capable of measuring slopes over the range 0.45 mV/sec to 450 V/sec and has been evaluated using the enzymatic determination of glucose[259] (Table 5) and the determination of

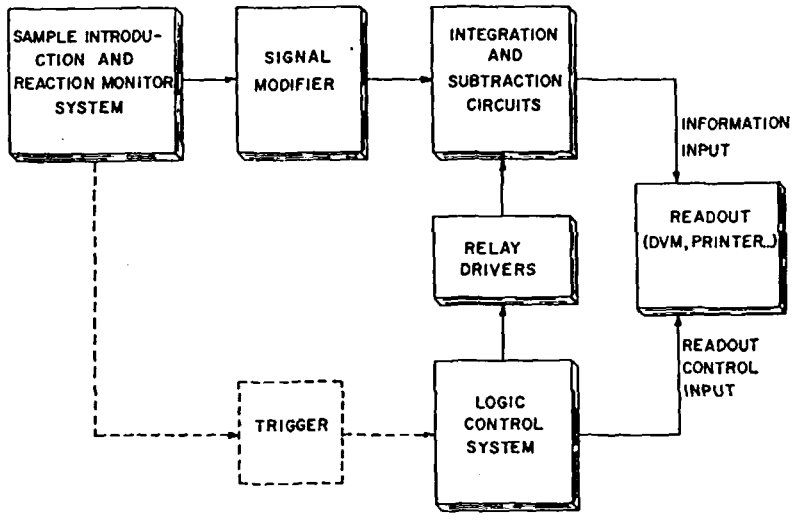

FIGURE 27. Block diagram of reaction-rate measurement system using an integration technique. (From Reference 258 courtesy of the American Chemical Society, Washington, D. C.)

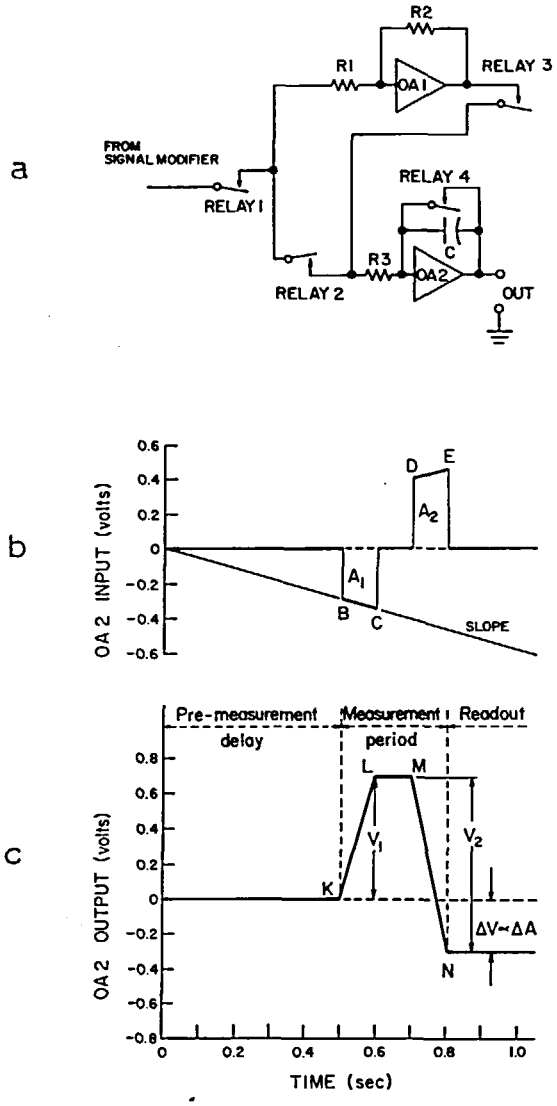

FIGURE 28. a) Integration and subtraction circuit of ratemeter. b) Oscilloscope trace of slope from ramp generator and input voltage of OA2. c) Oscilloscope trace of output voltage of OA2. (From Reference 258 courtesy of the American Chemical Society, Washington, D. C.)

phosphate using the 12-MPA method[26] (Table 6).

Recently, Ingle and Crouch[260] developed a digital integration procedure based on the same measurement principle. Rather than using analog integration and subtraction circuitry, a digital up-down counter is used. The transduced signal is directed to a voltage-to-frequency converter and the resultant pulses are counted by the up-down counter; during the second integration period it is set as a down-counter. At the end of the measurement period, the number of counts, which is proportional to the slope of the rate curve, is read out.

TABLE 5.

Automatic Results for Glucose[b]

Direct concentration readout[a]	Glucose concentration in μg/ml: Taken	Rel error, %	Rel std dev, %
5.0	5.0	0.0	1.6
10.0	10.0		1.0
15.1	15.0	+0.7	0.7
20.1	20.0	+0.5	0.8

[a]Averages of 5 results; 10.0 μg/ml standard used to set readout; integration time: 10 sec; premeasurement time: 30 sec.

[b]From Reference 258 courtesy of the American Chemical Society, Washington, D. C.

TABLE 6

Automatic Reaction-Rate Results for Phosphate[c]

p, μg/ml	Average Readout, mv	% Relative Standard Deviation[a]
0.50	54	1.01
1.00	103	0.79
1.25	125	0.85
1.50	149	1.05
1.75	172	0.97
2.00	200	0.54
2.25	226	0.40
2.50[b]	251	0.42
3.00	302	0.79
3.50	352	0.95
4.00	401	0.67
4.50	451	0.44
5.00	501	0.21

[a]The % relative standard deviation between averages of ten results.

[b]The 2.50 μg/ml standard was used to set the readout for direct digital concentration data.

[c]From Reference 26 courtesy of the American Chemical Society, Washington, D. C.

E. Considerations in Selecting Method of Reaction Rate Computation

Proper selection of measurement approach can play a very important role in determining the ultimate reliability of the rate method which is developed. For example, the derivative method, which allows measurement immediately after initiation of reaction and therefore provides a true measurement of initial reaction rate, can be used

only in the relatively few cases where the rate curve is accompanied by a very low noise level. The two-point methods, while frequently used, require by the very principle of the technique that the determination be extended over a certain period of time. While the ratio $\Delta P/\Delta t$ is a measure of the slope of the line which connects the two preselected points, this line does not coincide with the tangent of the rate curve at time $t = 0$. However, the ratio $\Delta P/\Delta t$ can be related to the tangent at time $t = 0$ using the basic kinetic equations relating concentration and time. Hence the relationship between initial concentration and the measured ratio $\Delta P/\Delta t$ depends upon the reaction order, reaction mechanism, the kinetic role of sought-for species (substrate, catalyst, etc.), and the signal-concentration relationship of the transducer used to monitor the reaction.

These factors have been discussed by Pardue[261] and Ingle and Crouch.[262] The latter workers begin their theoretical treatment with a statement of the fundamental rate equation involved. Assuming that the rate measurement is made at times t_1 and t_2, an explicit relationship between the initial concentration C_o and the remaining parameters of the equation is obtained.

1. Reaction Order (First Order Reactions)

For a first order reaction, the concentration C at any time t is given by:

$$C = C_o e^{-kt} \tag{36}$$

where C_o is the initial concentration and k is the first order rate constant. If the change in concentration ΔC is measured between t_1 and t_2, the following equation results:

$$-\Delta C = C_o(e^{-kt_1} - e^{-kt_2}) \tag{37}$$

which can be rearranged and solved for C_o:

$$C_o = \frac{-\Delta C}{(e^{-kt_1} - e^{-kt_2})} \tag{38}$$

Equation 38 shows that if the fixed-time method is used, the denominator is constant and a linear relationship exists between initial concentration and the measured quantity, ΔC. This linearity holds even when the rate curve is not linear over the measurement interval.

If the exponential terms in the denominator are expanded in a Maclaurin series and all terms but the first are neglected, Equation 38 becomes:

$$C_o = \frac{-\Delta C}{k\Delta t\, e^{-kt_1}} \tag{39}$$

where $\Delta t = t_2 - t_1$. Initial inspection of Equation 39 would indicate that the variable-time method could be used for the measurement since a linear relationship exists between initial concentration and reciprocal of the measurement time, $1/\Delta t$. However, Equation 39 is an approximation of Equation 38; hence an error is introduced in the measurement if the variable-time method is employed. The error which results depends upon the value of $k\Delta t$ and is approximately 1% when $k\Delta t = 0.02$ and the change in concentration (ΔC) is less than 2% during the measurement interval.

A similar treatment can be made for the integration method where the measured value is the difference between two areas under the rate curve (Figure 15). If the integration is made over the two time intervals $(t-\Delta t)$ to t and t to $(t + \Delta t)$, the difference between the two areas, ΔA, is given as:

$$\Delta A = C_o e^{-kt} \frac{(e^{k\Delta t} - 1)^2}{ke^{k\Delta t}} \tag{40}$$

which can be rearranged and solved for C_o:

$$C_o = \Delta A \frac{ke^{kt} e^{k\Delta t}}{(e^{k\Delta t} - 1)^2} \tag{41}$$

Since t and Δt are constants, there is always a linear relationship between initial concentration and the measured value. Equation 41 indicates that the linearity is valid in any region of the rate curve, and the sensitivity of the integration technique increases as the value of the fractional term decreases. This is accomplished by making the measurement at times close to the initiation of the reaction ($t \to 0$) or, for a given t, using long integration intervals.

2. Reaction Mechanism and Kinetic Role of Analyte (Catalytic Reactions)

The above considerations have been made on the basis of a simple reaction mechanism. Catalyzed reactions involve at least two successive reactions and exhibit a wide variety of mechanisms, making a generalized treatment somewhat difficult. Usually the rate is expressed by an equation of the form:

$$\frac{d[P]}{dt} = F([X_1], [X_2], \ldots [X_i])\,[C_o] \tag{42}$$

where P is the measured parameter, F is a function

of the rate constants and concentration of the reactants, and C_o is the initial catalyst concentration. Equation 42 can be rearranged and set up for integration:

$$\int_{[P_1]}^{[P_2]} \frac{d[P]}{F([X_1],[X_2],\ldots[X_i])} = \int_{t_1}^{t_2} [C_o]dt \qquad (43)$$

then Equation 43 becomes:

$$[C_o] = \frac{\{G([P_2]) - G([P_1])\}}{\Delta t} \qquad (44)$$

Equation 44 indicates that application of a variable-time method would be advantageous in this case since the numerator is held constant and $[C_o]$ depends linearly on $1/\Delta t$.

However, when the mechanism of the reaction is more complex and the relationship between reaction rate and concentration of sought-for species is nonlinear, the respective rate equations must be individually considered in selecting the proper measurement method. A case in point is an enzyme-catalyzed reaction which is generally represented by the Michaelis-Menten expression:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_2} P + E \qquad (45)$$

where E is the enzyme, S the substrate, and P the product. Steady-state treatment gives the rate equation:

$$\frac{d[P]}{dt} = \frac{-d[S]}{dt} = \frac{k_2[E_o]\,[S]}{K_s + [S]} \qquad (46)$$

where K_s, the Michaeles constant, equals $(k_{-1} + k_2)/k_1$. Integration of Equation 46 and rearrangement of the resultant expression yields the following expression for measurement of initial enzyme concentration by a two-point method:

$$E_o = \frac{-K_s \ln\left(\frac{[S_2]}{[S_1]} - \Delta[S]\right)}{k_2 \Delta t} \qquad (47)$$

where S_1 and S_2 are the substrate concentrations at times t_1 and t_2 and $\Delta t = t_2 - t_1$.

If substrate determination is intended, then Equation 47 can be rearranged and made explicit for S, assuming that $K_s \geqq 100\,[S_1]$:

$$\ln \frac{[S_2]}{[S_1]} = -K_1 \Delta t \qquad (48)$$

where

$$K_1 = \frac{k_2[E_o]}{K_s} .$$

Noting that Equation 48 is a characteristic expression for first-order reactions, the fixed-time procedure would be the method of choice for accurate substrate determination. On the other hand, inspection of Equation 47 indicates that for determination of enzyme activity, the variable-time procedure is more appropriate.

3. Noise Immunity

In addition to the relationship between initial concentration and the measured parameter, another criterion which should be considered in selecting the rate measurement technique is the noise susceptibility of the method. As was demonstrated, noise susceptibility seriously limits the derivative method, therefore in the majority of cases, two-point methods are used. The way in which noise may introduce an error in the measurement differs from method to method. In the case of the fixed-time method, the value of the selected parameter, determined at a time t, will vary about an average value, thereby introducing an error on the order of the amplitude of the noise superimposed on the rate curve. In the variable-time method, noise spikes can trigger the transduced signal interval detector at moments when the reaction has not yet reached the preselected level. This method is also particularly vulnerable to random spikes which originate in other portions of the measurement system (relays, switches, etc.). The greatest noise immunity is provided by the integration technique which, by virtue of the integration process, averages the noise. The average of the noise, for a relatively long integration period, is essentially zero.

A comparison can be made between a regular two-point method and the integration method. Assuming that the noise is sinusoidal as shown in Figure 29, the largest relative error, E_1, in determining the transduced signal value, V, at a time t is given by:

$$E_1(\max) = \frac{\Delta V}{V} = \frac{B}{\Delta t \sin\alpha} \qquad (49)$$

where B is the amplitude of the noise. In determining the area under the rate curve over the same time interval Δt, the maximum relative error, F_2, is given by:

$$E_2(\max) = \frac{\text{area of half cycle}}{\text{total area}} = \frac{2B}{\pi f(\Delta t)^2 \tan\alpha} \qquad (50)$$

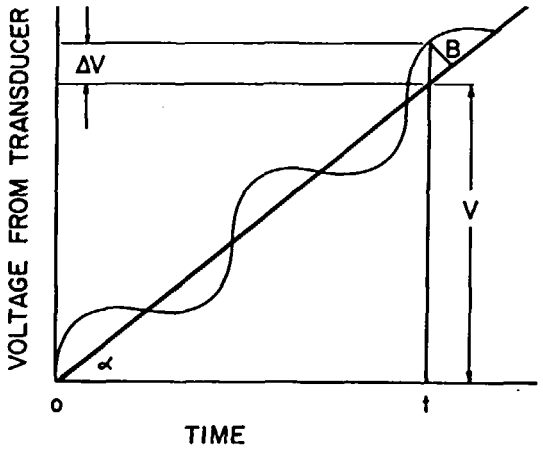

FIGURE 29. Expanded section of "noisy" rate curve with sinusoidal noise. (From Reference 258 courtesy of the American Chemical Society, Washington, D. C.)

where f is the frequency of the noise. The ratio of the two maximum errors is:

$$\frac{E_2(max)}{E_1(max)} = \frac{2\cos\alpha}{\pi f \Delta t} = \frac{kTn}{\Delta t} \qquad (51)$$

where T_n is the period of the noise and

$$k = \frac{2\cos\alpha}{\pi}$$

For a given phase angle, k is a constant which is less than unity. Equation 51 shows that if the integration time interval Δt is much larger than the period of the noise, the error introduced by the integration technique is much smaller than by the two-point method.

VIII. SOFTWARE SYSTEMS FOR DATA MANIPULATION AND EXPERIMENTAL CONTROL

The described measurement devices decode reaction-rate information by implementing a series of mathematical operations. The primary operation, measurement of the rate of change of a selected parameter, has generally been accomplished using analog circuitry whose output can be related to initial concentration of the rate-determining species. Since the relationship between readout and concentration is not always a simple function, frequently a number of data manipulation steps are required to provide an ultimate digital readout in the desired concentration units. These operations have been implemented using either analog circuits or appropriately programmed computers. In the past, computer application for data acquisition, reduction, and analysis has generally involved the passive use of a large computer system which sequentially performed these operations upon completion of the experiment. Only recently small dedicated laboratory-oriented computers have been made available for real-time data acquisition and processing. Furthermore, such small on-line computers are capable of interacting with the complete measurement system in much the same way as a human experimenter. That is, the computer can be used for surveillance of the entire process from reactant introduction to data reduction and for acquisition of analytical results in sufficient time to provide feedback control of the entire process by means of self-corrective or decision-making programming. This has extended the concept of automation from systems used solely for routine analyses to experimental systems used for chemical research. The use of such high speed "analytical tools" to perform routine experimental tasks and to automatically make defined real-time decisions regarding what to do next in an investigative experiment efficiently provides more meaningful and reliable results. Repetition of entire experiments due to improper choice in the course of action based on incomplete or preliminary evaluation of data is eliminated. The researcher is free to address himself to those tasks and decisions which require his immediate attention and scientific expertise.

A. Exclusively Software Approach

Computerized systems for on-line processing of reaction-rate data for quantitative analyses have been described by James and Pardue[263] and Willis et al.[271] In the first system the transduced signal is entered by means of an analog-to-digital converter (ADC) into computer memory where it is stored as a function of time. A functional diagram of the computer program is shown in Figure 30.

The acquired data points are processed in parallel branches by the variable-time method and

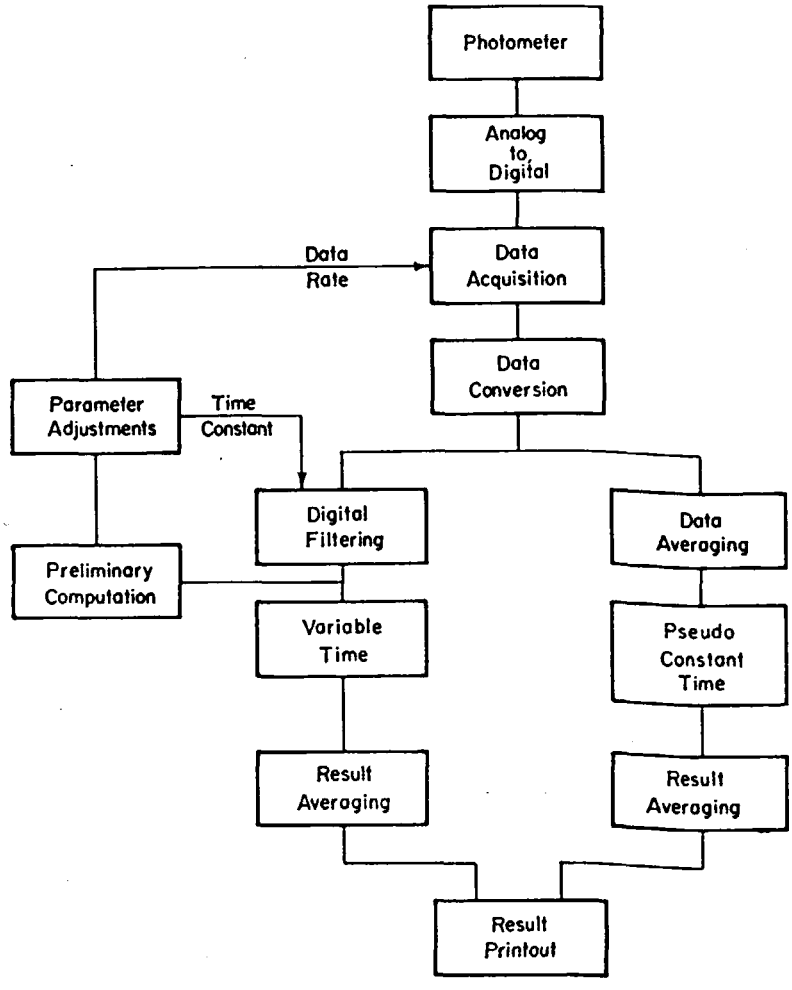

FIGURE 30. Functional diagram of computer program for reaction rate analyses. (From Reference 263 courtesy of the American Chemical Society, Washington, D. C.)

by a pseudoconstant time method which provides some versatility to the system. To insure that the available range of the ADC is not exceeded, a preliminary rate measurement is made by the variable-time program to eliminate noise pulses which could cause errors in the measurement. The time constant of this filter is also adjusted on the basis of such preliminary rate measurements. This allows an analysis of rates over a wide dynamic range without making any changes in hardware. After such preliminary computations and adjustments are completed, a series of independent measurements are made for each reaction. The results are averaged and printed out along with individual results on the teletype. To avoid any limitation in the rate of data acquisition, data conversions (e.g., transmittance to absorbance) are performed either prior to or after the data acquisition process.

The initiation procedure requires that information be entered into the computer in the form of decimal numbers or yes-no answers to a series of questions printed on the teletype by an initialization program. Since the program was written for general kinetic measurements, the experimenter must provide information regarding:

a. translation factors for conversion of ADC data to an appropriate transducer signal
b. direction of signal change
c. trigger level
d. number of multiple measurements to be made
e. information necessary to set up signal

intervals over which the measurements are to be made

f. a proportionality constant between the reciprocal time computed for an interval and the units to which that value is to be converted.

The computer-assisted system has been evaluated using two chemical systems. The results for determination of alkaline phosphatase activity indicate a relative standard deviation of about 0.3% and linearity (rate vs. activity) of 1%. Results from the catalytic determination of Osmium using the Ce(IV)-As(III) reaction system have relative standard deviations of about 5% at $10^{-11}M$.

The second software system referenced above[271] has been applied for on-line processing of stopped-flow data. The data acquisition and processing using ensemble averaging and quadratic smoothing are performed in real time. A number of parameters including A, ln $(A_\infty - A_t)$, and $d\ln(A_\infty - A_t)/dt$ are plotted vs. time and displayed on an oscilloscope, or a permanent record may be obtained on a punched paper tape or on a teletype print-out.

The systems described above use the computer to process data acquired at a computer-selected rate. While these operations are fully within the capability of analog systems, some are considered to be more easily implemented with a digital system. Such a software approach offers some advantages. These systems eliminate drift and offset problems associated with analog circuits. Smoothing routines can be applied, thereby eliminating the limitation of reduced response time associated with analog circuits used for damping and filtering signal noise. Digital systems offer greater versatility with regard to the manipulation of rate data. A choice in rate computation method (variable-time, fixed-time, etc.), final concentration or rate units, and print-out format can be programmed, thus eliminating major hardware changes associated with provision of such versatility in comparable analog circuits. Provision for data storage capability and feedback control based on self-corrective decision-making would require a rather complex hardware system.

B. Hybrid Hardware-Software Approach

On the other hand, exclusive use of a software approach requires that a computer with a large core memory be used, particularly in the case where feedback control of all aspects of the measurement process is desired. An alternate and more economical approach, in this case, consists of using a hybrid system which allows hardware-software interaction. Generally, an analog circuit is used for measurement of reaction rate while the computer is used to control all experimental equipment. Thus the computer is no longer committed to continuous monitoring, since it can determine when to read data and can spend the remainder of its time performing other tasks such as monitoring and controlling peripheral equipment and analyzing data. The rate is obtained directly rather than by calculation from many data points.

Such a hybrid approach has been taken by Eggert et al.[228] in developing a rather sophisticated system, ELLA. ELLA is capable of performing a kinetic experiment to a desired end point, making all necessary decisions and controlling all instrumentation via a special purpose time-sharing system. The interrelationship between the hardware and software of the system was illustrated in Figure 4 and discussed briefly during the description of sample handling systems. Reaction rate measurements are made by the integration technique using a ratemeter interface based on the design of Cordos et al.[258]

The overall flow diagram for the software of ELLA is shown in Figure 31. The software consists of four subprograms called the intialization routine, the on-line operating system, the updating system, and the clean-up routine. The first subprogram, the initialization routine, requests the experimenter to enter the volume of enzyme to be used and the incubation time. (The system has been applied to enzyme kinetic studies.) It then sets up five tubes containing an arbitrary volume of substrate, calibrates the reaction rate reading system, and prepares the blank reading tube automatically. When these operations are completed, the digital pipet is cycled to release the reagents which are subsequently mixed and transferred to the flow cell.

The on-line operating system performs many tasks simultaneously. To control the necessary time-sharing, a monitor system was developed to keep the reading, reagent preparation system, and decision-mak[illegible] systems from interfering with one another. Th[illegible]ow chart for the on-line operating system is given in Figure 32. First it takes readings of the rate of reaction and controls the reaction rate interface. When three points have been

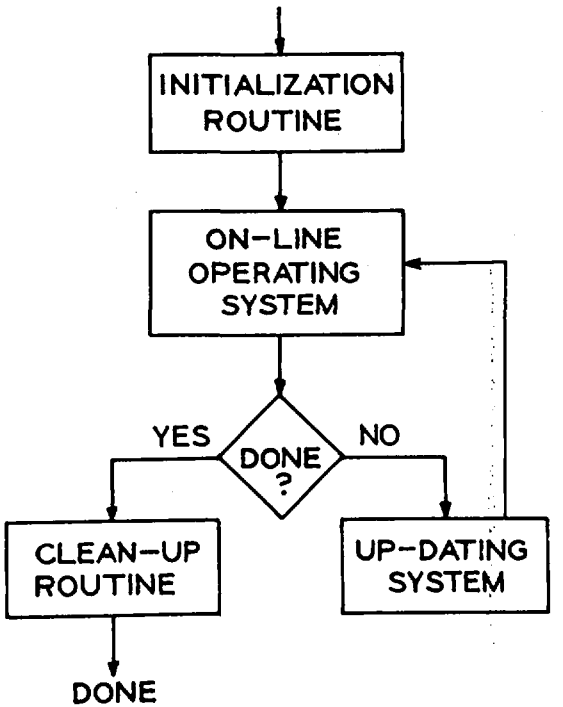

FIGURE 31. Flow chart of the experimental control and decision-making portion of ELLA. (From Reference 228 courtesy of the American Chemical Society, Washington, D. C.)

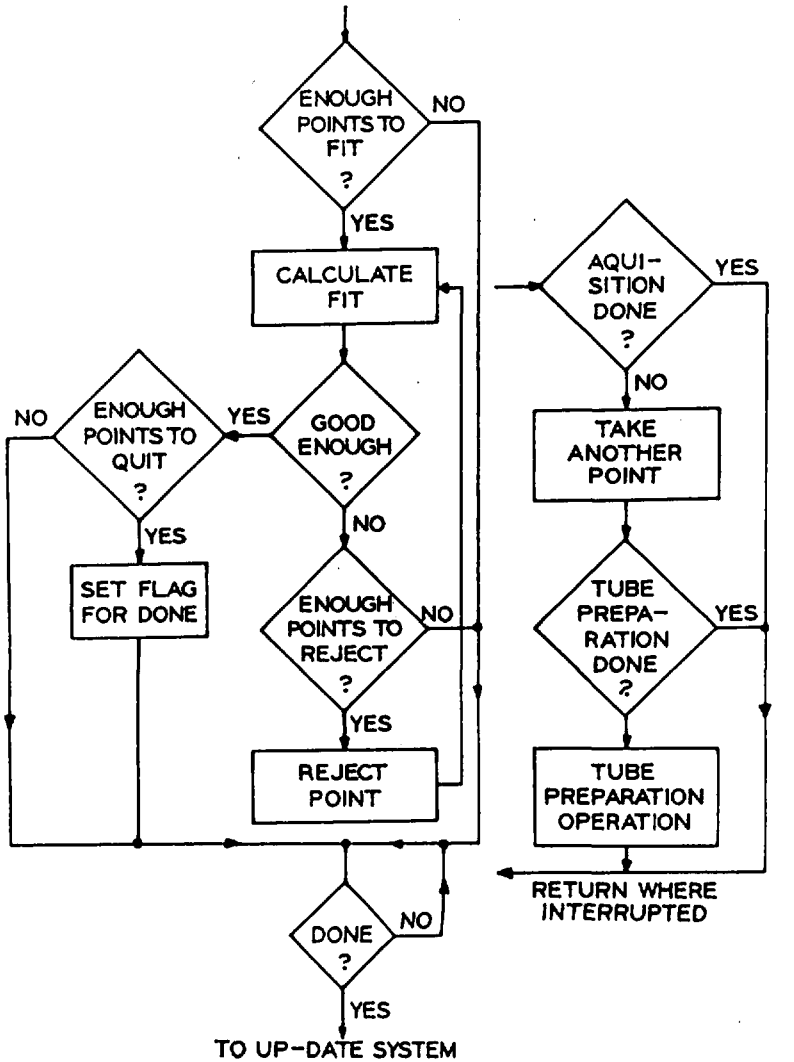

FIGURE 32. Flow chart of the on-line operating system of ELLA. (From Reference 228 courtesy of the American Chemical Society, Washington, D. C.)

obtained, they are fitted by a least-squares method to the Lineweaver-Burk Equation:

$$\frac{1}{v} = \frac{K_m}{V}\left(\frac{1}{S}\right) + \frac{1}{V} \qquad (52)$$

(where V is the maximum possible velocity, v the measured velocity, S the substrate concentration, and K_m the Michaelis constant), and the constants are determined. The error of estimation is compared with an error discriminator, the level of which has been determined and programmed by the experimenter. If the error estimation is below the discriminator, all points are retained; if not, a routine is called which rejects the worst point by a suitable criterion and the whole procedure is recycled. If six or more points are left in the system when the error of estimation falls below the discriminator, a flag is set indicating the experiment is completed. If less than six points remain, then a new substrate volume is computed which is based on the estimated constants of the equation. The necessary values are computed and stored in a preparation table. This procedure is repeated after every tube is run.

The up-dating program (Figure 33) runs after each tube to process information obtained from the previous tube and to set up for the next run. First the digital pipet is discharged and the timing circuitry is activated to set up this tube for reading and to re-initialize the hardware for preparation of the next tube. Next the flags set by the on-line program are examined to determine if the analysis is completed or if there is some reason to abort the experiment. Should this be the case, the reason is printed out. The program performs bookkeeping tasks and calculates the fit of the rate points. The calculated initial rate is corrected for blank and printed along with other relevant information. If the experiment is not completed, control is returned to the on-line operating system. If the experiment is completed, the cleanup routine is called which stores the data in the file on magnetic tape for successive experiments. The system then prints out this material upon command.

Two enzyme systems have been used to evaluate ELLA. The first, alkaline phosphatase, represents a nearly ideal system while the second, lactic dehydrogenase, can be readily made to behave nonideally, rendering it difficult for the human experimenter to study. For the ideal system, the relative standard deviations for the enzyme-substrate constants were 6 to 7% and

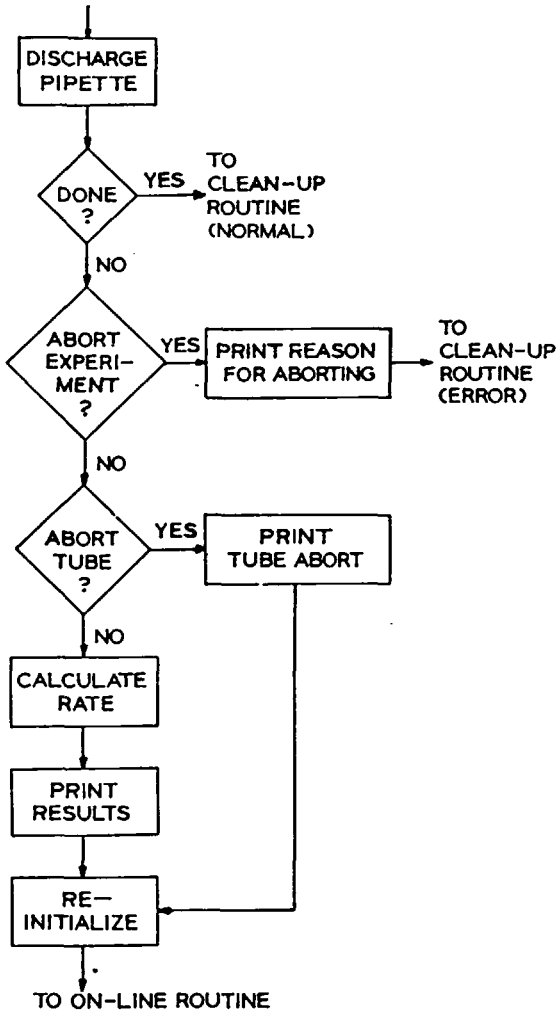

FIGURE 33. Flow chart of the up-dating system of ELLA. (From Reference 228 courtesy of the American Chemical Society, Washington, D. C.)

compared favorably with values obtained during humanly performed experiments under the same conditions (5 to 10%). Data collected for the nonideal system indicated ELLA's performance to be superior to that of the human experimenter.

IX. COMPLETE SYSTEMS FOR REACTION-RATE MEASUREMENTS

A. General Considerations

The basic components of a general automated reaction rate instrument can be used in a number of combinations to obtain desired characteristics. For example, one type of sample and reagent handling approach may be compatible with several different types of rate measurement systems. The characteristic desired may be that the complete

system is capable of measuring the rate of fast reactions as opposed to slow reactions, or that it is capable of performing parallel batch analyses as opposed to serial analyses. It is also possible to provide varying degrees of automation by means of feedback control of one or more processes from reactant preparation and introduction to readout. Furthermore, instruments can be designed to perform mechanistic investigations as well as simply carry out routine analyses.

The majority of commercially available "automated" instruments are only suitable for determination of rates of relatively slow reactions. With the exception of GeMSAEC, these systems perform the analyses serially. Such commercial units, in most cases, make spectrophotometric measurements which can be used for endpoint determinations in addition to kinetic techniques. The degree of sophistication, as well as the cost, is dependent upon whether a small computer and its peripherals are incorporated as part of the overall measurement and control system.

B. Diclan 240 (Honeywell®, Inc.)

For example, the system based on the analysis train approach to sample handling (Diclan 240, Honeywell, Inc.) uses a simple photometer module for the fixed-time measurement of initial rate. The photometer provides digital readout of the two absorbance values obtained, corrected for blank, and a sample identification number. The difference in absorbance must be manually computed and the concentration or enzyme activity determined from a suitable working curve.

C. Automatic Clinical Analyzer (E. I. du Pont® de Nemours and Co.)

Instruments are available which use small, ROM (read-only-memory) computers to interpret a code which specifies experimental conditions for the analysis, to automatically set the specified parameters, and to manipulate the data so that the final print-out is in desired units. In the case of the discrete reagent pack system (du Pont aca), the code is embossed on the header of the pack, allowing the tests to be performed in any order for a given sample. The ROM computer is also used to monitor the processing of sample. Should a malfunction be detected, it prints out a code indicating the source of error on the report sheet along with sample identification information and the test results. In addition to checking sample handling processes such as total sample and diluent volume delivered and analyzer processing temperature, the computer also detects deviations in decoding and readout processes such as optical cuvette formation and absorbance limits. To maintain a completely discrete processing of sample, after mixing and initiation of reaction the contents of the test pack are not transferred into a cuvette. Rather, upon entering the photometer station, a set of jaws close around the pack generating hydraulic pressure within the pack to form a precision cuvette between quartz windows, as shown in Figure 34. The excess fluid is channeled into a pressure relief cell. The initial rate is measured using the fixed-time method and the computer automatically supplies an appropriate conversion factor so that concentration or activity is printed out on the report sheet. However, this specific-purpose computer does not provide the versatility of the small dedicated general-purpose computers that can be programmed to meet new requirements.

D. ProgramaChem 1040 (American Monitor® Corp.)

A measurement system recently introduced by American Monitor Corporation, Indianapolis, Indiana, (ProgramaChem 1040) also uses a small computer to set the experimental parameters automatically. The instrument is programmed by insertion of a card containing the proper binary code for a given test. Samples are loaded on a rotating tray and the programmed test is performed sequentially on an aliquot dispensed from each sample cup. To perform another test, the program card corresponding to that test is inserted and the samples are processed again. Samples to be assayed are selected by pressing a button adjacent to the numbered sample cup. This system offers the advantage of computer manipulation of experimental parameters without locking the user into a closed system. Program cards can be readily fabricated in the laboratory from blank cards, thereby providing some flexibility in methodology. The rate measurement and conversion to appropriate units are made by the computer in a manner similar to the du Pont aca system. The type of test performed, sample cup number, and test result are printed out.

E. ELLA

The use of a small computer with a spectro-

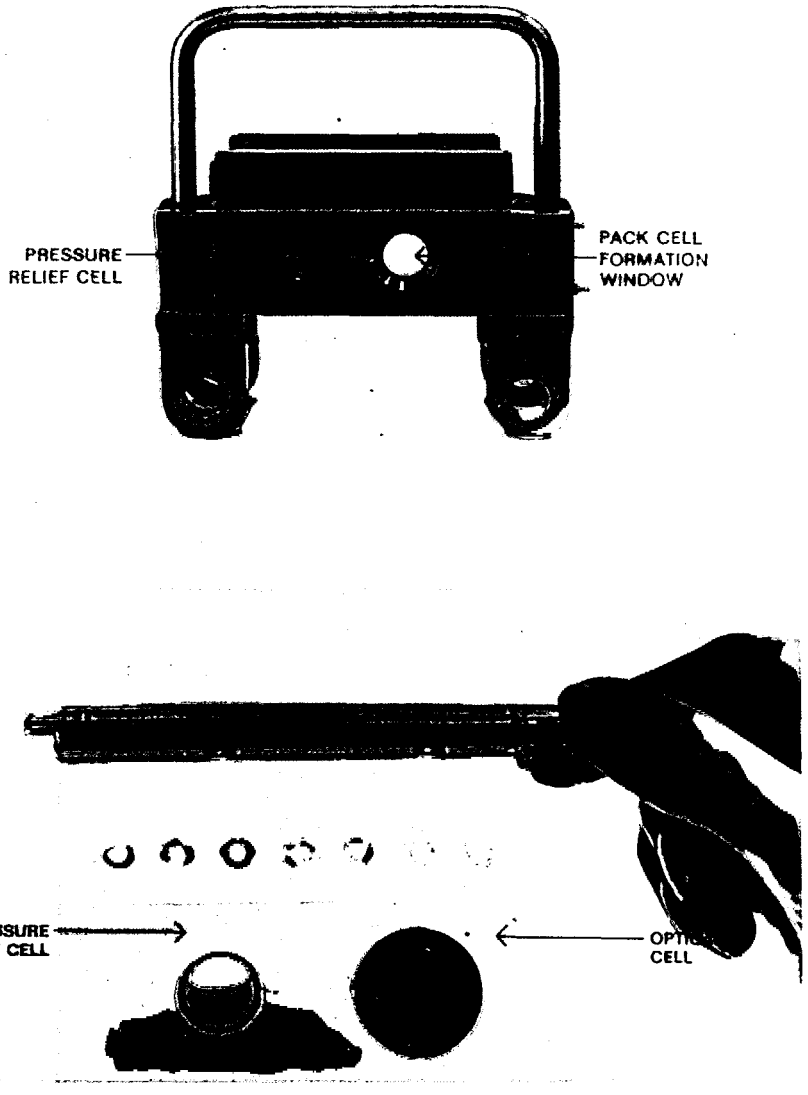

FIGURE 34. Upper – du Pont Automatic Clinical Analyzer cell forming die. Lower – test pack with formal optical cell. (Courtesy of E. I. du Pont de Nemours and Co., Wilmington, Delaware.)

photometric rate system can be extended to mechanistic investigations by programmed decision-making. Automation of such investigative experiments enhances the collection of data required for the development of new, reliable kinetic methods. This approach to automated instrumentation was discussed in some detail for a specific example (ELLA) in the previous section of this review. For such investigations, computer-controlled peripheral devices are useful for presentation of data in an easily interpreted final format. For example, Figure 35 illustrates a computer-generated Lineweaver-Burk plot obtained for an enzyme-substrate experiment using ELLA.

F. Automated Instrumental System

Recently, Deming and Pardue[264] have developed a computer-controlled instrumental system for characterization of chemical reactions. The system consists of four distinct elements: a programmable digital computer (controller), a spectrophotometer used to encode the chemical information into electrical information, an analog to digital converter, and a block for initiation and control of the experiment. The initiation and

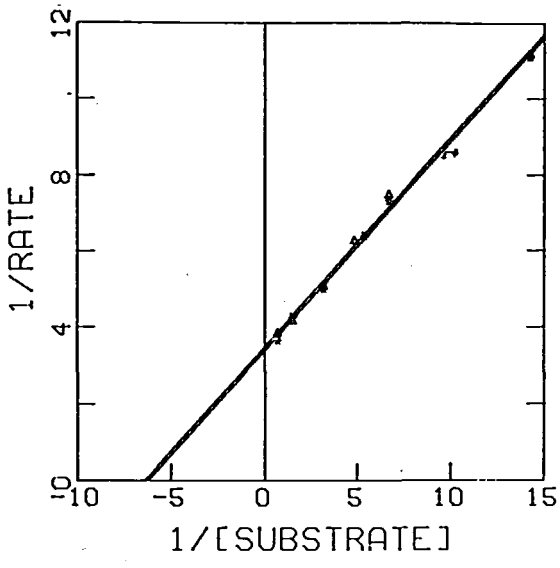

FIGURE 35. Computer generated Lineweaver - Burk plot of duplicate substrate curve determinations of alkaline phosphatase. (From Reference 228 courtesy of the American Chemical Society, Washington, D. C.)

control block is composed of a number of electrical and mechanical components which enable the system to introduce into the spectrophotometer cell the proper volume of reagents and diluents. Four stepping motors are used to drive four calibrated syringes which pull the reagents from a stock reservoir and dispense them. The volume of delivered solution is controlled by applying a corresponding number of pulses to the stepping motors. The system is also provided with the ability to remove the contents of the cell, rinse the cell, and mix the reagents.

The Automated Instrumental System has been evaluated for three types of operations: routine operations, experimental design, and data interpretation.

For routine operations, the experiments are preprogrammed into the information base and data are plotted on the storage scope and printed out on the teletype. In the experimental design operation the concentration of reagents and the limits over which they must be varied are introduced into the computer. Then the instrument plots the reaction rate vs. concentration of the species.

Data interpretation type of operation is accomplished using the results of precision experiments. These results are interpreted and new experiments are automatically designed to complete the characterization by obtaining additional information regarding, for example, substrate dependence, types of inhibition, etc.

The Automated Instrumental System allows the time required for the operations involved in the experiment preparation to be decreased. The efficiency of the experimenter is greatly increased by the fact that the results of a characterization are rapidly available and he then has more free time to explore other tasks that are more difficult to automate.

G. GeMSAEC Fast Analyzer

The GeMSAEC principle provides parallel mixing and transfer of reactants followed by a rapid sequential (pseudo-parallel) analysis of samples and standards under similar experimental conditions. The reactant mixtures are transferred into reaction chambers which also serve as the photometric cuvette. The cuvettes rotate past a stationary light beam which is completely interrupted between the cuvettes. The signal from the photomultiplier is continuously displayed on an oscilloscope which is synchronized with the rotor to provide an immediate visual monitor of the signals for all of the cuvettes. The photomultiplier signal is also passed through a buffer amplifier, directed to an analog-to-digital converter, and subsequently to a PDP 8/I computer. During each rotation of the rotor, the dark current value is obtained between each peak, and a blank peak value equivalent to 100% transmittance, in addition to a series of standard and experimental peak values, is determined. Figure 36a illustrates an oscilloscope trace showing the signal for a single cuvette while Figure 36b illustrates a typical display for a water blank and duplicate sets of standards.

When an endpoint determination is made, the readout is obtained by averaging the results for a number of rotor passes. For a reaction rate procedure a fixed-time technique is used. Two or more sets of digitized absorbance readings for each cuvette are stored simultaneously and the difference calculated and expressed as ΔA/min. One commercial version of GeMSAEC (CentrifiChem®, Union Carbide, Tarrytown, New York) incorporates digital switches which allow an appropriate conversion factor to be manually set so that readout is direct in concentration or enzyme units.

The CentrifiChem has been evaluated for the kinetic mode of operation using a method for the determination of serum creatinine based on the

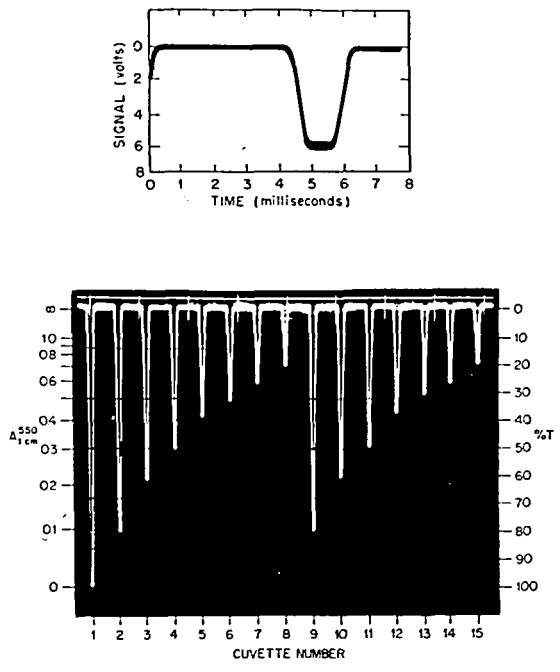

FIGURE 36. Upper – oscilloscope trace of signal for a single cuvette. (From Coleman, R. et al., *Amer. Lab.*, 3, 26, 1971. Courtesy of International Scientific Communications, Inc., Green Farms, Connecticut.) Lower – oscilloscope trace display for a water blank and duplicate sets of protein standards. (From Hatcher, D. and Anderson, N., *Amer. J. Clin. Pathol.*, 52, 645, 1969. Courtesy of J. B. Lippincott Co., Philadelphia, Pa.)

Jaffe reaction. [93] The working curve obtained in developing the method was linear for creatinine concentrations up to 100 μg/ml as shown in Figure 37. The family of curves shown correspond to data collected using the same time interval but different delay intervals from the time of initiation of reaction. The within-run precision for simultaneous analyses of 25 aliquots of a pooled serum as well as day-to-day precision during a 20 day interval for the same serum pool were evaluated. The results indicated a percent relative standard deviation of 7.1% and 5.9% for the within-run and day-to-day studies respectively.

H. Stopped-Flow System

The stopped-flow method has become the most popular approach to measurement of fast reaction rates. The most important component of the instrument, the sample handling system, has been described in detail earlier in this review. A schematic diagram of an automatic stopped-flow measurement system is shown in Figure 38. Upon initial activation of the system, reagent and sample

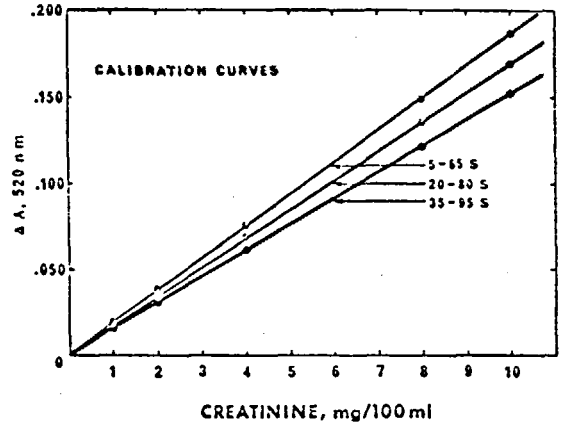

FIGURE 37. Working curves for the determination of serum creatinine. (From Reference 93 courtesy of the American Association of Clinical Chemists, Winston-Salem, North Carolina.)

are introduced at a rapid flow rate (1 m/sec) into the tangential mixing chamber. The reactant mixture flows through the observation tube until the check valve abruptly stops the flow of solution and the spectrophotometric rate measurement is rapidly made. The output of the current-to-voltage amplifier is directed to a digital ratemeter and control system [258] which also has been described earlier. The ratemeter, upon being triggered by the sample introduction system, automatically makes the rate measurement using the integration technique.

The output from the signal modifier is also directed to a storage oscilloscope to provide a continuous monitor of the reaction which may also be photographed for use in mechanistic investigations. A typical curve illustrating the rate of formation of 12-molybdophosphoric acid by reaction of phosphate and Mo(VI) in acid solution is shown in Figure 39. The apparent maximum is an indication of the old solution being flushed from the observation cell as the new reactant mixture is injected. Thus, a delay interval must be provided to insure that the rate measurement is made on the initial linear portion of the rate curve. The square waveforms shown in Figure 39 are provided by the logic circuit of the ratemeter and indicate over what segments of the rate curve the integration is made. The relative position and duration of the measurement intervals are determined by the variable delay and integration times selected.

This system has been further automated in our laboratories.[272] Once experimental parameters have been selected and samples and composite

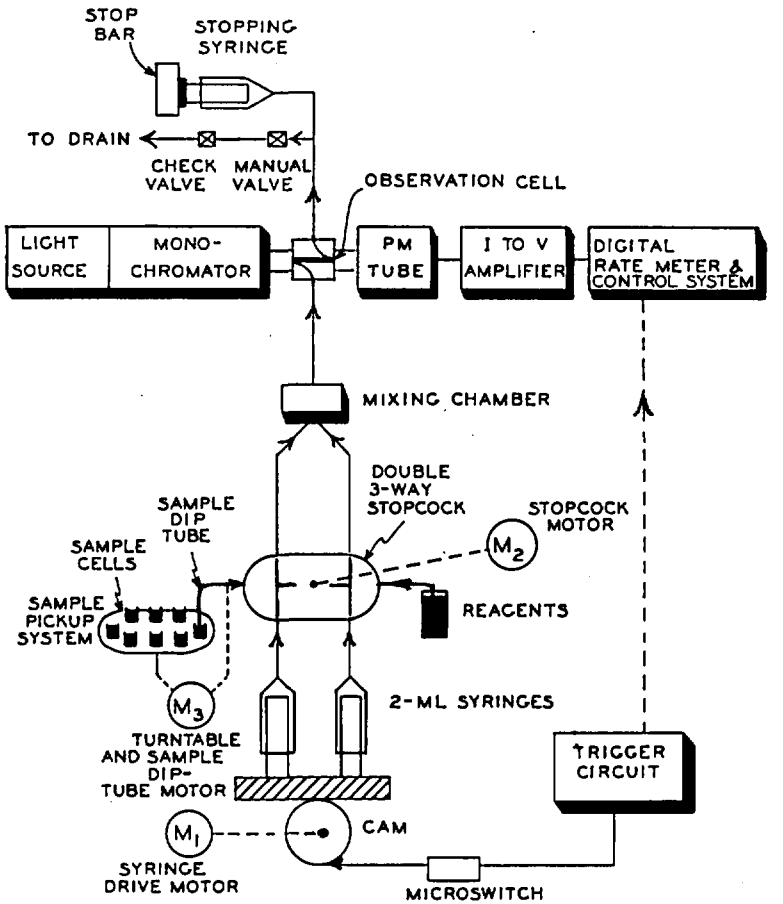

FIGURE 38. Schematic diagram of automatic stopped-flow system. (From Reference 26 courtesy of the American Chemical Society, Washington, D. C.)

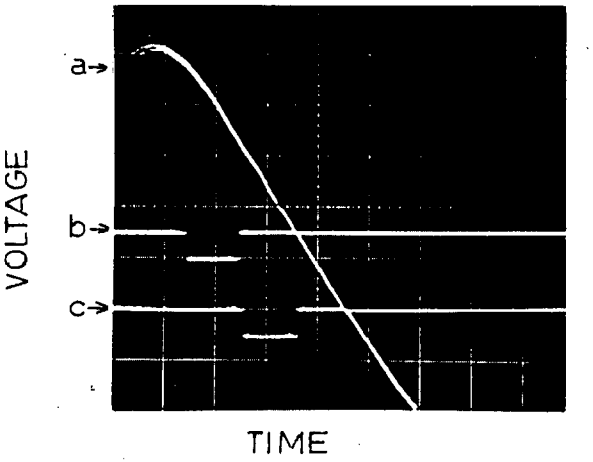

FIGURE 39. a) Typical reaction rate curve for phosphate and Mo(VI) in acid solution.
C_p = 3.0 μg/ml P
$C_{Mo\ (VI)_t}$ = 5.0 x 10^{-2} M
C_{HNO_3} = 0.4 M
Voltage Scale: 0.2 V/Div.
Time Scale: 0.2 sec/Div.
b) Ratemeter logic waveform for first integration period.
Voltage Scale: 5 V/Div.
Time Scale: 0.2 sec/Div. c) Ratemeter logic waveform for second integration period.
Voltage Scale: 5 V/Div.
Time Scale: 0.2 Sec/Div.

TABLE 7

Summary of Automatic Reaction-Rate Measurement Systems

Measurement system	Type of sampling	Sample preparation	Reagent preparation	Method of Aliquoting and mixing of reactants	Type of measurement (data domain conversion)
Honeywell® Inc. Diclan 240	Discrete	None	None	Syringe and pipet aliquot and dispense sample with diluent. Syringes, aliquot, and dispense reagents mixing via stirring rod.	Colorimetric. tungsten lamp; PM tube.
American Monitor Corp. Programa-Chem 1040	Discrete	None	None		Spectrophotometric
ELLA	Discrete	None	None	Digital pipet aliquots reactants and dispenses into mixing tube. Volumes controlled by computer. Mixing via magnetic stirring bar.	Spectrophotometric, tungsten bulb; PM tube
E. I. du Pont® de Nemours & Co. Automatic Clinical Analyzer	Discrete	Interferring substances removed by chromatographic columns	Reagents premeasured and sealed in test packs	Digital pipet aliquots dispenses sample, eluant and/or diluent. Reagents dissolved and mixed via mechanical oscillation of pack.	Colorimetric quartz-iodide lamp; PM tube
GeMSAEC analyzers	Discrete	None	None	Reactants manually aliquoted. Reactants transferred by centrifugal force and mixed via air stream.	Spectrophotometric tungsten lamp; PM tube
Stopped-flow system	Discrete	None	None	Reactants aliquoted and dispensed with syringes. Mixing via tangential mixing chambers.	Spectrophotometric tungsten lamp; PM tube
Technicon® Corp. Auto Analyzer	Continuous flow	Some interferring substances removed by dialysis	None	Reactants aliquoted and dispensed by peristaltic proportioning pump. Mixing via flow-thru mixing coils	Colorimetric PM tube

[a]Union Carbide (Centrifichem) uses hardware,
American Inst. Co. (Rotochem) uses PDP8/E with Termecomp tape deck,
Electro Nucleonics (GeMSAEC) uses PDP8/E,
Oak Ridge National Laboratories (Originator of GeMSAEC) uses PDP8/I with tape or disk storage.

TABLE 7 (continued)

Method of reaction-rate measurement	Data manipulation approach	Control system	Data output display	Time for one analysis	Number of analyses/hr
Fixed-time	Hardware, manual calculation ΔA and extrapolation from working curve.	None	Digital printer-displays sample number, A_{t_1} and A_{t_2}		100
Fixed-time	Software	Digital computer	Digital printer-displays test name, sample number, and result		200
Integration	Hybrid hardware-software	Digital computer μ-LINC 100(2K, 8 μsec) (Spear, Inc.)	Oscilloscope, teletype printout, Incremental plotter (Lineweaver-Burk Plots)	3 to 7 ½ min for 1 point; 20 to 30 min for/ E-S curve.	
Fixed-time	Software	Digital computer (ROM)	Digital printer-displays patient ID, test name, result, and error code	7 min	50
Fixed-time	Software	Digital computer[a] PDP 8/I or Hardware	Oscilloscope display A-t curve. Teletype printout A vs. t Teletype printout of test results	Average 5 min/disk. (16-place rotor)	Average 135
Integration	Hardware	Hardware-digital logic circuit	Oscilloscope displays %T-t curve. Digital voltmeter/printer displays test results	15 sec	240
Fixed-time				8 ½ min	40

reagent have been loaded on the sample tray, the stopped-flow measurement system operates automatically and continuously until reagents are exhausted. A logic circuit is used as a control system for a number of operations. At the end of a measurement cycle, the logic circuit provides a signal which activates the syringe drive circuit, thereby injecting sample and reagent automatically. The number of rate measurements per injection can be preset on the logic unit. Likewise, the number of injections per sample cup is programmed. A preselected number of injections are used to insure complete flushing of the flow system of previous reacted sample. During this flushing interval, the readout system is locked by the control circuit. After the preset number of injections and measurements have been made, the logic circuit activates the sample tray and the next sample cup is brought into position. A teletype is used to log the data and to generate a punched paper tape. The paper tape subsequently is used to input the data to a small computer (Digital Equipment Corporation PDP-8L). Results for standards and samples are computer averaged and corrected for blank if necessary. A least squares routine is used to provide printout of concentration of the samples.

Recently, Willis et al.[273] demonstrated the feasibility of simultaneous kinetic determination of mixtures by regression analysis using the on-line computer system for processing stopped-flow data cited earlier in this review.[271] Two and three-component mixtures of alkaline earths were determined at the 10^{-5} M level with percent relative standard deviations on the order of 2%.

The basic components of several automated measurement systems are summarized in Table 7. Presently there are three companies manufacturing GeMSAEC fast analyzers. These include American Instrument Co., (Rotachem) Silver Springs, Maryland; Electro-Nucleonics, Inc., Fairfield, New Jersey (GeMSAEC), and Union Carbide Research Institute, Tarrytown, New York (CentrifiChem). The three versions of GeMSAEC incorporate the same basic sample and reagent handling techniques, but differ in the control system and data output display approach. The three systems have somewhat different rate capabilities so the data in Table 7 are only a general indication for these types of analyzers. It should be again noted that while the du Pont Automatic Clinical Analyzer provides some sample and reagent preparation, there is no methodology flexibility. At present the user is dependent upon the company for method development as well as quality control.

The development of reliable reactant handling systems and the availability of small laboratory computers have made possible the development of completely automated reaction-rate methods. By using automated analyzers it is now feasible to conduct investigative experiments rapidly as well as perform routine analyses in a more reliable and efficient manner. Because of these new instruments it is expected that many more reaction-rate methods will become available.

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