A STUDY TO DETERMINE THE FEASIBILITY OF USING PHYSICAL METHODS FOR BIOCHEMICAL ANALYSIS UNDER SPACE FLIGHT CONDITIONS

FINAL REPORT

CONTRACT NASw -1560
16 JAN.-16 SEPT. 1967

PREPARED FOR
NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON, D.C.

HAYES
INTERNATIONAL CORPORATION
BIRMINGHAM, ALABAMA

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SUMMARY

Hayes International Corporation has conducted an eight-month study to determine the feasibility of using physical methods in biochemical analyses under space flight conditions.

The program was initiated with a series of literature searches, both computerized and manual, culminating in a massive search of Chemical Abstracts for the period from 1906 through 1966. The result of the literature searches is a comprehensive survey of analytical techniques and methodology, effectively defining the present state of the art relative to the biochemical constituents included in this study. The review of each constituent is complete in itself, including a merit evaluation of analytical methods with effective figures of merit and an annotated bibliography. This information is presented in summary form in a table. A concerted attempt was made to find or propose new physical methods and techniques of analysis. Three new analytical methods are presented in a separate section.

Based on the findings of this study, a number of recommendations concerning the further development of methodologies are offered.

CONCLUSIONS

As a result of this feasibility study, the following general conclusions have been drawn.

1. At the present time most analytical techniques, conceived and developed in terms of chemical analyses, cannot be readily altered to substantially reduce the amount of wet chemistry. In a few cases it has been suggested that filter paper be impregnated with reagents.
2. Several constituents, notably adrenocorticotrophic hormone, antidiuretic hormone, serotonin, and aldosterone, show little potentiality for effective assay in an orbiting laboratory. Indirect measurement, however, may in time become feasible.
3. The development of new physical methods, such as microcalorimetry and analyses of optical spectra, may permit determinations of many constituents with little or no chemical preparation. Very little attention has yet been given to these methods.
4. The introduction of physical techniques and methods into the procedures for biochemical analyses may permit simplification of the procedures for some constituents.
5. Clinical technology today is generally not advancing as rapidly as the increase of medical knowledge and industrial technology might indicate. A primary reason for this lag has been insufficient research directed toward new applications of basic research and the development of new analytical techniques.

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Section 1

INTRODUCTION

1.1 PROGRAM ADMINISTRATION

Hayes International Corporation, Birmingham, Alabama, under NASA Contract NASw-1560, has studied the feasibility of using physical methods to accomplish biochemical analyses under space flight conditions. The program was sponsored by the Space Medicine Directorate of the Office of Manned Space Flight, National Aeronautics and Space Administration. The technical representative for NASA headquarters was Dr. Sherman P. Vinograd, Director of Medical Science and Technology for the Space Medicine Directorate. The program monitor was Dr. Elliott Harris, Chief of Biochemical Research at the Manned Spacecraft Center, Houston, Texas.

The Hayes Program Manager was Dr. C. M. Askey, Chief of Research, and the Project Scientist was Ned D. Gilliam. The period of performance under the contract was 16 January - 16 September 1967. A group of eminent medical scientists from the University of Alabama Medical Center in Birmingham served as consultants and principal contributors to this study. These men, their fields of speciality, and their responsibility to this study were as follows:

Homer G. Biggs, Ph.D. Biochemistry	Amino nitrogen, BUN, Creatine, Creatinine, NPN, Total nitrogen, Bicarbonate, Chlorides, Phosphates, Pyrophosphates
G. William Cole, M.D. Hematology	Plasma thromboplastin component and other coagulation factors
Basil Doumas, Ph.D. Biochemistry	Fibrinogen, Hemoglobin, Methemoglobin, Mucoproteins, Proteins, Transferrins, Manganese, Sulfates, Zinc
Howard C. Elliott, Ph.D. Biochemistry	Adrenocorticotrophic hormone, Antidiuretic hormone, Catechol amines, Protein bound iodine, Serotonin, Thyroxine, Thyroxine binding prealbumin

Wells R. Moorehead, Ph. D. Biochemistry	Blood lactic acid, Sugar (glucose)
William Niedermeier, Ph. D. Biochemistry	Aldosterone, Alkaline phosphatase, Bilirubin, 17-Hydroxy corticosteroids, LDH isozymes, Calcium, Magnesium, Potassium, Sodium
Thomas A. Noto, M. D. Clinical Pathology	Immune bodies (complement and antibodies)
Kenneth M. Pruitt, Ph. D. Molecular Biology	General consultation
Jon V. Straumfjord, M. D., Ph. D. Clinical Pathology	Coordination and general consultation
Seymour S. West, Ph. D. Biomedical Engineering	Optical spectra
K. Lemone Yielding, M. D. Molecular Biology	Enzymatic reactions and general consultation
Edward L. Robinson, Ph. D. Nuclear Physics	Neutron activation

1.2 PURPOSE AND ORGANIZATION

The purpose of this study was to determine the feasibility of using physical methods for biochemical analyses under space flight conditions. The contract called for (1) a literature survey of industrial, Department of Defense, and academic sources with the submission of an annotated bibliography, and (2) a weighted evaluation of techniques and methods.

The literature survey, with which the program was initiated, included several automated searches by the National Library of Medicine, several by the Defense Documentation Center, inquiries to selected industrial sources, and a thorough search of Chemical Abstracts. The state of the art as revealed by the literature survey provided a meaningful context for evaluating new methods and variations or extensions of current methods for performing biochemical analyses. The limited duration of the contract and the time required to complete the literature survey, however, prevented as thorough an investigation of new methods as had been anticipated.

In the discussion of the various methods and techniques considered for the analysis of biochemical parameters, an attempt has been made to distinguish between currently proven and accepted methods, representing the present state of the art of clinical analysis, and extensions and variations of these methods which, along with new approaches to biochemical analysis, have not been fully explored and must be studied in more detail before designing flight instrumentation.

In studying the applicability of physical methods of analysis, one must realize that the distinction between physical and chemical methods is frequently a matter of degree. Most analytical methods require some chemical preparation (separation, reactions, etc.), and most methods employ physical measurement of the end point. The emphasis here has been on maximum reduction of wet chemistry.

Section 2

LITERATURE SEARCH

2.1 NATIONAL LIBRARY OF MEDICINE

The National Library of Medicine makes available to interested persons a literature search of journals abstracted in Index Medicus. This computerized search, known as Medical Literature, Analysis and Retrieval System (MEDLARS), is available only as far back as 1964, but it was felt that abstracts for these three years would provide a good introduction to recent work. Three search requests were submitted to the local MEDLARS office. The first request, submitted on 30 January 1967, asked for all references to analyses of body fluids for the biochemical constituents shown in Table 1. It also sought references to the use of physical methods for rendering biochemical analyses. A second request submitted at about the same time sought to determine what work had been done on the effects of space radiation (electromagnetic and particulate) on the parameters of Table 1. Because of a moratorium imposed on new MEDLARS searches by the National Library during December and January, these requests were not submitted by the local office until 24 February 1967. The returns from these requests were not received until 23 March 1967. Meanwhile, a third request was submitted relative to the analysis of all blood coagulation factors. The results of this third request were received about a month later.

At the time the MEDLARS searches were requested the format of the computer readout was not known, but a short abstract was believed to be included with each reference. When the returns were received, they contained only title, author, source, and key words. The absence of abstracts rendered the returns virtually useless, particularly in the remaining time. In the meantime, other literature searches had been initiated.

2.2 DEFENSE DOCUMENTATION CENTER (DDC)

At the same time the first MEDLARS searches were requested, similar requests were submitted to DDC. The first and second requests to DDC were

Table 1

CONSTITUENTS TO BE CONSIDERED FOR
PHYSICAL METHODS FOR BIOCHEMICAL ANALYSES

Creatine (S, U)	Pyrophosphates (U)
Creatinine (S, U)	NPN (S)
Proteins (S, U)	BUN (S)
Mucoproteins & related Biocolloids (S, U)	Sugar (WB)
Sodium (S, U, F, Sw)	Amino Nitrogen (S)
Potassium (S, U, F, Sw)	Total Nitrogen (U)
Chlorides (S, U, F, Sw)	Blood Lactic Acid (WB)
Phosphates (S)	Bilirubin (S)
Alkaline Phosphatase (S)	Protein Bound Iodine (PBI) (S)
Calcium (S, U, F, Sw)	17-Hydroxy Corticosteroids (U)
Magnesium (S, U)	Catecholamines (S)
Manganese (S, U)	Aldosterone (U)
Bicarbonate (S)	Antidiuretic Hormone (ADH) (S, U)
Zinc (S, U)	Serotonin (U)
Sulfates (S, U)	LDH Isozymes (S)
Thyroxine (S)	Transferrin (S)
Thyroxine Binding Prealbumin (TBPA) (S)	Hemoglobin (S, U)
Adrenocorticotrophic Hormone (ACTH) (S)	Methemoglobin (U)
Immune Bodies (Complement and Antibodies) (S)	Fibrinogen (U)
Plasma Thromboplastin Component (S)	

S = Serum or Plasma

U = Urine

WB= Whole Blood

F = Feces

Sw = Sweat

The same as those submitted to MEDLARS. The third request differed slightly. It requested data on flux densities and energy distributions of space radiation (again both electromagnetic and particulate), both inside and outside manned space capsules. Returns from the first two requests were received in about two weeks. As expected these requests produced very limited retrievals. The third request produced a bibliography of considerable length. The bibliographies, which included brief abstracts with each reference, were forwarded to the appropriate consultant for review and use if pertinent.

2.3 CHEMICAL ABSTRACTS

At the very beginning of the program the editorial office of Chemical Abstracts was contacted to determine whether or not computer searches of Chemical Abstracts were available. It was learned that computer tapes are currently being prepared, but automated searches are not expected to be available before 1969. At present the only means of reviewing Chemical Abstracts is manual. A very brief survey of subjects in Chemical Abstracts indicated that approximately fifty percent of the abstracts appeared in journals not abstracted by Index Medicus and hence would not be retrieved by MEDLARS. Furthermore, since MEDLARS covers only three years, some fifty-eight years of the literature abstracted by Chemical Abstracts would remain unreviewed without the manual search. At the first meeting with the consultants the matter of conducting a manual search of Chemical Abstracts was discussed at length. The consensus was that a complete review of Chemical Abstracts was requisite to adequately treat the problem. A system was set up to accomplish the formidable task of ferreting out every reference to the thirty-nine constituents in Table 1 plus related terms and reviewing every abstract relevant to the problem of applying physical methods to biochemical analyses.

Those pages of the cumulative indices which contained references to the thirty-nine biochemical constituents or related terms were xerographically copied and sent to the appropriate consultant. The consultant reviewed the indices, marking all abstracts he thought might be of interest. A graduate student or medical technologist assigned to assist the consultant retrieved the abstracts and read them. If he found that an abstract contained pertinent information on analytical techniques or instrumentation, he had it xerographically copied and submitted it to the consultant for further review. The index copying began with the volumes for 1906 and was carried through 1966. If an assisting searcher found an additional term which he considered important, the indices were checked and copied beginning with 1966 and proceeding backward to 1906 or until the term disappeared. During the course of the program specific papers were requested and copied. The total number of pages copied exceeded 9600.

2.4 INDUSTRY

From time to time during the program specific requests for literature references or reports were sent to selected individuals in the biomedical industry. In particular Dr. Robert Phillips, Vice President of G. K. Turner Associates, Palo Alto, California, manufacturers of fluorometric instruments, was contacted, apprised of the problem, and asked to send any pertinent reports or procedures that were available. He responded promptly and generously.

A similar request was sent to Dr. Gerald Soffen of the Jet Propulsion Laboratory at Pasadena, California. A copy of the Pace/Rho experiment* for analyzing animal urine in orbit was obtained.

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* See, for example, Biosatellite Project Document 883-12-06 (19 December 1966) and Biosatellite Fluorometry Experiment, JPL Technical Report (6 February 1967.)

Section 3

PHYSICAL METHODS OF ANALYSIS

3.1 INTRODUCTION

At the beginning of the program the biochemical constituents of Table 1 were divided into six somewhat arbitrary groups: proteins, protein metabolites, carbohydrates, steroids and enzymes, hormones, and inorganics. Each group was assigned to a consultant who became responsible for reviewing and evaluating the methods for each constituent in the group. Some subdivision of the groups was introduced during the early part of the program, and additional consultants were brought into the program. To provide a uniform format for the evaluation of analytical methods, the following outline was developed:

A. A brief statement of the principles of each significant method of analysis.

B. An evaluation of the suitability of analytical methods for use under space flight conditions.

   1. An evaluation of some fourteen merit parameters by assigning to each a rating between zero and ten, with zero representing the least desirable rating value and ten, the most desirable.

   2. A discussion of the merit table and the recommended techniques.

C. A discussion of areas deserving further research and development.

D. An annotated bibliography.

The merit table, as indicated above, presents some fourteen parameters, three of which are given actual values, while the remaining eleven are rated from zero to ten. The last item in the merit table is a Figure of Merit, which is calculated from the ratings assigned to the preceding eleven parameters and is intended to indicate the relative suitability of the method for use under space flight conditions. The Figure of Merit is calculated according to the formula

$$M = \frac{R}{4} \left(1 + 0.1\,G \right) \left(1 + 0.1\,S \right) \frac{\Sigma\, w_i \; P_i}{\Sigma\, w_i},$$

where R is the reproducibility, G is the suitability to null gravity, S is the

overall safety, and w_i is a weight attached to the merit parameter P_i. The values of the weights are listed in Table 2. The multiplicative factor 1/4 is a normalizing constant to provide a maximum value of 100 for the Figure of Merit. During the course of evaluating the various methods it was found that some merit parameters could not be quantitated for lack of information. In such cases a merit range is shown which corresponds to the range of the Figure of Merit with the indeterminate parameters set first to zero and then to ten. The mean Figure of Merit assumes a value of 0.5 for all indeterminate parameters.

Table 2

WEIGHTS ATTACHED TO MERIT PARAMETERS

Nontoxic reagents	1.0
Noncaustic reagents	1.0
Applicability*	0.8
Specificity	0.8
Insensitivity to environmental change	0.6
Reagent volatility*	0.6
Ease in training personnel	0.4
Degree of separation required (10=none)	0.4
Minimal handling by analyst	0.4
Common use of analytic equipment	0.2
Nondestructive of sample	0.2

* Not included in all merit tables

3.2 SUMMARY

In order to permit comparison of the best current methods of analysis, a summary is provided in Table 3. The order of the constituents has been

Table 3

BEST CURRENT METHOD FOR EACH DETERMINATION

Class.		Constituent	Method	Figure of Merit	Page
PROTEINS	Enzymes	Alkaline phosphatase	Phenolphthalein phosphate (Spectrophotometric)	59	11
		LDH isozymes	Electrophoretic separation	34	15
		Coagulation factors	Photometer Turbidimetric	39*	32
		Fibrinogen	Martinek	70	39
		Blood hemoglobin	Oxyhemoglobin	74	43
		Plasma hemoglobin	Martinek	42*	48
		Urine hemoglobin	Dip stick	87	52
		Immune bodies	Electrophoresis	29	56
		Methemoglobin	Martinek	59	71
		Mucoproteins	Protein-bound hexose	9*	75
		Serum albumin	Bromcresol green	75	78
		Total serum proteins	Refractometry	82	81
		Transferrin	Williams and Conrad	55	86
		Urinary proteins	Kutter (dip stick)	48*	91
	Hormones	Adrenocorticotrophic hormone	None		94
		Antidiuretic hormone	None		96
STER.		Aldosterone	Gas chromatography	3	98
		17-Hydroxycorticosteroid	Isotope dilution	57	100
NITROGENOUS COMPOUNDS		Catecholamines	"VMA" colorimetric	34*	107
		Serotonin	5-HIAA	4	110
		Thyroid function	Total thyroxine	22*	113
	Protein metab.	Amino nitrogen	β-Napthoquinonesulfonate	20*	119
		Blood urea nitrogen	Urease	48*	122
		Nonprotein nitrogen	Modified Kjeldahl	11*	130
		Total nitrogen	Modified Kjeldahl	11*	130
		Bilirubin	Iceterus index	34*	133
		Creatine	Modified Jaffe	14*	138
		Creatinine	Jaffe	29*	138
CARBO.		Lactic acid	Modified Loomis-Noll	79	144
		Glucose	Glucose oxidase	73	148
INORGANICS		Bicarbonate	Electrometric assay	65*	153
		Chlorides	Electrometric assay	68*	156
		Inorganic phosphate	Colorimetric	14*	158
		Inorganic pyrophosphate	Colorimetric	8*	158
		Manganese	Fernandez	7*	161
		Sodium, potassium, magnesium, calcium	Neutron activation	64	165
		Sulfates	Miller	36	168
		Zinc	Johnson	14*	172

*This figure represents a mean value of the Figure of Merit. Refer to page indicated. N.B. Care should be exercised in comparing

altered somewhat since the original grouping at the beginning of the program. The constituents have been divided into five major categories according to structure -- proteins, steroids, nitrogenous compounds, carbohydrates, and inorganics. Three minor categories group constituents with functional similarities -- enzymes, hormones, and protein metabolites. For methods having a merit range the mean figure of merit has been listed.

3.3 CURRENT ANALYTICAL METHODS

3.3.01 Alkaline phosphatase in blood serum

A. Principles of present methodology

1. Phenolphthalein phosphate method.[4, 5] (Spectrophotometric)

To a solution of phenolphthalein phosphate at pH 9.0 is added 0.2 ml. of blood serum. The solution is incubated exactly 30 minutes at 37° C during which time the enzyme present in the blood serum hydrolyzes the substrate, releasing free phenolphthalein which has a strong absorbance at 550 mμ. The reaction is first order and the absorbance at 550 mμ is proportional to enzyme concentration. The reagents have been prepared in the form of a stable compressed tablet to which only buffer solution and blood serum need be added to perform the test.[8]

2. Phenolphthalein phosphate method (Visual)

The test is performed as above, except that the color produced is compared with a color chart supplied by Warner-Chilcott, Moris Plains, N.J.

3. Test paper method

When mono alpha naphthylphosphoric acid (I) is treated with alkaline

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the figures of merit for various determinations. Because of the subjective aspect of evaluating analytical methods, the resultant figures of merit may not be absolutely consistent throughout the list; however, within a given category of constituents the figures of merit should give a reliable indication of the relative overall suitability of the methods for use in space.

phosphatase, alpha naphthol is liberated which will react with diazo-o-anisidine (II) to produce a compound with a blue violet color. The preparation of a test paper which consists of filter paper impregnated with I and II has been described.[7] The intensity of color produced when a drop of blood serum is placed on the test paper and incubated at room temperature in an atmosphere saturated with water vapor is compared with a standard color chart.

B. Applicability of present methodology to space flight conditions

1. Merit table

Alkaline Phosphatase in Serum

Merit Parameters	Phenolphthlein phosphate		
	Spectrophotometric	Visual	Test Paper
1. Sensitivity	7.5 Bodansky u/ml	7.5 Bodansky u/ml	unknown
2. Sample size	0.2 ml.	0.2 ml.	1 drop
3. Time required	15 min.	12 min.	5 min.
4. Reproducibility	10	5	5
5. Suitability for null gravity use	4	6	10
6. Overall safety	10	10	10
7. Nontoxic reagents	10	10	10
8. Specificity	10	10	10
9. Insensitive to environmental changes	10	10	10
10. Ease in training personnel	5	9	10
11. Degree of separation required (10=none)	10	10	10
12. Minimal handling by analyst	4	8	10
13. Common use of analytic equipment	10	8	8
14. Nondestructive of sample	0	0	0
15. Figure of merit	59	36	47

2. Discussion

Several reports attest to the simplicity and reliability of both the spectrophotometric and visual methods using phenolphthalein phosphate substrate.[1,3,6] Although the visual comparison gives only semiquantitative results, it appears to be adequate for diagnostic purposes. The procedures are generally accepted and diagnostic values are well established by these methods. Results obtained by the test paper method are less well documented. In view of its simplicity, further investigation of this method appears to be indicated. The application of test papers and compressed tablets to the problem of laboratory diagnosis has been discussed.[2]

C. Promising areas for research and development

The methods described appear adequate for clinical purposes and are sufficiently simple that adaptation to the space environment should pose no formidable problems. One promising area for future development would be thermal analysis based on the heat of reaction involved in hydrolysis of the substrate by alkaline phosphatase.

D. References

1. Babson, A.L.; Read, R.A.; Phillips, G.E.; Luddecke, H.F.; Use of a New Assay in Study of Serum Alkaline Phosphatase Levels in 2,000 Hospital Patients. Clin. Chem., Vol. 6, 1960, pp. 495 - 500.

Describes application of commercially available tablet (Phosphatabs)* for routine use in clinical laboratory. Results were found to be reliable and in agreement with those obtained by another established procedure.

2. Drevon, B.; Accelerated Biological Analysis; Use of Tablets and Reactive Papers; General Considerations. Ann. Biol. Clin., Vol. 21, 1963, pp. 453 - 456.

Discusses problems of application of tablets and reactive papers to the search for abnormal substances in urine.

3. Feldman, P.E.; A New Method for Alkaline Phosphatase Level Determinations. Am. J. Med. Tech., Vol. 25, 1959, pp. 143 - 144.

Results using "Phosphatabs"* were found to be reliable in both jaundiced and nonjaundiced patients. Test required 12 minutes to complete.

4. Hovels, O.; Laun, M.; On Determination of Alkaline Phosphatase Activity in Small Amounts. Z. Kinderheildt, Vol. 71, 1951, pp. 357 - 368.

Describes a micromethod based on the method of Huggins and Talalay using phenolphthalein phosphate as substrate. Method requires 0.05 ml of blood serum.

5. Huggins, C.; Talalay, P.; Sodium Phenolphthalein Phosphate as a Substrate for Phosphatase Tests. J. Biol. Chem., Vol. 159, 1945, pp. 399 - 410.

Describes a synthesis of the substrate and its application to determination of alkaline phosphatase. Simplicity and accuracy are discussed.

6. Klein, B.; Read, P.A.; Babson, A.L.; Rapid Method for Quantitative Determination of Serum Alkaline Phosphatase. Clin. Chem., Vol. 6, 1960, pp. 269 - 275.

Describes use of a compressed tablet and direct photometric measurement of phenolphthalein released from phenolphthalein phosphate. As many as 30 determinations were completed per hour.

7. Menske, R.; Method for the Rapid Detection of Alkaline Phosphatase. Naturwissenschaften, Vol. 46, 1959, pp. 668 - 669.

Describes the preparation and use of a test paper for semiquantitative determination of alkaline phosphatase in blood serum which gives clinically useful results.

8. Warner-Lambert Pharmaceutical Co. Serum Alkaline Phosphatase Diagnostic Preparation. Brit. Patent 863739, March 22, 1961.

Describes the preparation of a compressed tablet which contains phenolphthalein phosphate, magnesium sulfate and tris buffer which provides a simple method for determining alkaline phosphatase in blood serum.

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* Trademark of Warner-Chilcott, Morris Plains, N.J.

3.3.02 Lactate Dehydrogenase (LDH) Isozymes

A. Principles of present methods

1. Electrophoretic separation of isozymes.
2. React separated isozymes with a tetrazolium salt in presence of sodium lactate, phenazine methosulfate and niacin adenine dinucleotide (NAD).
3. Fix strip in an aqueous solution of 50% methanol and 10% acetic acid.
4. Determine intensity of reduced tetrazolium with a scanning microdensitometer similar to the Analytrol.*

B. Applicability of present methods to space flight conditions

1. Merit table

LDH Isozymes

Merit Parameters	
1. Sensitivity	-
2. Sample size	5 µl
3. Time required	2 hrs.
4. Reproducibility	8
5. Suitability for null gravity use	8
6. Overall safety	8
7. Nontoxic reagents	5
8. Specificity	10
9. Insensitive to environmental changes	5
10. Ease in training personnel	5
11. Degree of separation required (10=none)	0
12. Minimal handling by analyst	5
13. Common use of analytic equipment	5
14. Nondestructive of sample	0
15. Figure of merit	34

2. Discussion

The present state of the art requires separation of isozymes by

* Spinco Division, Beckman Instruments, Palo Alto, California

some physicochemical method prior to their quantitation. Electrophoresis on cellulose acetate appears to be the most satisfactory method for accomplishing this.[7,12] An electrophoretic cell similar to that manufactured by the Millipore Corporation* appears adaptable to the space environment. In this apparatus buffer soaked sponges serve as electrode vessels. Other electrophoresis media have been used for separation of LDH isozymes. These include Sepraphore III,[10] agar gel,[8,15] and starch gel.[1] Of these, Sepraphore III** which is a modified cellulose acetate might afford separation of the isozymes in less time than is required on cellulose acetate. The use of higher potentials might achieve the same end. Thin layer chromatography on DEAE Sephadex*** has also been used for separating LDH isozymes.[4]

The color reagent is added to the electrophoretic strip from a second cellulose acetate strip which has previously been impregnated with the reagent.

The choice of tetrazolium salts for the color reagent and the choice of operating conditions to obtain optimal results have been discussed.[13] Experiences in the clinical laboratory have been described.[9] Spectrofluorometric[8] and spectrophotometric[15] methods of detection have also been described.

C. Promising areas for research and development

Several reports indicate that the LDH isozymes have different heats of inactivation.[5,11,16] Development of a method of analysis based on these properties and on sensitivity to inhibitors[6,14] would obviate physicochemical separation of the isozymes. Recent advances indicate, for example, that a method based on heat sensitivity may soon be available for routine laboratory use.

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* Millipore Filter Corp., Bedford, Mass.

** Registered trademark of Gelman Instrument Co., Ann Arbor, Mich.

*** Registered trademark of Pharmacia, Inc., Stockholm, Sweden

D. References

1. Allison, M. J.; Gerszten, E., Sanchez, B.; A Method for Quantitation of Lactic Dehydrogenase in Vertical Starch Gels. Tech. Bull. Registry Med. Tech., Vol. 33, 1963, pp. 165 - 167.

Separated isozymes on starch gel and determined activity with phenazine methosulfate. Quantitation was achieved with a microscope photometer.

2. Barnett, H.; Electrophoretic Separation of Lactate Dehydrogenase Isozymes on Cellulose Acetate. Biochem. J., Vol. 84, 1962, pp. 83 - 84.

Identified five well defined bands on electrophoretic patterns with LDH activity.

3. Clarke, J. T.; Simplified Disk (polyacrylamide gel) Electrophoresis. Ann. N. Y. Acad. Sci., Vol. 121, 1964, pp. 428 - 436.

Separated isozymes on polyacrylamide gel and determined enzyme activity with Nitro Blue Tetrazolium.

4. Determan, H.; New Applications of Thin Layer Chromatography. I Chromatography on Dextran Gel. Experientia, Vol. 18, 1962, pp. 431 - 432.

Effectively separated isozymes on DEAE Sephadex in a simple gradient chamber.

5. Dubach, U. D.; Vareokojic; The Diagnostic Significance of the Lactic Dehydrogenase Isozymes (Heat Inactivation Method). Schweiz. Med. Wochschr., Vol. 93, 1963, pp. 1196 - 1200.

Studied heat inactivation of LDH isozymes in 300 patients and correlated results with clinical diagnosis.

6. Fritz, P.; Rabbit Lactate Dehydrogenase 5: A Regulatory Enzyme. Science, Vol. 150, 1965, p. 364.

Oxalacetate activated LDH 5 at low concentrations and inhibited at high concentrations, whereas LDH 1 was only inhibited.

7. La Mancusa, S. J.; Romel, W. C.; Electrophoresis of Lactic Dehydrogenase Isozymes in Serum on Cellulose Polyacetate. Lancet, Vol. 1, 1964, p. 668.

A simplified method is described.

8. Laursen, T.; Electrophoresis of Serum Lactic Dehydrogenase on Agar Gel with Fluorimetric Determination of the Enzyme Activity. Scand. J. Clin. Lab. Invest., Vol. 14, 1962, pp. 152 - 156.

   Describes electrophoretic separation of isozymes on agar gel followed by fluorimetric determination of activity with the diphosphopyridine nucleotide-pyruvate reaction.

9. Mager, M.; Blatt, W.F.; Abelmann, W.H.; The Use of Cellulose Acetate for Electrophoretic Separation and Quantitation of Serum Lactic Dehydrogenase Isozymes in Normal and Pathological States. Clin. Chim. Acta. Vol. 14, 1966, pp. 689 - 697.

   Describes experiences with the method in a clinical laboratory. A discussion of the diagnostic significance of the results is given.

10. Opher, A.W.; Collier, C.S.; Miller, J.M.; Rapid Electrophoretic Method for the Determination of the Isozymes of Serum Lactate Dehydrogenase. Clin. Chem., Vol. 12, 1966, pp. 308 - 313.

    A convenient procedure for the separation and quantitation of LDH isozymes is described. It used polyacetate Sepraphore III for separation and tetranitro blue tetrazolium followed by densitometry for quantitation.

11. Paunier, L.; Rotthauwe; Determination of Serum Lactic Dehydrogenase Isozymes by Relative Heat Stability Test. Enzymol. Biol. Clin., Vol. 3, 1963, pp. 87 - 96.

    Found that 20% of LDH activity was lost following incubation of serum at 57° C for 30 minutes. Noted differences in serum from diseased subjects.

12. Preston, J.A.; Briere, R.O.; Batasakis, J.G.; Rapid Electrophoretic Separation of Lactate Dehydrogenase Isozymes on Cellulose Acetate. Am. J. Clin. Pathol., Vol. 43, 1965, p. 256.

    Gives a complete description of the method recommended in the present report.

13. Starkweather, H.H.; Spencer, H.H.; Schwartz, E.L.; Schach, H.K.; Electrophoretic Separation of Lactate Dehydrogenase Isozymes and Their Evaluation in Clinical Medicine. J. Lab. Clin. Med., Vol. 67, 1966, pp. 329 - 343.

Describes a procedure for controlled separation of the isozymes on agar gel and discusses conditions for accurate quantitation. A discussion of the diagnostic significance of the results is given.

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LDH 1 and 5 showed differential heat lability and were protected in a differential manner by metabolic intermediates.

15. Wieme, R.J.; van Sande, M.; Karcher, D.; Lowenthall, A.; van der Helm, J.; A Modified Technic for Direct Staining with Nitro-Blue Tetrazolium of LDH Isozymes in Agar Gel Electrophoresis. Clin. Chim. Acta., Vol. 7, 1962, pp. 750 - 754.

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Found that LDH 1 was stable at 65° for 30 minutes, but LDH 5 was labile under these conditions. Correlated results with clinical observations.

3.3.03 Coagulation factors

A. Introduction

The only coagulation factors listed in Table 1 were plasma thromboplastin component and fibrinogen; however, conversations with NASA indicated that the real interest was in coagulation in general. Consequently, a more comprehensive study of coagulation factors and their analysis in vitro was undertaken.

Coagulation studies are readily justified in space principally because of the radiation hazard. Radiation in experimental animals as well as humans has been shown to alter a variety of coagulation factors.

A list of coagulation factors is given in Table 4. These factors circulate in an inactive state, and are for the most part either proteins or lipoproteins. Most are enzymes which can be accurately measured only by means of their activity in coagulation. Reliable assay is limited to their ability to accelerate clot formation. Fibrinogen is the sole exception.

Figure 1 demonstrates how these factors interact to form a fibrin clot. Coagulation is divided into four phases:

1. Thromboplastin generation
2. Prothrombin conversion to thrombin
3. Fibrinogen conversion to fibrin clot
4. Fibrinolysis or clot lysis

Phase I or thromboplastin generation is concerned with the formation of a substance (thromboplastin) which will accelerate the conversion of prothrombin to thrombin. It is an enzyme or aggregation of enzymes which, in the presence of calcium, is required for optimal prothrombin conversion. Thromboplastin formation is initiated when blood comes in contact with a foreign surface (i.e. glass). Glass will activate Factors XII and XI. These in turn activate the enzymes Factors V, VII, VIII, IX and X. Maximal thromboplastin generation is accomplished only when

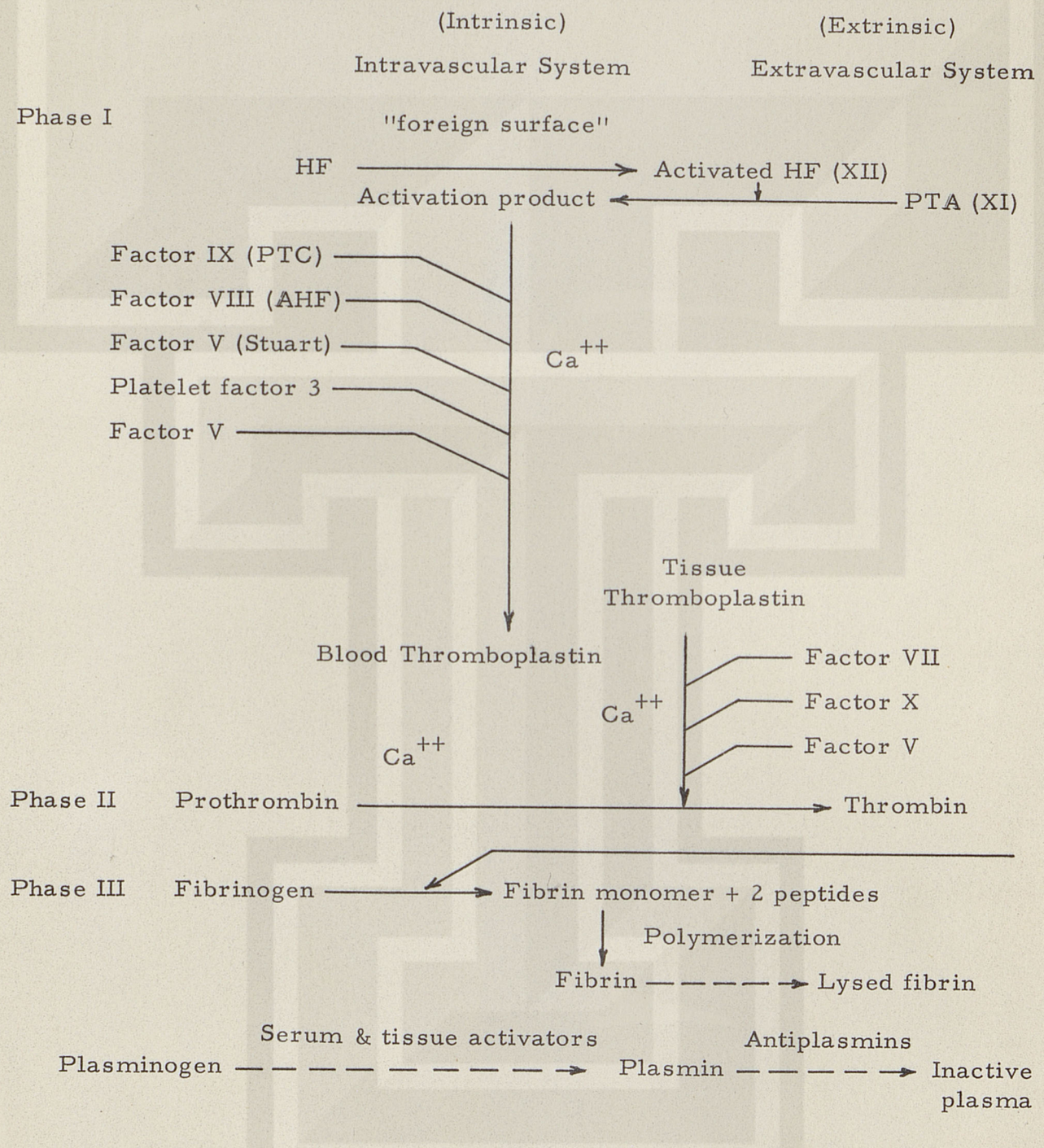

Figure 1. Interaction of Factors in Formation of Fibrin Clot.[2]

there are optimal amounts of these enzymes present.

Phase II or prothrombin conversion to thrombin will occur maximally if there are sufficient quantities of prothrombin and thromboplastin present. Calcium is an additional requirement.

Table 4

COAGULATION FACTORS

Factor	I =	Fibrinogen
Factor	II =	Prothrombin
Factor	III =	Thromboplastin
Factor	IV =	Calcium
Factor	V =	Labile Factor, Proaccelerin, Plasma Ac-globulin
Factor	VI =	Active Factor V, (Accelerin)
Factor	VII =	Stable Factor, Autoprothrombin I, SPCA
Factor	VIII =	Anti-Hemophiliac globulin (AHG) or Factor (AHF), Hemophilia A, Platelet Co-factor I
Factor	IX =	Plasma Thromboplastin Component (PTC), Autoprothrombin II, Christmas Factor, Hemophilia B
Factor	X =	Stuart-Power Factor
Factor	XI =	Plasma Thromboplastin Antecedent (PTA), Contact Factor
Factor	XII =	Hageman Factor, Contact Factor
Platelet Factor or Factors		
Tissue Thromboplastin = Incomplete Thromboplastin		
Blood Thromboplastin = Complete Thromboplastin		

Phase III or fibrin formation is dependent upon optimal quantities of thrombin and fibrinogen present. Thrombin is the active enzyme which splits the fibrinogen molecule in such a fashion that fibrin monomers are formed. These fibrin monomers align in an end to end and side to side manner so that a fibrin gel or clot is formed. Phase IV or fibrinolysin activity occurs under certain circumstances. Fibrinolysin or plasmin has a great affinity for fibrin. The fibrinolysin which is bound to the fibrin clot

during the process of coagulation exists usually in an inactive state (plasminogen). Certain factors will enhance the conversion of plasminogen to plasmin (fibrinolysin). The clot will undergo lysis when this occurs.

The in vitro study of coagulation is composed of tests which measure part of a single phase, a single phase, or a group of phases. There is no single test which will measure all phases.

The methods chosen, with their modifications are potentially suitable for the study of blood coagulation in space for the following reasons:

a. Minimal hazard to the physiological well being of the astronauts.
b. Limitation in laboratory space and equipment.
c. Minimal degree of technical skill and proficiency required.
d. Reliability and reproducibility of the methods are reasonably good.
e. Minimal reagents required.
f. No toxic chemicals are used.
g. Minimal influence on other tests to be performed.
h. Adaptability to null gravity environment.

In studying blood coagulation in vitro it is requisite that certain precautions be taken if reproducible results are to be obtained. Among them are the following:

1. Blood collection

A uniform and rapid method of collection is one of the most important aspects of coagulation study. The problem is mainly that of "contact activation." This problem can be overcome in several ways.

a. Collect blood in non-wettable vehicles such as siliconized glassware or smooth plastic containers.
b. Anticoagulation
c. Performance of tests immediately upon collection.

2. Anticoagulants

Sodium citrate or sodium oxalate are the only completely satisfactory anticoagulants. These chemicals bind calcium in such a fashion that coagulation is prevented and thromboplastin generation is inhibited. EDTA and heparin are not suitable or readily adaptable.

3. Temperature

Maximal acceleration of coagulation occurs at 37° C. However, coagulation reactions may be performed in a temperature range of 20 - 40° C. Tests may not be as reproducible at other temperatures but are workable. The temperature of the reaction must be the same each time the test is performed.

4. pH

Coagulation reactions are maximally reactant at a pH of 7.2 to 7.4. Very little latitude is possible. Blood must remain in a sealed container until ready for testing. This prevents the loss of CO_2 and tends to limit pH changes. The tests should be performed within two hours after collection if blood is kept at room temperature or sodium oxalate is used as an anticoagulant. Refrigeration at 4° C or sodium citrate as an anticoagulant permits a longer interval between collection and testing.

5. Fibrin endpoint

Several methods of coagulation study have been devised in recent years which measure several of the coagulation components by means other than a fibrin clot as an endpoint of the reaction. These methods are unproven and not desirable. For the purposes of this program the physiology of clotting is of greater interest than the biochemistry. Biochemical determinations that do not use fibrin end-point formation are difficult to correlate with in vivo coagulation. Therefore, the proposed methods are dependent upon a fibrin endpoint.

6. Duplicate testing and controls

There are no tests in coagulation which are so reproducible that

reliance can be placed wholly upon one determination. Duplicate and sometimes triplicate determinations are required for meaningful interpretation. Normal control plasma is generally employed as the method of assuring the realibility of a coagulation test. There are few suitable other standards.

7. Mixing of reagents

Interpretation and reliability of the methods are dependent to a great extent on uniform and rapid mixing of the reagents with patient plasma or blood.

Coagulation cannot be studied unless these requirements in whole or at least the greater part can be controlled.

In principle it is possible to study coagulation using either whole blood or plasma; however, plasma is preferable for two reasons. A fibrin endpoint is easier to detect, and inadvertent hemolysis of erythrocytes can be seen. Hemolyzed red cells alter the results of coagulation studies and yield uninterpretable results. The use of plasma permits more reproducible results. The four phases of coagulation and methods for studying them follow:

B. Principles of present methodology[1, 2, 3, 4, 5, 6]

1. The study of Phase I of coagulation (thromboplastin generation) in order of preference (only one required).

a. Activated partial thromboplastin time

This test measures moderate to severe changes in those factors (V through XII) concerned with thromboplastin generation. Severe depletions of prothrombin and fibrinogen will also prolong the clotting time. Minor changes in these factors will not be measured in this test. It is unlikely that clinical bleeding will occur, secondarily to a depletion of these factors, if this test is normal. It will not detect a deficiency of Factor VII or platelets.

Method: 0.1 ml plasma required

Cephalin (phospholipid platelet substitute), $CaCl_2$, kaolin or celite particles, and plasma are mixed together. The

time is recorded for clot formation. Normal time is under 45 seconds.

Reagents for this test are preserved in a dry or liquid form. The dried powder may be reconstituted in distilled water. Cephalin activity may be affected by temperature change. It is best preserved at 4° C. Lyophyllized control plasma would be desirable as a check on technique and reagent stability. If this test should become abnormally prolonged during space flight, the following procedures will further delineate the problem.

Three additional reagents are required.

(1) Lyophillized aged normal serum contains Factor IX and X activity. It does not have Factor V and VIII activity. Correction of a prolonged partial thromboplastin time by the addition of this serum to a patient plasma would indicate a deficiency of Factors IX or X. The Quick one stage prothrombin time (See Method for Phase II) will be prolonged by a Factor X deficiency but not by a Factor IX deficiency.

(2) Lyophillized $BaSO_4$ adsorbed plasma contains Factor V and VIII activity. It does not contain Factor IX or X activity. Correction of a prolonged partial thromboplastin time by adsorbed plasma would indicate a deficiency of Factor V or VIII. The Quick one stage prothrombin time is prolonged by Factor V deficiency but not prolonged by a Factor VIII deficiency.

(3) If both normal serum and adsorbed plasma correct the patient's plasma defect then depression of either Factor XI or XII would be expected. Both serum and adsorbed plasma contain these factors.

Fowl plasma has Factor XI activity but not Factor XII activity. Correction of the partial thromboplastin time with fowl plasma

would indicate a deficiency of Factor XII.

b. Alternative methods for Phase I

(1) Activated whole blood recalcification time

This method measures the same factors of coagulation as does the activated partial thromboplastin time. Two-tenths milliliter of anticoagulated blood is mixed with $CaCl_2$ and kaolin or celite particles. The time is recorded for clot formation. Normal: 90 - 150 seconds. Similar reagent substitutions might be used if desired in order to correct a prolonged clotting time. This would require considerable investigation in order to document the reliability and reproducibility.

(2) Capillary clotting time for Phase I

This test measures the same factors in coagulation as the activated partial thromboplastin time and the whole blood recalcification time. It is less reproducible for several reasons. Contact activation is not uniform and blood obtained by finger stick is contaminated by tissue juices. The latter may factitiously accelerate coagulation.

Blood is aspirated or allowed to flow into a 10 cm capillary tube. Every 30 seconds a segment of the tube is broken and the ends examined for fibrin thread. Normal time is 2 - 4 minutes.

2. The study of Phase II (prothrombin conversion to thrombin) by means of the Quick one stage prothrombin time method [7, 8, 15]

a. In plasma

This test is a measure of the prothrombin content of the blood. It is also a sensitive measure of Factors V, VIII and X activity. Severe depressions of substrate fibrinogen in patient plasma will also cause a prolonged prothrombin time. This test does not measure Factor VIII, IX, XI, XII, and platelet activity. Deficiences of these latter factors will go undetected if this method of measurement is used alone. It is preferable to the activated partial throm-

boplastin time when either prothrombin, Factor V or X deficiency are suspected, and is the only coagulation test that measures Factor VII activity.

Method: 0.1 ml plasma required

A small amount of $CaCl_2$ - thromboplastin mixture is added to the patient plasma, and the time is recorded for clot formation. Normal = 12 seconds. Tissue thromboplastin (an extract of animal brain or lung) contains Factor VIII, IX, XI, XII and platelet activity. Therefore, part of the first phase of coagulation is bypassed. Tissue thromboplastin combines with plasma Factors V, VII and X to form blood thromboplastin (complete thromboplastin). Prothrombin is then converted to thrombin which in turn converts plasma fibrinogen into a fibrin clot.

The reagents needed are an anticoagulant, $CaCl_2$ and tissue thromboplastin. Tissue thromboplastin is a thermolabile substance and requires refrigeration at 4° C for optimal activity (even in a lyophyllized state). A lyophyllized plasma control should be available (also thermolabile) that will serve as a check on reagents and technique.

b. Whole blood

These methods could be adapted to whole blood. Reliability and reproducibility would have to be further investigated.

3. Phase III * (Fibrinogen conversion to fibrin clot) [9]

a. Plasma - thrombin addition test

Plasma fibrinogen will convert maximally to fibrin in the presence of an optimal amount of thrombin. The rapidity of clot formation and the stability of the formed clot will roughly correlate with the fibrinogen concentration. A solid clot will form in a few sec-

* For chemical analysis of fibrinogen, see section 3.3.04.

onds following the addition of thrombin. Poor clot formation and stability occurs when the fibrinogen concentration falls below 100 mg%. Fibrinogen deficiency of lesser grades of severity will not result in clinical bleeding. Two-tenths millileter of plasma is required. This test does not measure other coagulation factors. A control fibrinogen (100 mg%) or a series of fibrinogen concentrations will assist the quantitation of the results.

b. Whole blood - thrombin addition test

The plasma method is probably easily adaptable to whole blood without much impairment of accuracy or reproducibility.

4. Phase IV - Fibrinolysis [10,11]

Clot lysis may be measured by a very simple method. A tube of blood is allowed to clot, and the clot is observed for clot lysis. However, differentiation between hypofibrinogenemia and elevated fibrinolysin activity is difficult. The following method is suggested.

a. Plasma - A heat-denatured fibrin plate or tube is required. A drop of patient plasma is added to this media. The fibrin plate is observed for lysis. The degree of lysis is roughly quantitative to the the amount of fibrinolysin present. Normally minimal lysis occurs over a period of several hours.

b. Whole blood - It is quite probable that the above method could be adapted for whole blood, but again will require special investigation.

5. Platelets

Platelets, although important in hemostasis, are generally not predictably important in blood coagulation. The ability of a clot to retract in a test tube is often proportional to the number and function of platelets present. This is not a good method to quantitate minor to moderate depression in platelet numbers. Therefore the following methods are suggested.

a. Indirect platelet count

An estimate of platelet numbers may be made by microscopic

examination of a blood smear. Reliability would be ± 20% of actual platelet numbers. The obvious disadvantage is that a microscope is required.

b. Adenosine diphosphate (ADP)[17, 18, 19] aggregation test

A few micrograms of ADP are added to a small volume of plasma. A 10X ocular is used to examine the platelet aggregation in the plasma. Deranged platelet function will be indicated by a failure of visible platelet aggregation.

It may be possible to correlate the degree of aggregation with platelet numbers. This test then would obviate the need for a microscope.

c. The use of plasma or whole blood would be determined by choice of the above methods. It may be possible to adapt the platelet aggregation test to whole blood.

6. Reagents and equipment

The following reagents and equipment would be required to perform the foregoing experiments.

a. Reagents

1. Sodium citrate of sodium oxalate
2. $CaCl_2$
3. Distilled H_2O
4. Control plasma
5. Fibrinogen
6. Cephalin
7. Tissue thromboplastin
8. Thrombin
9. Adenosine diphosphate (ADP)
10. Kaolin or celite

(Items 4 - 10 may be lyophyllized).

C. Applicability of present methodology to space flight conditions

1. Merit table

This table is applicable for all Coagulation Fractions except platelets. (This table appears on the following page.)

2. Discussion

A fibrin endpoint on each of the coagulation tests is preferable at our present state of knowledge. This will give us the truer physiological picture of clotting in space. The fibrin endpoint may be determined, usually, by photometer [12,13] or by change in electrical impedance.[16] A capillary method would probably be the easiest testing device, however it currently lacks accuracy and reproducibility. Visually observing the fibrin endpoint in a test tube or on a glass slide would rate next on the preferred list.

The additional requirement of a spectrophotometer makes the turbidimetric method somewhat less desirable yet adds little difficulty to the tests. Electrical impedance is probably not feasible since special equipment may well be required. Finally the correlation of esterase (enzymatic) activity[14] with coagulation activity has shown promise; however, we sacrifice the study of coagulation physiology by this method. It is worthy of future consideration. The use of plasma, though preferable to whole blood, is probably not required. If the blood were collected directly into a tubing or vial containing the active reagents, no anticoagulant would be necessary. Therefore, there is considerable flexibility in type specimen that may be used. Most of these tests can be done by direct vision, but are adaptable to turbidimetric methods.

These tests can be done in capillary tubes and only very small volumes of plasma or whole blood are required (0.1 ml to 0.2 ml per test).

The space environment introduces some problems in the study of coagulation, as in most other experiments. Careful design of appa-

Coagulation Factors

Merit Parameters	Present Methods					
	Visual Endpoint			Photometer Turbidimetric (Ref 9, 10, 11 12, 13)	Electrical Impedance (Ref 16)	Esterase Activity (Ref 14, 15)
	Mix in Test Tube (Ref 1-6)	Mix in Capillary Tube (Ref 1-6)	Mix on Slide or Watch Glass (Ref 7-8)			
1. Sensitivity	10	5	10	10	8	5
2. Sample size	.05 to 0.1 ml	.05 to 0.1 ml	.05 to 0.1 ml	0.3 ml	0.1 ml	0.3 ml
3. Time required	<10 min	<10 min	<10 min	1-30 min	1-30 min	1-30 min
4. Reproducibility	8	5	8	8	8	8
5. Suitability for null gravity use	3	8	5	7	7	7
6. Overall safety	-	-	-	-	-	-
7. Nontoxic reagents	10	10	10	10	10	8
8. Specificity	10	10	10	8	6	5
9. Insensitive to environmental changes	3	3	3	3	3	3
10. Ease in training personnel	6	6	6	8	8	3
11. Degree of separation required (10=none)	8	10	8	8	8	8
12. Minimal handling by analyst	8	10	8	8	8	6
13. Common use of analytic equipment	10	10	10	10	0	8
14. Nondestructive of sample	10	10	10	5	5	5
15. Merit range	21-42	15-31	25-50	26-52	23-46	19-38
16. Mean figure of merit	32	23	38	39	35	29

ratus with consideration of the following items will permit the experiments to be carried out in an orbiting laboratory.

a. Temperature requirements

Most of the reagents used are thermolabile and will require refrigeration or some method of preservation that will protect against loss of activity. The coagulation tests should all be carried out at a controlled fixed temperature.

b. Mixing reagents uniformly and rapidly is crucial and may be difficult in a null gravity environment.

c. Lability of reagents and reactants exposed to ionizing radiation.

d. Variability in collection.

The problems of collection and mixing require special consideration. The following suggestions might provide workable solutions to these problems.

(1) Plasma or blood could be collected directly into capillary tubes containing all the necessary reagents in a liquid or lyophyllized form. Preferably these reagents should be adherent to the inner wall of the capillary tube. Blood would solubilize the reagents, and the ends of the tube then sealed. The endpoint of the reaction would be the formation of a fibrin thread observed by successively breaking the tube at fixed time intervals.

(2) Capillary tubes could also be graduated and could serve as pipettes if desired.

(3) An alternative method would be to collect plasma or whole blood directly into a syringe containing the active reagents. The reagents could be in a liquid or lyophyllized form. Liquid form would be preferable for rapid mixing. Periodic attempts to express blood from the syringe might serve as the measurement. Clotted blood would be more difficult to express from the syringe.

(4) Reagents could be drawn into one end of a Y tube and plasma

the other end. When the reagents meet at the Y junction they would mix. Fibrin formation would be observed at this junction and beyond, as shown in Figure 2.

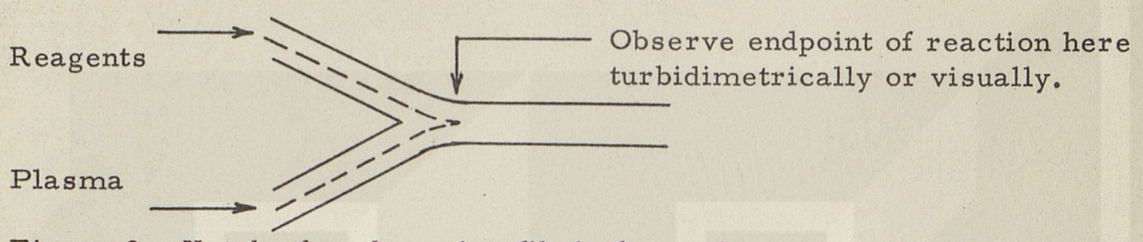

Figure 2. Y-tube for observing fibrin formation.

Finally, attempts to simulate the temperature changes, radiation effects, [20, 21, 22, 23, 24] gravitational alterations, or other variables encountered in the space environment which might handicap proposed methods of study and behavior of reactants should indicate what modifications need to be made. Normal values for the tests might also be established at this time.

Visual methods or spectrophotometric measurements of coagulation are the best approaches for the next two or three years.

D. Areas for research and development

The biochemical study of coagulation shows great potential. The ability of most clotting factors to exhibit enzymatic activity has been demonstrated. The development of specific substrates to measure individual factor activity would seem to be a desirable goal. Quantitation might then be more feasible than it is presently by our grosser, more qualitative measurements. It might also be possible to impregnate porous paper sticks with the active reagents and thus have a dip-stick of measuring the factors possessing enzymatic activity. This may be accomplished in 4 - 6 years with large scale investigations necessary.

Of course, specific molecular identification of these factors may lead to other methods of identification i.e. phospholipid and amino analysis of the clot accelerating lipoproteins. An estimated 7 years or longer will

elapse before this methodology can be developed.

E. References

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Methodology - Standard test tube methods of detecting clotting abnormalities are described.

1. Venous coagulation time
2. Clot retraction
3. Capillary bleeding time
4. Plasma recalcification time
5. One stage prothrombin time (macro & micro)
6. Partial thromboplastin time (macro & micro)
7. Thromboplastin generation test
8. Fibrinolysin
   a. Euglobulin lysis time
   b. Mixture of normal plasma and pot plasma lysis time.

2. Wintrobe; Clinical Hematology, Lea and Febiger, pp. 298 - 307.

Similar methods to reference 1 are described.

3. Dameshek and Stefanini; The Hemorrhagic Disorders, Grune and Stratton.

Similar methods as in reference 1.

4. Hougie; Fundamentals of Blood Coagulation in Clinical Medicine, Blakiston-McGraw.

Similar methods as in reference 1.

5. Biggs, McFarlane; Human Blood Coagulation and its Disorders, Blackwell Publishing Co., pp. 380 - 421.

Similar methods are described as in reference 1 with some modifications in technique.

6. Quick; Hemorrhagic Diseases and Thrombosis, Lea and Febiger, pp. 384 - 433.

Similar methods are described as in reference 1 but much less detail given.

7. Cole; Unpublished Results on the Pt. Time Using a Glass Slide.

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    Method described in this paper measures the esterase activity generated in the act of clotting as determined by the esterolytic action on TAMe (p-tosyl-L-arginine methyl ester). This is measured spectrophotometrically.

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