The use of platelet aggregation as a method of determining platelet concentration. The degree of platelet aggregation by ADP is proportional to the concentration of platelets present in the plasma. This can be done turbidimetrically.

20. Vasilevskii, N. M.; Blood Clotting Following Exposure to Ionizing Radiation. Zdravookhranenie Belorussii, Vol. 1, 1957, pp. 136 - 137.

21. Verwilghen, R. L.; J. M. Peremans; Changes in the Prothrombin Complex After Total-Body Irradiation. U.S. Armed Forces Med. J., Vol. 6, 1955, pp. 645 - 656.

22. Stender, H. S.; Elbert, O.; Effect Upon Blood Coagulation Factor After Whole Body Irradiation With Large Doses of Radiation. Strahlentherapie, Vol. 90, 1953, pp. 625 - 632.

23. Ponomarev. Yu. T.; Dynamics of Changes in Coagulative and Fibrinolytic System of Dog Blood in Acute Radiation Sickness. Radiobiolgiya, Russian, Vol. 5 (4), 1965, pp. 519 - 521.

24. Savitsky, J. P.; Sherry, S.; Changes in Clot Retraction Time and Platelet Adhesiveness Following Whole-Body Irradiation. Proc. Soc. Exptl. Biol. Med., Vol. 85, 1954, pp. 587 - 590.

References 20 - 24: Describes the changes in blood clotting induced by varying degrees of radiation exposure in experimental animals.

3.3.04 Fibrinogen (Plasma)

A. Principles of present methodology

1. Martinek[4]

Principle: Addition of 1.2 M phosphate buffer to plasma results in the precipitation of fibrinogen. The turbidity is measured at 450 mμ against a blank prepared by adding an equal volume of plasma to 0.9% NaCl solution. The relationship between absorbance values and fibrinogen concentrations is linear. The precipitating reagent is stable at room temperature for at least 2 years. Heparin, sodium citrate or EDTA are suitable as anticoagulants. Hemolysis must be avoided. Cuvettes with a light path greater than 1 cm will increase the sensitivity of the procedure. The instrument is calibrated with fibrinogen solutions of known concentrations. The total volume of plasma required is 0.5 ml but it probably can be reduced to 0.2 ml.

2. Ellis and Stransky[1]

Principle: Addition of a calcium-thrombin solution to plasma converts fibrinogen to fibrin. The degree of turbidity is directly related to the fibrinogen content of plasma.

Procedure: Plasma is diluted with 0.1 M barbital buffer. The solution is divided into two equal parts. One aliquot is used as the "blank" and the other as the "test". One drop of calcium-thrombin solution is added to the "test" and is allowed to clot for 20 minutes. The absorbance of the "test" is measured against the blank at 470 mμ in a spectrophotometer. The instrument is calibrated with fibrinogen solutions of known concentrations (calibration curve). A straight line is obtained when absorbance values are plotted versus fibrinogen concentrations.

Citrate is the only suitable anticoagulant. The calcium-thrombin solution is stable for at least 3 months at -20° C. Bilirubin, lipemia and moderate hemolysis do not interfere with the determination.

3. Rafferty et al.[5]

Principle: Fibrinogen is precipitated from plasma by the addition of ammonium sulfate. The resulting turbidity is measured in a nephelometer. Nephelometric techniques provide better sensitivity in measuring low concentrations of particles which are beyond the sensitivity of turbidimetric measurements. With this method it is possible to reduce the volumes of plasma required for the analysis of fibrinogen to approximately 0.05 ml.

B. Suitability of present methodology to space flight conditions

1. Merit table

Fibrinogen in Plasma

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Fair	Fair	Good
2. Sample size	0.5 ml	0.5 ml	.1 ml
3. Time required	3 min	25 min	5 min
4. Reproducibility	8	9	-
5. Suitability for null gravity use	9	8	9
6. Overall safety	10	9	10
7. Nontoxic reagents	10	8	10
8. Specificity	8	9	8
9. Insensitive to environmental changes	10	3	9
10. Ease in training personnel	10	6	9
11. Degree of separation required (10=none)	9	9	9
12. Minimal handling by analyst	9	5	8
13. Common use of analytic equipment	10	10	7
14. Noncaustic reagents	10	10	10
15. Nondestructive of sample	1	1	1
16. Merit range	-	-	0-83
17. Mean figure of merit	70	57	42

2. Discussion

Methods 1, 2, and 3 are turbidimetric, and a difficult aspect of

turbidimetry or nephelometry is the preparation of reproducible and stable suspensions. The rate of addition of reagent, the manner of mixing, and shaking and other variables must be kept under control.

Method 2 probably is more specific and should give more accurate results. Fibrinolysis is a potential source of error with this method as it may occur before the turbidity of the clot is measure; it can be avoided by allowing the plasma to stand at room temperature for several hours.

Numerous precipitating reagents, i.e., sodium sulfite, sodium sulfate, sodium chloride and heat, have been used for the measurement of fibrinogen. However, they do not offer any significant advantage over those already described.

Choice: The Method of Martinek should be the method of choice at the present. It is simple, rapid and requires a small volume of plasma.

Equipment

Method 1: Spectrophotometer and centrifuge

Method 2: Spectrophotometer and centrifuge

Method 3: Nephelometer and centrifuge

C. Areas for research and development

1. The nephelometric method should be explored. The available data with regard to its precision and standardization are meager. Its sensitivity is a very desirable feature.

2. Attempts have been made to determine fibrinogen by differential refractometry.[3] The difference between the refractive index of plasma and serum can serve as a means for estimating the fibrinogen concentration. However, the sensitivity, reproducibility, accuracy and normal values have not been reported.

D. References

1. Ellis, B.C.; Stransky, A.; A Quick and Accurate Method for the Determination of Fibrinogen in Plasma, J. Lab. Clin. Med., Vol. 58, 1961, pp. 477 - 488.

Fibrinogen is measured by the turbidity produced when it is polymerized to fibrin by thrombin.

2. Gleye, M.; Préparation et Utilisation d'un Immunsérum Spécifique du Fibrinogène. Compt. Rend., 1962, pp. 2685 - 2686.

Describes the preparation of an antiserum specific for human fibrinogen.

3. Leendertz, G. Eine Klinische Methode Zur Bestimmung des Blutfibrinogens. Klin. Wochschr., Vol. 2, 1963, pp. 746 - 747.

The difference in the refractive index between plasma and serum can be used for the estimation of fibrinogen.

4. Martinek, R.G.; Berry, R.E.; Micromethod for the Estimation of Plasma Fibrinogen. Clin. Chem., Vol. 11, 1965, pp. 10 - 16.

Fibrinogen is determined turbidimetrically after it is flocculated with 1.2 M phosphate buffer.

5. Rafferty, N.S.; Tyrol, A.G.; Parfentjev, I.A.; Turbidimetric Analysis of Fibrinogen and Gamma Globulin with Ammonium Sulfate by Different Photometric Technics. Clin. Chem., Vol. 4, 1958, pp. 185 - 193.

Fibrinogen is measured with a nephelometer after it is flocculated with $(NH_4)_2\ SO_4$.

3.3.05 Hemoglobin

I. In blood

A. Principles of present methodology

1. Oxyhemoglobin

There are numerous modifications of the oxyhemoglobin method but the procedure proposed by Collier[2] is the most attractive.

Principle: Whole blood is diluted and hemolyzed with a 0.3% solution of the tetrasodium salt of ethylenediaminetetraacetic acid ($EDTA\text{-}Na_4$) and the hemoglobin is fully oxygenated by exposure to air. The oxyhemoglobin is determined from its absorbance at 540 mμ.

Oxyhemoglobin standards are not available; this method should be standardized against the cyanmethemoglobin method or by determination of iron.

The only reagent required is $EDTA\text{-}Na_4$ which is stable and nontoxic.

The oxyhemoglobin color develops in a few seconds (the oxygenation of hemoglobin is very rapid) and stable for at least 16 hours.

In the absence of lipemia, the accuracy of the techniques is limited by the absence of abnormal hemoglobin pigments; methemoglobin and carboxyhemoglobin are not converted to oxyhemoglobin.

Due to the presence of EDTA the accuracy of the method is not affected by the presence of copper ions.

2. Cyanmethemoglobin[4]

Principle: Whole blood is diluted with a potassium ferricyanide-potassium cyanide ($K_3Fe(CN)_6\text{-}KCN$) solution that oxidizes hemoglobin to methemoglobin which is, in turn, converted to cyanmethemoglobin. The absorbance of cyanmethemoglobin is measured at 540 mμ. This procedure will measure hemoglobin, oxyhemoglobin, methemoglobin, and carboxyhemoglobin.

The Panel on the Establishment of a Hemoglobin Standard of the

of Medical Sciences, National Academy of Sciences, National Research Council has recommended universal adoption of the cyanmethemoglobin method because of its good accuracy and precision.

However, the presence of potassium cyanide is potentially hazardous to the health of the astronauts.

Cyanmethemoglobin standards are commercially available and very stable under refrigeration.

3. Azide methemoglobin

Vanzetti[5] proposed replacement of the toxic KCN with the much less toxic sodium azide.

Principle: Whole blood is diluted with potassium ferricyanide-sodium azide ($K_3Fe(CN)_6$-NaN_3) solution which converts hemoglobin to azide-methemoglobin. The absorbance of azide-methemoglobin is measured at 540 mμ. The method has not gained wide acceptance but the author claims that its accuracy and precision are identical with those of cyanmethemoglobin.

The absorption coefficients of cyanmethemoglobin and azide-methemoglobin are identical at 540 to 546 mμ but the absorption spectra of the two hemoglobin derivatives are not. A plateau is seen in the azide-methemoglobin curve at 575 mμ.

B. Suitability of present methodology to space flight conditions

1. Merit table

Hemoglobin in Blood

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	.2 g	.2 g	.2 g
2. Sample size	20 μl	20 μl	20 μl
3. Time required	1 min	20 min	10 min
4. Reproducibility	9	9	9
5. Suitability for null gravity use	9	9	9
6. Overall safety	9	1	5
7. Nontoxic reagents	10	1	5

Hemoglobin in Blood - continued

Merit Parameters	Method 1	Method 2	Method 3
8. Noncaustic reagents	9	9	9
9. Specificity	9	9	9
10. Insensitive to environmental changes	10	2	2
11. Ease in training personnel	10	10	10
12. Degree of separation required (10=none)	10	10	10
13. Minimal handling by analyst	9	9	9
14. Common use of analytic equipment	10	10	8
15. Nondestructive of sample	0	0	0
16. Figure of merit	74	30	46

2. Discussion

It seems that the method of choice for the determination of hemoglobin is the oxyhemoglobin method.

The only reagent needed is EDTA-Na_4 which is stable even in solution for several months at room temperature. This compound has the advantage over the KCN and NaN_3 that it is nontoxic and therefore safe to use. The oxyhemoglobin method is more rapid than the other two. The conversion of hemoglobin to oxyhemoglobin in the presence of air is almost instantaneous.

It has been argued that the oxyhemoglobin method does not measure any methemoglobin and/or carboxyhemoglobin present in the blood. However, both methemoglobin and carboxyhemoglobin absorb light at 540 mμ and the error introduced by significant quantities of methemoglobin and carboxyhemoglobin is no more than a few percent.[1]

Sodium azide is a known metabolic inhibitor and ingestion of the compound will cause hypotension. The fatal dose of NaN_3 for humans remains unknown. Studies on laboratory animals have indicated that it is 6 - 7 times less toxic than KCN.

Equipment

Oxyhemoglobin Method: Spectrophotometer or filter colorimeter

Cyanmethemoglobin Method: Spectrophotometer or colorimeter

Azide-methemoglobin Method: Spectrophotometer or colorimeter with narrow band width filter.

The present methods for the estimation of blood hemoglobin are precise, simple and require minimal handling. Only dilution of blood is necessary and that can be easily accomplished with an automatic diluting pipette. There is a possibility of measuring hemoglobin in undiluted blood samples by means of a cuvette with a very short light path. Such cuvettes with a light path of 0.07 mm have been constructed by Drabkin and Austin[3] but the accuracy and precision of their method was never evaluated.

C. Areas for research and development

(None)

D. References

1. Bell, G.H., et al.; The Routine Estimation of Haemoglobin as Oxyhaemoglobin. Biochem. J., Vol. 39, 1945, pp. 60 - 63.

At 520 mμ all the hemoglobin pigments likely to be found in the blood had approximately the same extinction coefficient.

2. Collier, H.B.; The Use of Sequestering Agent in Determination of Oxyhemoglobin. Am. J. Clin. Path., Vol. 25, 1955, pp. 221 - 222.

Suggests the replacement of dilute NH_4OH with tetrasodium versenate solution in the oxyhemoglobin method.

3. Drabkin, D.L.; Austin, J.H.; A Technique for the Analysis of Undiluted Blood and Concentrated Hemoglobin Solution. J. Biol. Chem., Vol. 112, 1935, pp. 105 - 115.

Describes spectrophotometry of hemoglobin in undiluted blood using a cuvette with narrow light path.

4. Hainline, A., Jr.; Hemoglobin, Standard Methods of Clinical Chemistry. Vol. 2, ed. D. Seligson, Academic Press, N.Y., 1958, pp. 49 - 60.

5. Vanzetti, G.; An Azide-methemoglobin Method for Hemoglobin Determination in Blood. J. Lab. Clin. Med., Vol. 67, 1966, pp. 116 - 126.

Describes the estimation of blood hemoglobin by conversion to azide-methemoglobin and spectrophotometric measurement at 542 mμ.

II. In plasma

The more sensitive method for the determination of plasma hemoglobin was introduced by Hanks et al.[2] and it is a modification of the method of Crosby and Furth.[1] Both methods are chemical.

A direct spectrophotometric method has been described by Martinek[3] which requires only 0.9% NaCl as reagent.

A. Principles of present methodology

1. Hanks et al.[2]

Principle: Hemoglobin catalyzes the oxidation of benzidine to a green product by oxygen released from hydrogen peroxide.

Procedure: Plasma is mixed with a solution of benzidine in glacial acetic acid. Hydrogen peroxide is added and the absorbance of the resulting color solution is measured after 3-1/2 minutes at 700 mμ.

The method can measure amounts of hemoglobin from 0 to 4 mg per 100 ml of plasma.

The time for color development is very critical and the benzedine solution must be used within 6 hours after its preparation.

The normal values for this procedure are the lowest ever reported; mean value 0.32 mg%(0.16 - 0.58 mg%.)

2. Crosby and Furth[1]

Principle: Same as in Method 1.

Procedure: Plasma is mixed with a benzidine solution in 90% acetic acid. Hydrogen peroxide is added and the mixture is incubated for 20 minutes at room temperature. It is diluted with 10% acetic acid and the absorbance is measured at 515 mμ. The precision of the method is poor when the hemoglobin level is below 4 mg%.

3. Martinek[3]

Principle: The absorbance of serum or plasma diluted with saline is measured at 412 - 415 mμ (Soret peak of oxyhemoglobin).

The spectrophotometer is calibrated with oxyhemoglobin solution of known concentrations. A correction is made for the interference caused by bilirubin by measuring the optical density of the diluted plasma at the peak of the bilirubin absorption, 455 - 457 mμ. The correction is based on the fact that the absorbance of bilirubin at 412 - 415 mμ is 80% of its peak absorbance. This method is very recent and has not been evaluated. However, the author claims that the reliability of the method has been established by comparison with the methods of Hanks, et al.[2]

Fasting blood specimens are mandatory with this procedure.

B. Suitability of present methodology to space flight conditions

1. Merit table

Hemoglobin in Plasma

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Excellent	Good	-
2. Sample size	0.4 ml	0.02 ml	0.1 ml
3. Time required	5 min	35 min	2 min
4. Reproducibility	8	8	-
5. Suitability for null gravity use	5	5	8
6. Overall safety	1	1	10
7. Nontoxic reagents	3	3	10
8. Noncaustic reagents	1	1	10
9. Specificity	9	9	8
10. Insensitive to environmental changes	0	3	10
11. Ease in training personnel	2	6	9
12. Degree of separation required (10=none)	9	9	9
13. Minimal handling by analyst	3	5	9
14. Common use of analytic equipment	10	10	10
15. Nondestructive of sample	1	1	8
16. Merit range	-	-	0-84
17. Mean figure of merit	13	15	42

2. Discussion

Benzidine has been reported to cause carcinoma of the bladder. Methods 1 and 2 require the use of glacial acetic acid which is highly undesirable. Therefore, they cannot be recommended.

A spectrophotometer and a centrifuge are required for all of the described methods.

C. Areas for research and development

Method 3 is very promising and should be evaluated. If reliable it will undoubtedly be the method of choice.

D. References

1. Crosby, W.H.; Furth, F.W.; A Modification of the Benzidine Method for Measurement of Hemoglobin in Plasma and Urine. Blood, Vol. 11, 1956, pp. 380 - 383.

Procedure for measuring hemoglobin levels in plasma above 4 mg%.

2. Hanks, G.E.; Casseli, M.; Ray, R.N.; Chaplin, H., Jr.; Further Modification of the Benzidine Method for Measurement of Hemoglobin in Plasma. J. Lab. Clin. Med., Vol. 56, 1960, pp. 486 - 498.

Describes a highly sensitive method capable of measuring amounts of hemoglobin from 0 to 4 mg per 100 ml of plasma.

3. Martinek, R.G.; A Simple Spectrophotometric Method for Quantitating Hemoglobin in Serum or Plasma. J. Am. Med. Technol., Vol. 28, 1966, pp. 42 - 58.

The method utilizes the absorbance of oxyhemoglobin at the Soret band, 412 - 415 mμ.

III. In Urine

One has to distinguish between hematuria and hemoglobinuria. The first term indicates the presence of an abnormal number of red blood cells in the urine. The second term refers to the presence in the urine of hemoglobin or its derivatives in solution.

Hematuria can be detected by microscopic examination of the sediment following centrifugation of a urine aliquot. According to Cook et al[3]. the number of erythrocytes must be close to 50 million per liter of urine before the presence of an abnormal content can be detected with certainty. The sensitivity of the microscopic examination will decrease with hemolysis of the erythrocytes in the urine. Adams et al.[1], and Leonards[5], reported that in over 95% of the urines containing blood some of the blood was hemolyzed. The extent of hemolysis may range from a small proportion of the total blood excreted to the extreme where all the excreted blood is hemolyzed. Therefore, microscopic examination of the urinary sediment cannot always give a reliable indication of the total amount of hemoglobin.

Methods for the detection of occult blood in urine must be able to detect both intact red blood cells and hemoglobin in solution. Reagents such as benzidine, guaiac and o-tolidine have been employed for the detection of occult blood in urine. The most sensitive of the three is o-tolidine.

A. Principles of present methodology

1. Stewart and Dunlop[6]

Principle: Hemoglobin and its derivatives catalyze the oxidation of o-tolidine to a blue colored product by oxygen released from hydrogen peroxide.

Specimen: Uncentrifuged urine heated to boiling point and cooled.

To a mixture of o-tolidine solution and hydrogen peroxide a few drops of boiled urine are added. In the presence of hemoglobin a blue color appears in approximately one minute.

The sensitivity of the test varies between samples because of the presence of inhibitors in urine.

True peroxidases which may be present in urine are destroyed by boiling the urine.

The stock o-tolidine solution (4 g in 100 ml of ethanol) is stable for a long time at 4° C.

The sensitivity of the test is decreased by high normal levels of ascorbic acid in urine and drastically decreased by therapeutically high levels.[3]

2. "Occultest" *

"Occultest" is a tablet containing o-tolidine, strontium peroxide, calcium acetate, sodium bicarbonate and tartaric acid and is manufactured by Ames Company.

The test for occult blood is performed as follows: One drop of uncentrifuged urine is placed in the center of the piece of a filter paper and an "Occultest" tablet is applied on the top of the moist area. Two drops of water are placed on the tablet. The appearance of a blue color on the paper within 2 minutes indicates the presence of blood. This test is very sensitive and in most cases a positive reaction is obtained with "Occultest" when one part of blood is present in 300,000 parts of urine.[3]

Free et al.[4], found good correlation between the results with the "Occultest" on uncentrifuged urine and those obtained by microscopic examination of the urine sediment.

3. "Hema-Combistix" * (dip stick)

The "Hema-Combistix" is a paper strip with four areas, one of which is used for the detection of occult blood in urine; the other three areas are used for the detection of glucose, protein and determination of urinary pH. The area used for the detection of occult blood is impregnated with o-tolidine, a peroxide and suitable buffers. A blue color appears in the area for the detection of

* Registered trademark of the Ames Co., Inc., Elkhart, Indiana.

blood when the dip stick is immersed in a urine specimen containing blood. The usefulness of the "Hema-Combistix" in the detection of occult blood in urine has been evaluated by several investigators.[2, 7] In general good correlation was found with the results of "Hema-Combistix" and those obtained by microscopic examination of the urinary sediment. However, in a few cases the dip-stick test was negative while there were from 5 to 14 red blood cells per high-power field. In some of these cases the "Hema-Combistix" was inhibited by a high ascorbic acid concentration in the urine.

B. Suitability of present methodology to space flight conditions

1. Merit table

Hemoglobin in Urine

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Excellent	Very Good	Very Good
2. Sample size	1 ml	.05 ml	.10 ml
3. Time required	5 min	1 min	1 min
4. Reproducibility	9	9	9
5. Suitability for null gravity	6	10	10
6. Overall safety	1	10	10
7. Nontoxic reagents	1	10	10
8. Noncaustic reagents	1	9	10
9. Specificity	8	8	8
10. Insensitive to environmental changes	2	8	10
11. Ease in training personnel	8	10	10
12. Degree of separation required (10=none)	10	10	10
13. Minimal handling by analyst	3	6	10
14. Common use of analytic equipment	3	7	10
15. Nondestructive of sample	0	2	10
16. Figure of merit	15	76	87

2. Discussion

The o-tolidine test is undoubtedly the most sensitive for the detection of occult blood in urine. However the test requires reagents which may be undersirable and dangerous, i.e., glacial acetic acid, ethanol, hydrogen peroxide; therefore, the "Occultest" and "Hema-Combistix" are much more suitable for urine hemoglobin.

Although the sensitivity of "Hema-Combistix" is inferior to that of the "Occultest", the former is preferable because of its simplicity and speed.

C. Areas for research and development

Efforts would be made to prepare a dip stick with greater sensitivity.

Equipment

Method 1:	Heater
Method 2	"Occultest" tablets
Method 3:	"Hema-Combistix" strips

D. References

1. Adams, E.C., Jr.; Fetter, M.C.; Free, H.M.; Free, A.H.; Hemolysis in Hematuria. J. Urol., Vol. 88, 1962, pp. 427 - 430.

Urine specimens containing occult blood were found to have hemolyzed blood in 90% of the cases.

2. Budinger, J.M.; Cavallo, M.; Detection of Hematuria with a Paper Strip Indicator. Am. J. Clin. Path., Vol. 42, 1964, pp, 626 - 629.

The "Hema-Combistix" gives a positive test when the number of erythrocytes per high-power field is approximately 3.

3. Cook, M.H., et al.; The Detection of Blood in Urine. Am. J. Med. Tech., Vol. 22, 1956, pp. 218 - 231.

Various methods of detecting blood in urine are compared with regard to sensitivity and accuracy.

Test for the Detection of Occult Blood in Urine. J. Urol., Vol. 75, 1956, pp. 743 - 752.

The "Occultest" is a simple and an accurate test for occult blood in urine.

5. Leonards, J.R.; Simple Test for Hematuria Compared with Established Tests., J.A.M.A., Vol. 179, 1962, pp. 807 - 808.

Hemolysis occurs in 95% of the cases in urines containing occult blood.

6. Stewart, C.P.; Dunlop, D.M.; Clinical Chemistry in Practical Medicine, Livingstone, London, 1958, pp. 287 - 305.

Application of the o-tolidine test for the detection of hemaglobin in urine.

7. Yoder, J.M.; Adams, E.C.; Free, H.M.; Simultaneous Screening for Urinary Occult Blood, Protein, Glucose, and pH. Am. J. Med. Tech., Vol. 31, 1965, pp. 285 - 290.

Good correlation exists between the results with "Hema-Combistix" and those obtained with microscopic examination of the urine sediment.

3.3.06 Immune bodies

This discussion will concentrate on the study of serum proteins, some of which have immunological activity. The laboratory procedures principally evaluated for this project include zone electrophoresis, immunoelectrophoresis and immunodiffusion.

I. Electrophoretic analysis

Electrophoretic analysis affords determination of a broader spectrum of serum proteins. Other chemical methods of serum albumin and globulin fractionation are not so sensitive as electrophoretic separation. Any changes observed with one or more of the five protein fractions obtained with zone electrophoresis can be a sensitive indicator of pathophysiologic changes.

A. Principles of present methodology

The physical principle underlying zone electrophoresis is stated as follows: particles carrying an electric charge are accelerated when placed in an electric field. The particles move at a constant speed proportional to their charge. This driving force is balanced by frictional forces arising in the medium.[10] These forces operate regardless of the type of medium (agar gel, starch gel, cellulose acetate) used.

B. Suitability of present methodology to space flight conditions

1. Merit table

Immune bodies

Merit Parameters	Agar Gel	Cellulose Acetate	Starch Gel (micro-methods)
1. Accuracy*	± 25%	± 4 to ± 15%	**
2. Sample size	0.006 ml	0.01 to 1.0 µl	0.015 ml
3. Time required	2 hrs.	approx. 1 hr.	2 hrs.
4. Reproducibility	8	8	7
5. Suitability for null gravity use	5	5	4

Immune bodies - continued

Merit Parameters	Agar Gel	Cellulose Acetate	Starch Gel (micro-methods)
6. Overall safety	4	4	4
7. Nontoxic, noncaustic reagents	2	2	2
8. Specificity	10	10	10
9. Insensitive to environmental change	8	8	8
10. Ease in training personnel	10	10	8
11. Degree of separation required (10=none)	10	10	10
12. Minimal handling by analyst	8	10	7
13. Common use of analytic equipment	3	3	3
14. Nondestructive of sample	0	0	0
15. Figure of merit	28	29	22

* This includes sensitivity for all of the separated fractions.

** It is difficult to report on the accuracy of this procedure since quantitation of these gels has not been very practical or definitive.

2. Discussion

The technique of choice for zone electrophoresis, for the primary investigations in this project, is the micromethod described by Grunbaum and Durum.[3, 4] This method makes use of cellulose acetate strips in a micro-electrophoresis apparatus. The Microzone Electrophoresis Cell (Model R - 101)[1] can be easily adapted and is available commercially.[+] After electrophoresis is completed, the strips have to be stained for the localization and identification of the protein fractions. Such stains as Amido-Black 10B, Bromphenol Blue and Ponceau S can be used. The separated protein fractions can be quantitated on a photodensitometer scanner-recorder. If desired, this can be done post-flight since the stained strips can be kept for a long period of time.

Microzone cellulose acetate electrophoresis has the shortest electrophoresis time (20 minutes) of the three methods compared

+ Beckman Instruments, Inc., Standard Industrial Park, Palo Alto, California.

here.

The rapid micromethods of starch gel electrophoresis are evaluated here. The starch gel for this purpose can be molded on either glass slides or in plastic trays.[6, 7] The electrophoresis time for these modified procedures can be reduced to about two hours or less. This is an advantage over the original method described by Smithies[8] which required 18 to 22 hours of electrophoresis time. One major drawback in the use of these gels is that they cannot be prepared in advance of the experiment. Personal experience as well as statements made in the literature[9] indicates that it is not feasible to store these gels for periods longer than one or two days at refrigerator temperatures. Consequently, these gels will have to be prepared during flight if the investigative period will extend into several days.

Electrical contact with gels or cellulose acetate strips is usually achieved by their direct immersion into compartments filled with a selected buffer solution. Major modifications may have to be made here for adapting these procedures to space flight. An ultramicromethod of electrophoresis described by Fischl and Gordon[2] offers several promising modifications for this project. The electrophoresis cell described is small, 40 x 30 x 12 mm. The electrode compartments are 20 x 40 mm and 4 mm deep. Pieces of cellulose sponge 16 x 4 mm and 3 mm, when dry, are placed into the electrode compartment and 0.35 ml of barbiturate buffer (0.05 M, pH 8.6) is added to both. The optimal conditions are 60 volts for 15 minutes or if very rapid results are required, 120 volts for 5 minutes is possible. Wet or dry storage batteries may be used for power supply. The batteries can be connected to the apparatus by copper foils.

For the initial experiments, zone electrophoresis utilizing cellulose acetate membranes appears to be the best approach.

Micro-methods of starch gel electrophoresis can be adapted at a later date if changes in electrophoretograms are noted in astronauts during flight. One major drawback to the use of these procedures is the staining process which requires the use of glacial acetic acid and methanol.

C. Area for research and development

The use of fluorescence depolarization methods in which the antigen is tagged with a fluorescent dye may permit detection of small amounts of antibody even under conditions where precipitation does not occur. Research in this area may lead to much more precise analysis. This approach may also prove useful in other applications of antigen-antibody reactions. For example it is also possible to employ fluorescent tagged antibody to detect antigens.[5]

D. References

1. Beckman Technical Bulletin, RB-TB-004, Model R-101, Microzone Electrophoresis Cell, Preliminary Instruction Manual, 1963.

This manual describes the commercial adaptation of the microelectrophoresis setup. A detailed outline of the procedure as used in laboratories is presented.

2. Fischl, J.; Gordon, C.; Ultramicro Electrophoresis. Clin. Chem., Vol. 12, 1966, pp. 887 - 889.

This article describes an ultramicro-electrophoresis apparatus. Important aspects of this setup are that it makes use of a few drops of buffer and a low voltage.

3. Grunbaum, B.W.; Collins, C.C.; A Self-Contained Microelectrophoresis Apparatus. Microchem. Journ., Vol. 7, 1963, pp. 283 - 286.

This paper describes a minature version of the microelectrophoresis procedure described in reference 4 below.

4. Grunbaum, B.W.; Zec, J.; Durrum, E.I.; Application of an Improved Microelectrophoresis Technique and Immunoelectrophoresis of the Serum Proteins on Cellulose Acetate. Microchem. Journ., Vol. 7, 1963, pp. 41 - 53.

Describes the adaptation of the microelectrophoresis technique and equipment using cellulose acetate for the study of protein fractions. The advantage of cellulose acetate over filter paper and gels is presented.

5. Haber, E.; Bennett, J.C.; Polarization of Fluorescence as a Measure of Antigen-Antibody Interaction, Proc. Nat. Acad. Sci., Vol. 48, 1935, pp.

Detection of antigen-antibody combinations was followed by the decrease in depolarization of fluorescence of fluorescent labeled antibody. This permitted a great increase in sensitivity for detection of soluble antigen-antibody complexes.

6. Marsh, C.J.; Jolleff, C.R.; Payne, L.C.; A Rapid Micromethod for Starch-Gel Electrophoresis. Amer. J. Clin. Path., Vol. 41, 1964, pp. 217 - 223.

A description of a rapid micromethod of starch-gel electrophoresis is presented. The gels are molded in plastic trays 3 mm in depth and 23 cm in length. Actual electrophoresis time is 80 minutes.

7. Ramsey, H.A.; Thin-Layer Starch Gel Electrophoresis on Glass Slides. Analytical Biochem., Vol. 5, 1963, pp. 83 - 91.

The advantages of thin layer starch gel electrophoresis over the use of a thicker gel are presented in this article. In this method the gel is poured as a thin layer on glass slides.

8. Smithies, O.; Zone Electrophoresis in Starch Gel and Its Application to Studies of Serum Proteins. Adv. Protein. Chem., Vol. 14, 1959, pp. 65 - 111.

This is a thorough and concise article that deals with starch gel electrophoresis and its application to studies of serum proteins.

9. Smithies, O.; Zone Electrophoresis in Starch Gels: Group Variations in the Serum Proteins of Normal Human Adults. The Bioch. Journ., Vol. 61, 1955, pp. 629 - 641.

This article deals with the advantages of starch gel electrophoresis such as its greater resolving power and sharper separation. Genetic variation of proteins is presented.

10. Wieme, R.J.; Agar Gel Electrophoresis, Elsevier Publishing Co.,

New York, 1965.

This is a well illustrated textbook discussing the physical aspects of electrophoresis. The applications of agar gel as a suitable electrophoretic medium are also presented.

II. Immunochemical analyses

In this section, two immunochemical methods will be compared, namely, immunoelectrophoresis and immunodiffusion. With either of these two sensitive methods, several specific proteins can be evaluated. Immunoelectrophoresis can be used as a screening procedure since it affords a qualitative measurement of at least 15 - 20 different serum proteins. With immunodiffusion, on the other hand, a quantitative estimation of several specific proteins (such as immunoglobulins, (IgG, IgA, and IgM), transferrin and one of the complement components, $\beta_1 c$) of interest in this project is possible.

A. Principles of present methodology

1. Immunoelectrophoresis

This involves the electrophoretic separation of mixtures of serum proteins in a medium followed by the diffusion of precipitating antibodies in the same medium at right angles to the electrophoresis axis.[6] During the diffusion phase the fractionated proteins react with specific antibodies to produce visible precipitin arcs.

2. Immunodiffusion (Oudin Diffusion Procedure)

The appropriate antiserum (that which contains antibody specific for the protein to be analyzed) is mixed with the buffered agar gel. The serum sample is inoculated on the surface of the solidified agar. The agar can be molded on either petri dishes, glass columns[3] and on 35 mm Cronar polyester photographic film* base strips.[1]

Diffusion of the serum sample is allowed to proceed in a moist chamber at room temperature or at 37° C for 18 to 24 hours. The endpoint of this reaction is a precipitation ring formed around the inoculation well when a flat surface is used, or if columns are used, an antigen-antibody front is observed and its distance of migration measured. The diameter of the precipitate is directly related to

* E.I. DuPont de Nemours & Co., 4560 Tuohy Ave., Chicago, Illinois.

the initial concentration of the antigen in solution when temperature and antibody concentration remain constant. The diameter of the precipitation rings of the standards are measured and plotted on semi-logarithmic paper as concentration in mg/100 ml vs. diameter in millimeters. The concentration of the specific protein under analysis is determined by referring to the standard curve.[3]

B. Suitability of present methodology to space flight conditions

1. Merit table

Immunochemical Analyses

Merit Parameters	Immuno-electrophoresis	Immuno-diffusion
1. Sensitivity	5 - 10 μg protein nitrogen/ml	5 - 10 μg protein nitrogen/ml
2. Sample size	0.004 ml	0.003 ml
3. Time required	20 - 24 hrs.	20 - 24 hrs.
4. Reproducibility	9	8
5. Suitability for null gravity use	4	4
6. Overall safety	4	4
7. Nontoxic, noncaustic reagents	2	2
8. Specificity	10	10
9. Insensitive to environmental changes	6	6
10. Ease in training personnel	8	8
11. Degree of separation required (10=none)	10	10
12. Minimal handling by analyst	7	7
13. Common use of analytic equipment	3	3
14. Nondestructive of sample	0	0
15. Figure of merit	27	24

2. Discussion

The coating of plates, or photographic film with agar gel for either of these two procedures can be done easily. The buffered agar gel can be prepared in advance and stored at refrigerator

temperature in covered vials. Usually sodium azide can be added to the buffer used so as to prevent bacterial growth during storage. The stored solidified agar can then be remelted in a hot waterbath (100° C) and then poured on the material that will support the gel.

The original method of immunoelectrophoresis described by Grabar[5, 6] utilized agar coated photographic glass plates. Micro-methods are described in which the hot agar gel is molded on 76 x 26 mm glass slides.[10] One major drawback with this modification is that the area in which the precipitin bands are formed is limited, thereby decreasing the sensitivity of the procedure. It should be stated that immunoelectrophoresis approaches the sensitivity of starch gel electrophoresis while at the same time it is technically less complicated.

Personal experience, as well as reports by Cawley et al.[2], have shown that these strips can be manipulated easily because of their flexibility. In addition, antigen wells for the application of the serum sample and the antibody trough used for the deposition of antiserum are easily made on the agar. Furthermore these film strips can be treated with several histochemical reagents without ill effects. These film strips can be kept as permanent records.

Cellulose acetate membranes can be adopted, if desired, as a medium for the immunoelectrophoretic identification of serum proteins.[4, 9] The patterns on cellulose acetate are generally similar to those observed on agar gel; however, some differences in position along the electrophoretic axis are reported. All methods using cellulose acetate for immunoelectrophoresis require long diffusion periods (46 hrs. to 72 hrs.)

Immunodiffusion techniques performed in glass colums involve less manipulation than agar gel strips. This is because the gel columns do not require washing. Washing of the strips is a necessity for immunoelectrophoresis and immunodiffusion prior to the

identification or quantitation of the precipitin bands. When cellulose acetate is used as a medium the washing time after diffusion can be reduced greatly. In order to eliminate staining, and thereby eliminate the use of glacial acetic acid and methanol, recording of results is done by photographing the wet strips, using dark field illumination techniques.[8]

Immunodiffusion appears to be the most practical method for the study of serum complement in this project. Laboratory procedures designed for the measurement of the complement system are intricate.[7] Measurements of complement with immunodiffusion are restricted to the third component of the hemolytic complement system (β_{1C}) in serum. However, in most clinical work there has been good correlation with the use of β_{1C} determinations as an indicator of in vivo antigen-antibody reactions and of hemolytic processes.

Antiserums for these procedures are available commercially. The use of goat antiserum is advised because of the nature of the precipitating antibodies formed in this species of animal.

C. Areas for research and development

The major disadvantage of these procedures in their present status of development is the prolonged diffusion period (20 - 40 hours) required. If these procedures are desired for this project, it would be advantageous to see if any factors in the space flight environment can lead to shortening of the diffusion time.

D. References

1. Cawley, L.P.; Eberhardt, L.; Wiley, J.L.; Double Immunodiffusion with Agar-Coated Plastic Film Base, Vox, Sang., Vol. 10, 1965, pp. 116 - 125.

The use of agar coated plastic film base for microimmunodiffusion is discussed in this article. The authors point out that a large number of micromethods may be carried out upon a single sheet of plastic.

2. Cawley, L. P.; Schneider, D.; Simplified Gel Electrophoresis, II Application of Immunoelectrophoresis. J. Clin. Lab. Med., Vol. 65, 1965, pp. 342 - 354.

This article discusses a simplified system of agar gel electrophoresis in which a low concentration of agar is supported on 35 mm Cronar polyester photographic film base leader strips. While the electrophoresis time is brief (30 to 45 mins.) the diffusion phase takes 24 hours at room temperature.

3. Claman, H. M.; Merrill, D.; Quantitative Measurement of Human Gamma - 2, Beta - 2A, and Beta - 2M Serum Immunoglobulins. J. Lab. and Clin. Med., Vol. 64, 1964, pp. 685 - 693.

This article presents a modification of Oudin's method of single precipitation in agar for measuring immunoglobulins in serum. The diffusion is carried out in glass tubing filled with agar and antiserum. The method presented is sufficiently simple and reproducible.

4. Feinberg, M. P.; Mann, L. T.; Blatt, W. F.; Cellulose Acetate Media in Immunochemical Tech. Amer. J. Clin. Path., Vol. 44, 1965, pp. 177 - 181.

The use of cellulose acetate membranes as a medium for immunoelectrophoresis is presented. Details of the apparatus used for electrophoresis and of the preparation of troughs and wells are given. Diffusion time is given 60 to 72 hours at 20° C.

5. Grabar, P.; The Use of Immunochemical Methods in Studies on Proteins. Adv. in Protein Chem., Vol. 13, 1958, pp. 1 - 33.

This article deals principally with the possibilities of immunochemical methods for the study of serum proteins. The chief limitations of immunochemical methods are given. The chief limitation given is the heterogeneity of the antibodies used.

6. Grabar, P.; Burtin, P.; Immuno-Electrophoretic Analysis, Elsevier Publishing Co., New York, 1965.

This is a complete monograph dealing with the principles of immuno-electrophoretic analysis and its applications to the study of human plasma in normal and pathologic states.

7. Kabat, E. A.; Mayer, M. M.; Experimental Immunochemistry. Second edition, Charles C. Thomas, Springfield, 1961, chapter 4.

This chapter discusses Complement and the various assay methods. This is a very theoretical presentation.

8. Nace, G.W.; Alley, J.W.; On the Photography of Unstained, Differentially Stained and Fully Stained Precipitation Lines in Agar. J. Biol. Photo. Assoc., Vol. 29, 1961, pp. 125 - 233.

This paper discusses the use of both transmitted light and dark field illumination for the photography of agar diffusion patterns. The value of the dark field technique is presented.

9. Nelson, T.L.; Stroup, G.; Weddell, R.; Immunoelectrophoretic Identification of Human Serum Proteins on a Cellulose-Acetate Medium. Amer. J. Clin. Path., Vol. 42, 1964, pp. 237 - 244.

The authors discuss the use of cellulose acetate as a medium for immunoelectrophoresis, emphasizing the small volume required (0.001 ml to 0.005 ml).

10. Wunderly, C.; Immunoelectrophoresis. Adv. in Protein Chem., Vol. 4, 1961, pp. 208 - 273.

This article is a review of the principles taking part in electrophoretic separation in Agar Gel. The sensitivity of these immunological reactions is given. Macromethods and micromethods of these immunochemical procedures are discussed.

III. The titration of serum anti-A and anti-B isoagglutinins

If a person has blood group A, anti-B agglutinins are present in the serum. A person of blood group B has anti-A agglutinins in the serum. A person of blood group O has both of these agglutinins. Individuals of blood AB have none of these agglutinins. Two molecular types of these agglutinins can be found normally in one individual, namely, the 19S and 7S types.

Complete absence of the agglutinins in persons who should possess them is very rare. This has been observed in hypogammaglobulinemia and in some hematologic diseases. Isoagglutinins may become weak or absent following any injury to the reticuloendothelial system.

A. Principles of present methodology

Present laboratory methods involve the addition of two-fold serial dilutions of serum to an equal volume of a 2 to 5% saline suspension of washed human A or B erythrocytes depending on the blood type of the individual. After the 19S agglutinins are estimated, the serum is neutralized by the addition of purified blood group A or B substances so that the 7S isoantibodies can be titered with antiglobulin serum. The 19S agglutinins are neutralized by these type specific substances.[3]

B. Suitability of present methodology to space flight conditions

1. Merit table

Anti-A, Anti-B Isoagglutinins

Merit Parameters	Serologic Quantitation of Anti-A and Anti-B Agglutinins
1. Accuracy	approx. 85%
2. Sample size	0.1 ml
3. Time required	1 hour
4. Reproducibility	9
5. Suitability for null gravity use	8
6. Overall safety	10
7. Nontoxic, noncaustic reagents	10

Anti-A, Anti-B Isoagglutinins - continued

Merit Parameters	Serologic Quantitation of Anti-A and Anti-B Agglutinins
8. Specificity	10
9. Insensitive to environmental change	10
10. Ease in training personnel	10
11. Degree of separation required (10=none)	10
12. Minimal handling by analyst	6
13. Common use of analytic equipment	0
14. Nondestructive of sample	0
15. Figure of merit	70

2. Discussion

This method while relatively simple requires a considerable degree of manipulation. This is because of the serial dilutions of the serum sample which have to be made. At best, this type of serologic test renders only an estimate of the 19S and 7S agglutinins present. The quantitative precipitin method[1] for determination of these agglutinins while more accurate than the method presented here is not practical for this project. This is because of the time requirements and of the manipulation involved.

The commercial preparations of antiserums (anti-A, anti-B, antiglobulin) and of type specific substances A and B can be used for this determination.

C. Areas for research and development

These areas do not necessarily involve this quantitation. The use of latex particles coated[2] with appropriate antigens can be investigated for the detection of other antibodies in the serum. These procedures are based either on the principle of agglutination - inhibition or direct agglutination. These tests could be employed as simple screening procedures or they can be designed for the serologic quantitation of a given substance in the serum. Applications of these tests

have been made in mycology, parasitology, and in general immunology.

D. References

1. Kabat, E.; Bezer, A.E.; Immunochemical Studies on Blood Groups: I. Estimation of A and B Isoantibodies in Human Serum by the Quantitative Precipitin Method. J. Exper. Med., Vol. 82, 1945, pp. 20 pp. 207 - 215.

The authors discuss a microprecipitin method for the quantitation of A or B isoantibodies in serum. The results are given in amounts of nitrogen precipitated with each type specific substance. The precipitation phase as presented in the paper involves a long period of time.

2. Kabat, E.A.; Mayer, M.M.; Experimental Immunochemistry. Second Edition. Charles C. Thomas, Springfield., 1961, p. 124.

The use of polystyrene latex particles in agglutination procedures is presented. Diagnostic areas in which these particles have been used are given.

3. Mollison, P.L.; Blood Transfusion in Clinical Medicine, F.A. Davis, Co., Philadelphia, 1967, pp. 246 - 255.

In the section noted here the author gives a thorough explanation of the ABO Blood System. Methodology is discussed briefly.

3.3.07 Methemoglobin

The measurement of methemoglobin in blood is rather difficult especially when this derivative of hemoglobin is present in small amounts. The only chemical difference between hemoglobin and methemoglobin is that in the former the iron atom is in the ferrous state (Fe^{++}) while in the latter the iron is oxidized to the ferric ion (Fe^{+++}).

A. Principles of present methodology

Method 1: Evelyn and Malloy[1]

Principle: Methemoglobin has an absorption maximum at 630 mμ. Addition of NaCN converts methemoglobin to cyanmetemoglobin and the absorption peak at 630 mμ disappears almost completely. The difference in absorbance at 630 mμ (A_{630}) is directly proportional to the concentration of methemoglobin.

Procedure: A sample of blood is diluted 1:51 with phosphate buffer, pH 6.6 and is allowed to lake. The solution is centrifuged to remove the cell debris and the absorbance A_1 of the supernatant is measured at 630 mμ against water. A drop of cyanide is added to the solution and its absorbance A_2 is measured again at the same wavelength. The difference of the two absorbances ($A_1 - A_2$) is proportional to the concentration of methemoglobin.

Turbidity due to lipemic blood samples will not affect the results.

Method 2: Martinek[4]

Principle: The determination of methemoglobin is made by measuring the absorbance of a diluted blood sample at two wavelengths. The two wavelengths λ_1 and λ_2 are selected in such a way that at λ_1 the ratio of the absorbances of methemoglobin to oxyhemoglobin, is the highest and at λ_2 the ratio is 1.0 (isobestic point). The calculation of methemoglobin concentration involves the solution of simultaneous equations.

Procedure: Heparinized blood is diluted 1:101 with distilled water and the the mixture is allowed to stand for a few minutes for complete

hemolysis. The cell fragments are removed by slow speed centrifugation and the absorbance of the clear supernatant is measured at 620 and 530 mμ. The amount of methemoglobin is expressed as a percentage of the total hemoglobin in blood.

Lipemia does not interfere with this method. It is preferable to use a buffer to control the pH; distilled water with a pH below 5 and above 7 is unsatisfactory.

The results obtained are in good agreement with those of Method 1.

Similar spectrophotometric techniques have been developed by other investigators. Zijlstra and Muller[5] prefer measurements of the absorbance at 558 and 523 mμ, while Hutchinson[3] suggested the wavelengths of 620 and 520 mμ.

B. Suitability of present methodology to space flight conditions

1. Merit table

Methemoglobin

Merit Parameters	Method 1	Method 2
1. Sensitivity	.2 g	.2 g
2. Sample size	.1 ml	.05 ml
3. Time required	20 min	15 min
4. Reproducibility	7	7
5. Suitability for null gravity use	9	9
6. Overall safety	2	10
7. Nontoxic reagents	2	10
8. Noncaustic reagents	10	10
9. Specificity	9	8
10. Insensitive to environmental changes	2	9
11. Ease in training personnel	9	9
12. Degree of separation required (10=none)	8	8
13. Minimal handling by analyst	7	8
14. Common use of analytic equipment	10	10

Methemoglobin - continued

Merit Parameters	Method 1	Method 2
15. Nondestructive of sample	0	2
16. Figure of merit	26	59

2. Discussion

The accuracy of both methods is not very good when the methemoglobin level is within the normal range, up to 0.5 g/100 ml of blood.

The method of Evelyn and Malloy is preferred by many investigators but it suffers from certain disadvantages, i.e., the high intoxicity of NaCN reagent. Perhaps the NaCN can be replaced by sodium azide which is far less toxic and quite stable.

The differential spectrophotometric method is more attractive and easier to perform. It should be emphasized that the validity of this method depends on the presence of no more pigments in blood than are assumed to be present.

Further experimentation will be required to determine the optimum wavelengths for the spectrophotometric procedures. The method of Martinek[4] or some other modification[3,5] is preferable for the present time.

Equipment

Method 1: Spectrophotometer, centrifuge

Method 2: Spectrophotometer, centrifuge

C. Areas for research and development

It may also be possible to measure the methemoglobin content of blood by electrophoretic techniques.

D. References

1. Evelyn, K.A.; Malloy, H.T.; Micro-determination of Oxyhemoglobin Methemoglobin and Sulfhemoglobin in a Single Sample of Blood. J. Biol. Chem., Vol. 126, 1938, pp. 655 - 662.

Describes the simultaneous determination of oxyhemoglobin and some of its derivatives.

2. Henry, R.J.; Determination of Methemoglobin and Sulfhemoglobin in Clinical Chemistry: Principles and Technics, Harper and Row Publishers, New York, 1964, pp. 75 - 83.

   A book devoted entirely to the principles and techniques of chemistry. The author generally discusses several methods in some detail and then selects one or two and describes them in great detail. Provides values of accuracy, precision and normal values for selected procedures.

3. Hutchinson, E.B.; The Measurement of Methemoglobin, Am. J. Med. Tech., Vol. 26, 1960, pp. 75 - 83.

   Methemoglobin is measured in blood by direct spectrophotometry in two wavelengths.

4. Martinek, R.G.; Spectrophotometric Determination of Abnormal Hemoglobin Pigments in Blood. Clin. Chim., Acta 11, 1965, pp. 146 - 158.

   Methemoglobin is measured spectrophotometrically in a diluted blood sample without any additions.

5. Zijlstra, W.G.; Muller, C.J.; Spectrophotometry of Solutions Containing Three Components with Special Reference to the Simultaneous Determination of Carboxyhemoglobin and Methemoglobin in Human Blood. Clin. Chim. Acta, Vol. 2, 1957, pp. 237 - 245.

   Method for methemoglobin by spectrophotometry.

3.3.08 Mucoproteins

Many complexes of carbohydrate with proteins are classified as mucoproteins and glycoproteins.

Mucoproteins are characterized by the fact that the carbohydrate-protein linkage is easily broken by hydrolysis or by an electric field suggesting an ionic or polar-type linkage.

Glycoproteins are defined as having at least 0.5% hexosamine and having no hexuronic or sulfate esters. The carbohydrate-protein bond can be split by drastic treatment, strong alkali, or acid.

Mucoids are defined as mucoporteins having at least 70% carbohydrate while mucoproteins have considerably less.

The fraction which is usually isolated from serum by precipitation with phosphotungstic acid, after removal of the serum proteins by perchloric acid, is called seromucoid.

Two fractions are usually measured in serum, (1) Seromucoid, (2) Protein- bound Hexose.

A. Principles of present methodology

1. Seromucoid in serum[2]

Principle: Serum proteins are precipitated with 1.2 M perchloric acid. After removal of the precipitate by centrifugation, the seromucoid is precipitated in the supernatant by phosphotungstic acid. The precipitated seromucoid is dissolved in dilute NaOH and quantitated colorimetrically at 485 or 520 mμ by the orcinol reaction. Results are expressed as mg of seromucoid per 100 ml of serum in terms of a galactose-mannose standard.

2. Protein-bound hexose in serum[3]

Principle: Protein-carbohydrate conjugates containing hexose are precipitated from serum by addition of ethanol. The hexose is measured in the precipitate by the orcinol reaction.

3. Acid mucopolysaccharides (AMP) in urine[1]

Principle: Uromucoid and acid mucopolysaccharides are

selectively precipitated from urine by cetyltrimethyl-ammonium bromide (CTV). The precipitates are centrifuged, the supernatant is discarded and the CTV-AMP complexes are dissociated by treatment with ethanol saturated with NaCl. The AMP is dissolved in NaOH and the various carbohydrate moieties are measured by color reactions employing specific reagents.

B. Suitability of present methodology to space flight conditions

1. Merit table

Merit Parameters	Mucoproteins		
	Sero - Mucoid	Protein - Bound Hex.	AMP Urine
1. Sensitivity	-	-	-
2. Sample size	0.5 ml	0.1 ml	40 ml
3. Time required	1 hour	1 hour	2 days
4. Reproducibility	6	6	6
5. Suitability for null gravity use	-	-	-
6. Overall safety	0	0	0
7. Nontoxic reagents	5	5	5
8. Noncaustic reagents	0	0	0
9. Specificity	7	7	7
10. Insensitive to environmental changes	6	6	6
11. Ease in training personnel	2	4	1
12. Degree of separation required (10=none)	4	6	3
13. Minimal handling by analyst	2	3	1
14. Common use of analytic equipment	9	9	9
15. Nondestructive of sample	1	1	2
16. Merit range	11	6-13	5-11
17. Mean figure of merit	8	9	8

2. Discussion

Strong sulfuric acid is required for the color reactions involved in the methodology presented above with the exception of Protein-

Bound Hexose which can be measured by turbidimetry.

Equipment

All three determinations: heating bath, centrifuge, spectrophotometer

C. Areas for research and development

Electrophoretic and immunological studies may prove to be valuable in these determinations. Infrared spectra of isolated fractions may also provide valuable information.

D. Comments

No method of any kind can be recommended until it is defined what type of polysaccharide is to be measured.

E. Reference

1. Clausen, J.; Asboe-Hansen, G.; Urinary Acid Mucopolysaccharides in Mastocytosis. A New Technique for Quantitative Estimation. Clin. Chim. Acta, Vol. 13, 1966, pp. 475 - 483.

Acid mucopolysaccharides are precipitated by cetyltrimethyl-ammonium bromide and quantitated colorimetrically.

3.3.09 Serum proteins

I. Serum albumin

A. Principles of present methodology

1. Method 1

Albumin can be estimated by any one of the numerous electrophoretic techniques with simultaneous determination of the total serum proteins or by one of the three methods described under part II, "Total Serum Proteins." Ultraviolet scanning of the electropherograms permits the estimation of albumin as percentage of the total serum protein without drying or staining.[2]

2. Method 2

Albumin can also be measured by a dye binding method. Rodkey[3] proposed the use of bromcresol green for the estimation of serum albumin. Unlike other dyes, bromcresol green binds specifically with albumin and there is no interference from other serum proteins. Albumin values by this dye-binding method are in very good agreement with those obtained with electrophoretic techniques. Bartholomew[1] modified Rodkey's method and developed an excellent procedure for the measurement of serum albumin. When serum is added to a solution of bromcresol green in 0.05 M citrate buffer, pH 4.0, the optical density of the solution increases and this increase is directly proportional to the albumin concentration. Absorbance measurements are made at 620 - 640 mμ. The method is very sensitive, specific and can be easily automated.

B. Suitability of present methodology to space flight conditions

1. Merit table

Serum Albumin

Method Parameters	Method 1	Method 2
1. Sensitivity	Good	Excellent
2. Sample size	1 μl	10 μl
3. Time required	45 min	20 min
4. Reproducibility	7	9

Serum Albumin - continued

Method Parameters	Method 1	Method 2
5. Suitability for null gravity use	-	9
6. Overall safety	6	10
7. Nontoxic reagents	-	10
8. Noncaustic reagents	9	10
9. Specificity	10	10
10. Insensitive to environmental changes	-	6
11. Ease in training personnel	5	9
12. Degree of separation required (10=none)	9	9
13. Minimal handling by analyst	2	8
14. Common use of analytic equipment	5	10
15. Nondestructive of sample	0	0
16. Merit range	14-45	-
17. Mean figure of merit	27	75

2. Discussion

Both methods are very suitable for the measurement of serum albumin; however, the dye-binding method is preferable because of its simplicity, specificity, and excellent reproducibility. The compiler believes this is the method of choice.

Equipment

Method 1: Electrophoresis apparatus

Method 2: Spectrophotometer, centrifuge, automatic diluting pipette.

C. Areas for research and development

(None)

D. References

1. Bartholomew, R. J.; Sulphonephthaleins are Specific Reagents for Albumin. VI International Congress of Clinical Chemistry (Abstract) Enzym. Biol. Clin., Vol. 6, 1966, p. 168.

Bromcresol green at an acid pH is a specific reagent for albumin.

2. Ressler, N.; Photoelectric Ultraviolet Photometry Applied to Protein Electrophoresis. J. Lab. Clin. Med., Vol. 54, 1959, pp. 291 - 299.

Electrophoretic patterns are scanned at 200 mμ without drying or staining. The technique permits the estimation of albumin and other serum protein fractions.

3. Rodkey, F. L.; Direct Spectrophotometric Determination of Albumin in Human Serum. Clin. Chem., Vol. 11, 1965, pp. 478 - 487.

Serum albumin is measured directly by a dye-binding method with bromcresol green.

II. Total serum proteins

There are several methods which permit rapid and reliable measurements of the total serum proteins.

A. Principles of present methodology

1. Refractometry

The estimation of serum proteins by measurement of the refractive index was investigated by Sunderman[5] and has been reviewed by Naumann.[4]

Most of the commercially available instruments require a sample of serum from 0.05 to 0.30 ml. The protein content of serum is given by the following equation.[5]

$$\text{Protein content in g\%} = 510\ (RI_S - RI_{H_2O}) - 1.81$$

where: RI_S = the refractive index of serum

RI_{H_2O} = the refractive index of pure water.

The above equation has recently been corrected by Sundermann[6] as follows:

$$\text{Protein content in g\%} = 533.6\ (RI_S - RI_{H_2O}) - 1.89$$

The correction is valid and based on the fact that the Kjeldahl nitrogen factor for serum proteins is 6.54 rather than the traditionally accepted value of 6.25.

The results obtained by refractometry are in good agreement with those of the biuret and Kjeldahl techniques.[2,4] The same serum sample may be used for analysis of other serum components. A possible source of error is the temperature effect but that is negligible in methods determining the difference of the refractive indices of serum and water as long as both are at the same temperature.[2,5]

The method has good precision, adequate sensitivity, and does not require highly trained personnel.

280 mμ due to the presence of aromatic amino acids. The intensity of absorption in this spectral region depends on the aromatic amino acid content of a particular protein and various proteins differ widely in their specific absorptivity. However, in the spectral region between 200 and 240 mμ, absorption of light is principally due to peptide bonds and proteins differ relatively little in their specific absorptivity. Furthermore, the absolute absorbance in the 200 - 240 region is much greater than at 260 - 280 mμ.

In a procedure proposed by Tombs, et al.[7], serum is diluted 1:2000 with 0.9 percent sodium chloride solution and absorbance measurements are made at 210 mμ. A linear relationship exists between protein concentration and optical density.

The specific extinction of total serum protein at 210 mμ is:

$$E^{1\%}_{1\,cm} = 205 \pm 1.4$$

In the method developed by Waddell[8] total serum proteins are determined from the difference in absorbances of a 1:1000 dilution of serum at 215 and 225 mμ. The use of the difference, rather than of the absorbance at a single wavelength, almost eliminates the error from non-protein serum constituents, since the difference in absorbance, at these two wavelengths, of human plasma ultrafiltrates was found to be negligible. Moreover, the use of somewhat longer wavelengths in the Waddell method results in significantly lower stray light errors than those that exist at 210 mμ.

Both methods are highly sensitive and require very small serum samples. The high dilution of serum eliminates essentially all potential interference from non-protein substances absorbing between 215 and 225 mμ.

To minimize potential errors, the serum dilution must be prepared with extreme care and the volume of the sample must be measured with meticulous attention. Highly accurate pipettes

should be employed; a well-calibrated mechanical pipetting-diluting machine would be preferable to hand pipetting.

3. Biuret

This method is probably the most widely used for the determination of total serum proteins, because of its simplicity and reproducibility.

Principle: A sample of serum is mixed with the biuret reagent[3] and the optical density of the resulting colored complex is measured at 545 mμ. The optical density is directly proportional to the protein content of serum. Many biuret reagents have been proposed but the one described by Huerga et al.[1] has been found to be very stable. It incorporates copper sulfate, sodium-potassium tartrate, sodium hydroxide and potassium iodide in a single solution.

B. Suitability of present methodology to space flight conditions

1. Merit table

Total Serum Proteins

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	.05 g	1 μg	.05 g
2. Sample size	.05 ml	1-5 μl	20 μl
3. Time required	2 min	5 min	20 min
4. Reproducibility	9	9	9
5. Suitability for null gravity use	10	9	9
6. Overall safety	10	10	6
7. Nontoxic reagents	10	10	7
8. Noncaustic reagents	10	10	5
9. Specificity	8	8	10
10. Insensitive to environmental changes	9	9	6
11. Ease in training personnel	10	8	9
12. Degree of separation required (10=none)	9	9	9
13. Minimal handling by analyst	9	6	9
14. Common use of analytic equipment	2	9	10

Total Serum Proteins - continued

Merit Parameters	Method 1	Method 2	Method 3
15. Nondestructive of sample	10	0	0
16. Figure of merit	82	73	50

2. Discussion

Any of the three described methods may be applicable to space flight conditions. Method 1 is preferred if an instrument capable of measuring the refractive index with a precision of 0.0001 unit is available. The required handling of the specimen is minimal and no reagents are necessary.

Lipemic sera must be avoided with any of the three methods. The length of time for analysis listed under each method does not include the time required for centrifugation of the specimen to accomplish the separation of serum from the blood clot.

Equipment

Method 1: Refractometer, centrifuge

Method 2: Ultraviolet spectrophotometer, centrifuge automatic pipetting-diluting equipment

Method 3: Spectrophotometer of photoelectric colorimeter, centrifuge

It is possible to avoid the high dilution in the spectrophotometric method by using cuvettes of a very short light path.

C. Areas for research and development

Absorption spectra of sera in the ultraviolet may provide additional useful information. Specific immunologic reactions may also be employed to quantitate specific proteins either by precipitation techniques or by more sensitive means such as fluorescence depolarization.

D. References

1. De la Huerga, J.; et al., In Serum Proteins and the Dysproteinemias, J.B. Lippincott, Co., Philadelphia, 1964, pp. 52 - 62.

Discusses the determination of serum proteins by the biuret method.