3.3.21 Non-protein nitrogen (NPN) and total nitrogen

Essentially all specificity is lost when these two groups of nitrogenous compounds are to be analyzed. The only aspect in common for the several different materials assayed in these two groups is that they all contain nitrogen. In the instance of NPN it is the total nitrogen in protein-free blood solutions and for total nitrogen of urine it is all the nitrogenous urinary components. The nitrogen in both cases is in various forms, including ammonia, amines, amides and as constituents of heterocyclic rings. To assay nitrogen in the various forms it seems necessary to convert it first to a common form. The technique suggested is conversion to ammonia, a compound readily assayed.

A. Principle of present methodology

1. NPN

The sample of serum plasma or whole blood is treated with an alkaloidal reagent (usually trichloroacetic or tungstic acid) to prepare a protein-free solution. The filtrate (supernatant solution of dialyzate) is then treated as urine for total nitrogen as described below.

2. Total nitrogen

The classical and probably the best technique for converting nitrogen in biological samples to ammonia is a modification of the Kjeldahl technique.[1] An aliquot of the protein-free solution or urine is subjected to wet acid digestion. Sulfuric and selenious acids are generally the main reagents, although other reagents, such as syrupy orthophosphoric acid, are often added to raise the boiling point and thus shorten digestion time. The ammonium ions produced can be quantitated in various ways, but for convenience and economy nesslerization appears most suitable as described above for the analysis of urea.

B. Suitability of present methodology to space flight conditions

1. Merit table

Non-protein Nitrogen and Total Nitrogen

Merit Parameters	NPN	Total Nitrogen
1. Sensitivity	Good	Good
2. Sample size	100 µl	10 µl
3. Time required	60 min	60 min
4. Reproducibility	9	9
5. Suitability to null gravity use	-	-
6. Overall safety	0	0
7. Nontoxic reagents	1	1
8. Noncaustic reagents	1	1
9. Specificity	9	9
10. Applicability	0	0
11. Reagent volatility	1	1
12. Sensitivity to environmental changes	8	8
13. Analyst training	4	3
14. Manipulation	3	3
15. Common use of analytic equipment	10	10
16. Merit range	7-15	7-14
17. Mean figure of merit	11	11

2. Discussion

As indicated previously it seems necessary to convert all the nitrogen to a state where a single technique can be applied for quantitation. All existing methods for the degradation of organic materials require either wet digestion or combustion; processes which do not appear entirely desirable under the conditions specified.

NPN and total nitrogen determinations under the prescribed conditions do not appear feasible. The wet acid digestion requires the continuous removal of volatile components and a failure in this removal could lead to serious environmental contamination.

Measurements of NPN for evaluations of kidney function can be largely, and often better, replaced by BUN assays. Assessment of metabolic states, which can perhaps be measured in part by NPN, can probably be done as well with estimations of blood amino acid nitrogen.

There is apparently no substitute for the measurement of urinary nitrogen. Over an extended period the nitrogen balance can be approximated from a knowledge of the nitrogen intake and body weight. Also urine urea can be used as a crude measure of nitrogen balance in that normally the majority of the urinary nitrogen occurs as carbamide.

Conditions and Equipment

NPN assay requires equipment to prepare protein-free solutions. Both procedures need digestion equipment with provision for the removal of toxic fumes, and a colorimeter.

C. Areas for research and development

(None)

D. Reference

1. Kjeldahl, J.; Neue Methode zur Bestimmung der Stickstoffs in Organischen Korpern, Z. Anal. Chem., Vol. 22, 1883, pp. 366 - 378.

A description of the author's original technique for measuring organic nitrogen.

3.3.22 Bilirubin in Blood serum or plasma

A. Present methodology

1. Icterus index[9]

With the exception of carotene, bilirubin is the only normally occurring substance found in blood serum which has a strong absorbance at 455 mμ. The commonly used method is to compare the absorbance of a sample of blood serum in a colorimeter or spectrophotometer with that of a series of artificially prepared standards[1, 2, 3, 7, 15]

2. Van den Bergh Method

This test is based on the spectrophotometric determination of the diazo derivative which results from treatment of bilirubin with diazotized sulfanilic acid.[10, 11, 14]

3. Rutkowski-deBaare Method[12]

This is an ultramicro modification of the van den Bergh method. It requires the use of only 20 microliters of blood serum with no apparent sacrifice of accuracy.

B. Applicability of present methodology to space flight conditions

1. Merit table

Bilirubin

Merit Parameters	Icterus Index	van den Bergh	Rutkowski
1. Sensitivity	< 0.1 mg%	< 0.1 mg%	< 0.1 mg%
2. Sample size	0.25 ml.	0.2 ml.	0.02 ml.
3. Time required	3 min	5 min	5 min
4. Reproducibility	5	8	8
5. Suitability for null gravity use	-	-	-
6. Overall safety	10	5	5
7. Nontoxic reagents	10	3	3
8. Specificity	6	9	9
9. Insensitive to environmental changes	10	10	10

Bilirubin - continued

Merit Parameters	Icterus Index	van den Bergh	Rutkowski
10. Ease in training personnel	10	10	8
11. Degree of separation required (10=none)	10	10	10
12. Minimal handling by analyst	10	8	8
13. Common use of analytic equipment	10	10	10
14. Nondestructive of sample	10	0	0
15. Merit range	23-46	22-44	22-44
16. Mean figure of merit	34	33	33

2. Discussion

The proposed methods are based on established time-tested procedures, and normal values by these methods are well established.

a. Icterus index

Carotene, lipemia and hemolysis interfere with accuracy. The method does not allow for differentiation between "free" and conjugated bilirubin which would be useful in the differential diagnosis of biliary obstruction and hepatocellular or hemolytic jaundice. These disadvantages may be offset, however, by the simplicity of the test.

b. Van den Bergh test

This method is more specific than icterus index but slightly more involved. The reagents have been prepared in the form of a compressed tablet[13] which should be simple to handle in a space environment. The use of this method has been reported to give excellent results.[8] In order to determine indirect (conjugated) bilirubin, methyl alcohol or a similar organic solvent is generally used.

c. Rutkowski-deBaare method

Because of the small sample of blood serum required and

the apparent sensitivity and accuracy of this method, it is preferred over the standard van den Bergh method. It suffers the disadvantage of requiring four reagent solutions, one of which is 60 percent phosphoric acid.

C. Areas for research and development

The simplicity and time-honored usefulness in clinical medicine of the presently employed tests for bilirubin, suggest that for purposes of monitoring the health of astronauts, these methods may be adequate.

More refined methods based on the fluorescence of oxidation products of bilirubin might be a useful approach to the development of more specific, highly sensitive methods of analysis.[4, 5, 6]

Other porphyrins also yield fluorescent oxidation products which may interfere. It should be noted that these studies were reported before the art of fluorescence analysis was well developed. Careful examination of the fluorescence spectra of bilirubin and related porphyrins, using different wavelengths for excitation may prove fruitful.

D. References

1. Bilissis, P.K.; Speer, R.J.; A Stable "Bilirubin" Standard Clin. Chem., Vol. 9, 1963, p. 552.

An aqueous solution of N-(1 naphthyl) ethylenediamine - di - H Cl is a stable standard for bilirubin determination.

2. Bolshev, I.N.; Apparatus for Determining Bilirubin in Small Amounts of Blood, Sovrem. Probl. Gematol. i Perelin. Krovi, Vol. 36, 1964, p. 183.

Determined bilirubin by icterus index in 3 mm capillary tubes by visual comparison with artificial standards prepared from bromthymol blue. Claims the method to be accurate and applicable for studies on newborn infants.

3. Cantarow, A.C.; Trumper, M.; Hepatic Function in Clinical Biochemistry, W.B. Saunders Co., 4th edition, 1950, p. 425.

A discussion of the various clinical chemical methods for

determining bilirubin, including interpretation of the results.

4. Dhere, C.; Roche, J.; Fluorescence of Pigments of the Urobilin Group and Determination of their Fluorescence Spectrum, Bull. Soc. Chim. Biol., Vol. 13, 1931, p. 987.

   Compares the fluorescence spectra of several bilirubin-related compounds.

5. Dhere, C .; The Red Fluorescence which Certain Bilirubin Derivatives Show in Ultraviolet Light, Compt. Rend. Soc. Biol., Vol. 103, pp. 371 - 374.

   Describes the fluorescence spectrum of the product resulting from treatment of bilirubin with ammonia and zinc acetate.

6. Dhere, C.; Spectroscopical Study of a Bilirubin Derivative with Red Fluorescence, Arch. Intern. Pharmacodynamic, Vol. 38, 1930, pp. 134 - 139.

   The fluorescence spectra of compounds prepared by action of zinc acetate, iodine and potassium iodide were examined.

7. Fog, J. Serum Bilirubin and the Yellow Color of Serum, Scand. J. Clin. Lab., Invest., Vol. 10, 1958, pp. 251 - 256.

   Obtained good correlation between icterus index and bilirubin concentration of normal serum.

8. Hart, C.; Plaut, D.; Evaluation of a Bilirubin Screening Test, Am. J. Clin. Pathol., Vol. 45, 1966, pp. 510 - 511.

   Compressed tablet preparation containing sulfanilic acid and sodium nitrite was found to be efficient, rapid and reliable for routine clinical bilirubin determination.

9. Henry, R.J.; Golub, O.J.; Borkman, S.; Segalove, M.; Am. J. Clin. Pathol., Vol. 23, 1953, p. 841.

   Describes a widely used procedure for doing icterus index of blood serum.

10. Martinek, R.G.; Improved Micromethods for Determination of Serum Bilirubin, Clin. Chim. Acta., Vol. 13, 1966, pp. 161 - 170.

    A simple, rapid and precise diazo method is described which

requires 0.1 ml. of serum. Interferences were resolved by improved kinetics and stoichiometry. Reliability of the method was established by demonstrating the lack of effect of lipemia and hemolysis.

11. Rand, R.N.; Di Pasqua, A.; A New Method for the Determination of Bilirubin, Clin. Chim. Acta., Vol. 8, 1962, pp. 570 - 578.

The use of a 2-4 dichloroaniline instead of sulfanilic acid provided a simple method of determination. May be used for direct and indirect reading bilirubin.

12. Rutkowski, R.B.; de Baare, L.; An Ultramicro Colorimetric Method for Determination of Total and Direct Serum Bilirubin, Clin. Chem., Vol. 12, 1966, pp. 432 - 437.

Describes a method which requires only 20 µl of blood serum. An improved diazo coupling reagent is utilized and phosphoric acid is used to prevent turbidity in the reaction mixture. Coefficient of variation of 15 replicate determinations on a 5 mg% standard was 3.4%.

13. Sherman, L.; Diagnostic Preparation for Determination of Serum Bilirubin, U.S. Patent 2, 737, 501, March 6, 1956.

A stable composition in tablet form which contains sodium sulfanilate and sodium nitrite in a water soluable binder is described for the determination of serum bilirubin.

14. Van den Bergh, A.A.H.; Miller, P.; Direct and Indirect Diazo Reaction with Bilirubin, Biochem. Z., Vol. 77, 1916, pp. 90 - 103.

The original description of the van den Bergh method.

15. With, T.K.; Spectral Absorption of Bilirubin Measurements in Pure Aqueous Solutions Containing Human Serum. Acta., Physiol. Scand., Vol. 10, 1945, pp. 172 - 180.

In pure aqueous solutions, maximum absorbance is at 420 mµ. In solutions containing serum, maximum absorbance is at 455 mµ. Change is due to formation of bilirubin-serum complex.

3.3.23 Creatine and creatinine

A. Principles of present methodology

Creatine can be measured in urine and protein-free blood solutions by the Voges and Proskauer reaction.[6] A fluorometric procedure has also been described which involves the reaction of creatine and ninhydrin in an alkaline solution.[4] The latter method is reported not to be very specific.[7]

For the present application creatine and creatinine are both to be measured and it would appear most economical and convenient to utilize a single technique for both. This can be done by employing a method for measuring creatinine. Creatine can be readily and essentially quantitatively converted to creatinine. Thus in one aliquot of urine, creatinine is measured and in another portion creatine is dehydrated to creatinine and creatinine is re-measured. The difference in the two analyses represents the creatinine formed from creatine.

There are three methods (colorimetric) which have been used to measure creatinine. One involves the use of 3,5-dinitrobenzoate in an alkaline solution.[2] This technique has not enjoyed great popularity because of its lack of specificity and the limited concentration range where it may be used. The procedure of Van Pilsum, et al.,[9] is probably the best method for measuring creatinine, but the large number of manipulations involved and the required use of an unstable reagent preclude its use for the present application.

The technique apparently most suitable for the present situation is one employing the Jaffe reaction.[8] This consists of the reaction of picrate and creatinine in an alkaline medium. A protein-free blood filtrate or unmanipulated urine is used for the reaction.

The application of this technique for the measurement of urine creatine and creatinine has received considerable study.[1] It is not so specific as some other methods,[5] but it is generally considered to be valid enough for most applications. A very desirable aspect of this

is the simplicity and the reagent stability. This is the only method the compiler can recommend for the current application.

B. Suitability of present methodology to space flight conditions

1. Merit table

An evaluation of the selected method for measuring creatine and creatinine is shown tabulated below. Dimensionless numbers are merit values.

Creatine and Creatinine

Merit Parameters	Serum Creatinine	Urine Creatinine	Urine Creatine
1. Sensitivity	Fair	Very Good	Fair
2. Sample size	100 µl	30 µl	30 µl
3. Time required	20 min	10 min	45 min
4. Reproducibility	7	9	6
5. Suitability for null gravity use	-	-	-
6. Overall safety	5	5	4
7. Nontoxic reagents	2	2	2
8. Noncaustic reagents	1	1	1
9. Specificity	5	8	7
10. Applicability	3	9	6
11. Reagent volatility	10	10	10
12. Insensitive to environmental changes	1	1	1
13. Analyst training	5	10	7
14. Manipulations	5	10	3
15. Common use of analytic equipment	10	10	10
16. Merit range	10-20	19-38	9-19
17. Mean figure of merit	15	29	14

2. Discussion

The method which appears most promising for near-future use is one utilizing the Jaffe reaction[6] for creatinine and creatine.

Creatine and creatinine are in equilibrium in solution. The

equilibrium constant for the reaction is known to be a function of pH and temperature.[3] It seems reasonable that conditions could be found which would favor the accumulation of creatine. If the creatine formed could be "trapped" by the formation of a derivative or complex, it would then be possible to effect a quantitative conversion of creatinine to creatine and then the Voges and Proskauer[6] reaction or perhaps even a fluorometric technique[4] could be employed for measuring creatine and creatinine. The advantage of this approach is that the Voges and Proskauer reaction is probably much more specific for creatine that the Jaffe reaction[8] is for creatinine. The "trapping" of the creatine might be accomplished by the use of a cationic exchange resin. The amidine group is a strong base and should form a stable complex with acidic resins.

C. Areas for research and development

The mono-substituted amidine group of creatine and the disubstituted amidine of creatinine provide quite reactive sites for the formations of derivatives with characteristic optical properties. Perhaps techniques suggested for forming derivatives of the amino acids could be utilized.

D. References

1. Biggs, H.G.; Cooper, J.M.; Modified Folin Methods for the Measurement of Urinary Creatine and Creatinine, Clin. Chem., Vol. 7, 1961, pp. 655 - 664.

Presents a simplified method for converting creatine to creatinine and describes a method of using the Jaffe reaction to measure both.

2. Bollinger, A.; The Colorimetric Determination of Creatinine in Urine and Blood with 3,5-Dinitrobenzoic Acid, Med. J. Australia, Vol. 2, 1936, pp. 818 - 821.

Describes a procedure for the assay of creatinine using 3,5-dinitrobenzoate and 1 N NaOH. Best range is 2 - 15 mg%.

3. Brinkerink, P.C.; Determination of Creatine in Urine, Clin. Chim.

Acta, Vol. 6, 1961, pp. 532 - 537.

Studied the equilibrium of creatine and creatinine under the various conditions, particularly as related to measurement of creatinine.

4. Conn, R. B.; Fluorometric Determination of Creatine, Clin. Chem., Vol. 6, 1960, pp. 537 - 548.

Assay of creatine by fluorescence following reaction with ninhydrin in an alkaline media.

5. Cooper, J. M.; Biggs, H. G.; An Evaluation of Four Methods of Measuring Urinary Creatinine, Clin. Chem., Vol. 7, 1961, pp. 665 - 673.

Compared the methods of Folin, Hare, Van Pilsum and Sullivan and Irreverre. Found the latter method to yield erroneous results whereas the others provided essentially the same results.

6. Ennor, A. H.; Stocken, L. A.; The Application of the Diacetyl Reaction to the Estimation of Creatine in Urine, Biochem. J., Vol. 55, 1953, pp. 310 - 314.

Applied the Voges and Proskauer reaction for the measurement of creatine in urine. Avoided all references to Voges and Proskauer.

7. Henry, R. J.; Clinical Chemistry, Principles and Techniques, Hoeber Medical Division, Harper and Row, New York, 1964, p. 291.

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.

8. Jaffe, M.; Uber den Niederschlag welchen Pikrinsaure in normalen Harn erzeugt, und uber eine neue Reaction des Kreatinim, Z. Physiol. Chem., Vol. 10, 1886, p. 391.

Described the chromogenic reaction of picric acid and creatinine in an alkaline medium.

9. Van Pilsum, J. F., et al.; Determination of Creatine, Creatinine, Arginine, Guanidoacetic Acid, Guanidine and Methyl Guanidine in

Biological Fluids, J. Biol. Chem., Vol. 222, 1956, pp. 225 - 236.

Measured creatinine by degradation to methyl-guanidine with o-nitrobenzaldehyde and Sakaguchi reaction. Creatine was determined by prior dehydration to creatinine.

3.3.24 Lactic acid in serum

A. Present methodology with modifications

1. Method 1

Compared to many other methods for measuring lactic acid, the fluorometric method of Loomis[1] has a distinct advantage since no protein precipitating step is required. The native fluorescence of different sera is not significantly different, and only one serum blank is needed for a series of tests. The reaction is as follows:

$$\text{L-lactate} + \text{NAD}^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$$

The reaction is carried to completion by conducting it at pH 10.5 and by using semicarbazide to trap the pyruvate. The method has good precision and adequate sensitivity. However, the reaction rate is slow (requires a 90 minute incubation period at room temperature) and for this reason modifications are recommended (see Method 3.)

2. Method 2

A method for measuring lactic acid recently developed by Noll[2] requires only a 20 minute incubation period. This rapid reaction rate was achieved by coupling the lactate dehydrogenase (LDH) and glutamate-pyruvate transaminase (GPT) reactions whereby pyruvate is converted to L-alanine by reaction with excess glutamate. Pyruvate is therefore rapidly removed from the equilibrium of the lactate dehydrogenase reaction:

pH 8.9

$$\text{L-lactate} + \text{NAD}^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$$

$$\text{L-glutamate} + \text{pyruvate} \xrightleftharpoons{\text{GPT}} \alpha\text{- ketoglutarate} + \text{L-alinine}$$

In addition to being required in the reaction, glutamate also serves as the buffer. As in the previous method, the product that is measured is NADH, but in this case it is measured spectrophotometrically at 366 mμ. The method has good precision and adequate sensitivity. Its primary disadvantage is that the analysis is per-

formed on a protein free filtrate. For this reason modifications are recommended (See Method 3.)

3. Method 3

Proposed modified Loomis-Noll method for measuring lactate. Loomis[1] has demonstrated that it is not necessary to deproteinize serum before fluorometric analysis of its lactate content. Noll[2] has demonstrated that the time required for the reaction to go essentially to completion can be significantly shortened. Thus it appears that the good points of the two methods can be combined to yield a method that is better than either. It also appears that the required reagents can be premixed in solution, apportioned into the tubes, and lyophilized before being carried into space. A general outline of the proposed modified method is as follows:

a. A solution (pH 8.9 glutamate buffer) containing the proper amounts of NAD^+, LDH, L-glutamate and glutamate pyruvate transaminase would be prepared on earth and apportioned into tubes that would ultimately be used as cuvettes for the fluorometer.

b. The contents of the cuvettes would immediately be lyophilized, sealed, and stored under refrigeration until used in space.

c. In space, the lyophilized material could be reconstituted by adding the proper amount of water at the time the experiment is to be conducted.

d. Serum would then be added (no protein precipitation required) to the reaction mixture and incubated at room temperature for 20 minutes.

e. The NADH produced during incubation would be measured fluorometrically.

f. A single serum blank and appropriate standards would also

be required. The amount of serum required for this modified procedure should be 50 µl or less.

The method should have good precision, adequate sensitivity, and only a minimum of time and training will be required. Consequently, this proposed procedure is recommended for measuring lactate during space flight.

B. Suitability of present methodology to space flight conditions

1. Merit table

An evaluation of the methods for lactic acid is presented below in tabular form. Dimensionless numbers are merit values; the greater the value, the more desirable.

Lactic Acid

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	Good
2. Sample size	100 µl	200 µl	50 µl
3. Time required	100 min	20 min	20 min
4. Reproducibility	9(± 2%)	9(± 2%)	9(± 2%)
5. Suitability for null gravity use	8	7	9
6. Overall safety	10	10	10
7. Nontoxic reagents	10	10	10
8. Noncaustic reagents	10	10	10
9. Specificity	10	10	10
10. Insensitive to environmental changes	9	9	9
11. Ease in training personnel	7	6	9
12. Degree of separation required (10=none)	9	7	9
13. Minimal handling by analyst	9	7	9
14. Common use of analytic equipment	10	10	10
15. Nondestructive of sample	0	0	0
16. Figure of merit	73	66	79

2. Discussion

In the modified method (Method 3) recommended for measuring blood lactate, the reaction is carried out under essentially the same conditions as recommended by Loomis.[1] Quantitation is accomplished by fluorometric measurement of the native fluorescence of the NADH produced as recommended by Loomis.

In theory the modifications should result in an improved method, but this can be verified only by laboratory experiments. Since the modifications are relatively minor, only a short period of laboratory experimentation should be required to perfect the method for space use.

In the method of Loomis large volumes are used (0.1 ml of serum diluted to a final volume of approximately 16 ml before measurement in the fluorometer). By reducing the volumes used, one should need only 25 - 50 µl of serum for each determination.

Equipment

Method 1: Fluorometer

Method 2: Spectrophotometer

Method 3: Fluorometer

Method 4: Densitometer (See part C)

A centrifuge will be needed to separate the serum from the blood clot.

C. Areas for research and development (Method 4)

Another possible approach to the problem of measuring lactate is one similar to that which is commonly used to measure LDH activity. This approach is briefly outlined below:

1. Impregnate filter paper with the following reagents:
   a. Phosphate buffer (pH 7.4)
   b. Sodium cyanide
   c. Nitro blue tetrazolium

d. Phenazine methosulfate

e. NAD^+

f. Lactic dehydrogenase

2. The dried substrate should be stable in filter paper if properly stored.

3. When serum is applied to filter paper impregnated with substrate the following reactions should occur:

$$\text{lactic acid} + NAD^+ \xrightleftharpoons{LDH} \text{pyruvic acid} + NADH + H^+$$

$$NADH + H^+ + \underset{\text{tetrazolium}}{\text{nitro blue}} + \underset{\text{methosulfate}}{\text{phenazine}} \rightleftharpoons \underset{\text{formazan}}{\text{violet}} + NAD^+$$

With a densitometer one should be able to make a semi-quantitative estimate of the amount of lactate present by comparing the intensity of the formazan spot produced by serum with that produced by a standard lactate solution.

D. References

1. Loomis, M. E.; An Enzymatic Fluorometric Method for the Determination of Lactic Acid in Serum, J. Lab. Clin. Med., Vol. 57, 1961, pp. 966 - 969.

This method is a simplified adaptation to fluorometry of an existing spectrophotometric method for the determination of serum lactic acid. The analysis is performed directly on serum without protein precipitation.

2. Noll, F.; Methode zur quantitativen Bestimmung von L (+) -Lactate mittels Lactat-Dehydrogenase und Glutamat-Pyruvat-Transaminase, Biochem. Z., Vol. 346, 1966, pp. 41 - 49.

The author achieved a very rapid reaction rate by coupling the lactate dehydrogenase and glutamate-pyruvate transaminase reactions whereby pyruvate is converted to L-alanine by reaction with excess glutamate. Pyruvate is therefore rapidly removed from the equilibrium of the lactate dehydrogenase reaction. The analysis is performed on a protein free filtrate. One of the products of the reaction (NADH) is measured spectrophotometrically at 366 mμ.

3.3.25 Blood sugar (glucose)

A. Present methodology and recommendations

1. Method 1

A modification of the method of Cawley et al.,[1] is recommended for measuring blood glucose. The reactions involved in this method are outlined below:

$$\text{glucose} + H_2O + O_2 \xrightarrow[\text{peroxide}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} 2H_2O + \text{oxidized o-dianisidine}$$

In this method serum or plasma may be used directly without a protein precipitating step. The product of the above reactions (oxidized o-dianisidine) is stable and is measured at 400 mμ. The method requires only 25 μl of serum or plasma and values obtained compare favorably with those obtained by the classical methods for measuring glucose.[1]

Hemolyzed and icteric serum or plasma cannot be used in this procedure. Other interferring substances commonly found in serum and plasma do not cause significant error under the conditions of the procedure (the serum is diluted with excess enzyme.)

In order to render the method more adaptable to space use, the following modifications are recommended:

1. A solution containing the proper amounts of the required enzymes and reagents should be prepared on earth, apportioned into tubes that will ultimately be used as cuvettes, frozen, lyophilized, and stored under refrigeration until used in space. The premixed lyophilized reagents should be stable since they are essentially the same as those used in the "dip stick" method for glucose. In "dip sticks" the premixed reagents have been found to be stable if the proper amount of gelatin is included in the reagent mixture.

2. The lyophilized material can be reconstituted in space by adding the proper amount of water at the time a glucose determination is to be made. Serum can now be added and incubation commenced.
3. It is recommended that the incubation be carried out at 37° C for 30 minutes rather than under the conditions used by Cawley et al. The longer incubation time at a higher temperature permits the reaction to go essentially to completion and should eliminate the necessity of stopping the reaction with a drop of HCl provided measurements are made within a short time after incubation is completed.

The method has good precision, adequate sensitivity and only a minimum of training is required.

2. Method 2

Thompson[3] has published a colorimetric glucose oxidase method which is approximately four times as sensitive as similar glucose oxidase methods (those requiring a protein free filtrate). In this procedure the peroxidase reaction is eliminated. H_2O_2 produced by the glucose oxidase reaction reacts with a molybdate catalyst and with I^- to yield I_2, which in turn reacts with o-tolidine to yield a chromogen that is measured at 620 mμ.

When considered for space use the method has the following disadvantages:

a. A protein precipitating step is required.
b. The stability of the reagents when premixed and lyophilized is unknown. It may be possible to eliminate the protein precipitating step just as Cawley, et al.,[1] have done, and the premixed, lyophilized reagents may be stable. These uncertainties can be resolved only by experimentation. If the method can be modified to eliminate the above disadvantages, it should be superior to Method 1.

3. Method 3

Despite the limitations of the "dip stick" method[2] for measuring glucose, simplicity makes it attractive. Therefore the possibility of improving its accuracy should be investigated.

Increasing the enzyme concentration of the "dip stick" should help to minimize the effect of interferring substances present in blood. A search for better chromogenic oxygen acceptors is also needed.

It would probably be worthwhile to test the accuracy of "dip sticks" on earth on each individual who will later need glucose measurements in space. The wide variation in glucose values of certain individuals as measured by "dip sticks" may reflect biological differences that one would not find in a healthy astronaut population. In its present state, however, the method appears to have neither adequate precision nor adequate sensitivity for quantitative glucose measurements.

B. Suitability of present methods to space flight conditions

1. Merit table

An evaluation of the methods for glucose is presented on the following page. Dimensionless numbers are merit values; the greater the value, the more desirable.

2. Discussion

The recommended method and modifications thereof should be adequately tested in the laboratory before being used in space. Since the modifications are minor, this should require only a short period of time. The following major instruments would be needed:

Method 1	Spectrophotometer
Method 2	Spectrophotometer
Method 3	None
Method 4	Fluorometer

A centrifuge will also be needed to separate the serum from the blood clot.

Glucose

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	Good
2. Sample size	25 µl	200 µl	20 µl
3. Time required	35 min	60 min	1 min
4. Reproducibility	8(± 3%)	8(± 3%)	2(± 20%)
5. Suitability for null gravity use	8	7	9
6. Overall safety	10	10	10
7. Nontoxic, noncaustic reagents	10	10	10
8. Specificity	10	10	10
9. Insensitive to environmental changes	9	9	9
10. Ease in training personnel	9	7	9
11. Degree of separation required (10=none)	9	7	10
12. Minimal handling by analyst	9	7	9
13. Common use of analytic equipment	10	10	10
14. Nondestructive of sample	0	0	0
15. Figure of merit	73	65	17

C. Areas for research and development

Method 4 (to be developed)

In theory it should be possible to measure glucose by the following reaction:

$$\text{glucose} + \text{NAD}^{+} \xrightarrow[\text{dehydrogenase}]{\text{hepatic glucose}} \text{gluconic acid} + \text{NADH} + \text{H}^{+}$$

The NADH produced could be measured fluorometrically and would be directly related to the amount of glucose present in the serum. Such a fluorometric procedure should be much more sensitive than the method recommended for measuring glucose. However, in order to develop such a method one would first have to purify hepatic glucose dehydrogenase since it is not commercially available in a highly purified form. Consequently a great deal of time and effort would probably be required for development of the method.

D. References

1. Cawley, L.P.; Spear, F.E.; Kendall, R.; "Ultramicro Chemical Analysis of Blood Glucose with Glucose Oxidase," Am. J. Clin. Path., Vol. 32, 1959, pp. 195 - 200.

A coupled enzyme system of glucose oxidase and peroxidase is used in combination with a chromogenic oxygen acceptor, and the analysis performed directly on 25 µl of serum or plasma without protein precipitation. The product of the reaction is measured at 400 mµ. The effect of common interferring substances is essentially eliminated by incubating the serum in a large excess of enzyme.

2. Joyner, R.E.; Reagent-Strip Method of Blood Glucose Determination, J. Occupational Med., Vol. 7, 1965, pp. 512 - 515.

In most patients the Dextrostix* value for glucose was found to be within ± 10 mg% of the glucose value obtained by classical methods. However 20 of 70 patients were outside the ± mg% range with five values being in error by more than 20 mg%.

3. Thompson, R.H.; Colorimetric Glucose Oxidase Method for Blood Glucose, Clin. Chim. Acta., Vol. 13, 1966, pp. 133 - 135.

In this procedure the peroxidase reaction is eliminated. H_2O_2 produced by the glucose oxidase reaction reacts with a molybdate catalyst and with I^- to yield I_2 which in turn reacts with o-tolidine to yield a chromogen that is measured at 620 mµ.

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

3.3.26 Bicarbonate in serum

A. Principles of present methodology

There are several volumetric, manometric, and colorimetric procedures for the measurement of bicarbonate. The preferred method however, would be the non-destructive electrometric assay. The usual technique is to use pH sensitive electrodes in a very dilute carbonic acid bicarbonate buffer solution.[1] This solution is separated from the specimen to be analyzed by a membrane permeable to carbon dioxide. The carbon dioxide in the specimen and the buffer will equilibrate and the resulting pH (carbon dioxide concentration) of the specimen. A concomitant direct measurement of the specimen pH allows a simple calculation of the bicarbonate concentration in that they are related as follows:

$$pH = pK + \log \frac{[HCO_3]}{[H_2 CO_3]} \qquad (1)$$

where pH for serum = 6.1 and brackets indicate concentration. Since

$$[H_2 CO_3] = pCO_2 \times 0.03,$$

equation (1) can also be written as

$$pH = pK + \log \frac{[HCO_3]}{[pCO_2 \times 0.03]} \qquad (2)$$

There are other methods utilizing electrometric measurements, but the one described appears most expedient. The equipment is commercially available and quite reliable.

B. Suitability of present method to space flight conditions

Bicarbonate in Serum

Merit Parameters	Electrometric Assay
1. Sensitivity	Good
2. Sample volume	50 µl
3. Time required	5 min
4. Reproducibility	10

Bicarbonate in Serum - continued

Merit Parameters	Electrometric Assay
5. Suitability for null gravity use	-
6. Overall safety	10
7. Nontoxic reagents	9
8. Noncaustic reagents	8
9. Specificity	9
10. Applicability	10
11. Reagent volatility	9
12. Insensitive to environmental changes	9
13. Analyst training	8
14. Manipulation	9
15. Common use of analytic equipment	2
16. Merit range	44-87
17. Figure of merit	65

C. Areas for research and development

Future efforts should probably be directed toward further development of the electrometric technique. Instrument stability and maintenance are areas which undoubtedly need the greatest attention.

One of the problems encountered with the electrometric measurement of bicarbonate is the slow response time. The time required for a valid measurement is apparently due to the fact that the reactions

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad (3)$$

are slow to reach equilibrium.

The use of enzymes in conjunction with electrometric assays has been described[2] and in one application carbonic anhydrase has been used with a pCO_2 electrode system.[3] The technique utilizes carbonic anhydrase and bicarbonate incorporated in methylated cellulose. The enzyme catalyzes the hydration-dehydration of carbon dioxide,

$$CO_2 + H_2O \underset{\text{anhydrase}}{\overset{\text{carbonic}}{\rightleftharpoons}} H_2CO_3, \qquad (4)$$

so that the equilibrium for reaction (3) is reached more quickly.

A difficulty with the use of carbonic anhydrase is instability of the enzymes. A low pCO_2, which results in an elevated pH in the buffer-electrolyte solution, inactivates the enzyme.[3] Two possible ways of avoiding this problem would be to use an electrolyte with a greater buffer capacity so that the pH changes would be less, or to employ a more pH-resistant carbonic anhydrase. The enzymes used have been obtained from erythrocytes. Other sources, perhaps parsley,[3] might yield a more resistant enzyme.

The use of carbonic anhydrase in this system requires a considerable investigation before it can be recommended.

D. References

1. Jensen, O.J.; Direct pO_2 and pCO_2 Measurement. Labatorium No. 3, 1963, pp. 2 - 7.

Discusses the principles of the direct measurement of pO_2 and pCO_2.

2. Clark, L.C., Jr.; Lyons, Champ; Electrode Systems for Continuous Monitoring in Cardiovascular Surgery, N.Y. Acad. Sci., Vol. 102, 1962, pp. 29 - 45.

Authors describe the use of certain enzymes in conjunction with electrometric techniques.

3. Clark, L.C., Jr. Dept. of Surgery, Univ. of Ala. Medical Center, Birmingham, Alabama; Personal communication, 1967.

3.3.27 Chloride in serum, plasma, urine, feces, and sweat

A. Principles of present methods

There are several colorimetric procedures for estimating chloride. Chlorides can also be assayed amperometrically. However, for the present application it appears that the method of choice would be electrometric. This is a non-destructive physical-chemical technique which presumably could be used for all the specimens. A recent publication describes the automated potentiometric determination of four inorganic ions including chloride.[1] The author did not experience "protein fouling" with his electrodes which has been a previous problem with similar systems. Electrometric methods measure activity instead of concentration, but the difference should not be great and the results should not be difficult to interpret.

B. Suitability of present method to space flight conditions

Chloride

Merit Parameters	Electrometric Chloride Assay
1. Sensitivity	-
2. Sample volume	200 µl *
3. Time required	60 sec
4. Reproducibility	10
5. Suitability for null gravity use	-
6. Overall safety	10
7. Nontoxic reagents	9
8. Noncaustic reagents	10
9. Specificity	10
10. Applicability	10
11. Reagent volatility	10
12. Insensitive to environmental changes	9
13. Analyst training	9
14. Manipulation	8

* Estimated from Dahms' data.

Chloride - continued

Merit Parameters	Electrometric Chloride Assay
15. Common use of analytic equipment	2
16. Merit range	45-90
17. Mean figure of merit	68

C. Areas for research and development

Future efforts should probably be directed toward further development of electrometric devices. This approach seems well-suited for the intended application: non-destructive, fast, accurate and requires small amounts of material. The long term stability of the electrodes and maintenance problems need evaluation.

D. Reference

1. Dahms, H.; Automated Potentiometric Determination of Inorganic Blood Constituents (Na^+, K^+, H^+Cl^-), Clin. Chem., Vol. 13, 1967, pp. 437 - 450.

Describes an automated system for electrometric measurements. Fast and accurate data processing interfacing also.

3.3.28 Phosphate and pyrophosphate in serum and urine

A. Principles of present methodology

There are numerous techniques which have been employed to assay inorganic phosphate in biological materials. Most of these which are sensitive enough for the micro-sampling required for the current application are colorimetric.

The literature search revealed few procedures for the estimation of inorganic pyrophosphate in the same sample with the same reagents.[1] The differentiation is made on the basis of time. When a sulfhydryl compound (cysteine) is incorporated as a reagent, the color due to orthophosphate is produced maximally in 5 - 7 minutes, whereas the color due to pyrosphosphate is not fully developed for 90 minutes. Thus the same specimen, under the same conditions can be used to colorimetrically measure orthophosphate at 7 minutes and the difference in color intensity (absorbance) at 90 - 7 minutes can be used as a measure of pyrophosphate.

B. Suitability of present method to space flight conditions

Phosphate and Pyrophosphate

Merit Parameters	Inorganic Phosphate	Inorganic Pyrophosphate
1. Sensitivity	Fair	-
2. Sample volume	100 µl*	87 µg **
3. Time required	30 min	110 min
4. Reproducibility	8	-
5. Suitability for null gravity use	-	-
6. Overall safety	2	2
7. Nontoxic reagents	1	1
8. Noncaustic reagents	1	1
9. Specificity	9	-
10. Applicability	2	2
11. Reagent volatility	3	3

* Volume of serum with concentration of 3.5 mg% inorganic phosphate which would would provide an absorbance of 0.09.[1]

Phosphate and Pyrophosphate - continued

Merit Parameters	Inorganic Phosphate	Inorganic Pyrophosphate
12. Sensitivity to environmental changes	7	7
13. Analyst training	5	5
14. Manipulation	5	6
15. Common use of analytic equipment	10	10
16. Merit range	9-19	0-24
17. Mean figure of merit	14	8

The mechanism of the color reaction for pyrophosphate is apparently not known, but a sulfhydryl compound is required and pyrophosphate is not hydrolyzed to orthophosphate.

Difficulty with this procedure will arise if the amount of pyrophosphate is small as compared with the amount of phosphate because the absorbance of the latter will tend to obscure the absorbance of the former. This in turn will yield little accuracy and precision for the assay of pyrophosphate.

2. Discussion

The estimation of urine pyrophosphate would require only a colorimeter, unless the urine contains protein or certain other abnormal constituents.

C. Areas for research and development

The procedure described above for phosphate and pyrophosphate analyses is not very desirable because of the reagents and the probable lack of sensitivity for pyrophosphate.

Neutron activation and analysis of the products would appear to be an area worthy of exploration. However, this type of analysis must be preceded by a separation of the two compounds. Ion-exchange[3] and differential precipitation[2] techniques have been used for separation.

** Amount of inorganic pyrophosphate required to yield an absorbance of 0.050.[1]

It should be simple to determine inorganic pyrophosphate directly by employing the enzyme pyrophosphatase and measuring phosphate before and after enzymic digestion.

Should colorimetric procedures evolve as more applicable, separation of the phosphorus compounds prior to analyses should provide greater sensitivity and probably greater accuracy and precision, particularly for pyrophosphate.

D. References

1. Flynn, R. M.; Jones, M. E.; Lipmann, F.; The Colorimetric Determination of Inorganic Pyrophosphate. J. Biol. Chem., Vol. 211, 1954, pp. 791 - 796.

A procedure is described in which inorganic phosphate and inorganic pyrophosphate are measured sequencially. Differentiation is based on time; pyrophosphate reacts slower in presence of sulfhydryl.

2. Hoffmann, E.; Saraez, A.; Use of Chloranilic Acid Salts in Microanalysis, Z. Anal. Chem., Vol. 190, 1962, pp. 326 - 329.

Chloranilic acid can be used to precipitate pyrophosphate, leaving orthophosphate in solution. Orthophosphate can be measured in supernatant solution and pyrophosphate can be quantitated on basis of absorbance of chloranilic acid released on acidification and solubilization of precipitated chloranilopyrophosphate.

3. Lindenbaum, S.; Peters, T. V.; Rieman, W.; Analysis of Mixtures of Condensed Phosphates by Ion-Exchange Chromatography, Anal. Chim. Acta., Vol. 11, 1954, pp. 530 - 537.

Presents a technique for separating several polyphosphates by ion-exchange. Procedure requires 5 hours.

3.3.29 Manganese in whole blood or serum

A. Principles of present methods

1. Method[1] - Papavasiliou and Cotzias[3]

Upon neutron bombardment, the stable isotope of manganese, Mn^{55}, becomes Mn^{56}, which decays by emission of beta and gamma rays. If activation conditions are constant the Mn^{56} produced is proportional to the parent element Mn^{55}. The Mn^{56} must be chemically separated from the mixture of radioisotopes (e.g. K, Na, Cl, etc.) which exert a masking effect. Separation is facilitated by the addition of carrier.

Procedure: The samples of blood or serum are dried at 85° C in polyethylene tubes. The tubes are sealed and introduced in the reactor for activation (2 hours). The contents of the tubes are washed in glass containers with hot HNO_3. Carrier is added to ($MnSO_4$) and the solutions are digested and oxidized to MnO_4^-. The MnO_4^- is precipitated with tetraphenylarsonium chloride. The precipitate is filtered, washed and placed on planchets for assay of the radioactivity with a gamma-ray spectrometer.

The method has good reproducibility and specificity. The levels of manganese in blood and serum by this method are extremely low. Cotzias[1] reported mean values of manganese for serum and whole blood of 0.587 μg/L and 8.44 μg/L, respectively.

2. Method 2 - Fernandez, et al.[2]

Manganese ions in the presence of sodium periodate catalyze the oxidation of Malachite Green (MG) to colorless products. The disappearance of MG is measured at 620 mμ in a spectrophotometer. The reaction may be illustrated as follows:

$$Mn^{++} \xrightarrow{NaIO_4} Mn^{oxidized}$$

$$\text{Malachite green} \xrightarrow{Mn^{ox}} \text{colorless products}$$

The reaction is of first order and therefore requires a kinetic

analysis. Absorbance measurements are taken at various time intervals and the logarithms of the absorbances are plotted against time. The manganese concentration is calculated from the slope of the line by comparison to the slope obtained from a known quantity of manganese:

$$m\mu g\ Mn = \frac{Kx - b}{Ks - b} \times S$$

Where:

$m\mu g$ = millimicrograms

Kx = slope of the sample

Ks = slope of the standard

S = $m\mu g$ of Mn in the standard

b = slope of the blank

Procedure: Serum is dried at 116° C and then ashed in a muffle furnace at 540° C, until no carbon particles are visible.

The residue is dissolved in HCl and the pH of the acid solution is adjusted to 3.6 with a buffer. Malachite green and $NaIO_4$ are added and the samples are incubated at 26°. Spectrophotometric readings are taken every hour for five hours. The readings are commenced three (3) hours after the addition of $NaIO_4$. Blanks and standards are run along with the samples. The normals of values values for this method are similar to those reported by Cotzias, et al.[1] The normal range was .360 - .900 µg/L. The recovery of known amounts of Mn added to serum was approximately 76%.

B. Applicability of present methods to space flight conditions

1. Merit table

Manganese

Merit Parameters	Method 1	Method 2
1. Sensitivity	Good	Good
2. Sample size	.1 ml	.2 ml
3. Time required	-	12 hours

Manganese - continued

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

2. Discussion

Equipment

Method 1: Heater, reactor for neutrons, gamma-ray spectrometer, pulse height analyzer, oven, centrifuge, amplifier and power supply.

It may also be worthwhile to investigate the effects of serum manganese on enzymes requiring manganese, e.g., isocitric dehydrogenase.

Method 2: Centrifuge, spectrophotometer, muffle furnace, oven.

Neither method can be recommended.

C. Areas for research and development

The isolation of Mn from serum must be achieved without acid digestion or ashing. Following its isolation the catalytic properties of the element can be applied for its measurement.

D. References

1. Cotzias, G.C.; Miller, S.T.; Edwards, J.; Neutron Activation Analysis: The Stability of Manganese Concentrations in Human Blood and Serum, J. Lab. Clin. Med., Vol. 67, 1966, pp. 836 - 849.

Normal values of Mn in whole blood and serum are given. The serum level of Mn appears to be very constant.

2. Fernandez, A.A.; Sobel, C.; Jacobs, S.L.; Sensitive Method for the Determination of Submicrogram Quantities of Manganese and its Application to Human Serum, Anal. Chem., Vol. 35, 1963, pp. 1721 - 1724.

3. Papavasiliou, P.S.; Cotzias, G.C.; Neutron Activation Analysis: The Determination of Manganese, J. Biol. Chem., Vol. 236, 1962, pp. 2365 - 2369.

Manganese was measured in blood, plasma and tissues by neutron activation analysis.

3.3.30 Sodium, potassium, magnesium, and calcium

A. Principles of present methodology

1. Flame photometry[3, 4]

Atomize a solution of the sample to be analyzed into a flame in order to effect ionization of the atoms of the metal present in the sample. Measure the quantity of light of characteristic wavelength which is emitted with a photomultiplier tube.

2. Atomic absorption[1]

The emission from a hollow cathode lamp which contains Na, K, Ca and Mg is passed through a flame into which a solution of the sample for analysis is aspirated. The quantity of light from the emission of the hollow cathode with characteristic wavelength of the element being determined which passes through the flame will be inversely related to the concentration of atoms of that element present in the flame.

3. Neutron activation analysis

The sample to be analyzed is placed in a high flux of thermal neutrons for a period of several minutes. Radionuclides of the elements present in the sample are produced as a result of reactions with neutrons. The energy of radiation emitted when these nuclides decay is characteristic of that element. By determining the decay rate of each radioactive element produced, one can calculate the concentration of the element present in the sample.

B. Applicability of present methodology to space flight conditions

1. Merit table

Merit Parameters	Flame Photometry	Atomic Absorption	Neutron Activation
1. Sensitivity	> 1 ppm	> 0.1 ppm	> 0.1 ppm
2. Sample size	< 0.1 ml	< 0.1 ml	< 0.1 ml
3. Time required	3 min	3 min	5 min

Sodium, potassium, magnesium, and calcium

Merit Parameters	Flame Photometry	Atomic Absorption	Neutron Activation
4. Reproducibility	10 (> 1%)	10 (> 1%)	10 (> 1%)
5. Suitability for null gravity use	5	5	10
6. Overall safety	5	5	5
7. Noncaustic, nontoxic reagents	7	7	5
8. Specificity	8	9	10
9. Insensitive to environmental changes	8	8	10
10. Ease in training personnel	8	8	10
11. Degree of separation required (10=none)	10	10	10
12. Minimal handling by analyst	9	9	10
13. Common use of analytic equipment	5	5	6
14. Nondestructive of sample	0	0	10
15. Figure of merit	42	43	64

2. Discussion

If the atmosphere in the spacecraft is 100% oxygen, a means must be devised for containing the burner used in flame photometry and atomic absorption. Also a means must be provided for eliminating the products of combustion. Lasers might be substituted for flames. Hollow cathode lamps have been described[2] into which a sample may be placed and ionized electrically in an inert atmosphere. This might serve as a better source of excitation than a flame. Shielding of personnel from the neutron source and of the radiation detector from cosmic radiation appear to be the major problems related to neutron activation analysis.

C. Areas for research and development

1. Investigation of the use of lasers in flame photometry and atomic absorption.
2. Specific ion electrodes.

This category of approach to sodium, potassium, calcium, and magnesium was placed in the promising area for research and

development because at the present time information regarding their clinical use is sketchy. This problem, however, should be easily solved. This area undoubtedly represents the most fruitful approach for research and development for clinical measurements under space flight conditions. Equipment required is relatively simple[5]. A well designed high impedance voltmeter could serve for multiple measurements. It is possible that variations of specific ion electrodes can be used for other methodologic approaches.

D. References

1. Elwell, W. T.; Gidley, J. A. F.; Atomic Absorption Spectrophotometry, McMillan, New York, 1962.

A book which reviews the theory and application of atomic absorption.

2. Goodfellow, G. I.; Simple Interchangeable Hollow-Cathode Lamps for Use in Atomic-Absorption Spectrometry. Applied Spectroscopy, Vol. 21, 1967, pp. 39 - 42.

Describes the design of demountable hollow-cathode lamps into which the sample may be introduced and ionized in an electrical discharge. Lamps are operated in a reduced atmosphere of argon.

3. Polucktov, N. S.; Technics in Flame Photometric Analysis, Van Nostrand, Princeton, N. J., 1966.

A book which reviews methods of analysis by flame photometry.

4. Pungar, E.; Flame Photometry Theory, Van Nostrand, Princeton, N. J., 1967.

A book which reviews the theory of analysis by flame photometry.

5. Rechnitz, G. A.; Ion-Selective Electrodes, Chem. and Eng. News, Vol. 45, June 12, 1967, pp. 146 - 158.

A very recent review of advances in the development of ion-selective electrodes. Three types of electrodes are discussed: glass, solid-state or precipitate, and liquid-liquid membrane. Ion-selective electrodes, which measure activity rather than concentration, have been developed for such difficult ions as sodium, potassium, calcium, ammonium, magnesium, and fluoride.

3.3.31 Sulfates

A. Principles of present methods

1. Method 1, Kleeman et al.[3] modified by Henry[2]

Serum is deproteinized with uranyl acetate. Addition of benzidine to the filtrate precipitates the sulfate as benzidine sulfate. The benzidine in the precipitate (benzidine sulfate) is quantitated colorimetrically by reaction with sodium β-naphthoquinone-4-sulfonate.

Procedure: Benzidine is added to the protein-free filtrate of serum and the mixture is allowed to stand at 4° C for 3 hours. The benzidine sulfate is separated by centrifugation and is washed with alcohol-ether. The benzidine sulfate is dissolved in borate buffer with heat at 60° C. This addition of naphthoquinone reagent results in the formation of a colored complex the absorbance of which is measured at 485 mμ. The method is sensitive and has a good precision. However, it requires the use of volatile reagents (alcohol, ether, acetone) and the naphthoquinone reagent is very unstable.

Uranyl acetate has been selected as the protein precipitating reagent because it also precipitates the interfering phosphates. The method is applicable to urine.

2. Method 2, Miller et. al.[4]

A protein free, phosphate-free filtrate is obtained by mixing plasma with a solution of uranyl acetate. A standard $Ba^{133}Cl_2$ solution is added to the filtrate resulting in $BaSO_4$ precipitation, which is allowed to proceed for 4 hours at 5° C.

The mixture is centrifuged and an aliquot of the supernatant is removed and counted in a scintillation well counter. The concentration of sulfate is calculated by the equation

$$M_s = \frac{CoVb - C(Vs + Vb)}{CoVb} \times \frac{(Vp + Vu)\,Vb}{VsVp} \times Mb,$$

where

Co = counting rate of the standard $Ba^{133}Cl_2$ solution.

C = counting rate of the Ba^{133} in the supernatant solution.

Vp = volume of plasma

Vu = volume of uranyl acetate solution

Vs = volume of supernatant counted

Vb = volume of barium solution added

Mb = concentration of $Ba^{133}Cl$ solution

Ms = concentration of sulfate in plasma

If Vp = 1, Vu = 2, Vs = 1, and Vb = 0.5, the equation becomes

$$Ms = 1.5 \times \frac{Co - 3C}{Co} Mb.$$

This method requires less handling than Method 1 and the use of volatile reagents is avoided. The authors claim that their results are in good agreement with those of Kleeman et al.[3] The specific activity of the $BaCl_2$ used was 0.12 mc per gram of Ba.

3. Method 3, Berglund and Sorbo[1]

Serum is deproteinized with trichloroacetic acid. To the clear filtrate a $BaCl_2$ - gelatin reagent is added which precipitates the sulfate as $BaSO_4$. The absorbance of the turbid solution is measured at 360 mμ and compared to that of a standard sulfate solution. The relationship between sulfate concentration and absorbance is linear. The same procedure can be employed for the measurement of inorganic sulfate in urine. The specificity and precision of this method need to be evaluated.

B. Applicability of present methods to space flight conditions

1. Merit table

Sulfates

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	Fair
2. Sample size	1 ml	1 ml	1 ml
3. Time required	5 hours	5 hours	1 hour

Sulfates - continued

Merit Parameters	Method 1	Method 2	Method 3
4. Reproducibility	8	8	-
5. Suitability for null gravity use	2	7	7
6. Overall safety	5	5	6
7. Nontoxic reagents	4	9	8
8. Noncaustic reagents	9	9	5
9. Overall safety	5	5	6
10. Insensitive to environmental changes	2	9	3
11. Ease in training personnel	1	2	7
12. Degree of separation required (10=none)	2	6	8
13. Minimal handling by analyst	1	4	6
14. Common use of analytic equipment	8	2	8
15. Nondestructive of sample	1	1	1
16. Merit range	-	-	0-46
17. Mean figure of merit	17	36	20

2. Discussion

Serum: None of the methods can be recommended at the present time. However the method of Berglund is relatively simple and it could be applicable if its reliability, sensitivity, and precision are established.

Urine: Method 3 is recommended. The sensitivity of the method is more than adequate.

Equipment

Method 1: Centrifuge, spectrophotometer, heating bath, refrigerator

Method 2: Scintillation well counter, centrifuge, refrigerator

Method 3: Spectrophotometer, centrifuge

C. Areas for research and development

Certain anaerobic bacteria can utilize sulfate as their biologic oxidant. It may thus be possible to employ such microorganisms in as-

saying sulfate content.

D. References

1. Berglund, F.; Sorbo, B.; Turbidimetric Analysis of Inorganic Sulfate in Serum Plasma and Urine, Scand. J. Clin. Lab. Invest., Vol. 12, 1960, pp. 147 - 153.

Addition of $BaCl_2$ - gelatin reagent to a protein free filtrate results in the precipitation of $BaSO_4$ which is measured by turbidimetry.

2. Henry, R.J.; Determination of Sulfate in Clinical Chemistry, Principles and Technics, Hueber Med. Division, Harper and Row Publishers, 1964, pp. 417 - 421.

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. Kleeman, C.R.; Taborsky, E.; Epstein, F.H.; Improved Method for the Determination of Inorganic Sulfate in Biologic Fluids, Proc. Soc. Exp. Biol. Med., Vol. 91, 1956, p. 480.

Describes a method for the estimation of serum inorganic sulfate using benzidine and naphthoquinone sulfonate.

4. Miller, E. et al.; The Use of Radioisotopes to Measure Body Fluid Constituents 1. Plasma Sulfate, J. Lab. Clin. Med., Vol. 58, 1961, pp. 656 - 661.

Describes the use of Ba^{133} for the determination of inorganic sulfate.

3.3.32 Zinc in blood, serum, or urine

The determination of zinc in biological fluids presents serious difficulties. Zinc can be determined colorimetrically with dithizone, by neutron activation analysis, emission spectrometry, atomic absorption spectrophotometry and x-ray fluorescence.

A. Principles of present methodology

1. Method 1, Burns et al.[1]

Principle: The method depends upon the formation of radioactive zinc by bombardment of the sample with a flux of neutrons. The radioactive zinc is then measured by a sensitive device.

Procedure: Blood is collected in quartz ampoules and first is dried at 70° C and then at 110° C for a total of 13 hours. The ampoules are sealed and irradiated in a thermal neutron flux of 2×10^{12} to 2×10^{13} $n/cm^2/sec$ for 3 days. After a decay interval of 2 days the organic material is digested with H_2SO_4-H_2O_2. The various elements present in the ash are separated by distillation upon addition of hydrobromic acid (HBr), passage through ion-exchangers and selective elution. The gamma-spectrometric measurements are made with a 512-channel pulse height analyzer.

2. Method 2, Johnson[3]

Principle: Zinc analysis is made on a deproteinized sample at pH 9.5 by titrating with tetraethylenepentamine (Tetren) potentiometrically, using dual, gold, polarized electrodes to the respective end-point. The latter is determined from the inflection point on a graphic plot or where the potential is at a minimum.

Procedure: Serum is deproteinized with 1% picric acid solution. Picric acid is removed from the protein free filtrate by absorption on a Dowex column. The zinc ions are eluted from the column by elution with deionized water. The pH of the eluate is adjusted to 9.5 with NH_3-NH_4NO_3 buffer and NaCN and the zinc is titrated with

a solution of Tetren. The author claims very good precision for this method. The results obtained are in good agreement with colorimetric methods. The presence of sulfates and halide ions is undesirable.

3. Method 3, Helwig, et al.[3]

Principle: Serum or urine are digested with HNO_3 -H_2SO_4 -$HClO_4$ mixture. The acid-digest is neutralized with ammonia and then the pH is adjusted to 5.7, with thiosulfate-cyanide-acetate buffer. The zinc is extracted from the solution with a carbon tetrachloride solution of dithiocarbazone (dithizone). The absorbance of the zinc dithizonate is measured at 525 mμ in a spectrophotometer. If urine is analyzed a volume of 5 ml is required.

B. Suitability of present methodology to space flight conditions

1. Merit table

Zinc

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

Zinc

Merit Parameters	Method 1	Method 2	Method 3
16. Merit range	3-5	12-16	6-8
17. Mean figure of merit	4	14	7

2. Discussion

Choice of method: Method 2 if modified extensively could be applicable. The deproteinization step may be altered by absorption of the serum proteins on a Sephadex column. The burette can be replaced by a syringe-type burette and titration can be performed in a closed system.

Equipment

Method 1: Heater, neutron flux, ion-exchange columns, 512-channel pulse height analyzer, quartz ampoules

Method 2: pH-meter, magnetic stirrer, centrifuge, mercury electrode, chromatography columns, burette

Method 3: Heater, spectrophotometer

C. Areas for research and development

The titration method is attractive and should be thoroughly investigated.

Atomic Absorption Spectrophotometry is quick and specific but the flame requirement makes the method unacceptable.

D. References

1. Brune, D.; Samsahl, K.; Wester, P.O.; A Comparison Between the Amounts of As, Au, Br, Cu, Fe, Mo, Se and Zn in Normal and Uraemic Human Blood by Means of Neutron Activation Analysis, Clin. Chim. Acta., Vol. 13, 1966, pp. 285 - 291.

Zinc and other elements were measured in blood by gamma-spectrometric analysis after blood is dried, irradiate, digested, etc.

2. Helwig, H.L., et. al.; Modified Zinc Analysis Method and Serum and Urinary Zinc Levels in Control Subjects, Am. J. Clin. Path., Vol. 45, 1966, p. 45.

Zinc is measured colorimetrically with dithizone.

3.4 NEW ANALYTICAL METHODS

3.4.1 Introduction

The analytical methods discussed in Section 3.3 present essentially the current state of the art. These methods have not necessarily been proven for all the analyses studied during this program, but the technology is established and the problems remaining involve primarily engineering to achieve compatibility with the space environment. The major engineering effort would have to be devoted to the designing of (1) a suitable spectrophotometer for use in the ultraviolet, visible, and near infrared portions of the spectrum, (2) a centrifuge for separating serum and plasma from whole blood and electrophoretic or chromatographic apparatus for protein separation or removal, and (3) apparatus for transfer of samples and reagents from one closed system to another without endangering the crew. Given suitable instrumentation in these three areas, it seems entirely reasonable that all the practical methods discussed so far could be implemented within a period of one to three years. This period includes the launching of the Apollo Applications Program and the establishment of an orbiting research laboratory.

Admittedly the methods of Section 3.3 do not greatly advance the state of the art in clinical analysis, which in some areas has not changed significantly in two decades. There are a number of reasons for this lag in clinical technology, but perhaps the most important has been the inability of medical research centers to obtain support for research which would ultimately advance the state of clinical technology. It is through promotion of research, particularly the development of new applications, methods, and analytical techniques, that private foundations and government agencies such as NASA can contribute significantly to advancing clinical technology. Such efforts could provide direct support for the space program while simultaneously easing the developing crisis in clinical laboratories. A good example of an analytical technique that is currently showing significant advances is the ion-selective electrode.* Development of new glasses permeable to specific ions has made possible a whole family of electrodes capable of identifying ions in solution, many of which have heretofore

* See reference 5 under section 3.3.30