2. Drickman, A.; McKeon, F.A.; Determination of Total Serum Proteins by Means of the Refractive Index of Serum, Am. J. Clin. Path., Vol. 38, 1962, pp. 392 - 396.

Compares values of serum protein obtained with the biuret method and refractometry.

3. Kingsley, G.R.; The Direct Biuret Method for the Determination of Serum Proteins as Applied to Photoelectric and Visual Colorimetry, J. Lab. Clin. Med., Vol. 27, 1942, pp. 840 - 845.

Application of the biuret reaction for the determination of serum proteins.

4. Naumann, H.N.; In Serum Proteins and the Dysproteinemias, J.B. Lippincott Co., Philadelphia, 1964, pp. 86 - 101.

Principles of determination of serum proteins by refractometry.

5. Sunderman, F.W.; A Rapid Method for Estimating Serum Proteins. Formula for Calculating Serum Protein Concentration from the Refractive Index of Serum, J. Biol. Chem., Vol. 153, 1944, pp. 139 - 142.

The Abbé refractometer was used for the determination of serum proteins.

6. Sunderman, F.W.; Revised Calculations for Total Serum Proteins by Refractometry and for Total Base (and Sodium) by Conductivity, Am. J. Clin. Path., Vol. 46, 1966, pp. 679 - 683.

The use of the Kjeldahl N factor of 6.54 requires revision of refractometry formulas.

7. Tombs, M.P., et al., The Spectrophotometric Determination of Protein at 210 mμ, Biochem. J., Vol. 73, 1959, pp. 167 - 171.

Absorption measurements of diluted serum at 210 mμ can be employed for the estimation of total proteins.

8. Waddell, W.J.; A Simple Ultraviolet Spectrophotometric Method for the Determination of Protein, J. Lab. Clin. Med., Vol. 48, 1956, pp. 311 - 314.

Describes a micromethod for the determination of serum protein by ultraviolet spectrophotometry.

3.3.10 Transferrin

Transferrin is a serum protein which combines with iron and serves as a vehicle for iron transport. Approximately 25 - 40% of the transferrin exists in serum in combination with iron and the remainder is free transferrin. Concentrations of transferrin are expressed in terms of micrograms of iron per 100 ml of serum under conditions of full saturation.

The maximum amount of iron that can be bound to transferrin is known as Total Iron Binding Capacity (TIBC). The amount of free transferrin in serum is known as Unsaturated Iron Binding Capacity (UIBC).

There are three approaches for estimating the concentration of transferrin in serum. Two of them are chemical and the third immunochemical.

A. Principles of present methodology

1. Williams and Conrad[4]

a. Principle

This approach measures serum iron (SI) and the amount of free transferrin (UIBC). Then SI + UIBC = TIBC. The UIBC can be measured by adding a known quantity of iron to saturate the transferrin and then measure the excess iron with a reagent which reacts with the free iron but not with the iron bound to transferrin.

b. Method

The pH of serum is lowered to approximately 2.2 by addition of a KCl-HCl buffer containing hydroxylamine hydrochloride as the reducing agent. At this low pH the iron is released from the transferrin and upon addition of 2,4,6-tripyridyl-s-triazine (TPTZ), it reacts to give a colored complex the absorbance of which is measured at 595 mμ. The absorbance reading is used to determine the iron concentration of the serum. The same solution is used for the determination of UIBC.

The solution is made alkaline, (pH 8-9) by the addition of "Tris" buffer. At this pH the iron dissociates from TPTZ and recombines with the transferrin. A known amount of iron is now added to the solution to saturate the transferrin. The excess iron is measured by the addition of 2, 2', 2'' tripyridyl which reacts only with the free iron to give a colored complex the absorbance of which is measured at 552 mμ. The UIBC is determined by subtracting the excess iron from the known amount which was added to the solution. Both determinations of SI and UIBC are performed in a single tube. Addition of SI and UIBC gives the TIBC. Reliable serum iron concentrations and UIBC can be obtained in specimens containing up to 15 mg% of bilirubin or 150 mg% of hemoglobin.

2. Goodwin, et al.[2]

   a. Principle

      Iron in excess is added to serum and allowed to combine with transferrin. The unbound iron is removed by addition of solid magnesium carbonate. After removal of the magnesium carbonate by centrifugation, the amount of iron is measured in the supernatant. The content of iron in micrograms per 100 ml of serum represents the TIBC.

   b. Method

      A known quantity of iron is added in excess to a serum sample to saturate the transferrin. The unbound iron is precipitated by the addition of solid $MgCO_3$. The mixture is centrifuged to remove the $MgCO_3$. The iron content of the supernatant solution is measured and this represents the total iron binding capacity (TIBC). The supernatant solution is mixed with acetate buffer, pH 4.5, to release the iron from the transferrin. The liberated iron is complexed with 4, 7-diphenyl-1, 10-phenathroline disulfonic acid, disodium salt.

The resulting pink color is measured at 535 mμ. The procedure requires 1 ml of serum but it can be scaled down so that a volume of 40 μl is adequate.[3]

3. Burrows[1]

a. Principle

Serum is placed in immuno-diffusion plates containing antiserum specific for human transferrin. A precipitating ring is formed in the agar gel.

b. Method

This procedure employs the "Immuno-Plate"* which is an agar gel diffusion plate containing antiserum specific for human transferrin. Serum is placed in small wells of the diffusion plate and incubated at 37° C for 4 hours. The diameter of the precipitin ring around each well is measured. The concentration of transferrin in the serum is determined by comparison with standard solutions of transferrin treated in the same manner. The concentration of transferrin is proportional to the diameter of the precipitin ring formed, and it is expressed in micrograms of iron-binding capacity per 100 ml of serum.

This method is simple and very specific but unfortunately grossly underestimates the iron-binding capacity of serum as judged by comparison with results by other methods. However, the approach is sound and it is possible that the method can be improved.

B. Suitability of present methodology to space flight conditions

1. Merit table

Transferrin

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	-
2. Sample size	.05 ml	1 ml	-

* "Immuno-Plate" is a registered trademark of Hyland Laboratories, Los Angeles, California.

Transferrin - continued

Merit Parameters	Method 1	Method 2	Method 3
3. Time required	1 hour	1 hour	4 hours
4. Reproducibility	8	8	-
5. Suitability for null gravity use	8	8	8
6. Overall safety	10	10	10
7. Nontoxic reagents	9	9	10
8. Noncaustic reagents	9	9	10
9. Specificity	8	8	9
10. Insensitive to environmental changes	8	7	-
11. Ease in training personnel	4	3	8
12. Degree of separation required (10=none)	9	9	9
13. Minimal handling by analyst	5	4	8
14. Common use of analytic equipment	10	10	10
15. Nondestructive of sample	1	1	1
16. Merit range	-	-	0-82
17. Mean figure of merit	55	54	38

2. Discussion

Method 1 is preferable because all determinations are performed in a single tube. All optical measurements can be made in the reaction tube or cuvette. Fasting blood samples should be obtained because lipemic sera may seriously interfere. Hydroxylamine hydrochloride may be used as a reducing agent because it is far more stable than ascorbic acid.

Equipment

Method 1 and 2: Spectrophotometer, disposable iron-free glassware or plastic tubes, centrifuge, incubator.

Method 3:
a. "Immuno-Plates"
b. Incubator

C. Areas for research and development

Method 3 should be investigated; its simplicity can provide a very

suitable procedure.

D. References

1. Burrows, S.; Comparison of Methods Designed to Measure Transferrin and Iron-Binding Capacity of Serum. Am. J. Clin. Path., Vol. 47, 1967, pp. 326 - 328.

Compares values of transferrin by an immunological method and by procedures measuring total iron-binding capacity.

2. Goodwin, J.F.; Murphy, B.; Guillemette, M.; Direct Measurement of Serum Iron and Binding Capacity, Clin. Chem., Vol. 12, 1966, pp. 47 - 57.

Serum iron, total iron binding capacity, and unsaturated iron binding capacity are measured with bathophenanthroline.

3. Watkins, D.K.; Butler, E.B.; Micromethod for the Determination of Serum Iron and Total Iron-binding Capacity and its Application in Pregnancy, Clin. Chim. Acta, Vol. 13, 1966, pp. 448 - 456.

TIBC is measured in 40 µl of serum.

4. Williams, H.L.; Conrad, M.E.; A One Tube Method for Measuring the Serum Iron Concentration and Unsaturated Iron-Binding Capacity, J. Lab. Clin. Med., Vol. 67, 1966, pp. 171 - 176.

Serum iron and UIBC are measured without precipitating the serum proteins.

3.3.11 Urinary proteins

In normal individuals the amount of protein excreted in the urine is undetectable by routine methods. A qualitative test for urinary protein is probably more than adequate.

A. Principles of present methodology

1. Kutter[2]

The simplest way to detect protein in the urine is probably by means of dipsticks supplied by Ames Company, under the trade-names "Hema-Combistix" and "Labstix." These dipsticks are quite sensitive in that they can detect protein levels as low as 7 mg/100 ml urine.[2] They suffer from the disadvantage of reacting only with albumin, but proteinuria without albuminuria will be encountered very rarely.

Both dipsticks are paper strips impregnated with bromphenol blue buffered at an acid pH. In the presence of albumin bromphenol blue undergoes a color change from yellow to blue. Semi-quantitative results can be obtained with the dipsticks. A false positive result may be obtained with stale urines or urine specimens with high alkalinity.

2. Dannenberg[1]

Another convenient way for the detection of urinary proteins had been proposed by Dannenberg.[1] A small volume of urine is added to a tube containing a dry mixture consisting of 85 parts sea sand and 15 parts of crystalline sulfosalicyclic acid. The supernatant will show turbidity if the urine contains protein. However, under null gravity conditions low speed centrifugation will be required to separate the sand from the turbid supernatant.

B. Suitability of present methodology to space flight conditions

1. Merit table

Urinary Proteins

Merit Parameters	Kutter	Dannenberg
1. Sensitivity	7 mg%	5 mg%
2. Sample size	0.1 ml	0.5 ml
3. Time required	0.5 min	10 min
4. Reproducibility	-	-
5. Suitability for null gravity use	10	3
6. Overall safety	10	5
7. Nontoxic reagents	10	9
8. Noncaustic reagents	10	5
9. Specificity	9	9
10. Insensitive to environmental changes	8	9
11. Ease in training personnel	10	10
12. Degree of separation required (10=none)	10	10
13. Minimal handling by analyst	10	3
14. Common use of analytic equipment	10	1
15. Nondestructive of sample	10	0
16. Merit range	0-96	0-35
17. Mean figure of merit	48	18

2. Discussion

It seems that the use of dipsticks is the method of choice. Hundreds of paper strips can be stored in a small bottle for long periods of time.

Another advantage of the dipstick is that one can also test for ketone bodies (acetoacetic acid and acetone), glucose, hemoglobins, and pH of the urine.

C. Areas for research and development

(None)

D. References

1. Dannenberg, E.; Rapid Analysis: Detection of Protein with Dry Reagent, Munch. Med. Wochschr., Vol. 95, 1953, p. 102.

Urine protein can be detected by the addition of urine to a dry mixture of sea sand and sulfosalicylic acid. Turbidity in the supernatant solution indicates protein.

2. Kutter, D.; Simultaneous Determination of Glucose, Protein and pH in Urine. Therap. Gegenwart, Vol. 104, 1965, pp. 496 - 498; 506 - 507.

Evaluates the "Hema-Combistix" for the detection of protein in urine.

3.3.12 Adrenocorticotrophic hormone (ACTH)

A. Principles of present methodology

There are three estimations which depend on various biological assays. These include the (1) repair test-determination of the re-appearance of lipid material in regressed adrenal cortices of hypophysectomized rats following injections of preparations containing ACTH; (2) maintenance test-estimation of quantity of ACTH material required to maintain the adrenal weights of rats injected immediately after hypophysectomy; (3) decrease in adrenal ascorbic acid - one hour after i.v. injection of ACTH containing material the decrease in ascorbic acid content of the adrenal glands of a hypophysectomized rat is determined.

In man various indirect tests have been proposed including: measurement of blood and urine levels of corticosteroid following (1) ACTH injection; (2) blockage of ACTH secretion by drugs (SU-4885-Ciba); (3) following administration of a synthetic corticosteroid.

B. Applicability of present methodology to space flight conditions

The presently available assays seem to be technically impossible under present space flight conditions.

C. Areas for research and development

At the present time there is no practical clinical laboratory procedure[1, 3, 4, 5] available for adrenocorticotrophin on earth -- much less under conditions of space flight. However, two approaches may be studied. The indirect approach requires the development of methodology for corticosteroid determination under space flight conditions. The second approach would be the development of radioimmunologic assay[2] as described by Rosselin, et al. This method would not apply any new physical principles but depends on the preparation of iodinated I^{131} ACTH and the production of specific antibodies before measuring ACTH in plasma.

D. References

1. DeBarbieri, A.; Urinary Corticotropin, Folia Endocrinol., Pisa, Vol. 7, 1954, pp. 719 - 726.

Active corticotropic substance was extracted from 200 - 300 liters of urine and assayed by decrease of ascorbic acid in the adrenal gland of hypophysectomized rats.

2. Rosselin, G., et al.; Radioimmunologic Assay of Protein Hormones by the Method of Berson and Yalow. Assay of Protein and of their Antibodies in the Plasma of Man, Vol. 26, 1965, pp. 449 - 464.

A number of protein hormones in plasma may determine precisely and specifically by radioimmunological techniques. The techniques can be used to measure antibodies to hormones, in particular antibodies to insulin.

3. Rubin, B. L., et al.; Adrenocorticotropic Hormone in Urine, J. Clin. Endocrinol. and Metabolism, Vol. 14, 1954, pp. 154 - 169.

Fresh urine samples were tested for ACTH activity by i.v. injection into hypophysectomized rats. Minimum amount of ACTH detected after injection was 0.004 I.U.

4. Ruf, K.; Determination of ACTH in Man, Schweiz. Wochschr., Vol. 96, 1966, pp. 684 - 687.

A review with 68 references.

5. Sayers, G.; Blood Adrenocorticotropin (ACTH), J. Clin. Endocrinol., Vol. 15, 1955, pp. 751 - 759.

A review of the difficulties encountered in the determination of ACTH in blood. 22 references.

3.3.13 Antidiuretic hormone (ADH)

A. Principles of present methodology

Pressor activity is assayed by comparing the rise in blood pressure following i.v. injection in anesthetized dogs or rats with that produced by known amounts of a standard. Antidiuretic activity is assayed by comparing the reduction of urine volume following i.v. injection of an extract in hydrated unanesthetized rats or rabbits with that produced by a standard preparation.

B. Applicability of present methodology to space flight conditions

Biological assays seem to be technically impossible under space flight conditions within the next three years. The extremely small quantities of the hormone which may be found in either plasma or urine make it quite unlikely that a method based on physical measurements will be developed in the foreseeable future.

C. Areas for research and development

It may be possible that the immunochemical approach will be productive. Sylvana now has available Guinea pig anti-human growth hormone which is sensitive enough to detect 0.025 millimicrograms of human growth hormone using a trace label of I^{131} labeled growth hormone for radioimmunoassay. The reaction between "unknown" serum and the antibody must be carried out for 7 days at 4° C. It seems possible that in time other antibodies will become available. There is no way to estimate development time in this area.

D. References

1. Bisset, G.W.; Assay of Oxytoxin and Vasopressin in Blood and the Mechanism or Inactivation of these Hormones by Na Thioglycolate. Oxytoxin, Proc. Intern. Symp., Montevideo, 1959, (Pub. 1961), pp. 380 - 399.

This is a review article containing some new material on experiments performed on the response of isolated strips of rat uterus.

2. Ginsberg, M.; A Method for the Assay of Antidiuretic Activity, Brit. J. Pharmacol., Vol. 6, 1951, pp. 411 - 416.

A method based on a four point assay using a regimen of water administration described by Birnie, et al., (Chemical Abstracts, Vol. 43, p. 3084e.)

3. Hickey, R.C.; Hare, K.; The Renal Excretion of Chloride and Water in Diabetes Insipidus, J. Clin. Invest., Vol. 23, 1944, pp. 768 - 775.

The functional capacity of the neurohypophysis is tested by following the liberation of the antidiuretic hormone in response to the injection of hypertonic saline.

4. Tsukamoto, S.; Antidiuretic Substance in Serum. I. Antidiuretic Substance and its Diurnal Variation. II. Antidiuretic Substance in Urine and its Physiological and Pathological Significance. Kumanoto Daigaku Taishitsu Igaku Kenkyusho Hokoku, Vol. 13, 1962, pp. 236 - 255.

Variation of the "antidiuretic substance" with time of day and season of the year. Diurnal variation was observed.

3.3.14 Aldosterone

A. Principles of present methodology

Current methods depend on solvent extraction of the steroid, followed by chromatographic isolation. Even under ideal laboratory conditions, reproducible results are difficult to obtain. No well developed methods appear to be available which would be directly applicable in a space environment. Gas chromatography appears to be the method that presently offers the most promise.[1, 2, 3]

B. Suitability of present methodology to space flight conditions

1. Merit table

Aldosterone

Merit Parameters	Gas Chromatography
1. Sensitivity	0.4 μg
2. Sample size	100 ml
3. Time required	> 1 hr
4. Reproducibility	5
5. Suitability for null gravity use	0
6. Overall safety	0
7. Nontoxic reagents	0
8. Specificity	5
9. Insensitive to environmental changes	4
10. Ease in training personnel	1
11. Degree of separation required (10=none)	0
12. Minimal handling by analyst	0
13. Common use of analytic equipment	5
14. Nondestructive of sample	0
15. Figure of merit	3

2. Discussion

Use of albumin (as described in Section 3.3.15 on 17-hydroxycorticosteroids) might circumvent the need to use organic solvents

in the extraction procedure.

The author of this report is of the opinion that the satisfactory analysis of aldosterone is contingent on the development of new methods.

C. Areas for research and development

1. Isotope dilution method similar to that described for 17-OH-CS in which albumin, which has a strong binding affinity for aldosterone is substituted for CBG.
2. Immunochemical investigation of natural and synthetically prepared aldosterone-protein conjugates.
3. Investigation of the optical rotatory dispersion spectrom of aldosterone and its simple derivatives.
4. Enzymatic methods. Search for enzymes specific for aldosterone.

D. References

1. Kliman, B.; Foster, D. W.; Analysis of Aldosterone by Gas-Liquid Chromatography, Anal. Biochem., Vol. 3, 1962, pp. 403 - 407.

The 18-, 21 diacetate was produced which yielded a single symmetrical peak over a wide range of column temperatures. The eluted steroid was not identical to the injected diacetate.

2. Merits, I., Gas-Liquid Chromatography of Adrenal Cortical Steroid Hormones, Lipid Research, Vol. 3, 1962, pp. 126 - 127.

The steroid was oxidized with periodic acid (15 hours) to produce the lactone of aldosterone which could be separated from the other steroids due to its insolubility in sodium bicarbonate. The procedure involved an ether extraction. The gamma lactone of aldosterone was stable under conditions of chromatography.

3. Woitz, H.H., Naukkarinen, I., Carr, H.E., Jr., Gas Chromatography of Aldosterone, Bio. Chim. et Biophys. Acta, Vol. 53, 1961, pp. 449 - 452.

Aldosterone, acetylated at the 18-, 21-hydroxyl functions could be chromatographed in the vapor phase without decomposition. As little as 0.4 μg could be detected. Separation from cortisone was achieved. Chromatography required 15 - 20 minutes.

3.3.15 17-Hydroxycorticosteroid (17-OH-CS) in blood serum or urine

A. Principles of present methodology

1. Isotope dilution method for 17-OH-CS in blood serum

The method most likely to be adaptable to space flight conditions is based on the capacity of 17-OH-CS to bind selectively to a blood serum alpha-1 globulin which has been called transcortin[8] or cortical steroid binding globulin (CBG).[1] The method is based primarily on two earlier reports.[4, 5] Methods for the preparation of CBG have been described.[6, 7] The method is based on the competition between isotopically labeled cortisol and cortisol endogenously present in 1 ml of blood serum for the binding sites on CBG. It is assumed that the isotopically labeled cortisol and the cortisol endogenously present in blood serum have an equal binding affinity for the sites on CBG. The number of isotopically labeled molecules which bind to the CBG will be a function of the total (labeled plus unlabeled) number of cortisol molecules present. It is only necessary then to add a known amount of isotopically labeled cortisol plus the cortisol present in 1 ml of blood serum to a standard quantity of CBG, remove the unbound cortisol and determine the radioactivity of the solution. By using known quantities of unlabeled cortisol in place of blood serum, a reference curve can be constructed from which cortisol concentrations in blood serum can be determined. Unbound cortisol is removed by adsorption on dextran coated charcoal.[5]

2. Colorimetric method for 17-OH-CS in urine

The steroids are extracted from urine as albumin-steroid complexes. In one method[9], egg or serum albumin is added to urine. The albumin is precipitated by saturating the solution with ammonium sulfate. The precipitated albumin-corticosteroid complexes are re-dissolved in water, and the albumin precipitated by organic solvents, leaving the free steroids in solution. Colorimetric

determination using 2, 4 dinitrophenylhydrazine or tetrazolium blue is done on the supernatant.

3. Enzymatic method for determination of 17-OH-CS in blood or urine

This method depends on reduction of the 20-oxo group of the corticosteroids by 20 β-hydroxycorticosteroid dehydrogenase and the simultaneous oxidation of stoichiometric amounts of reduced diphosphopyridine nucleotide (DPNH). As little as 0.25 microgram of steroid was determined with an accuracy of $\pm$ 5%. Both 17-hydroxy and 17-desoxy steroids are determined by the method, but by selective solvent extraction the method was made specific for the 17-OH-CS. Apparently a 20 β-hydroxycorticosteroid dehydrogenase specific for 17-OH-CS has not been described in the literature. If such an enzyme could be isolated, and used in the above procedure, a simple, highly specific procedure could be developed that does not require solvent extraction.

B. Suitability of present methodology to space flight conditions

1. Merit table

17-Hydroxycorticosteroids

Merit Parameters	Isotope dilution (Blood serum)	Colorimetric (Urine)	Enzymatic (Blood serum or urine)
1. Sensitivity	1 μg.	1 μg.	0.25 μg.
2. Sample size	1.0 ml.	0.3 ml. urine	0.3 ml. urine
3. Time required	1 hr.	45 min.	?
4. Reproducibility	10	5	8
5. Suitability for null gravity use	5	5	8
6. Overall safety	10	5	5
7. Nontoxic, noncaustic	10	3	3
8. Specificity	10	3	5
9. Insensitive to environmental changes	3	5	5
10. Ease in training personnel	5	5	5

17-Hydroxycorticosteroids - continued

Merit Parameters	Isotope dilution (Blood serum)	Colorimetric (Urine)	Enzymatic (Blood serum or urine)
11. Degree of separation required (10=none)	8	1	10
12. Minimal handling by analyst	5	4	6
13. Common use of analytic equipment	8	10	10
14. Nondestructive of sample	10	0	0
15. Figure of merit	57	10	27

2. Discussion

There are three major problems in the development of suitable methods of analysis of 17-OH-CS in urine. The first of these stems from the fact that the 17-OH-CS are excreted in conjugated froms. Most schemes of analysis begin with hydrolytic procedure, involving either acid or enzymatic hydrolysis. This is a time consuming step, requiring up to 24 hours. The second major problem lies in the fact that the methods for determination of these compounds are not specific, and accurate analytical data can only be obtained after multiple extractions with highly volatile organic solvents. The third problem with which we are faced with regard to the determinations of these compounds in urine results from the observation that although only three corticosteroids (cortisol, corticosterone and aldosterone) appear in blood serum, multiple metabolic products as well as several conjugated forms of the otherwise unchanged steroids are found in urine.

It is suggested that a knowledge of the 17-OH-CS concentrations in blood serum might be of more value than their rate of excretion. In view of the multiple problems concerned with their determination in urine, it is suggested that the isotope dilution method for determination of 17-OH-CS in blood serum be given prime consideration.

This method might also be adapted for the determination of unmetabolized 17-OH-CS in urine. This method probably would not detect the tetra-hydro derivatives of 17-OH-CS found in urine, however. Results obtained by the isotope dilution have been compared with other methods with very favorable results.[2]

C. Areas for research and development

1. Enzymatic

Search for 20-β-hydorxycorticosteroid dehydrogenase or other enzymes specific for 17-OH-CS.

2. Immunochemical methods

a. Investigate antigenicity of naturally occurring protein conjugates.

b. Prepare synthetic protein conjugates and investigate their antigenicity and specificity.

3. Vapor phase chromatography

Investigate stability and chromatographic behavior of trimethylsilyl ethers, acetates and other derivatives.

D. References

1. Doughaday, W.H.; Binding of Corticosteroids by Plasma Proteins, J. Clin. Invest., Vol. 37, 1958, pp. 511 - 518.

Studied binding of corticosteroids and related hormones to transcortin by equilibrium dialysis, using 4-C^{14} labeled steroids.

2. Jones, J.A.; Mason, J.W.; A Critical Comparison of a Chromatographic and an Isotope Dilution Method of Plasma 17-Hydroxycorticosteroid Measurement, J. Clin. Endocrinol. and Metab., Vol. 26, 1966, pp. 1010 - 1111.

Results obtained by the isotope dilution method compared favorably with those obtained by a conventional chromatographic method.

3. Margoff, C.O.; Weichselbaum, T.E.; A Method for the Enzymatic Determination of Corticosteroids in Extracts of Whole Blood, Plasma, and Urine, Steroids, Vol. 2, 1963, pp. 143 - 154.

An enzymatic procedure based on reduction of the 20-oxo group of corticosteroids by 20 β-hydroxysteroid dehydrogenase and simul-

taneous oxidation of stoichrometric amounts of reduced DPN was applied to extracts from biological fluids. Solvent extraction was used to increase the specificity of the method.

4. Murphy, B.P.; Engelberg, W.; Pattee, C.J.; Simple Method for the Determination of Plasma Corticoids, J. Clin. Endocrinol. and Metab., Vol. 23, 1963, pp. 293 - 300.

   Describes a highly specific method for determining plasma corticoids using C^{14} labeled cortisol and corticosteroid binding globulin by a dialysis technique. One ml. of blood serum was used. A standard deviation of $\pm$ 1 µg. was obtained over a range of 1 to 10 µg/ 100 ml.

5. Nugent, C.A.; Mayes, D.M.; Plasma Corticosteroids Determined by Use of Corticosteroid Binding Globulin and Dextran Coated Charcoal, J. Clin. Endocrinol. and Metab., Vol. 26, 1966, p. 1116.

   Used dextran coated charcoal to adsorb cortisol which was not bound to corticosteroid binding globulin in an analytical technique using C^{14} labeled cortisol and 1 ml. of blood serum. Results compared favorably with older accepted chromatographic-fluorometric technique.

6. Seal, U.L.; Doe, R.P.; Corticosteroid-Binding Globulin: Species Distribution and Small Scale Purification, Endocrinol., Vol. 73, 1963, pp. 371 - 376. (CA 60, 2018a).

   Binding activity of CBG was studied and found to be tempera-dependent. A method of isolation and purification using hydroxylapatite is described.

7. Seal, U.S.; Doe, R.P.; Corticosteroid-Binding Globulin I Isolation from Plasma, J. Biol. Chem., Vol. 237, 1962, pp. 3136 - 3140.

   A procedure for preparation is described which results in a 1000-fold purification in 75% yield. The protein appeared homogenous. Its physicochemical properties are described.

8. Slaunwhite, W.R.; Sandberg, A.A.; Transcortin: A Corticosteroid Binding Protein of Plasma, J. Clin. Invest., Vol. 38, 1959, p. 3841.

   This paper represents the original description of transcortin. Its binding affinity for different corticosteroids is described.

9. Zumoff, B.; Bradlow, H.L.; Quantitative Extraction and Separa-

tion of Conjugated Steroid Metabolites from Human Urine, J. Clin. Endocrinol., Vol. 23, 1963, pp. 799 - 804.

Describes a procedure for quantitatively extracting conjugated steroid metabolites from urine using albumin. Conjugates were further studied by chromatography. It was concluded that the procedure does not introduce artifacts and that it yields quantitative results.

3.3.16 Catecholamines

A. Principles of present methodology[1, 2, 3, 4, 5, 6, 7, 8, 9, 10]

Biological, colorimetric and fluorometric techniques are available for the assay of catecholamines. Fluorometry seems to be the most simple and practical. Biological assays lack sensitivity and colorimetric tests lack both sensitivity and specificity. Fluorometry involves the isolation of the amines by an adsorption procedure, elution of the amines with acid, and then conversion to a fluorescent derivative. A symposium which appears in Pharmacological Reviews, Vol. 2, 1959, pp. 233 - 304, is an excellent review of the field and contains an extensive bibliography.

B. Suitability of present methodology to space flight conditions

1. Merit table

Catecholamines

Merit Parameters	Catecholamines in Urine Fluorescence	Catecholamines in Blood Fluorescence	"VMA" Colorimetric
1. Sensitivity	very good	very good	good
2. Sample size	24 hr. sample	13 ml. plasma	1.0 ml. urine
3. Time required	approx. 2 hrs.	approx. 2 hrs.	8
4. Reproducibility	4	3	8
5. Suitability for null gravity use	-	-	-
6. Overall safety	8	8	8
7. Nontoxic, noncaustic reagents	8	8	8
8. Specificity	5	5	4
9. Insensitive to environmental changes	8	8	10
10. Ease in training personnel	2	1	4
11. Degree of separation required (10=none)	1	1	3
12. Minimal handling by analyst	2	1	5

Catecholamines - continued

Merit Parameters	Catecholamines in Urine Fluorescence	Catecholamines in Blood Fluorescence	"VMA" Colorimetric
13. Common use of analytic equipment	8	8	10
14. Nondestructive of sample	6	4	8
15. Merit range	10-19	7-14	23-46
16. Mean figure of merit	14	10	34

2. Discussion

As is true of many analyses of substances from biological sources it is necessary to perform certain isolation and purification steps. Currently, the use of alumina as an adsorbent results in recoveries of about 85%. This many be due in part to losses at neutral pH. It may be better to use a cation exchange resin such as Dowex-50, which will adsorb the catechol amines under acid conditions and allow the removal of the amines under stronger acid conditions. All the methods involve a large number of manipulations and consequently the number of determinations which can be accomplished is limited. Some estimate 5- 8 per day.

C. Areas for research and development

A simplified approach to this problem might be the combination of paper chromatographic separation by adsorption followed by direct fluorometry on paper using equipment like the chromatogram door supplied with the Turner Fluorometer.

Some investigators have chosen to use the urinary excretion of 3-methoxy-4-hydroxymandelic acid (VMA) as an index of catechol amine metabolism and excretion. It can be determined by either chromatography or solvent extraction followed by colorimetric determination.[4, 5, 8] Simple modifications of methods for VMA should be

possible which would enable the determination under space flight conditions.

D. References

1. Brunjes, Shammon; Wybenga, D.; Differential Fluorimetry in Catechol Amine Determination: A Simplified Method of Calculation. Clin. Chem., Vol. 9, 1963, pp. 626 - 630.

A method for solving two simultaneous equations for the differential fluorimetry of epinephrine and norepinephrine using internal standards for urine analysis.

2. Callingham, B. A.; Cass, R.; The Determination of Catechol Amines in Biological Materials. West European Symp. Clin. Chem., Vol. 2, 1963, pp. 19 - 30.

For the extraction and purification of the catechol amines in plasma and urine adsorption and ion-exchange (Dowex 50) are used. To obtain sensitivity with specificity, fluorimetric methods are necessary.

3. Cardona, R.; Soehring, K.; Thin-layer Chromatographic Detection of Micro Amounts of Catechol Amines and their Derivatives, Med. Exptl., Vol. 10, 1964, pp. 251 - 257.

Method for preparation of plates and the separation of 0.05 gamma of catecholamines and their derivatives is described.

4. Connelian, T. P.; Godfrey, J. M.; Routine Determination of Urinary 4-hydroxy-3-methoxymandelic Acid, Clin. Chem. Acta, Vol. 9, 1964, pp. 410 - 412.

Modification of the method of Pisano, et al. (Chemical Abstracts, Vol. 57, p. 1147g) so that critical pipetting operations were reduced to 2 by using a single set of 100 ml. cylindrical short stemmed separate funnels. The new technique increased overall recovery, eliminated the need for microcuvets and reduced analysis time.

5. Crymble, G.; A Comparison and Evaluation of Colorimetric Procedures for 3-methoxy-4-hydroxymandelic Acid, Can. J. Med. Technol., Vol. 26, 1964, pp. 188 - 197.

The extraction of the title compound from acidified urine into ethyl acetate and reextraction into aqueous $K_2 CO_3$ was found essential. The adsorption of Florisil approached the extraction into

ethyl acetate. The absorption curves of processed urines did not approach those of pure title compound and many phenolic acids may be measured with the title compound.

6. Kawai, S.; Nagatsu, T.; Imanari, T.; Gas Chromatography of Catecholamines and Related Compounds, Vol. 14, 1966, (Eng.), pp. 618 - 621.

   A satisfactory separation of epinephrine, norepinephrine, doapmine, metanephrine and normetanephrine was achieved through trimethylsilylation with hexamethyldisilazane followed by condensation with 2-penatanone.

7. Kirschner, N.; Goodall, McC.; Separation of Adrenaline, Noradrenaline and Hydroxytyramine by Ion-Exchange Chromatography, J. Biol. Chem., Vol. 226, pp. 207 - 212.

   A procedure is described for the separation of adrenaline, noradrenaline and hydroxytyramine by use of Amberlite IRC-50. Recoveries from pure solutions ranged from 85 - 97%.

8. Mahler, D.J.; Humoller, F.L.; Comparison of Methods for Determining Catechol Amines and 3-methoxy-4-hydroxymandelic Acid in Urine, Clin. Chem., Vol. 8, 1962, pp. 47 - 55.

   Methods for the evaluation of pheochromocytoma were compared. The results obtained by bioassay, fluorimetry, and spectrophotometry in normal subjects are reported.

9. Mattok, G.L.; Wilson, D.L.; Separation of Catechol Amines and Metanephrine and Nor-Metanephrine Using a Weak Cation-Exchange Resin, Biochem., Vol. 11, 1965, pp. 575 - 579.

   The title compounds were adsorbed on Amberlite IRC-50 and the catechol amines eluted by a boric acid solution and metanephrine and normetanephrine by H_2SO_4.

10. Ritzel, G.; Hunzinger, W.A.; Determination of Catechol Amines in Urine, Klin. Wochschr., Vol. 41, 1963, pp. 419 - 423.

    The catechol amines were oxidized to adrenochrome and reduced to the corresponding trihyroxyindoles and determined fluorimetrically. Normal data for the excretion of adrenaline, noradrenaline and vanilmandelic acid are listed.

3.3.17 Serotonin

A. Principles of present methodology

The unique fluorescence charactheristics of serotonin (5-hydroxy-tryptamine) make possible its direct determination in blood after removal of protein. This method lacks specificity in that all 5-hydroxy-indoles have the same general fluorescence characteristics. Serotonin, however, is the only hydroxyindole found in tissues. In the normal subject only 0.05 - 0.2 micrograms/ml. are found which makes direct measurement impossible.[2, 6]

A number of methods for the assay of 5-HIAA, a metabolite of serotonin, are available in the literature.[1, 3, 4, 5] A semiquantitative assay is based on a color reaction with 1-nitroso-2-naphthol.[4] This reaction can also be used in a more quantitative extraction procedure.[5]

B. Suitability of present methodology to space flight conditions

1. Merit table

Serotonin

Merit Parameter	Serotonin	5-HIAA
1. Sensitivity	< 0.035 µg/ml.	40 µg/1 (urine)
2. Sample size	?	3
3. Time required	1 hour	20 minutes
4. Reproducibility	2	3
5. Suitability for null gravity use	0	0
6. Overall safety	1	1
7. Nontoxic reagents	2	3
8. Specificity	2	8
9. Insensitive to environmental changes	0	0
10. Ease in training personnel	0	5
11. Degree of separation required (10=none)	0	3
12. Minimal handling by analyst	1	3
13. Common use of analytic equipment	5	5

Serotonin - continued

Merit Parameters	Serotonin	5-HIAA
14. Nondestructive of sample	0	0
15. Figure of merit	1	4

2. Discussion

The simple test reproted by Sjoerdsma[6] and Elliott[4] should have direct application. Since this determination has generally been used for the diagnosis of carcinoid tumors of the intestine, however, its significance in evaluation of the effect of space flight on man appears doubtful. The concentration of 5-HIAA in normal urine is not detectable by this method.

C. Areas for research and development

Strum[6] has suggested that amounts of 5-hydroxytryptamine normally found in plasma can be quantitated by paper electrophoresis of deproteinized serum, treatment with Ehrlich's reagent, direct elution and measurement of absorption at 275 mμ. The method recently reported by Contractor[1] seems to hold the most promise for the simple determination of 5-HIAA. The 5-HIAA is selectively absorbed on Sephadex G-10*, and interfering fluorophores are then leached out and the 5-HIAA is eluted with NH_4OH and determined with spectrophotofluorimetry.

D. Comments

It seems that several of these methods could be applicable immediately, if methods can be developed for handling liquids and volatile solvents in a space environment.

E. References

1. Contractor, S. F.; A Rapid Quantitative Method for the Estimation of 5-hydroxyindoleacetic Acid in Human Urine, Biochem. Pharmacol., Vol. 15, 1966, pp. 1701 - 1706.

An adsorption separation followed by elution and spectrophotofluorometry.

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

2. Crawford, N.; Rudd, B. T.; A Spectrophotofluorometric Method for the Determination of Serotonin in Plasma, Clin. Chim. Acta, Vol. 7, 1962, pp. 144 - 121.

A method for analysis of platelets or platelet rich plasma. Ether extraction is used or purify the serotonin before analysis.

3. Deux, C.; Delauneus, B.; Critical Testing of Biochemical Methods for the Investigation of Serotonin Metabolism, Presse. Med., Vol. 72, 1964, pp. 2925 - 2930.

Methods for the determination of 5-HIAA in urine are reviewed. 19 references.

4. Elliott, H.C.; Casey, A.E.; Experience With a Simple Screening Test for Serotonin, Sou. Med. J., Vol. 51, 1958, pp. 836 - 840.

A modified colorimetric procedure for 5-HIAA was used to screen 4,517 urine samples from routine hospital admissions.

5. Kirberger, E.; Significance and Method of Detection of Increased Urinary Excretion of 5-HIAA, Deut. Med. Wochschr., Vol. 91, 1966, pp. 2128 - 2130.

A modification of the method of Sjoerdsma (see 6.)

6. Sjoerdsma, A.; Weissbach, H.; Udenfriend, S.; Simple Test for the Diagnosis of Metastic Carcinoid, J.A.M.A., Vol. 159, 1955, pp. 397 - 399.

A color reaction for semiquantitative estimation of 5-HIAA.

3.3.18 Thyroxine, thyroxine binding prealbumin, protein bound iodine

Measurement of the thyroid hormones in the serum has been the most valuable method of assaying thyroid function. Indirect measurement by determination of protein-bound iodine[3, 4, 6, 12] has been the method of choice for some years. It can be demonstrated that the PBI level is directly proportional to the serum thyroxine level. Recently it has been possible to develop highly specific assay for thyroxine[5, 7, 8, 9] based upon work with thyroxine-binding globulin.

A. Principles of present methodology

The Barker method for determination of PBI depends upon the steps of removal of inorganic iodide from serum, conversion of organic iodine into inorganic iodide, and the determination of the iodide formed by measuring its catalytic effect on the reduction of Ce^{+4} to Ce^{+3} by As^{+3}.

The thyroxine-binding globulin[2, 11, 13] (TBG) is generally used as a measure of the thyroid hormone transport system. The bound thyroid hormones are in continuous equilibrium with a small amount of circulating free hormone. Ultimately these plasma carrier proteins exchange thyroid hormone with binding proteins of the tissues, and it is probably the free moiety of thyroid hormone which is physiologically active. Thyroxine (T-4) is bound preferentially to triiodothyronine (T-3), and free T-4 will replace bound T-3. Free T-3, however, will not replace bound T-4. This equilibrium and preferential binding of T-4 over T-3 is the basis of two thyroid function tests involving thyroid hormone transport. TBG measures the total thyroxine binding capacity and indicates the quantity of TBG in circulation. The procedure is performed by the addition of excess of I^{131} labeled thyroxine to the subjects serum, followed by electrophoretic separation of the TBG inter-alpha band.

Assay for total thyroxine[7, 10] depends on the equilibrium previously mentioned. In the test, a pooled serum is saturated with I^{131} thyroxine

so that an equilibrium is established between free and bound radioactive thyroxine. A measured amount of this serum is added to an alcholic extract of the patient's serum. This extract contains the patient's total thyroxine. The amount of thyroxine in this extract will upset the equilibrium in the serum and establish a new equilibrium; dependent upon the amount of thyroxine in the extract. After passing the serum solution through a sephadex gel, the amount of bound thyroxine is measured and the patient's total thyroxine level is calculated by comparing the radioactivity of the serum treated with the extract from the subject's serum with serums treated with known amounts of pure thyroxine.

B. Suitability of present methodology to space flight conditions

1. Merit table

Thyroid Function

Merit Parameters	PBI	Total Thyroxine	TBG
1. Sensitivity	Excellent	Good	Unknown
2. Sample size	All would require several ml. of subject's serum.		
3. Time required	2 hrs.	Unknown	Unknown
4. Reproducibility	9	5	5
5. Suitability for null gravity use	10	8	8
6. Overall safety	2	8	8
7. Nontoxic, noncaustic reagents	0	9	9
8. Specificity	7	6	6
9. Insensitive to environmental changes	-	-	-
10. Ease in training personnel	2	4	3
11. Degree of separation required (10=none)	4	3	2
12. Minimal handling by analyst	1	2	2
13. Common use of analytic equipment	*	*	**
14. Nondestructive of sample	0	3	3
15. Merit range	11-22	18-26	17-25
16. Mean figure of merit	18	22	21

* Not weighted so that merit ratings comparable. (Photometry)
** Depends on availability of adequate counting equipment.

2. Discussion

The two methods currently used for PBI chemical determination (Chaney and Barker) are quite difficult technically. They involve, among other difficulties, either wet or dry ashing of a serum sample, and the use of very strong sulfuric acid solution. It does not seem likely that such procedures could be accomplished in space. Other hormone transport measurements involve the use of I^{131} labeled thyroxine and triiodothyronine and therefore fairly sophisticated counting equipment. This obstacle would not appear to be insurmountable however. The most likely approach will be to simplify in some way the techniques of electrophoretic separation and isotope counting.

C. Areas for research and development

It would seem that in the current state of knowledge concerning thyroid physiology the determination of either total thyroxine[7] or free thyroxine[5, 7] would be the method of choice for development. Determination of free thyroxine would be technically much more complex simply because it is present in serum in very small amounts (3 - 6 mμg/100 ml.)

It may also be possible that the electron probe x-ray analyzer can be applied after a preliminary separation of organic and inorganic iodine.[1]

D. References

1. Bowen, H.J.M.: Cawse, P.A.; The Determination of Inorganic Elements in Biological Tissue by Activation Analysis, U.K. At. Energy Authority Rept. AERE-R 4309, 1963, p. 39.

Neutron activation was used for determining Ca, Cl, Cu, I, Mg, etc., in biological tissue. A very selective and sensitive analysis achieved by chemical separation before counting. Accuracy ± 5%.

2. Braverman, L.E.; Dawber, N.A.; Ingbar, S.H.; Binding of Thyroid Hormones in Sera of Normal Subjects of Varying Ages,

J. Clin. Invest., Vol. 45, 1966, pp. 1273 - 1279.

The average endogenous thyroxine (T_4) distribution on T_4-binding globulin (TBG), albumin, and prealbumin (TBPA) was determined for age groups from 2 years to 70 years in 10-year intervals. In the group 31 - 40 the distribution was TBG 39.5%, albumin 15.8%, TBPA 44.6%.

3. Gardiner, E.; Burns, A.; Micromethod for the Rapid Determination of Serum Protein-Bound Iodine and Total Serum Iodine, Clin. Chem., Vol. 10, 1964, pp. 1137 - 1146.

A modification of the colorimetric chloric acid method which requires less than 0.5 ml serum for duplicate determination.

4. Hellauer, H.; Significance and Application of the PBI Determination, Wien, Klin, Wochschr., Vol. 78, 1966, pp. 553 - 556.

Free thyroxine in serum calculated from the degree of discussion of the protein complex of I^{131} labeled hormone and PBI. Eleven procedures for determining PBI are compared and 6 for determining total thyroid hormone binding proteins.

5. Henry, R.J.; Golub, O.J.; Determination of the Free Thyroxine Content of Serum, J. Clin. Endocrinol. Metab., Vol. 24, 1964, pp. 486 - 495.

After equilibration of thyroxine ^{131}I with serum protein bound iodine the specific activity of the free thyroxine is the same as that of protein bound thyroxine. Free thyroxine was separated from bound by Sephadex G-25. Free thyroxine was calculated from net count rate of Sephadex bound ^{131}I, net count rate of protein-bound ^{131}I, and serum thyroxine I.

6. Lerner, S.R.; Iodinated Amino Acid Chromatography on Polystyrene Resins. Arch. Biochem. Biophys., Vol. 103, 1963, pp. 36 - 41.

Iodinated amino acids eluted from Dowex-50-X4 using formamide buffers.

7. Murphy, B.P.; Pattee, C.J.; Determination of Thyroxine Using the Property of Protein Binding. J. Clin. Endocrinol. Metab., Vol. 24, 1964, pp. 187 - 196.

Use of I^{131} labeled thyroxine to determine protein binding

globulin and measurement of equilibrium displacement as more T_4 is added.

8. Nakajima, H.; Kuramochi, M.; Horiguchi, T.; A New Simple Method for the Determination of Thyroxine in Serum, Nippon Naibumpi Gakkai Zasshi, Vol. 40, 1965, pp. 1303 - 1307.

Method based on use of pooled serum of known triiodothyronine binding capacity. Amberlite IRA-400 used to separate bound and unbound I. Linear relation established between amounts of thyroxine in serum and resin bound I^{131} thyroxine.

9. Oppenheimer, J.H.; Martinez, M.; Berstein, G.; Determination of the Maximal Binding Capacity and Protein Concentration of Thyroxine Binding Prealbumin in Human Serum, J. Lab. Clin. Med., Vol. 67, 1966, pp. 500 - 509.

An improved method for determining the maximum binding capacity of TBPA using paper electrophoresis. Mean level in a group of normal adults was a maximum binding capacity of 274 gamma/100 ml.

10. Pileggi, V.J.; Lee, N.D.; Golub, O.J.; Determination of Iodine Compounds in Serum. I. Serum Thyroxine in the Presence of Some Iodine Contaminants, J. Clin. Endocrinol. and Metabolism, Vol. 21, 1961, pp. 1272 - 1279.

A Dowex-1 column is used to isolate more than 80% of added I^{131} in the first 10.0 ml of eluate.

11. Rich, C.; Bearn, A.G.; Localization of the Thyroxine-Binding Protein of Serum by Starch Gel Electrophoresis, Endocrinology, Vol. 62, 1958, pp. 687 - 689.

Thyroxine binding protein migrated ahead of albumin in a sharp band.

12. Schorn, H.; Winkler, C.; Thin Layer Chromatographic Analysis of Thyroid Hormones, J. Chromatography, Vol. 15, 1965, pp. 69 - 75.

Triiodothyronine and thyroxine were separated in 2 hrs. by thin layer chromatography on silica gel G. Use of thin-layer chromatography in a clinical study of hyperthyroidism is discussed.

13. Tata, J.R.; Purification of Thyroxine Binding Globulin (TBG) and Thyroxine Binding Prealbumin (TBPA), Clin. Chim. Acta, Vol. 6, 1961, pp. 819 - 832.

Methods described for isolating these two major thyroxine binding proteins from human serum. Fractions were characterized by ultracentrifugation, immunoelectrophoresis, gel diffusion and zone electrophoresis.

3. 3. 19 Amino nitrogen

To measure accurately the amino acid concentration of a biological sample it is first necessary to isolate and measure each of the twenty or so compounds and quantitate each individually. This is required because there are large differences in the molecular weights, reactivities, and abundances of the various amino acids. To adequately isolate and measure the various amino acids would require equipment and techniques which appear to be too complex and cumbersome for the current application.

A. Principles of present methodology

Methods commonly used to measure amino nitrogen generally employ colorimetric techniques which assay total amino nitrogen as compared with a selected amino acid standard. The three most popular techniques employ protein-free solutions.

1. One method depends upon the color produced by reaction of amino compounds with ninhydrin.[4] This procedure yields values greater than other techniques and thus there is some question about the specificity.
2. The method of choice is one utilizing the color produced by reaction of amino acids with β-naphthoquinonesulfonate.[3, 5]
3. The third procedure involves the addition of cupric phosphate to the sample. Copper forms soluble complexes with amino groups and the measurement of copper in the supernatant is then a measure of amino nitrogen. To obtain satisfactory results with blood samples it is necessary to isolate the amino compounds with an ion-exchange resin prior to equilibration with the cupric phosphate. The numerous manipulations required for this procedure eliminate it from further consideration.

B. Suitability of present methodology to space flight conditions

1. Merit table

Amino Nitrogen

Merit Parameters	Method 1	Method 2
1. Sensitivity	Good	Good
2. Sample size	300 µl	500 µl
3. Time required	45 min	45 min
4. Reproducibility	10	9
5. Suitability for null gravity use	-	-
6. Overall safety	0	6
7. Nontoxic reagent	0	1
8. Noncaustic reagent	1	1
9. Specificity	-	7
10. Applicability	6	6
11. Reagent volatility	0	4
12. Insensitive to environmental changes	1	1
13. Ease in training personnel	7	6
14. Minimal handling by analyst	5	4
15. Common use of analytic equipment	10	10
16. Merit range	5-17	13-26
17. Mean figure of merit	10	20

2. Discussion

All three of the methods presented for the measurement of amino nitrogen require equipment for the preparation of protein free solutions (centrifuge, dialysis device, etc.) and colorimeter.

C. Areas for research and development

Perhaps the most fruitful area for exploration would be gas-liquid chromatography. This technique can now be used to afford complete separation and quantitation of the naturally occurring amino acids. Many support media are stable and many times reusable. Probably the greatest problem would be encountered in preparing derivatives; however, the operation could undoubtedly be largely or entirely automated. The instrument output would be best interfaced with data

processing equipment to provide digital values for the individual constituents.

Another approach would be to form derivatives with the amino acids which would have characteristic optical properties. Such a compound currently enjoying considerable popularity is 1-dimethylaminonaphthalene-5-sulfonyl halide, often disguised as the acronym dansyl. This compound has had frequent application for N-terminal end-group analyses of proteins[1] and for "tagging" amino acids.[2] Another possible compound for derivatizing amino acids is 2-chloro-3, 5-dinitropyridine. This substance can be used to form derivatives in aqueous solutions under very mild conditions.

A method employing amino acid oxidase might also be explored. L-Amino acid oxidase catalyzes the oxidative deamination of most of the amino acids with the release of ammonia. The ammonia produced could be estimated using one of the methods discussed under Blood Urea Nitrogen. One great disadvantage of this approach is the great variation in reaction rates for the different amino acids. This aspect might be resolved by using long incubation times to approach complete deamination for all the amino acids.

It may also be possible to employ bacterial amino acid auxotrophs to devise a simple microbiologic assay.

D. References

1. Crombrugghe, B.; Edelhoch, H.; The Properties of Thyroglobulin XIV, Biochem., Vol. 5, 1966, pp. 2238 - 2245.

Used 1-dimethylaminonaphthalene-5-sulfone derivatives of thyroglobulin to study structure.

2. Deranleau, D. A.; Neurath, H.; The Combination of Chymotrypsin and Chymotrypsinogen with Fluorescent Substrate and Inhibitors for Chymotrypsin, Biochem., Vol. 5, 1966, pp. 1413 - 1425.

Employed amino acid ester derivatives of 1-dimethylaminonaphthalene-5-sulfone to study interaction of enzyme and substrate.

3.3.20 Blood urea nitrogen (BUN)

A. Principles of present methodology

Most clinical measurements of urea employ either a direct chromogenic reaction with diacetyl or the action of urease followed by a technique to quantitate the ammonia produced.

1. Diacetyl

a. The use of the diacetyl reaction to measure urea colorimetrically has become popular with continuous flow automated equipment. The reaction is quite sensitive and difficult to control when employed in manual procedures; however, with automated equipment, where conditions are very reproducible, the method is quite good.

The reaction is not specific in that apparently most ureido-compounds will react to yield chromogens. Other disadvantages are that the color produced fades quickly, is photosensitive, does not show a linear relationship between concentration of urea and absorbance, and the heating time required for maximum color development depends upon the urea concentration.[5]

b. Diacetyl can also be used in a fluorometric procedure.[7] When urea and diacetylmonoxime are heated with sulfuric acid and irradiated at 380 mμ the reaction products show fluorescence at 420 and 520 mμ.

2. Urease

The utilization of urease in the measurement of urea is a well established procedure. Urease is extremely specific, has great activity, is easily prepared, and is stable for long periods in certain solutions or when lyophilized. Most methods that utilize urease measure the ammonia liberated by nesslerization or by the Berthelot[3] reaction.

a. Nessler's reagent, $(KI)_2$ HgI, is easily prepared and very stable. The colored product of nesslerization is colloidal and

generally requires a protective colloid for photometry.

b. The Berthelot reaction is about ten times more sensitive to ammonia than nesslerization and the colored product is soluable and quite stable. All of the reagents required are stable except for the hypohalite (usually hypochlorite).

3. Xanthydrol

Xanthydrol is a very specific reagent for urea and can be employed in several ways.

a. In a turbidimetric method[6] a sample of serum in acetic acid is mixed with an alcoholic solution of xanthydrol. After 30 minutes the turbidity is measured at 546 mμ. Xanthydrol can also be employed in colorimetric procedures.

b. In one procedure[1] a protein-free filtrate of blood is reacted with xanthydrol. The dixanthydryl urea product is collected by filtration, washed, and dissolved in 50% H_2SO_4. The absorbance of the yellow dixanthydryl urea solution is then measured and compared with similarly treated standard urea solutions.

c. Another colorimetric procedure involves the reaction of the separated dixanthydryl urea with hydrogen peroxide and phenol reagent.[2, 8]

4. Coulometry can also be used in the analyses of urea, following the conversion to ammonia. However, the ammonia must be isolated from interfering substances prior to oxidation. Isothermic distillation (diffusion) has been employed for the separation of the ammonia.[4]

5. There are commercially available "dipsticks" which can be used for very crude evaluations of urea concentrations. It is possible that these could be modified to provide better quantitation. These devices will not be considered further because they are,

at best, semi-quantitative.

B. Suitability of present methodology to space flight conditions

1. Merit table

Blood in Urea Nitrogen

Merit Parameters	1a	1b	2a	2b	3a	3b	3c	4
1. Sensitivity	good	very good	very good	excel-lent	good	fair	-	good
2. Sample size	50 µl	10 µl	10 µl	20 µl	100 µl	500 µl	-	50 µl
3. Time required	30 min	40 min	20 min	70 min	40 min	60 min	-	150 min
4. Reproducibility	8	-	10	8	-	-	-	-
5. Suitability to null gravity	-	-	-	-	-	-	-	-
6. Overall safety	2	2	6	5	8	2	2	8
7. Nontoxic reagents	2	2	5	3	4	2	3	6
8. Noncaustic reagents	1	1	8	6	5	1	3	8
9. Specificity	6	7	9	9	9	9	9	9
10. Applicability	5	5	8	6	9	5	5	5
11. Reagent volatility	1	1	10	3	3	10	9	10
12. Sensitivity to environmental changes	10	10	10	2	10	10	10	10
13. Ease in training personnel	8	8	8	7	9	8	7	6
14. Manipulations	5	5	8	7	10	1	2	1
15. Common use of analytic equipment	10	7	10	10	8	10	10	1
16. Merit range	10-21	0-27	32-65	16-33	0-62	0-33	0-35	0-60
17. Mean figure of merit	16	10	48	24	23	12	13	22

2. Discussion

It would seem that the best procedure for immediate use would be method 2a. The method enjoys the specificity of urease and the reagents are stable. The Nessler's reagent is toxic, but it is not volatile and not very caustic. Quantitation is by photometry

(colorimetry), measuring technique which will undoubtedly be common for many other procedures.

The technique consists of adding the serum sample to lyophilized urease. The buffering capacity of the serum precludes the use of additional buffer. By using very active urease, incubation at room temperature for 5 - 10 minutes is adequate for complete urea hydrolysis. Following deproteinization the protein-free solution is reacted with Nessler's reagent. The absorbance of the resulting colored solution, stabilized by a protective colloid (usually gum ghatti) is measured at around 400 mμ. The absorbance of the sample is compared with a similarly treated standard. One good aspect of nesslerization is that if the absorbance is too great at lower wavelengths, valid measurements can be made at longer wavelengths (450 - 550 mμ) where the extinction coefficient of Nessler's compound is less.

The approaches which would appear most promising for future application would probably be those discussed in paragraphs C. 2, C. 3, and C. 1 in that order. If the substance used to prepare the urea derivative was such that it did not interfere with subsequent determinations, the approach would be essentially non-destructive.

Conductivity measurements, discussed in paragraph C. 3, should involve simple equipment and following the measurement, the sample should be suitable for other determinations.

The electrometric techniques seem to present more problems in that the alkalinization for ammonia release or acidification for carbon dioxide liberation might interfere with subsequent measurements with the sample. Also the isolation of the ammonia or carbon dioxide would appear to be difficult under the conditions of the present application.

Equipment

a. Method 1a

A centrifuge (protein free solution), heating bath and colorimeter. Reagents stable.

b. Method 1b

A centrifuge (protein free solution), heating bath and fluorimeter. Reagents stable.

c. Method 2a

Centrifuge and colorimeter. Reagents stable.

d. Method 2b

Colorimeter only required. Hypochlorite reagent unstable for prolonged periods.

e. Method 3a

Colorimeter or nephelometer (fluorimeter) required.

f. Method 3b

Centrifuge and colorimeter.

g. Method 3c

Centrifuge and colorimeter.

h. Method 4

Coulometer with amperometric detector and isothermic distillation apparatus or similar device (ion-exchange resin system or aeration system are possibilities).

C. Areas for research and development

1. The enzymic hydrolysis of urea with urease produces ammonia and carbonic acids. If either of these two products could be isolated and introduced into solutions with known buffer capacities then pH measurements could conceivably serve for quantitation.

Ammonia can be readily separated from solutions by alkalinization and distillation. Isothermic distillation, where the ammonia is collected as non-volatile ammonium ion, has enjoyed

extensive application in clinical chemistry. The major problem with this technique and the present application is that of keeping the solutions (sample and collecting solution) separated. This might be done with membranes which would be permeable to ammonia and perhaps vaporized water, but would be impermeable to the sample solution. To reduce distillation time the operation might be done with very thin films with large surface areas.

Perhaps more promising would be a method to measure the carbon dioxide produced. Carbon dioxide can be distilled from samples by acidifying the sample and collecting the volatile gas in alkali. Another approach would be to use a membrane permeable to carbon dioxide to separate the sample and the buffer solution, such as described below for the measurement of serum bicarbonate. The increase in pCO_2 from initial to final would then be related to the amount of urea originally present. Aeration could also be used in place of distillation, but a solution barrier (hydrophobic membrane permeable to gases) would be required to separate sample and receiving solutions.

2. The optical properties of urea derivatives might also provide simple specific methods of quantitation. An obvious example would be to use xanthydrol and measure the absorbance and/or fluorescence at characteristic wavelengths. Derivatives formed by reaction with phthalyl halide or p-nitrobenzyl halide might also be worth investigating.

3. Conductivity measurements before and after hydrolysis of urea by urease would appear to be a meaningful approach. Conductivity can be measured on a very small sample, and except for the addition of urease and the destruction of urea, the sample suffers no alterations. It would seem that an initial conductivity measurement (blank) would not be required so long as several values of conductivity were made during hydrolysis so that an initial conductivity

value could be estimated by extrapolation. The increase in conductivity as a result of the formation of ammonium carbonate from urea would then be a measure of urea concentration.

D. References

1. Beattie, F.; Micro-method for the Colorimetric Determination of Urea in Blood, Biochem., J., Vol. 22, 1928, pp. 711 - 712.

Describes a colorimetric technique for urea using xanthydrol.

2. Carr, J.J.; Alkaline and Acid Phosphatase, Standard Methods of Clinical Chemistry, Vol. 1, 1953, pp. 75 - 83.

Describes the preparation of phenol reagent as used in phosphatase assay.

3. Chaney, A.L.; Marbach, E.P.; Modified Reagents for Determination of Urea and Ammonia, Clin. Chem., Vol. 8, 1962, p. 131.

Combined reagents for Berthelot reaction and found they were quite stable when stored under proper conditions.

4. Christian, G.D.; Knoblock. E.C.; Purdy, W.C.; A Coulometric Determination of Urea Nitrogen in Blood and Urine, Clin. Chem., Vol. 11, 1965, pp. 700 - 707.

Presents a coulometric analysis for urea, but requires isolation of ammonia.

5. Henry, R.J.; Clinical Chemistry, Principles and Technics, Harper and Row Publishers, New York, 1964, pp. 263 - 264.

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.

6. Kachani, Z.F.Ch.; Simplified Determination of Urea in Serum, Aergtl. Lab., Vol. 9, 1963, pp. 81 - 86.

Describes a direct turbidimetric method for measuring urea with xanthydrol.

7. McCleskey, J.E.; Fluorometric Method for the Determination of

Urea in Blood, Anal. Chem., Vol. 36, 1964, pp. 1646 - 1648.

8. Yoshimatou, S.; A New Colorimetric Urea Method with 0.1 ml. of Blood, Tohoku J. Exptl. Med., Vol. 13, 1929, pp. 1 - 5.

Presents a colorimetric procedure for urea using xanthydrol, hydrogen peroxide and phenol reagent.