ANNUAL REPORT
Contract No. NAS 9-11118

July 1, 1974 through June 30, 1975

Lee R. Brown, Ph.D.

ANNUAL REPORT

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

Contract No. NAS 9-11118

July 1, 1974 through June 30, 1975

TITLE: STUDY TO DEFINE AND VERIFY THE PERSONAL ORAL HYGIENE REQUIREMENTS FOR EXTENDED MANNED SPACE FLIGHT - ORAL PHYSIOLOGY AND MICROBIOLOGY IN SKYLAB MANNED SPACE MISSIONS

by

Lee R. Brown, B.S., M.S., Ph.D.
Professor and Member, The University of Texas
Dental Branch and Dental Science Institute
Houston, Texas

Oral Physiology and Microbiology in Skylab Manned Space Missions

SUMMARY

This report details the findings pertaining to:

- Methods to establish metabolic profiles for fingerprinting microorganisms associated with oral pathoses; and,
- the effects of Skylab missions on salivary electrolyte levels.

High resolution gas liquid chromatographic (GLC) and pyrolysis-GLC procedures were used to obtain distinctive metabolic profiles of closely related genera, species and strains of oral microbes associated with dental caries and periodontal disease. Emphasis was placed on serologically unique strains of Streptococcus mutans. Bacterial filtrates of spent media from mid-log cultures were processed for extraction of volatile organic compounds by a headspace concentration technique using a solid adsorbent. The evolved vapors were trapped onto a porous polymer adsorbent trap (Tenax GC 35/60 mesh). Lyophilized or washed cells from the cultures were used for pyrolysis-GLC. High resolution capillary columns were employed to elicit the microbial metabolic profiles. The samples were initially pyrolized under flowing helium, and sample products were analyzed in a SE-30 coated capillary column.

- The high resolution GLC techniques disclosed pronounced metabolic distinctions between two physiologically related genera of the same bacterial family, and between two closely related oral species of the same genus.
- Differences between and within serologically unique strains of the same species were detected, but were much less striking than between inter-species profiles.
- The GLC procedures provided a rather practical and reproducible means of obtaining metabolic markers for identifying closely related strains of pathogenic oral microbes unattainable by conventional techniques.

Fractions of stimulated whole saliva samples from the prime and back-up crews of the three Skylab missions were used to measure saliva electrolyte concentrations. All the electrolytes previously reported as having increases in urine and feces during the missions were assessed in saliva. Sodium, potassium, calcium, and magnesium were analyzed by atomic absorption utilizing a Pye-Unican 1900 double beam spectrophotometer. Phosphorous was assessed by a referenced micromethod using a Beckman 150 microspectrometer, and chloride was determined by a referenced micro-titration procedure. Data obtained during the three phases of the Skylab missions (pre-flight/conventional diet, pre-flight/space diet, and recovery/space diet) were compared by an unbalanced two-way analysis of variance.

- The effects of the Skylab missions on salivary electrolyte levels in crew members were limited to a statistically significant decrease in sodium, and an increase in magnesium.

- The mineral imbalance attributable to the mission-related increases in urinary electrolytes were not detectable in this study, perhaps because: a) saliva does not play a decisive role regulating water and electrolyte balance since it is essentially recycled by absorption from the gut; b) saliva values were based on single sample evaluations while urinary values were based on 24-hour collections; and, c) there were no in-flight saliva samples to compare with in-flight urine samples.

Oral health studies of the Skylab series of manned space flights were both clinically and basic science oriented. The clinical part encompassed a prevention program, pre- and post-flight restorative procedures, measurements of oral health indices, and in-flight provisions for emergency dental care. The basic science aspect involved the pre- and post-flight monitoring of changes in the oral microflora and secretion of specific salivary components. The clinical, oral microbiologic, and immunological changes found during each of the Skylab missions have been included in previous progress reports. This report details the data relating to: 1) the establishment of metabolic profiles for fingerprinting microorganisms associated with oral pathoses; and, 2) the effects of Skylab missions on salivary electrolyte levels.

A practical and reproducible method is needed to definitively identify closely related strains of oral microorganisms associated with dental disease that are presently difficult to distinguish by conventional methodology. High resolution gas liquid chromatographic techniques have been used to characterize soil samples and tissue exudates. These procedures provide a means to effectively establish metabolic profiles (metabolic fingerprints) between physiologically and serologically closely related, potentially pathogenic oral microbes. Such metabolic fingerprints might yield explicit recognition patterns to definitively identify closely related strains of microbial species.

Saliva plays an important role in the maintenance of oral health. Changes in saliva composition and volume may reflect local and systemic disorders. Changes in the concentration of salivary electrolytes may be due to emotional stress, drugs, drug toxicity, metabolic changes, and

systemic disease. If the mineral imbalance revealed by the urinary and fecal elevations in specific electrolytes during the Skylab missions is also evidenced by electrolyte changes in saliva, prolonged space flight might have an impact on the buffer systems in saliva and thereby upset a basic homeostatic mechanism in the oral cavity.

The specific aims of this study were twofold: 1) to adapt and/or develop analytical chemical procedures to establish metabolic profiles for fingerprinting microorganisms associated with oral pathoses; and, 2) to assess the effects of the 28-, 59- and 84-day Skylab missions on the salivary electrolytes.

PROCEDURES

1. Gas Liquid Chromatography Procedures for Establishing Microbial Metabolic Profiles. Uninoculated culture media and pure cultures of known strains of the same and different serotypes of cariogenic and noncariogenic Streptococcus mutans, Strep. sanguis, and Lactobacillus fermenti, and uncharacterized strains of Strep. mutans isolated from crew members of the Skylab missions were analyzed. Emphasis was placed on the definitive characterization of Strep. mutans since this microorganism has been directly implicated in the etiology of dental caries and indirectly associated with periodontal disease. Although strains of this organism can be characterized into four different serological groups, biochemical reactivity among the strains is for the most part indistiguishable. Growth characteristics and carbohydrate fermentation do not differ in gross detail from the other oral streptococci or lactobacilli. A high prevalence of Strep. mutans in the crew members of the Skylab missions provided a specific interest for fingerprinting the different strains of this organism.

The study was designed to delineate: a) the compounds present in the media (background); and, b) the metabolites produced by the test organisms. Cell-free filtrates from both glucose and sucrose containing broth were obtained at the mid-log phase of growth for chromatographic analyses of the metabolites in spent media (culture supernatants), and washed cell suspensions were used for chromatographic analysis of pyrolyzed cells.

Bacterial filtrates (20-50 ml) were heated to 100°C and continuously purged with helium or nitrogen at 20 ml/minute for one hour for extraction of volatile organic compounds by a high resolution (headspace concentration) technique using a solid adsorbent. The evolved vapors were trapped onto the porous polymer adsorbent trap (Tenax GC 35/60 mesh). Moisture was removed with a water cooled condensor in series with the Tenax trap. Following the sampling period, the trap was stored in a teflon lined screwcapped test tube. The analytical procedure involved the insertion of the trap into a modified injector port of a Perkin-Elmer 900 gas chromatograph. The volatile compounds were heat desorbed at 200°C for 20 minutes at helium flow of 20 ml/minute. In order to simulate a syringe injection technique, the volatiles were trapped into a dry ice cooled pre-column. An 8-port valve joining the separating column and the pre-column was turned to direct the sample from the pre-column to the separating column. Chromatography began when the pre-column was removed from the dry ice. Chromatographic conditions were: carrier, He 7 ml/min; air, 300 ml/min; H_2, 14 psi; detector, flame ionization; and, column, 465 feet x 0.02 inch I.D. nickle capillary needle stock coated with 10% DC 200.

For pyrolysis-gas liquid chromatography, lyophilized or washed cells (0.1 - 1 mg) were prepared. High resolution capillary columns were used to increase the probability of obtaining profiles unique to a specific organism. The samples were initially pyrolyzed in a flowing helium environment at 200°C and 500°C (60 seconds each), and analyzed for products on a SE-30 coated glass capillary column (60 meters x 0.3 minute I.D.). These parameters were believed to be the likely ones for detection of pyrolytic profile differences for the various strains analyzed.

2. Procedures for Salivary Electrolyte Determinations. Over 100 saliva samples from the prime and back-up crew members of the three Skylab missions were available at the Dental Science Institute for assay. Since other investigators associated with the Skylab missions reported a mineral imbalance as displayed by increased fecal and urinary excretion of calcium, phosphorous, chloride, potassium, magnesium and sodium, the emphasis in this study was placed on quantitative estimates of these electrolytes in stimulated whole saliva.

For comparative purposes, the saliva samples were grouped into three categories corresponding to the various phases of each mission: 1) pre-flight/conventional diet; 2) pre-flight/space diet; and, 3) post-flight/space diet.

Atomic absorption analysis, utilizing a Pye-Unican 1900 double beam spectrophotometer equipped with an emission mode and digital read out was used for the quantitative determination of sodium, potassium, calcium and magnesium in all samples. Sodium and potassium were determined by emission spectrometry according to the manufacturer's procedure. Calcium and magnesium were measured in 0.1 ml of saliva diluted to 10 ml with 0.125% Lanthanum chloride solution.

Phosphorous determinations were made by the micromethod of Fiske and Subbarow (J. Biol. Chem. 66:375-400, 1941) in a Beckman 150 micro-spectrometer. Chloride was determined by microtitration with mercuric nitrate using a diphenylcarbozone indicator according to the method of Schales and Schales (J. Biol. Chem. 140:879-883, 1941).

All data were recorded for computerization and statistical analyses using an unbalanced analysis of variance for multiple comparisons of individual, paired and grouped data. Comparisons were made among the three segments of data (pre-flight/conventional diet, pre-flight/space diet, and recovery/space diet).

RESULTS

1. Gas Liquid Chromatography Procedures for Establishing Microbial Metabolic Profiles. It was thought that volatile substances in a complex bacterial medium could easily be detected in this study, but it was not known to what extent these compounds would interfere with the production of metabolic profiles from bacteria. The first sample analyzed was the media control and as expected, a complex chromatogram resulted. However, the contribution of volatiles from the bacteria could be distinguished from media volatiles by background subtraction (this may be automated in future work). Previous experience has shown that classes of compounds such as alcohols, ketones, aldehydes, sulfur compounds and hydrocarbons may be concentrated and analyzed by the Tenax procedure. These compounds are of lower molecular weight (<250 M.W.) and range from C_8 - C_{16} (aliphatic hydrocarbons).

After the three bacterial filtrates were analyzed (first series), comparisons were made by roughly determining which peaks in the filtrates

were not indigenous to media peaks. Eleven peaks were tentatively assigned to the three filtrates that did not appear in the control. Of the differences found in the three filtrates, the most distinguishable were those between the Lactobacillus and Streptococcus genera. Two streptococcal strains were very similar and difficult to distinguish, although some differences were noted.

Initial work (not statistically valid) was therefore viewed with some promise, even though background from the media appeared to be a formidable problem. It was then decided to analyze a second series of samples which included the addition of glucose (as opposed to sucrose) to the media. The dextrans produced by bacteria grown in a sucrose broth made filtration very difficult. This problem did not exist when glucose was added. It was hoped that glucose supplemented broth could be substituted for the previously used sucrose broth. Accordingly, three additional samples (second series) were analyzed using the glucose medium control and two Strep. mutans glucose-spent media. The glucose medium control appeared less complex than the sucrose medium.

Seven more samples (third series) were analyzed including a repeat of the original samples. Several of the samples in the third series also included comparisons of modified extraction (e.g., salt vs. no salt, sample size - 5 ml, 20 ml, 50 ml) techniques. Salt $\overline{(NH_4)_2SO_4}$ had been used in similar work to increase sample volatilization and was used up to this series. It was hoped that eliminating this step would enhance the sampling technique. It was found that salt did increase volatilization. Therefore, salt was eliminated from future work.

The third series produced quite interesting data which in normalized

representation (skeletons) showed different profiles for the three organisms tested. Although a major peak was found in the first series that was absent in the third series, data were reproducible when duplicates were run of each separate series.

Analyses employing pyrolysis-GLC have been performed on a small number of pure cultures. The metabolic differences obtained from two closely related species (Strep. mutans and Strep. sanguis) are shown by 12 distinguishable peak differences in Figure 1. Efforts are presently underway to establish the reproducibility of the pyrolysis-GLC technique and compare it with that obtained by the headspace technique.

2. Effects of Skylab Missions on Salivary Electrolytes. Saliva electrolyte data monitored during each of the three Skylab missions are summarized in Figure 2. The values (milliequivalents/liter) are expressed as cumulative means of the nine prime crew members of the missions. The data of the combined missions are representative of those obtained during the individual missions. Similarly, the pre-flight values of the prime crew members are comparable to those obtained from the back-up crew members. Except for sodium and magnesium, no statistically significant changes were found among the three segments of data compared (pre-flight/conventional diet vs. pre-flight/space diet vs. post-flight/space diet). Salivary sodium demonstrated a statistically significant ($P = .01$) decrease concomittantly with a statistically significant increase ($P < .01$) in saliva magnesium. Both changes were relatively persistent through the period of study. Salivary levels of potassium, calcium, and chloride showed some tendency to increase during the missions, but only the increase of potassium during the 59-day mission was statistically significant ($P < .04$)

by a two-way analysis of variance. There were statistically significant inter-astronaut differences in saliva levels of most of the electrolytes during each mission. There also was considerable intra-astronaut variation among samples within data segments. This was particularly evidenced by levels of K^+ and $PO_4^=$ which fluctuated noticeably at the time of space diet incorporation (approximately F-20, Figure 1).

DISCUSSION

The gas liquid chromatographic techniques employed in this study to obtain metabolic profiles for differentiating closely related organisms showed rather pronounced metabolic distinctions between two physiologically related genera (Streptococcus and Lactobacillus of the same bacterial family (Lactobacillaceae). Metabolic differences also were quite distinct between closely related oral species of the genus Streptococcus (mutans and sanguis). Although chromatograms demonstrated several differences between and within serologically distinguishable strains of Strep. mutans, the differences were much more subtle than between the interspecies profiles.

The problem of background subtraction of peaks contributed by culture media may be eliminated by the use of a suitable synthetic medium for culturing cariogenic bacteria. Such a medium has been tried and it has greatly reduced the problem of background interference.

The headspace concentration technique offers an alternative approach to fingerprint bacteria metabolically and to determine the nature and quantity of the volatile organic metabolites produced. Metabolite identification may be best achieved by combining high resolution gas chromatography with mass spectrometry.

High resolution capillary columns are superior to packed columns for the separation of complex biological mixtures of similar polarity, as the results reported here demonstrate. Another important reason for using capillary columns is that the degree of complexity may readily be ascertained. In some cases, this may dictate the use of other types of columns including packed columns. The addition of mass spectrometry will serve two purposes: a) to identify significant compounds; and, b) to enhance resolution of overlapping GC peaks.

This investigation stresses the convenience of using a solid adsorbent for concentrating trace metabolites (sampling, storage, analysis) and the potential for expanding information on metabolic profiles or fingerprints of dental caries organisms. This has never been previously approached from the aspect of using a solid adsorbent in combination with capillary columns.

Although analyses employing pyrolysis-GLC have been performed on only a small number of pure cultures, results obtained by this procedure are very encouraging. The potential of this procedure for distinguishing inter-strain differences within species, however, awaits the establishment of reproducibility and comparison with the headspace technique.

The effects of the Skylab missions on salivary electrolyte levels in crew members were limited. Although magnesium, calcium, potassium and chloride demonstrated modest increases during the period of assessment, only the change in magnesium was statistically meaningful. The magnesium increase was observed during each mission and showed less inter-individual and intra-cell variation than the other salivary partitions monitored.

The magnesium increase as well as the statistically significant decrease in salivary sodium may have been related to the increase of adrenocortical steroids, particularly the aldosteronism evidenced by urinary increases of aldosterone during each mission. Both primary aldosteronism and pseudoprimary aldosteronism and hypertension have been shown to decrease sodium in parotid and submaxillary saliva secretions (Wotman, S. and Mandel, I.P., Postgrad. Med. 53:73-82, 1973). In addition to the administration and/or elaboration of excessive amounts of adrenocortical steroids, low salt diets are associated with sodium depletion. However, a deficit in sodium intake could not account for the salivary sodium decrease observed in this study since the dietary intake of electrolytes was closely monitored, and supplemented as necessary, to compensate for a dietary electrolyte imbalance during Skylab missions.

The salivary electrolyte changes did not parallel the increased urinary electrolyte excretion observed during each mission. This indicates that saliva does not play a decisive role in regulating the water and electrolyte balance of the body. However, the lack of correlation between the salivary and urinary changes in electrolyte concentration may simply stem from the difference in comparing a 24-hour collection of urine with a few minutes collection of saliva.

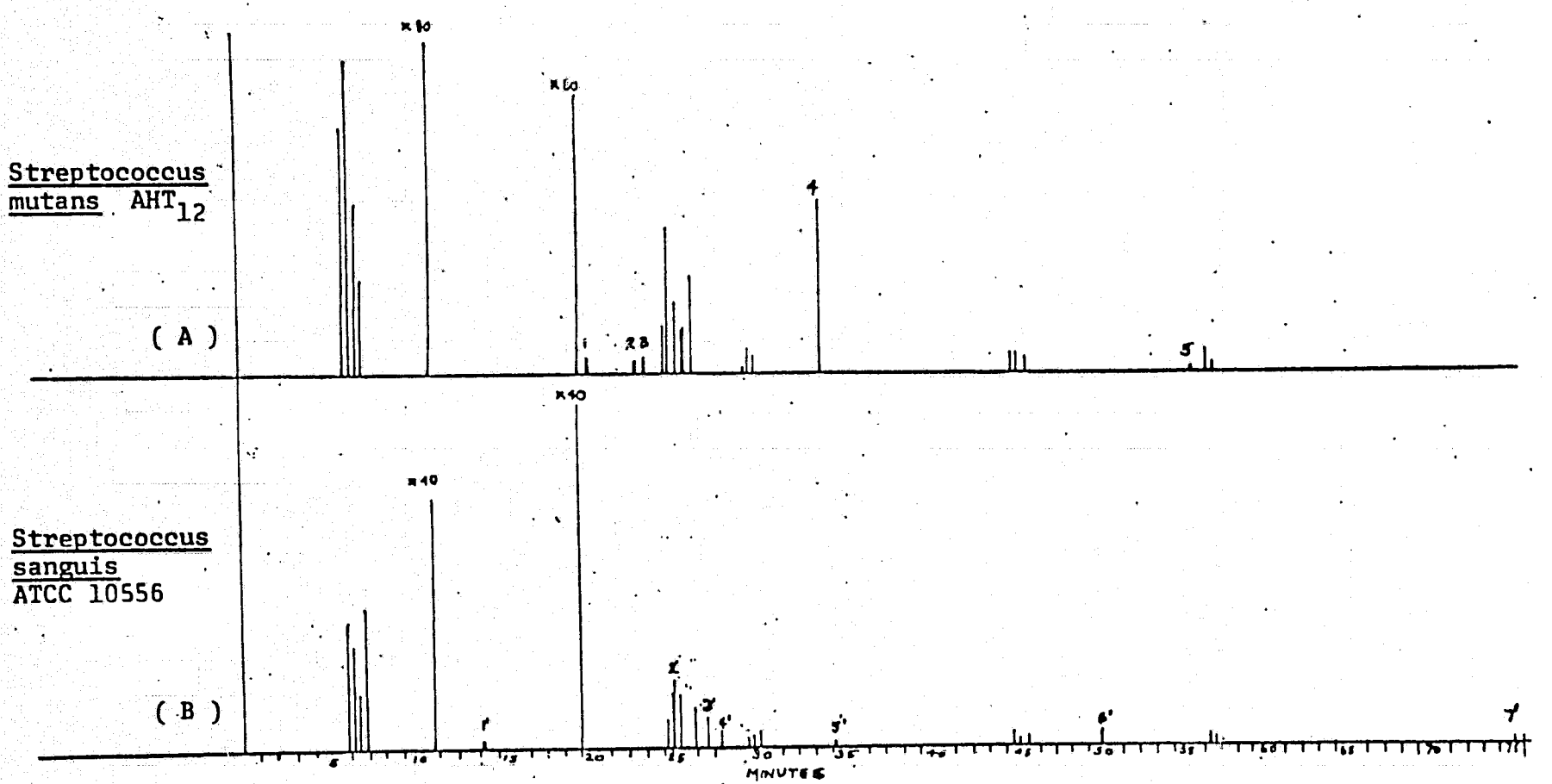

Figure 1. Histogram of absolute peak heights and retention time demonstrating metabolic differences between Streptococcus mutans AHT_{12} (A) and Strep. sanguis ATCC 10556 (B). Strep. mutans (A) displayed 5 compounds (as numbered) not found in (B), and Strep. sanguis (B) demonstrated 7 compounds not found in (A).

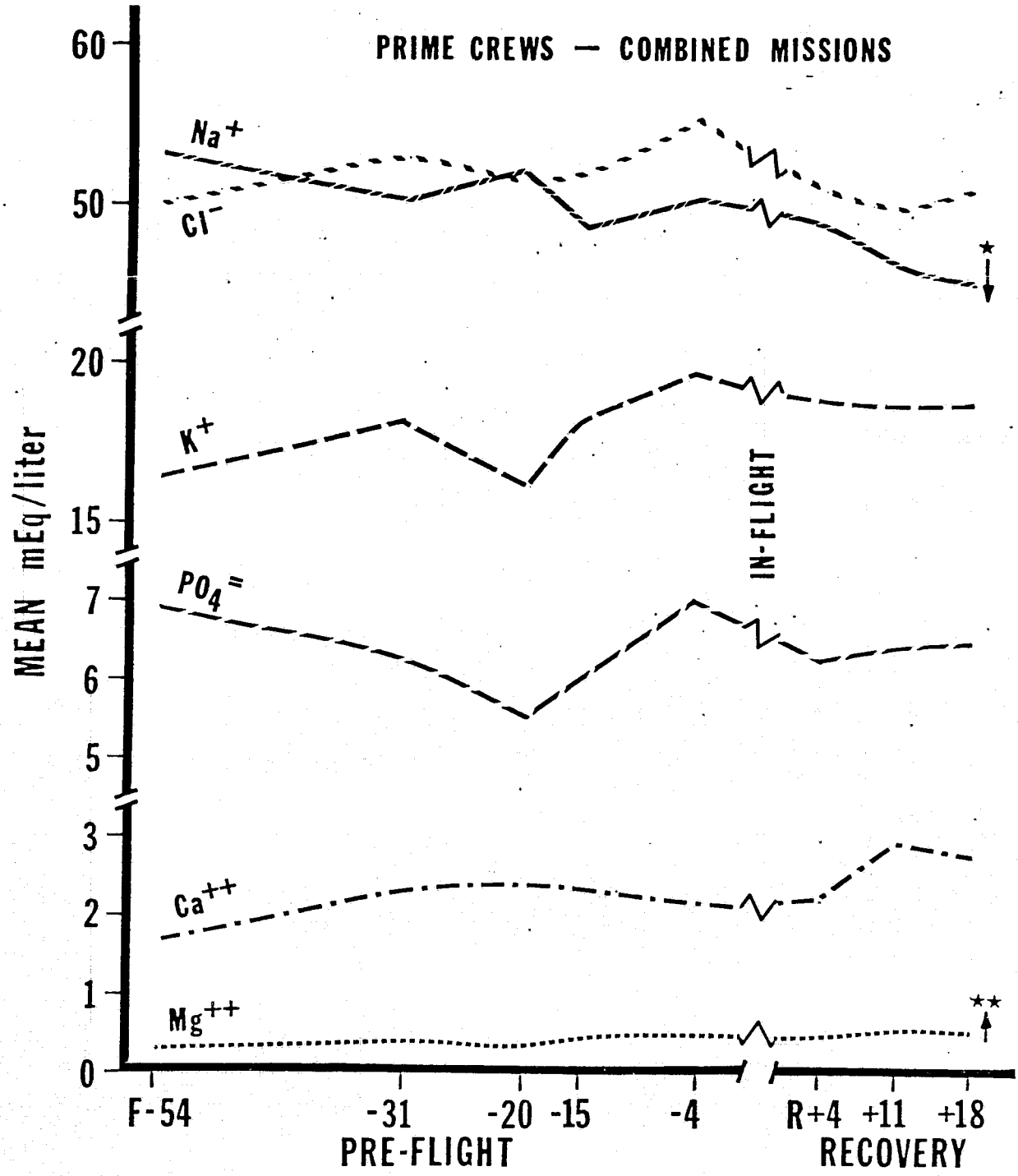

Figure 2. Cumulative means of salivary electrolyte estimates from nine astronauts combined from the 28-, 59-, and 84-day Skylab missions. *Probability of Na^+ decrease =1% by unbalanced two-way analysis of variance with 2 degrees of freedom (df), a 128.9 mean square (MS), and 4.7 F ratio. **Probability of Mg^{++} increase =1% with 2 df, .08 MS, and 5.0 F ratio.