16613

Problems in Determining the Bacterial Flora of the Pharynx.*

COMMISSION ON ACUTE RESPIRATORY DISEASES.†
(In collaboration with Walter A. Mickle and Thomas J. Oliver.)
From the Respiratory Diseases Commission Laboratory, Regional Station Hospital, Section 2, Fort Bragg, N. C.‡

The role of bacteria in the pathogenesis of the common respiratory diseases has not been clearly defined. Attempts have been made to correlate the pharyngeal flora with the presence or absence of upper respiratory infection and to determine whether or not changes in flora occur during the course of illness.[1-14] The results have been inconstant and difficult to interpret. Consideration of the methods employed suggests that technical and nutritional limitations may have been responsible, in some instances, for apparent differences in flora, while in other instances, such limitations may have obscured changes in flora that were present.[12] A study has therefore been made to define some of the problems of interpreting the results of pharyngeal cultures and to develop a technic that would yield reproducible as well as interpretable data. The investigations were carried out in conjunction with other studies on respiratory disease.[15-18]

This report presents certain of the results under 3 headings: a) technic, b) quantitative growth and nutritional interdependence, and c) use of a selective medium for detecting *beta*-hemolytic streptococci.

Technic. Two of the technical difficulties

* This investigation was supported through the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Office of the Surgeon General, U. S. Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation and the International Health Division of the Rockefeller Foundation to the Board for the Investigation and Control of Influenza and Other Epidemic Diseases for the Commission on Acute Respiratory Diseases.

† Members and professional associates of the Commission on Acute Respiratory Diseases were: John H. Dingle, Director; Theodore J. Abernethy; George F. Badger; Joseph W. Beard; Norman L. Cressy; A. E. Feller; Irving Gordon; Alexander D. Langmuir; Charles H. Rammelkamp, Jr.; Elias Strauss; Hugh Tatlock.

‡ Now located at the Department of Preventive Medicine, School of Medicine, Western Reserve University, Cleveland, Ohio.

1 Bloomfield, A. L., *Johns Hopkins Hosp. Bull.*, 1921, **32**, 33.

2 Bloomfield, A. L., *Johns Hopkins Hosp. Bull.*, 1921, **32**, 121.

3 Mudd, S., Grant, S. B., and Goldman, A., *Ann. Otol., Rhin., and Laryng.*, 1921, **30**, 1.

4 Shibley, G. S., Hanger, F. M., and Dochez, A. R., *J. Exp. Med.*, 1926, **43**, 415.

5 Burkey, E. L., and Smillie, W. G., *J. Exp. Med.*, 1929, **50**, 643.

6 Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1932, **55**, 445.

7 Kneeland, Y., Jr., and Daws, C. F., *J. Exp. Med.*, 1932, **55**, 735.

8 Dochez, A. R., *Medicine*, 1933, **12**, 245.

9 Hoyle, L., *J. Hyg.*, 1934, **34**, 195.

10 Mulder, J., *Acta Med. Scandinav.*, 1938, **94**, 98.

11 Straker, E., Hill, A. B., and Lovell, R., Ministry of Health, Reports on Pub. Health and Med. Subjects, 1939, No. 90.

12 Dingle, J. H., Abernethy, T. J., Badger, G. F., Buddingh, G. J., Feller, A. E., Langmuir, A. D., Ruegsegger, J. M., and Wood, W. B., Jr., *Am. J. Hyg.*, 1944, **39**, 269.

13 Cecil, R. L., Plummer, N., and Smillie, W. G., *J.A.M.A.*, 1944, **124**, 8.

14 Julianelle, L. A., and Siegel, M., *Ann. Int. Med.*, 1945, **22**, 10.

15 Commission on Acute Respiratory Diseases, *J.A.M.A.*, 1944, **125**, 1163.

16 Commission on Acute Respiratory Diseases, *Am. J. Pub. Health*, 1945, **35**, 675.

17 Commission on Acute Respiratory Diseases, *Johns Hopkins Hosp. Bull.*, 1946, **79**, 97.

18 Commission on Acute Respiratory Diseases, *Medicine*, 1947, **26**, 465.

TABLE I.
Comparison of Results with Two Methods* of Examining Throat Swabs.

Organism	Dry swab positive Suspension positive	Dry swab positive Suspension negative	Dry swab negative Suspension positive	Dry swab negative Suspension negative
	No. of swabs			
Beta-hemolytic streptococci	2	1	4	69
S. aureus	1	0	3	72
H. influenzae	51	0	11	14
H. hemolyticus	52	3	4	17
Total	106	4	22	172

* See text.

in culturing the throat reside in (*1*) the transference of bacteria from the cotton swab to the culture plate in a manner such that the resultant growth will reflect quantitatively the various species of bacteria on the swab, and (2) the visual identification of species by their colonial morphology prior to picking the colony and identifying the pure culture so obtained.

The problem of transferring bacteria from the swab to the medium was approached by comparing the results obtained by steaking the swab directly on the medium in the manner previously employed,[12] with those obtained by suspending the material on the swab in broth prior to inoculation of the medium.

Each of 76 swabs, rubbed thoroughly over the tonsils and oropharynx, was streaked directly onto a blood agar plate. Each swab was then immersed in 5 ml of tryptose-phosphate broth. With the swab in place, the broth was drawn into and expelled from a 5 ml pipette 20 times. From this tube 0.5 ml was then transferred to a second 5 ml portion of broth, and mixed as above with the same pipette. Using a 1.0 ml pipette, 0.1 ml of the suspension was transferred to the surface of a blood agar plate (tryptose-phosphate agar base). The inoculum was spread with a glass rod which had been immersed in 95% alcohol and flamed. Spreading was facilitated by revolving the plate on a turntable. After incubation at 37°C for 24 hours the plates inoculated by each method were examined with a wide field binocular microscope ("colony" microscope). Typical colonies were picked and identified by standard confirmatory procedures.

Table I shows the results obtained in respect to 4 bacterial species. In 22 instances one or more of these 4 species was detected by the broth suspension method and not by the dry swab method, while the reverse situation obtained in only 4 instances. These data indicate that the bacterial contents of a swab can be determined better by preparing a suspension in broth than by direct inoculation from the swab.

Further experiments were designed to determine the variations encountered in the broth suspensions. In each experiment, a throat swab was obtained as described above and a broth suspension prepared. Each of 5 plates was then inoculated with 0.1 ml of the suspension. After incubation, the plates were examined with a "colony" microscope and representative colonies picked and identified. The plates were given code numbers so that the examiner was not aware of which plates were inoculated from each suspension. The results of 35 experiments are shown in Table II.

The variations encountered were greater than should arise from a homogeneous suspension, but were less than those observed in similar earlier experiments where mixing was known to have been inadequate. It is apparent that certain organisms were recovered from many of the suspensions with such low frequency that they would have been missed on a single plate regardless of the method of mixing employed.

In order further to evaluate the degree of homogeneity that could be achieved in a bacterial suspension, experiments were carried out with pure cultures. A satisfactory method of

TABLE II.
Relative Frequency of Various Organisms in a Series of Experiments in Which Five Plates Were Prepared from Each Swab.

Swab No.	Readings* Plate No. 1	2	3	4	5
	Beta-hemolytic streptococci (a)				
1	0	0	0	0	1
9	0	0	0	0	1
15	+	+	+	+	+
20	5	3	1	3	0
22	6	13	3	3	6
30	+	+	+	+	+
33	+	+	+	+	+
	H. influenzae (a)				
4	NR	0	15	15	0
5	7	0	0	5	0
8	4	0	13	0	0
12	1	0	0	0	0
13	2	0	0	3	3
14	6	4	0	2	2
15	3	0	5	1	2
16	2	3	0	0	0
18	0	1	0	7	1
21	+	+	+	+	+
22	0	1	1	0	0
23	11	6	12	7	9
24	0	1	0	0	2
28	+	+	+	+	+
29	10	14	15	9	18
30	0	0	3	0	2
31	++++	++++	++++	++++	++++
32	10	5	8	2	14
34	+++	+++	+++	+++	+++
35	+	+	+	+	+
	S. aureus (b)				
1	0	11	0	2	2
5	1	0	0	0	0
8	0	0	0	0	+
11	2	1	0	0	0
17	0	8	0	0	0
18	0	0	0	0	1
20	0	0	3	0	0
	H. hemolyticus (c)				
11	0	1	0	4	0
12	0	3	0	0	3
13	3	7	12	11	8
14	0	0	1	0	0
16	5	1	3	6	2
19	0	2	0	0	0
20	3	2	4	0	1

* NR = Not Read. Numerals indicate number of colonies of the organisms, which were counted if less than 20.

\+, ++, +++, ++++ } If more than 20 colonies of the organisms; this scale is an estimate of the relative number of colonies of this organism to the total number of colonies on the plate.

(a), (b), (c); plates examined for these organisms were from individuals numbered: (a) 1-35; (b) 1-20; (c) 11-20.

suspending organisms would be one resulting in a random distribution of colony-producing units (whether they be single or clumped organisms) within the resulting suspension. The distribution of such units in a suspension may be determined by making plate counts on measured aliquots from the suspension. The total variation in plate counts should arise from a combination of technical errors or limitations, and sampling variation.

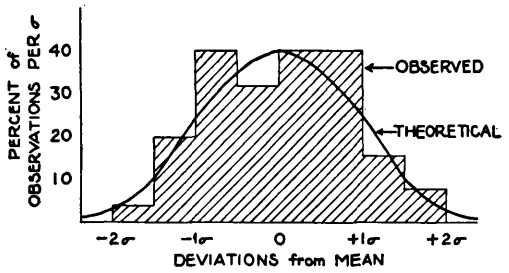

FIG. 1.

Variation in plate counts in a series of experiments in each of which several plates were inoculated with portions of a given suspension.

A loopful of broth culture of *beta*-hemolytic streptococci was transferred to 10 ml of broth and shaken a few times by hand. One loopful was then transferred to 3 ml of broth. The inoculated broth was mixed 20 times with a 5 ml pipette. Five pour plates were prepared using 0.5 ml of the suspension for each plate. Tryptose-phosphate agar with peptic-digested blood was used as the medium. Colonies were counted after the plates had been incubated at 37°C for 24 hours. The experiment was repeated 10 times for a total of 50 plates.

The results of the 10 experiments have been combined into a single experience for the purpose of measuring the variation in successive samples (Figure 1). A theoretical distribution curve is also shown for comparison with the observed results. The theoretical curve shows the results expected if the only source of variation were sampling from a homogeneous suspension.§ The agreement of observed and expected results is close, permitting the conclusion that the suspensions were essentially homogeneous.

Similar experiments were carried out with each of 3 other organisms (*Staphylococcus aureus, Hemophilus influenzae,* and a gram-negative coccus). Variations in plate counts were somewhat greater than expected due to sampling, but not grossly so.

The second technical difficulty investigated —that of the need of visual aid in identifying the colonial characteristics of bacteria—is apparent from Table III. The first 2 columns show the accuracy of naked eye identification of colonies as they appeared on blood agar plates inoculated directly with throat swabs. The last 2 columns show the improvement accomplished by using the wide-field binocular microscope (magnification 15x) to examine plates prepared by the broth-suspension technic.

The advantage of microscopic examination is that it facilitates the accurate differentiation of colonial types and, therefore, leads to the detection of colonies which might have been missed by the naked eye. The fact that it makes possible an increased accuracy of naming colonies is important when a survey is being made for the presence or absence of a particular organism.

The above results indicated that the preparation of a broth suspension from a swab and the use of a microscope in reading plates provided an improved, though not yet ideal, cultural technic, and decreased considerably the errors incurred when the swab was rubbed directly on the culture medium. The data further indicate, however, that the inoculation of only a single plate from the broth suspension, in contrast to multiple plates, considerably decreases the likelihood of determining reliably the presence or absence of an organism of relatively low frequency.

Quantitative growth and nutritional interdependence. The preceding data indicated that a broth-suspension of a single bacterial species can be prepared that for practical purposes is homogeneous, as indicated by growth on the surface of a plate. Similar experiments were then carried out using a mixture of pure cultures of 2 species of bacteria to determine whether or not the growth of each would be quantitative. If such were the case, the colonies of the 2 species should be scattered at random over the surface of the medium. In one set of experiments, *S. aureus* and *beta*-hemolytic streptococci were used; in the second set, *H. influenzae* and *beta*-hemolytic streptococci were employed, because of the nutritional dependence of *H. influenzae* on

§ If the only source of variation were sampling from a homogeneous suspension the counts in a given experiment should have formed a hypergeometric distribution with a standard deviation equal to the square root of 5/6 of the mean plate count.

TABLE III.
Accuracy of Identification of Colonies with a) No Visual Aid and b) Using the Microscope.

Colony tentatively identified as	(a) No visual aid		(b) Using microscope	
	No. of colonies	% confirmed	No. of colonies	% confirmed
Beta-hemolytic streptococcus	75	40	182	92
S. aureus	20	95	142	96
Pneumococcus	66	5	337	51
H. influenzae	174	53	566	99
H. hemolyticus	94	82	486	97

TABLE IV.
Distribution of Fields of 100 Colonies of a) a Mixture of *Beta*-hemolytic Streptococci and *S. aureus*, and b) *a* Mixture of *Beta*-hemolytic Streptococci and *H. influenzae*, According to Number of Streptococci per Field.

Deviation* from mean number of Strep. per field	a) Mixture of *beta*-hemolytic streptococci and *S. aureus* No. of fields		b) Mixture of *beta*-hemolytic streptococci and *H. influenzae* No. of fields	
	Observed	Expected	Observed	Expected
−2.0 or more		3.3		1.2
−1.5 to −1.9	1	6.4		2.2
−1.0 to −1.4	12	13.3		4.6
−0.5 to −0.9	28	21.7	8	7.5
0 to −0.4	25	27.8	19	9.6
0 to 0.4	42	27.8	17	9.6
0.5 to 0.9	24	21.7	6	7.5
1.0 to 1.4	12	13.3		4.6
1.5 to 1.9	1	6.4		2.2
2 or more		3.3		1.2
Total	145	145	50	50

* In units of $\sqrt{npq}$

other bacteria, including *Streptococcus hemolyticus*.[19]

In the first group of experiments a 3 ml broth suspension of *S. aureus* and *beta*-hemolytic streptococci was mixed with a 5 ml pipette 20 times. One-tenth ml of this mixture was placed in the center of each of 5 blood agar plates. Each plate was then rotated, using a mechanical turntable, while a sterile glass spreader distributed the inoculum evenly. After incubation at 37°C for 24 hours, the plates were examined with a wide field binocular microscope.

In order to learn whether or not the two organisms were distributed at random over the surfaces of the plates, colonies were counted differentially in fields of 100 colonies each. Forty-five to 50 such fields were counted on the 5 plates. This experiment was repeated 3 times, for a total of 145 fields.

[19] Davis, D. J., *J. Infect. Dis.*, 1921, **29**, 178.

If the colonies of streptococci and staphylococci were distributed at random, the distribution of the number of streptococci per field should be a normal one with a standard deviation of $\sqrt{npq}$ (where p is the proportion of streptococcal colonies, q the proportion of staphylococcal colonies, and n, the number of colonies per field = 100).

Table IV summarizes the differential counts of 145 fields in terms of deviation from the mean‖ and shows that less variation was observed than expected. For example, the expected number of fields with a deviation

‖ For example, in 50 fields counted from one series of 5 plates the average number of streptococci per field was 24.6, and the standard deviation was 4.3 streptococci per field. Eight of these fields showed 27 colonies of streptococci; this was 2.4 colonies more than the average. On a scale of standard deviations these 8 observations fall at $\frac{2.4}{4.3} = +.56$ standard deviation from the mean.

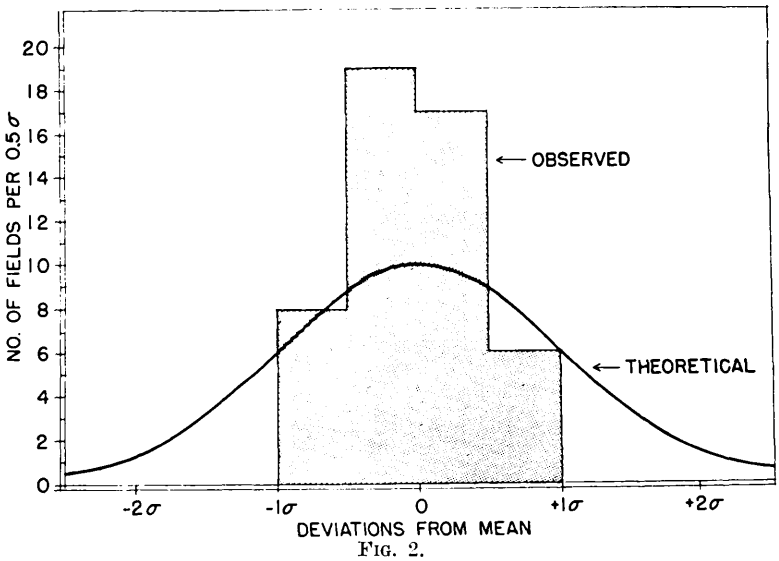

FIG. 2.

% distribution of fields of 100 colonies of a mixture of *Beta*-hemolytic streptococci and *H. influenzae*, according to number of streptococci per field.

of −1.5 standard deviations or greater was 10 while actually only one was observed. Again at the other end of the scale, fields with a relatively high proportion of streptococci were observed less frequently than was expected.

Such lack of variation between the differential counts in various fields is indicative of bias. It suggests that the colonies grew as if the organisms had been placed in relationship to one another in an orderly distribution and not at random, and indicates a relationship, nutritional or otherwise, between the numbers of bacteria of each species which develop into colonies. Probably the presence of one species enhanced the growth of the other.

The second set of experiments was performed in the same manner with a mixture of pure cultures of *H. influenzae* and *beta*-hemolytic streptococci. A total of 50 fields was counted differentially. The lack of variation in differential counts (Table IV and Figure 2) was more marked than with the mixture of *beta*-hemolytic streptococci and *S. aureus*, indicating even a greater dependence of one upon the other.

These observations suggest that the growth on a plate is a resultant of the nutritional inter-relationship of the organisms and the adequacy of the media. The total number of colonies of a given species may be influenced to an undetermined degree by the relative presence or absence of other species. With the large variety of bacteria found in a throat culture this relationship is probably very complex, since not only symbiosis but also antagonism may be involved. Unless these relationships can be circumvented, possibly by selective media or by media adequate for the nutritional demands of all pharyngeal flora, it seems impossible to interpret quantitatively the growth which appears on the usual culture plate.

Thus the absolute or relative growth of colonies on the plate does not necessarily reflect the occurrence and distribution of bacteria in the pharynx. This consideration assumes importance in trying to determine whether organisms normally present in the throat are increased in number in the presence of infection.

Use of a selective medium for detecting beta-*hemolytic streptococci.* Selective media as yet provide a means of detecting the presence of only a limited number of bacteria found in the upper respiratory tract. Because of special interest in *beta*-hemolytic

TABLE V.
Comparison of Two Methods for Detecting *Beta*-hemolytic Streptococci.

	No. positive by Pike method	No. negative by Pike method	Total
Positive by direct method	21	3	24
Negative by direct method	31	195	226
Total	52	198	250

TABLE VI.
Number of Isolations of *Beta*-hemolytic Streptococci from Men Cultured Three Times.

Group and type of streptococcus	1st culture (direct)	2nd culture (Pike)	3rd culture (direct)
Group A, no type*	47	118	48
" A, typable	19	24	7
" B	3	63	1
" C	15	49	27
" F	1	3	5
" G	7	32	12
No group	3	4	2
Not done	1	0	1
Total	96	293	103

* Sera for types 1, 2, 3, 4, 5, 6, 10-12, 11, 14, 17, 18, 19, 22, 23, 24, 26, 28, 29, 30, 31, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 44, 46, and 47 were employed throughout most of the study. Types 8, 9, 13, 15 and 32 were available for part of the period.

streptococci, two studies were made to test the advantage of Pike's selective medium for this organism.[20,21]

In the first study a total of 250 throat swabs was obtained by culturing 125 well soldiers on each of two occasions. Each swab was streaked directly on a blood agar plate, and then treated according to Pike's method. The results are shown in Table V.

The superiority of the selective medium is shown by the fact that *beta*-hemolytic streptococci were found by Pike's technic in 52 of the 250 swabs (20.8%), but in only 24 (9.6%) by the direct plating method. The Pike method detected the organism from 31 swabs which the direct plating method missed, while 3 swabs were positive by the direct method of plating and negative by the Pike method.

In the second study cultures were obtained on 3 occasions from a battalion of approximately 1050 men. The first and third cultures, obtained 3 weeks apart, were rubbed directly onto blood agar plates. The second cultures, taken during the intervening period, were treated by the Pike technic. Grouping of *beta*-hemolytic streptococci was performed by the capillary tube precipitin technic, using commercial rabbit serums and bacterial extracts prepared by Fuller's method.[22] Typing was done by the capillary tube modification of the Lancefield method.[23] ¶

Table VI shows the number of isolations of *beta*-hemolytic streptococci in men who were cultured 3 times. The swabs examined by Pike's method showed an increased number of streptococci in every group. The greatest increase was for Group B, the least for typable strains of Group A. Since the 3 cultures were not taken simultaneously, the results are not strictly comparable. Several facts, however, suggest that the conclusions are valid. The Pike cultures were interposed between the other two during a period when

[20] Pike, R. M., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 186.

[21] Pike, R. M., *Am. J. Hyg.*, 1945, **41**, 211.

[22] Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.

[23] Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 127.

¶ We are grateful to Drs. Homer F. Swift and Rebecca C. Lancefield for generous supplies of typing sera, capillary tubes, and serum containers; and to Dr. Chester S. Keefer, Director, Commission on Streptococcal Infections, Army Epidemiological Board, for additional sera.

there was no indication of an unusual occurrence of streptococcal infection in the study group. Furthermore, the difference in carrier rate as determined by the two methods in this experiment is compatible with Pike's observations[20,21] and with the results of the cultures of 125 well soldiers (Table V).

Summary. A technic for examining throat swabs which appears to have certain advantages is described. This method involves suspending the material on the swab in broth, inoculating blood agar plates with this suspension, and examining the resultant growth with the aid of a microscope. The experiments which led to the adoption of this technic are presented.

This and other similar technics for determining pharyngeal flora, however, have definite limitations, as indicated by the observation that inoculation of blood agar plates with a mixture of only two bacterial species yielded colonies which were not distributed at random on the surface of the medium, but rather in a manner indicating interdependence of the organisms. Thus, the growth on a plate does not necessarily reflect the relative proportion of each species present in the inoculum.

The value of selective media as a means of detecting organisms present in low frequency has been demonstrated by utilizing Pike's medium for the isolation of *beta*-hemolytic streptococci. The carrier rate for *beta*-hemolytic streptococci was increased from 9.6%, as determined by usual cultural methods, to 20.8% by the use of Pike's method. The increase was greatest for Group B streptococci and least for the typable strains of Group A.

16614 P

Production of Increased Ketonemia in a Normal Dog by Adrenocorticotropic Hormone.*

LESLIE L. BENNETT, JOSEPH F. GARCIA, AND CHOH HAO LI.

From the Division of Physiology and The Institute of Experimental Biology, University of California, Berkeley.

Previous work[1] from this laboratory has shown that pure adrenocorticotropic hormone increased the ketonemia of fasted normal rats. The present experiments have been undertaken to establish whether adrenocorticotropic hormone would have a similar effect in a normal dog.

Methods. All the experiments were carried out on one female mongrel dog weighing approximately 15 kg. Two control experiments were done: one in which the dog was given 50 mg of serum albumin, the other in which the dog was given 50 mg of a crude alkaline anterior pituitary extract which had been heated to 100°C for 1 hour. The adrenocorticotropic hormone was prepared according to the previously published method,[2] and 3 experiments were done in which it was given at a dose of either 50 mg or 25 mg.

The dog was fasted 72 hours before each experiment and an interval of at least 2 weeks elapsed between experiments. An initial blood sample was taken and then the hormone or control foreign protein was administered by a single intraperitoneal injection. At 1½, 3, 4½ and 6 hours after the injection additional blood samples were taken. Total blood ketone bodies were determined by the method

* Aided by grants from the National Institute of Health, RG 409, and The Research Board, University of California.

1 Bennett, L. L., Kreiss, R. E., Li, C. H., and Evans, H. M., *Am. J. Physiol.*, 1948, **152**, 210.

2 Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1943, **149**, 413.