and ultra-violet irradiation will help to improve calcium metabolism, while infra-red rays often give much relief from pain. Breathing exercises are of the greatest importance in preventing, so far as is possible, the tendency of the costo-vertebral joints to ankylose; they also have a beneficial effect on the general condition. Deep x-ray therapy has been credited with good results in some cases. I have not found drugs of any service except for relief of pain, for which aspirin, or in severe cases tab. codein. co., will be useful. Gold, as already stated, is quite ineffective in the adollesc form, and the same may be said for vaccines.

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A STUDY OF THE ACCURACY OF SERUM PROTEIN ESTIMATIONS AND OF DIURNAL VARIATIONS IN THEIR LEVEL*

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The object of the present investigation was: (i) to estimate the degree of accuracy obtainable in our hands with two methods of serum protein estimation in common use; (ii) to determine whether there is any diurnal variation in the level of serum proteins in the normal individual under ordinary conditions of activity and food intake.

Methods

Serum was used in preference to plasma for the following reasons: (a) haemolysis is less likely to occur in serum than in plasma; (b) there is a theoretical possibility that the presence of oxalate may, by withdrawing water from the cells, dilute the plasma, while the presence of heparin, which contains nitrogen, must make any estimation of protein inaccurate; (c) in another investigation in progress, necessitating the estimation of proteins, it is more convenient to use serum than plasma. It was therefore preferable to have a base-line in terms of serum than of plasma proteins. The methods used were:

(i) Proteins were precipitated with zinc sulphate and caustic soda and estimated by the micro-Kjeldahl method as described by Pregl (1937) and Niederl and Niederl (1938). In the samples taken at two-hourly intervals the result recorded represents the mean of two determinations. (ii) The method described by King and his co-workers (King, Haslewood, and Delory, 1937; King, Haslewood, Delory, and Beall, 1942), in which Kjeldahl digestion is followed by Nesslerization and colorimetric estimation. The colorimetric readings were made with a Hellige colorimeter in a dark-room. Owing to war conditions a Klett violet filter, as advised in the original paper, was unobtainable; an Ilford blue filter was therefore used. In the samples taken at two-hourly intervals the result recorded represents the mean of two determinations.

Results

Accuracy of the Method.—To test the accuracy of the method it would have been satisfactory to estimate the protein content of 100 samples of the same serum on the same day. Owing to the time and apparatus required for the micro-Kjeldahl method this was not practicable. Ten estimations were therefore made on each of 10 samples of the same serum by both methods. This procedure was carried out for 10 different sera, giving 100 estimations in all (Table I). These figures were treated in two different ways to determine what difference in any two results should be attributed to technical error. First working on the basis that any two of these values observed for a given serum might have occurred in actual

practice, the distribution of differences between the 10 values was determined. Each 10 results thus gave 45 differences (or 90 if read both ways). There were in all, therefore, 450 differences. Each 45 based on a separate experiment. The number of differences of various sizes was very similar for each of the 10 sera, though the

TABLE I.—Estimations from Ten Samples of the Same Serum, using King's Method and the Micro-Kjeldahl Method. Estimated with 10 Different Sera

Serum	King's Method			Micro-Kjeldahl Method		
	Mean Reading	Mean Difference	Max. Difference	Mean Reading	Mean Difference	Max. Difference
1	7.48	0.14	0.3	7.40	0.056	0.14
2	7.23	0.18	0.4	6.79	0.054	0.16
3	6.91	0.16	0.4	6.69	0.086	0.22
4	6.27	0.17	0.4	6.26	0.069	0.17
5	6.45	0.24	0.5	6.38	0.067	0.18
6	7.10	0.16	0.4	6.80	0.049	0.14
7	6.06	0.16	0.4	6.03	0.070	0.18
8	6.97	0.14	0.4	6.95	0.053	0.14
9	6.66	0.09	0.2	6.67	0.072	0.22
10	6.76	0.10	0.3	6.75	0.079	0.21

average value of the latter varied from 6.06 to 7.48 with King's method and from 6.03 to 7.4 with the micro-Kjeldahl method. Within this range it seems, therefore, that errors of observation will be of the same magnitude. Adding them together for the 10 sera gives the figures shown in Tables II and III. With King's method (Table II) it seems that differences as large as 0.3 and even 0.4 will

TABLE II: KING'S METHOD.—Grouped Differences in Value of Serum Proteins, using 10 Samples of the Same Serum and 10 Different Sera, compared with Similar Differences observed on 5 Samples withdrawn at Two-hourly Intervals from Each of 20 Normal Subjects

Differences in g.	Differences between Estimations on the Same Serum		Diurnal Variations	
	No. of Diffs. Observed	% Frequency	No. of Diffs. Observed	% Frequency
0.0	113	25	47	25
0.1	121	27	78	41
0.2	123	27	32	17
0.3	50	11	25	13
0.4	37	8	8	4
0.5	6	1	1	0.5
0.6	—	—	1	0.5

TABLE III: MICRO-KJELDAHL METHOD.—Grouped Differences in Value of Serum Proteins, using 10 Samples of the Same Serum and 10 Different Sera, compared with Similar Differences observed on 5 Samples withdrawn at Two-hourly Intervals from Each of 20 Normal Subjects

Differences in g.	Differences between Estimations on the Same Serum		Diurnal Variations	
	No. of Diffs. Observed	% Frequency	No. of Diffs. Observed	% Frequency
0.00	175	39	58	30
0.05	171	38	59	31
0.10	77	17	40	21
0.15	20	4	22	12
0.20	7	2	6	3
0.25-0.35	—	—	7	4

occur quite frequently with the same serum, for 11% of them were 0.3 and 8% were 0.4. A difference as large as 0.5 is rare, for there was only 1 in 100. With the micro-Kjeldahl method, on the other hand (Table III), it appears unlikely that differences over 0.2 will be obtained in testing the same serum (only 2 in 100), and fairly unlikely that any over 0.15 (only 6 in 100) will occur. As a satisfactory rule, according to this method of analysis, changes of under 0.15 should be ignored with the micro-Kjeldahl method as too likely to be due to errors inherent in the technique, changes of 0.15 to 0.2 as probably significant, and those over 0.2 as almost certainly significant.

The same figures were treated in a different manner with approximately the same results. An average standard deviation from the total 100 values was obtained by calculating the sum of the squared deviations from the observed means in each set of 10 tests on the same serum, adding all these squares together, and dividing by 90 (the number of degrees of freedom left). This standard deviation from the 100 values for King's method was 0.1416 and for the micro-Kjeldahl method 0.0574. The test of significance between any two observed values was then measured by the standard deviation multiplied by $2\sqrt{2}$. Differences as large as this value would be expected to occur by chance only once in 20 trials. With King's method, therefore, the difference between two values has to be at least 0.4 and with the micro-Kjeldahl technique at least 0.16 to be significant.

Either method of treating the figures shows that the micro-Kjeldahl technique is the more accurate.

* A report to the Medical Research Council from the N.W. London Blood Supply Depot.

It should be noted, however, that if a number of estimations were made on the same serum, apparently as accurate an answer would be obtained by King's method as by the micro-Kjeldahl, as the means in the above series with the exceptions of sera 2, 3, and 6 are in close agreement. King's method presumably involves larger chance errors, which swing either side and therefore balance out. It is, however, in practice not possible to make a number of estimations, and therefore the micro-Kjeldahl is preferable.

Diurnal Variations

To determine whether there is a significant diurnal variation under ordinary working conditions 5 samples were taken, at two-hourly intervals, from 20 healthy members of the staff between the ages of 18 and 50. With one exception these subjects were all living under approximately the same conditions as regards diet—i.e., they all had some meals during the week in a canteen or restaurant in addition to their own ration. The exception was an unmarried woman living alone. All subjects were working in the N.W. London Blood Supply Depot and carried on their normal active duties. The first sample was withdrawn from a vein in the ante-cubital fossa between 9 and 10 a.m.; the subject then continued normal work throughout the day, further samples being withdrawn at two-hourly intervals. The mean figures for serum protein estimations made at two-hourly intervals on each of 20 normal individuals are shown in Table IV. The figures were treated in the same manner as described for the estimations on samples on the same serum. The values

TABLE IV.—*Diurnal Variations of Serum Protein Concentration in g. per 100 c.cm. (Two-hourly Samples) in 20 Normal Subjects*

No. of Subject	King's Technique			Micro-Kjeldahl Technique		
	Mean Reading	Mean Difference	Max. Difference	Mean Reading	Mean Difference	Max. Difference
1	7.08	0.26	0.6	7.25	0.144	0.27
2	7.44	0.06	0.1	7.39	0.092	0.18
3	7.40	0.08	0.2	7.41	0.070	0.14
4	7.02	0.21	0.4	7.11	0.080	0.19
5	7.42	0.10	0.2	7.08	0.056	0.11
6	7.32	0.04	0.1	7.21	0.088	0.20
7	6.88	0.14	0.3	7.08	0.074	0.14
8	7.38	0.10	0.2	7.24	0.096	0.20
9	6.94	0.16	0.4	6.93	0.070	0.16
10	7.14	0.10	0.2	7.17	0.096	0.18
11	7.18	0.18	0.3	7.16	0.087	0.14
12	6.46	0.16	0.3	6.31	0.048	0.11
13	7.08	0.22	0.5	6.96	0.142	0.27
14	6.92	0.22	0.4	7.02	0.144	0.32
15	7.16	0.14	0.3	7.11	0.086	0.16
16	7.20	0.21	0.4	7.17	0.172	0.35
17	7.23	0.18	0.3	7.17	0.040	0.07
18	7.30	0.08	0.2	7.15	0.046	0.10
19	7.40	0.08	0.2	7.28	0.044	0.09
20	7.38	0.04	0.1	7.29	0.070	0.13
Mean ..	7.17	0.14	0.29	7.12	0.088	0.176

for the 20 individuals show immaterial changes during the day by either King's or the micro-Kjeldahl method (Tables II and III). On the other hand, the distribution of pairs of differences for each individual shows that with the micro-Kjeldahl method rather more relatively large differences occur than were found in the tests of experimental error—19% are over 0.15 against 6% (Table III). Therefore, if tests are being done at different times of day a slightly larger difference for significance should probably be demanded than that previously given. Differences of more than 0.2 g. should be regarded as very suspicious and differences over 0.25 g. as most likely significant.

The average standard deviation method gives much the same answer, since its significance level for the micro-Kjeldahl technique will be a difference of 0.22 g. between two values, the standard deviation for the combined figures being 0.0781. King's method gives a rather lower variability in the diurnal tests than in those of experimental error, the standard deviation in the former being 0.128 and in the latter 0.142. In either case the significance level for the difference between two observations will be about 0.4.

The mean of each series of five values on 20 normal individuals ranged from 6.31 to 7.41 g. per 100 c.cm. (micro-Kjeldahl) and 6.46 to 7.44 g. per 100 c.cm. (King). The mean for the whole series was 7.12 ± 0.008 (micro-Kjeldahl) and 7.17 ± 0.013 (King). The only figure for serum protein below 6.59 g. per 100 c.cm. occurred in a single middle-aged woman living alone.

Discussion

Two facts regarding the interpretation of serum protein estimations emerge from a consideration of the figures given above:

(i) That there is an error inherent in the method of estimation which must be considered in drawing any conclusion from the results, and, further, that this error varies from method to method.

(ii) That if tests of serum protein are done at different times of day on subjects leading a normal active life a slightly larger

difference for significance should be demanded than if repeated estimations are made on the same sample or on samples taken very close to one another in time. The variation, however, is slight. A difference greater than 0.169 per 100 c.cm. appeared to be significant on repeated estimations on the same sample by the micro-Kjeldahl technique, while on samples taken at intervals throughout the day under standard conditions differences of over 0.25 g. are probably significant.

Perera and Berliner (1943) report rather larger differences on repeated samples taken throughout the day from 10 a.m. to 10 p.m. on 10 normal individuals whom they describe as ambulatory. This may be due to the fact that their normal subjects took more exercise or altered their posture more than the subjects in the present study, since many observers (Böhme, 1911; Cipriani and Moracchini, 1925; Thompson, Thompson, and Dailey, 1928; Bock *et al.*, 1927; Youmans *et al.*, 1934) have emphasized the effect of both posture and exercise on protein levels. Perera and Berliner themselves record a consistent fall in serum proteins of about 0.8 g. % in ambulatory patients during the night. In persons staying up all night and sleeping during the day the fall occurred during the day, making it evident that the fall was associated with rest in the recumbent position. Bed-rest alone appears sufficient to reduce serum protein levels to what are often regarded as pathological figures.

The effects of possible changes in posture and activity have in the last few years to a large extent been ignored by workers who have stressed variations in the level of serum or plasma protein as a method of calculating changes in the balance of both fluid and protein between tissues and circulating fluid (Black, 1940; Ebert and Stead, 1941; Ebert, Stead, and Gibson, 1941; Beattie, 1942; Beattie and Collard, 1942a, 1942b).

It is clearly essential to standardize both the activity and posture of the subjects and the methods employed if small variations in serum protein are to be considered significant. Single estimations should not be made after prolonged rest in the recumbent position.

Summary

Observations have been made on the accuracy of King's method and the micro-Kjeldahl method in the estimation of serum protein.

The figures obtained suggest that differences of more than 0.25 g. obtained by the micro-Kjeldahl technique are almost certainly significant, while with King's method differences must be greater than 0.4 g. to be significant.

No great variation in serum protein was found in 20 normal individuals leading an ordinary working life between the hours of approximately 9 a.m. and 6 p.m.

It is suggested that many variations in serum protein recorded in the literature are not significant and that changes in the character of tissue and circulating fluid cannot be calculated from changes in serum protein unless accurate methods with a known technical error are employed and standard conditions of posture and exercise adopted.

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H. J. Gareau (*Canad. med. Ass. J.*, 1942, **46**, 51) gives a report on 75 cases of acro-dynia, in 57 of which the tonsils and adenoids were removed in the course of treatment. In these cases, the author states, the average total duration of the illness was less than 3½ months, and as this is less by 2½ months than the usual course he presumes that acro-dynia is due to a toxæmia caused by infected tonsils and adenoids.