XXVI. A NOTE ON THE USE OF INDICATORS FOR THE COLORIMETRIC DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF SERA.

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During the progress of a series of investigations on the concentration of antitoxic sera it was found necessary to adjust the reaction of the sera to certain fixed values in order to get concordant and consistent results. In the experimental work, owing to lack of data with regard to the reliability of indicators in the presence of the serum proteins, the hydrogen ion concentration of the sera was determined by the electrical method. Since the latter method cannot be conveniently used in a general routine laboratory, experimental work was undertaken to ascertain whether determinations of sufficient accuracy for practical purposes could be made by the colorimetric method.

In this connexion to several samples of different sera were added varying quantities of acid or of alkali. The hydrogen ion concentration $[H^+]$ of each of the samples was estimated by the electrical method and the values thus found were compared with those obtained by the colorimetric method using the stated indicators and Sørensen's standard solutions in a Walpole's colorimeter, the colour system being as follows:

<i>Eye.</i>	
Left	Right
10 cc. serum + x drops of Indicator	10 cc. serum + no Indicator
10 cc. water + no Indicator	10 cc. Sørensen's standard solution + x drops of Indicator

Source of Light.

In the colorimetric determination of the values for the $[H^+]$ care must be taken to use a constant amount of the specific indicator. It is a matter of experience that, both with the standard solutions and with the test sera, the intensity of the colour reaction at a definite hydrogen ion concentration can be varied by alterations in the amount of indicator added, so that, even with indicators which may prove sufficiently reliable for use with sera, unless the amount of indicator be kept constant, neither consistent nor comparable results will be forthcoming.

For my purpose litmus, methyl violet, methyl orange and Henderson and Forbes' indicator were useless with serum as I was unable to match the two colour systems. In these cases the colour reaction between the serum and each of the above-mentioned indicators seemed to me to be different from that between the indicator and the standard solutions.

With *p-nitrophenol* and *tropaeolin* 000. it was found that, although it was possible to match the two colour systems, the colour changes were neither sufficiently sharp nor in sufficient contrast to the colour of the serum itself for me to obtain satisfactory readings.

With *phenolphthalein* the colorimetric determinations gave values not sufficiently near those obtained by the electrical method to be of any service even for approximate values for routine work. Thus, the colour reaction between the indicator and serum corresponding to that with the standard solutions at P_H 8.25 was not evidenced until the true reaction of the serum had been brought to values of the order of P_H 10.5.

On the other hand, contrary to expectation, *neutral red* proved sufficiently reliable for routine determinations with sera. It was found that with the addition of five drops (Pasteur capillary pipette) of a 0.05 % solution of this indicator in alcohol to 10 cc. of the Sørensen's standard solutions the useful range was between P_H 5.6 and 7.8. The same amount of indicator was then added to 10 cc. of samples of the different sera and the colour reaction thus produced was matched with the colour scheme obtained by viewing the standard solutions and indicator through serum in the colorimeter: in serum the useful range was between P_H 5.9 and 7.6. The results thus obtained for the values of the $[H^+]$ of the sera together with those obtained by the electrical method have been embodied in Table I.

TABLE I.

A comparison between the values obtained for the hydrogen ion concentration (P_H) of various sera by the electrical method and by the colorimetric method, using neutral red as the indicator.

[To 10 cc. of the test solutions were added five drops of a 0.05 % solution of neutral red in alcohol.]

Plasma No.	Percentage of protein in the sample of plasma taken	P_H of the plasma determined by the colorimetric method	P_H of the plasma determined by the electrical method
1	6.73	7.6	7.7
1 a	4.92	7.14	7.14
2	6.16	6.98	7.02
2 a	4.62	between 6.98 and 6.81	6.82
2 b	4.62	6.60	6.40
3	6.16	5.91	6.29
4	8.12	7.35	7.46
4 a	6.09	between 7.64 and 7.35	7.50
4 b	4.06	7.34	7.40
5	6.09	5.91	6.02
5 a	4.06	5.91	6.02
6	6.56	7.34	7.40
6 a	6.56	5.90	5.92
6 b	4.92	6.97	6.98
6 c	4.92	6.60	6.40

A study of Table I shows that the $[H^+]$ of sera can be determined between P_H 5.9 and 7.6 with sufficient accuracy for practical purposes by the colorimetric method using neutral red as indicator.

The studies of Sørensen and Palitzsch [1910] have shown the value of *α-naphtholphthalein* as an indicator. It was tried with sera as its useful range, using three drops of a 0.10 % solution with the standard solutions, was from P_H 7.6 to 8.5.

From the data given in Table II it will be seen that the determinations by the electrical method are consistently higher than those by the colorimetric method using this indicator. Thus, the slightest tinge of green colorimetrically corresponding to that for P_H 7.6 with the standard solutions does not show until the true value for P_H is of the order of 8.5. However, with this indicator the "buffer" action of the proteins is not sufficiently marked to be too serious a drawback to its use for routine work. Thus, it was found that, if to 10 cc. of the serum containing three drops of the solution of *α-naphtholphthalein*

ammonia was added until the appearance of the faintest green tinge in the serum was detected, the reaction of the serum thus treated was approximately P_H 8.5.

TABLE II.

A comparison between the values for the P_H of various sera obtained by the electrical method and by the colorimetric method, using α -naphtholphthalein as the indicator.

[To 10 cc. of the test solutions were added three drops of a 0.10 % solution of the indicator in alcohol.]

Plasma No.	Percentage of protein in the plasma	P_H determined by the colorimetric method	P_H determined by the electrical method
7	6.73	7.64	8.29
7 a	4.92	7.64	8.04
8	8.12	7.86	8.65
8 a	6.09	7.64	8.44
8 b	4.06	7.64	8.55
8 c	2.03	7.65	8.60
9	6.56	7.64	8.27
9 a	4.92	7.64	8.48
9 b	4.92	8.04	8.71
10	6.16	7.64	8.52
10 a	4.62	7.64	8.45
10 b	4.62	7.86	8.25

For routine work in which a value of about P_H 8.0 was desired it was found that the following procedure would approximately give the desired result: To an aliquot part of the serum was added the necessary volume (x cc.) of a standard solution of ammonia to bring the $[H^+]$ to the value P_H 7.6, using neutral red as the indicator. To a similar volume of serum was added the necessary volume (y cc.) of the ammonia solution to bring the $[H^+]$ to the value P_H 8.5 (faint green tinge with α -naphtholphthalein).

The addition of $\frac{x+y}{2}$ cc. of the standard solution of ammonia to the stated volume of serum brought the $[H^+]$ approximately to the value of P_H 8.0. This is a purely empirical arrangement which has given results of sufficient accuracy for routine work for the reason that, while an excess of alkali is undesirable, slight variations in the reaction round about P_H 8.0 are not followed by the marked variations in the heat denaturation of the serum proteins so characteristic of sera more acid than P_H 5.5.

It was next desired to find reliable indicators for the determination of the $[H^+]$ of sera more acid than could be tested by neutral red. For this purpose,

owing to the unreliability of litmus, methyl orange, methyl red, Henderson and Forbes' indicator, and also to my inability to match the colour reaction with *p*-nitrophenol, resource was had to the sulphonephthalein indicators, the use of which has been so strongly advocated by Mansfield Clark and Lubs [1915]. The brilliancy of the colour reactions of these substances suggested that besides possibly furnishing indicators for the desired acid ranges they might also replace the indicators already in use on the alkaline side.

In this connexion, the following indicators in 0.05 % alcoholic solution, were used.

	Useful range P_H
Thymol-sulphonephthalein *	8.0-9.6
<i>o</i> -Cresol-sulphonephthalein *	7.2-8.8
Phenol-sulphonephthalein	6.8-8.4
Dibromothymol-sulphonephthalein *	6.0-7.6
Dibromo- <i>o</i> -cresol-sulphonephthalein *	5.2-6.8
Methyl red	4.4-6.0
Tetrabromophenol-sulphonephthalein	3.0-4.6

* Samples of these indicators were very kindly sent to me by Dr Mansfield Clark as some delay was experienced in obtaining the chemicals necessary for their preparation.

The useful range of each indicator was tested against Sørensen's standard solutions which were also checked by the electrical method. A comparison was then made between the values of the $[H^+]$ of the various sera obtained by the colorimetric method using the above mentioned indicators and also by the electrical method. The data with regard to these observations have been embodied in Table III.

Thymol-sulphonephthalein was found to be unreliable with serum. Thus, the colour reaction corresponding to P_H 8.3 with the standard solutions did not appear in the serum until the value P_H 10.3 had been reached.

The colour changes with *o*-cresol-sulphonephthalein in serum could not be detected by me with sufficient ease for its adoption in routine work.

The colour reactions of *phenol-sulphonephthalein* and of *bromothymol-sulphonephthalein* in serum could be readily matched throughout their useful ranges with the standard solutions, but, the values for the $[H^+]$ of the sera thus determined were consistently lower than the true values determined by the electrical method. As it was found that, in sera containing from 4 to 10 % of protein, these deviations from the true values were constant for the same worker, use was made of these indicators for the determination of the $[H^+]$ of sera between the ranges P_H 6.8 and 9.0 as follows.

To the test serum were added six drops of a 0.05 % solution of the indicator. The colour reaction was then matched against standard solutions containing

TABLE III.

A comparison between the values obtained for the P_H of various sera by the electrical method and by the colorimetric method using the indicators of the sulphonephthalein series.

Plasma No.	Percentage of protein in the plasma	Indicator used	P_H of the plasma determined by	
			the colorimetric method	the electric method
11	8.12	Thymol-sulphonephthalein	8.03	10.34
12	6.16	" "	8.03	10.21
13	6.56	" "	8.03	10.33
14	4.40	" "	8.03	10.55
15	6.73	Phenol-sulphonephthalein	6.81	7.65
15	6.73	" "	7.14	8.00
15	6.73	" "	7.34	8.25
15	6.73	" "	7.64	8.55
15	6.73	" "	7.86	8.70
15	6.73	" "	8.03	8.97
16	4.92	" "	7.64	8.43
17	6.16	" "	7.64	8.46
18	8.12	" "	7.64	8.25
19	6.09	" "	7.86	8.70
20	4.06	" "	7.44	8.27
20 <i>a</i>	4.06	Bromothymol-sulphonephthalein	6.23	6.86
21	6.09	" "	6.46	7.31
22	4.92	" "	6.46	7.58
23	4.40	" "	6.46	7.38
24	8.12	" "	6.81	7.86
24	8.12	" "	7.10	8.10
24	8.12	" "	7.30	8.66
25	6.97	" "	7.30	8.60
26	9.90	" "	7.30	8.70
27	6.73	Methyl red	4.98	4.00
28	4.92	"	6.00	5.24
29	8.12	"	5.60	4.80
30	8.12	Bromo- <i>o</i> -cresol-sulphonephthalein	5.2	5.14
31	6.09	" "	5.2	5.06
32	6.56	" "	5.2	5.14
33	4.40	" "	5.2	5.23
34	6.73	" "	5.4	5.50
35	7.56	" "	5.4	5.58
36	8.12	" "	5.4	5.65
37	6.56	" "	5.4	5.32
38	6.73	Bromophenol-sulphonephthalein	3.0	2.90
39	8.12	" "	3.0	2.87

a corresponding amount of indicator, the colour produced with the latter being viewed through serum. The value thus obtained was transmuted to the true value according to the following table:

Phenol-sulphonephthalein		Bromothymol-sulphonephthalein	
Value for P_H found by the colorimetric method	True value for P_H of the serum (approximately)	Value for P_H found by the colorimetric method	True value for P_H of the serum (approximately)
6.81	7.65	6.23	6.86
7.14	8.00	6.46	7.40
7.34	8.25	6.81	7.80
7.64	8.55	7.10	8.10
7.86	8.70	7.30	8.60
8.03	8.95		

For the colorimetric work with serum, I found it more easy to match the yellowish green to blue colour reactions of bromothymol-sulphonephthalein than the orange to reddish purple colour reactions of phenol-sulphonephthalein. For this reason in routine work the reaction of sera was approximately adjusted to the value P_H 8.0 by the addition of ammonia until the colour reaction of bromothymol-sulphonephthalein with the serum matched that with a standard solution at P_H 7.1, the latter being viewed through serum in the colorimeter. However, although fairly consistent results were obtained, I found that I was not always as successful with bromothymol-sulphonephthalein as with α -naphtholphthalein, the sensitiveness of my colour vision to the delicate colour changes of the former being very much influenced by personal fatigue.

With *dibromo-o-cresol-sulphonephthalein* and serum it was found somewhat difficult to match the various shades of purple through the ranges of the indicator. However, in practice, it was an easy matter to bring the serum to the degree of acidity at which the indicator showed a slight greenish tinge, *i.e.* just before the acid limit of the useful range of the indicator was reached. The reaction of the sera so adjusted was found to be approximately P_H 5.5.

If acid were added to serum containing bromo-*o*-cresol-sulphonephthalein until the final slight green tinge had just disappeared it was found that approximately the value P_H 5.2 had been reached.

For the adjustment of the sera between the values P_H 5.5 and P_H 4.5 the following arbitrary method was adopted which gave results of sufficient accuracy for routine work, provided the colorimetric determinations were made by the same observer.

Sera containing from 5 to 8 % of protein were brought to P_H 5.5 (circ.) by the addition of the requisite amount (x cc.) of acetic acid to a stated volume of serum containing a fixed amount of indicator until only the faintest tinge of green was detected. Since the colour of serum is affected by its acidity or alkalinity, the comparison in all cases was made with the same volume of serum containing no indicator but to which had been added

a corresponding amount of acetic acid. It was found that the further addition of acetic acid to the serum beyond this point gave results of the following order:

Volume of <i>N</i> acetic acid (or equivalent) added to 100 cc. of the serum	P_H of the acidi- fied serum (approximately)
x cc.	5.5
$x + 0.5$ cc.	5.4
$x + 1.0$	5.3
$x + 1.5$	5.2
$x + 2.0$	5.1
$x + 2.5$	5.05
$x + 3.0$	4.9
$x + 4.0$	4.7
$x + 5.0$	4.6

The value of such an arbitrary method depends on the acuteness of the colour vision of the individual and, for this reason, in order to obviate the personal error in obtaining the determination of the initial value depending on the colour tint, which, for me, approximated to the value P_H 5.5, it must be urged that each investigator should work out his own table.

Methyl red proved to be unsatisfactory with serum, as the colorimetric determinations gave results not sufficiently near those obtained by the electrical method to justify its use in routine work, slight changes in the reaction of sera on the acid side of P_H 5.5 leading to marked variations in the heat denaturation and precipitation of the serum proteins.

Difficulty was also experienced in matching the purple colour of *bromophenol-sulphonephthalein* in serum. It was however found that, with the sera taken, the faint green tinge just before the useful range of the indicator on the acid side was passed (*i.e.* at P_H 3.0) could be regulated with approximate accuracy.

During the course of the investigation it was found that dilution of the serum was followed by sharper changes in the colour reactions throughout the useful ranges of all the above-mentioned indicators. But, even where the dilution had been of the order of 1 in 4, there was no appreciable variation in the values obtained for P_H by the colorimetric method.

SUMMARY.

During the course of the investigation it has been demonstrated that the hydrogen ion concentration of sera can be determined, with sufficient accuracy for routine work, by the colorimetric method with the use of certain indicators.

It was my experience that:

1. Neutral red gave reliable results with serum throughout the ranges P_H 5·9 to 7·6.

2. The results obtained with α -naphtholphthalein were consistently higher than the true values. Thus, the colorimetric determination of the values for P_H 7·6 was not given until the reaction of the sera had been adjusted to the value P_H 8·5.

3. By a combination of the use of neutral red and of α -naphtholphthalein the adjustment of routine batches of sera to P_H 8·0 can be arranged with sufficient ease for practical purposes.

4. Of the indicators of the sulphonephthalein series thymol-sulphonephthalein and *o*-cresol-sulphonephthalein proved unsatisfactory with serum. Phenol-sulphonephthalein and bromothymol-sulphonephthalein gave results which were consistently higher than the true values. Bromo-*o*-cresol-sulphonephthalein could be used with ease for P_H 5·4 and 5·2 while bromophenol-sulphonephthalein was satisfactory for values about P_H 3·0.

5. By a purely arbitrary arrangement based on my own observations, I was able to adjust the reaction of sera approximately to values between P_H 7·6 and 8·95 with the use of phenol-sulphonephthalein; to values between P_H 6·8 and 8·6 with the use of bromothymol-sulphonephthalein and to values between P_H 5·5 and 4·5 with the use of bromo-*o*-cresol-sulphonephthalein.

6. In view of the personal error involved in the determination of colour reactions with sera it is advisable that each worker using the colorimetric method should ascertain the degree of approximation of his own values to the true values determined by the electrical method.

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