[CONTRIBUTION FROM THE BUREAU OF PLANT INDUSTRY, U. S. DEPARTMENT OF AGRICULTURE.]

ABNORMALITIES IN THE FORMOL TITRATION METHOD.

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Next to Van Slyke's[1] very accurate nitrous acid method and the exceedingly sensitive colorimetric method,[2] the formol titration method elaborated by Schiff[3] and perfected by Sörensen[4] and his co-workers,[5] is extensively used in biochemical investigations because it offers a convenient means for the quantitative estimation of amino acids, polypeptides, etc., which usually occur in biological substances. The disadvantage of the latter method consists in certain inaccuracies which found their expression in the literature without any attempt to explain them. Thus, Levene and Van Slyke[6] pointed out that the "gasometric method has decided advantages over the well-known Sörensen formol titration" in that, for instance, the volume of nitrogen evolved is 5 times that of the 0.2 *N* alkali required for the formol titration of the same quantity of amino nitrogen.

Harding[7] and MacLean found that a comparison of the 3 methods applied to protein hydrolysis in very dilute solution, showed the colorimetric and the Van Slyke methods to be preferable to Sörensen's method.

Cook[8] stated that the formol method gave lower results for amino nitrogen than Van Slyke's method and that from the standpoint of accuracy the latter method was unquestionably superior to the formol method.

By Abderhalden,[9] on the other hand, the proposition was made to check up the gasometric method by the formol method in view of the fact that conjugated amino acid (polypeptides, etc.) might be hydrolyzed by the continued action of nitrous acid.

The writer[10] was among the first to apply the formol titration method to the quantitative estimation of diamino and monoamino acids. Having had later[11] several times occasion to use the formol method, he could not

[1] *Ber.*, **43**, 3170 (1910); *J. Biol. Chem.*, **9**, 185 (1911); **10**, 287 (1911); **12**, 275 (1912); **16**, 121, 125 (1913–14).

[2] V. J. Harding and R. M. MacLean, *J. Biol. Chem.*, **20**, 217 (1915); **24**, 503 (1916).

[3] *Ann.*, **310**, 25 (1900); **319**, 59, 287 (1901); **325**, 348 (1902).

[4] *Biochem. Z.*, **7**, 45, 407 (1907–08).

[5] V. Henriques, *Z. physiol. Chem.*, **60**, 1 (1909); V. Henriques and S. P. L. Sörensen, *Ibid.*, **64**, 120 (1910).

[6] *J. Biol. Chem.*, **12**, 301 (1912).

[7] *Ibid.*, **24**, 512 (1916).

[8] THIS JOURNAL, **36**, 1555 (1914).

[9] *Z. physiol. Chem.*, **96**, 8 (1915).

[10] Iowa Agr. Expt. Sta., *Research Bull.* **1**, 3–46 (1911).

[11] S. L. Jodidi, THIS JOURNAL, **33**, 1226 (1911); **34**, 94 (1912); Jodidi and Wells, Iowa Agr. Expt. Sta., *Research Bull.* **3**, 113–154 (1911); Jodidi, Kellogg and True, *Jour. Agr. Research.*

help being impressed with the need of a satisfactory interpretation of hitherto unexplained differences between theory and actual results obtained by this method. In proteins, and especially in polypeptides, in which these amino acids occur in considerable proportions, the errors obtained with the formol method may be too serious to be overlooked and, indeed, may even be of such magnitude as to exclude the application of this method to the quantitative estimation of the amino acids under consideration. Sörensen gives a satisfactory explanation as to why the formaldehyde method leads to too high results in the case of tyrosine,[1] this being due, as he correctly points out, to the presence of the negative phenol group in the tyrosine molecule. We wish to add that this opinion of Sörensen is also corroborated by the fact that the homocyclic compound, phenylalanine, not containing the phenol group, gives accurate results. Sörensen and his collaborators fail, however, to throw light on the anomalies of other amino acids. The main data in question published by Sörensen[2] found their way, unchanged, into standard text-books.[3]

An interpretation of the abnormalities incident to the formol method, as offered here, seems to be desirable because it shows the cases in which the irregularities occur pointing, in part, to the limits of the accuracy of this method.

Proline.—In the case of the simple amino acids, such as alanine, glycocoll, leucine, etc., the addition of formaldehyde leads to a monobasic acid, without any other groups modifying its acidity, as can be seen from the general equation

$$R\begin{cases}NH_2\\COOH\end{cases} + CH_2O = R\begin{cases}N=CH_2\\COOH\end{cases} + H_2O.$$

Amino acid. Methylene amino acid.

The resulting acid contains the two groups R = and —N = CH_2, which are neither basic nor acid, and, therefore, do not modify the COOH group present. For this reason the formol titration of such amino acids yields results as accurate as those generally obtained with the titrimetric methods in vogue. The conditions are quite different in the case of *proline*. The latter is an *imino* acid and, so far as the =NH group is concerned, a secondary base. The reaction between secondary bases and formaldehyde takes place according to the following general equation:

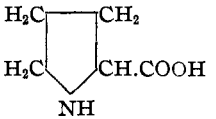
$$2R_2NH + CH_2O = \begin{matrix}R\\R\end{matrix}\!\!>\!N - CH_2 - N\!<\!\!\begin{matrix}R\\R\end{matrix} + H_2O$$

[1] 109 and 137.5% of tyrosine are indicated when phenolphthalein and thymolphthalein, respectively, are used as indicators. See *Biochem. Z.*, 7, 59 (1907–08).

[2] *Compt. rend. de Laboratoire de Carlsberg*, 7, 1 (1907); *Biochem. Z.*, 7, 45 (1907–08).

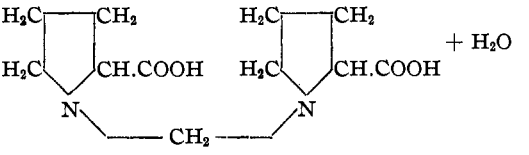
[3] See, *e. g.*, Abderhalden's *Handbuch Biochem. Arbeitsmethoden*, 6, 262 (1912).

In the case of proline this reaction should lead to the equation



(2 mol) + CH_2O =

Proline.



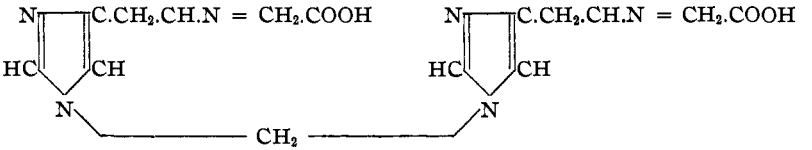
Methylene-proline.

while the negative $=CH_2$ group is able to convert the basic $—NH_2$ group into the neutral $—N = CH_2$ group, as shown by all *amino* acids, its acidifying influence is not sufficient to fully neutralize *two imino* groups. The group $=N—CH_2—N=$, resulting from the action of formaldehyde upon proline, is still somewhat alkaline and as such neutralizes in part the COOH group of proline. It is for this reason that the formaldehyde titration gives too low[1] results.

What is true of proline holds undoubtedly good for *hydroxyproline*, which stands in the same relation to proline as serine to alanine, both of which when formol titrated, give reasonably accurate results (99 and 98%, respectively).

Histidine.—The alkaline reaction of the histidine molecule is due to the presence in it of the imino group, the amino group being neutralized by the neighboring COOH group. The reaction of histidine with formaldehyde may be expressed as follows:

$2C_6H_9N_3O_2 + 3CH_2O = 3H_2O +$



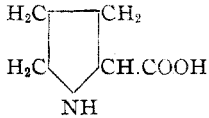
Inasmuch as the amino group of histidine is by the action of formaldehyde converted into the neutral group $—N = CH_2$, the imino group acting with formaldehyde as in the case of proline, we should expect the formol titration of histidine to give a result similar to that of proline. Actually, however, the formol titration of the former yields a more satisfactory result, namely, 89%[2] with phenolphthalein as indicator, as against 80% for proline. It seems reasonable to ascribe the better result in the

[1] Sörensen found 80 and 92%, using as indicator phenolphthalein and thymolphthalein, respectively. See *Biochem. Z.*, **7**, 59 (1907–08).

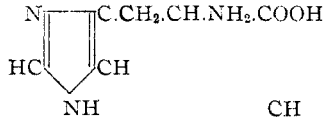
[2] *Biochem. Z.*, **7**, 79 (1907–08).

case of histidine to the slightly acidifying influence of the azole[1] group present in its molecule.

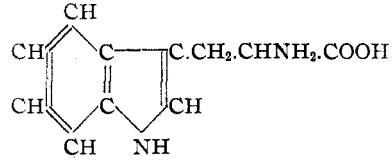
The formol titration of *tryptophane*, so far as we are aware, has not been given yet in the literature. Judging from the presence in its molecule of the =NH group, it seems safe to state that its formol titration will give a result below the theory, just as in the case of proline and histidine, to which it is chemically related as can be seen from their structural formulas



Proline.

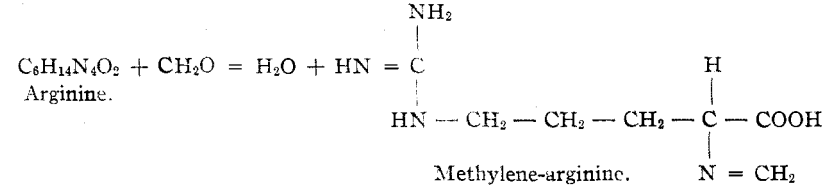


Histidine.



Tryptophane.

Arginine owes its alkaline reaction to the presence in its molecule of the guanidine nucleus, while the amino and carboxyl groups neutralize each other. When acted upon by formaldehyde the guanidine group in arginine behaves like guanidine itself, as pointed out already by Sörensen,[2] *i. e.*, it remains indifferent toward formaldehyde. We thus can write the reaction as follows:



Arginine. Methylene-arginine.

The resulting *methylene-arginine*, in which the amino group has been converted into the neutral —N =CH_2 group, is *neutral* because the free carboxyl group is now neutralized by the guanidine group which remained unchanged. It is for this reason that salts of arginine with acids, when treated with formaldehyde, can accurately be titrated as if the acids alone were present.

Inasmuch as *lysine* contains in its molecule only 2 amino groups, while other groups which could modify the carboxyl are totally absent, we should expect the formol titration of lysine to give an accurate result in accordance with the equation

[1] The slightly negative character of the azole is best illustrated by the fact that while the monazole, pyrrol, is practically neutral, the tetrazole turns litmus red.

[2] *Biochem. Z.*, **7**, 60 (1907-08).

$$H_2N.CH_2.CH_2.CH_2.CH_2.CH.NH_2.COOH + 2CH_2O =$$
(ω) Lysine. (α)

$$2H_2O + CH_2 = N.CH_2.CH_2.CH_2.CH_2.CH.N = CH_2.COOH.$$
Dimethylene-lysine.

In other words, after each of the 2 NH_2 groups has been converted into the neutral group $CH_2 = N—$, the dimethylene-lysine should represent a monocarboxylic acid. Actually, however, the formol titration of lysine[1] yields 92.5% (with phenolphthalein as indicator). It is suggested that in this case the *distance of one NH_2-group from the COOH group* comes into play. While the amino group in the α-position is strongly basic, neutralizing as it does the neighboring carboxyl group (as in the case of α-monoamino acids), the distant amino group (in the ω-position) is comparatively feebly basic. For this reason the former completely unites with the CH_2-group of the formaldehyde, while the weak amino group in the ω-position undoubtedly remains partly dissociated (free) and as such neutralizes the COOH group, at least in part, which explains the too low result obtained by the formol titration of lysine. This interpretation is supported, *e. g.*, by the fact that the formol titration of ornithine and α,ϑ-diamino adipic acid, in both of which the distant amino group is closer to the COOH group, yields more satisfactory results (98 and 99%, respectively), as compared with lysine and α,ϵ-diamino pimelic acid, whose formol titration gives 92.5 and 96%, respectively.

Summary.

The reasons were outlined as to why the formol titration method yields:

(1) Accurate results in the case of such amino acids which contain in their molecule *amino* and *carboxyl groups only*.

(2) Too low results in the case of amino acids which contain also the *imino* group.

(3) Too high or too low results in the case of amino acids which, in addition to amino and carboxyl groups, contain also *other* groups (the phenol or guanidine group).

(4) Too low results in the case of diamino acids in which (though they do not contain any modifying groups) the *distance* of one amino group from the carboxyl plays a role (*e. g.*, in lysine).

WASHINGTON, D. C.

[1] *Biochem. Z.*, **7**, 79 (1907–08).