# SOME OBSERVATIONS CONCERNING THE CHEMICAL REACTIONS OCCURRING BETWEEN FORMALDEHYDE AND PEPTONE

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The uptake of formaldehyde by peptone has been studied. Relatively stable powders (formol-peptones) can be prepared by exposing peptone powder to the vapour phase over formalin for four days and drying the product *in vacuo*. Different batches of such powders, obtained from the same sample of peptone are of similar composition but formol-peptones obtained from different makes of peptone may differ considerably in composition. The state of binding between the formaldehyde and the peptone in powder and in solution has been investigated by means of the chromotropic acid and Vorländer's reactions. The firmness with which formaldehyde is bound to peptone in solution depends not only on the quantities of formaldehyde and peptone present but also on the previous relationship of the two substances. The reactions between formaldehyde and peptone have been found to be complex. Equilibrium is not readily attained either in solution or when peptone powder is exposed to formalin vapour.

IT has been known for a long time that the disinfectant activity of such substances as formaldehyde (HCHO) is reduced by the presence of organic matter such as peptone. Bullock and Rawlins found that during the spray-drying process while 0·02 per cent formaldehyde ensured a sterile product in the absence of peptone, 0·4 per cent was necessary in the presence of peptone[1]. In later work, when studying the disinfection of powders by formaldehyde vapour, it was found that even small amounts of peptone reduced considerably the effectiveness of the HCHO[2]. Since formaldehyde is used as a disinfectant both in solution and as vapour it was decided to investigate the reactions occurring between peptone powder and formaldehyde vapour as well as the reactions occurring in solution and if possible, to relate the types of combination between these two substances with the disinfectant power of the products. These observations are the subject of a separate report[3].

The subject has interest because of the use of HCHO in the preparation of toxoids as well as its use as a disinfectant in solution and as vapour in the sterilisation of surfaces and powders.

## EXPERIMENTAL

*Peptone.* Three commercial brands of peptone A, B and C were used. A fourth powder D was obtained by dissolving a quantity of A in water and spray-drying the solution. A comparison of powders A and D was used to determine whether the physical characteristics of a spray-dried powder exerted any effects. Since no such effects were observed no further reference to powder D will be made.

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*Solution of Formaldehyde B.P.* (Formalin) found to contain 38·2 per cent HCHO by the B.P. assay process was used in the preparation of formol-peptones and after dilution and neutralisation to phenolphthalein, in the formol titrations.

*Solution of Formaldehyde A.R.* (Formalin A.R.) found to contain 38·4 per cent HCHO by the B.P. assay process was used in all other cases.

*Chromotropic acid and dimedone* (5 : 5-*dimethyl*cyclo*hexane*-1 : 3-*dione*) were both of commercial quality (B.D.H.).

*Formol-peptone* was prepared by placing a quantity of formalin in the bottom of a desiccator and above it a thin layer of peptone on a clock glass. After four days in the closed desiccator, the peptone, now a sticky paste, was transferred to a second desiccator and dried over $P_2O_5$ under reduced pressure. After one week's drying the resultant "formol-peptone" was powdered. Such formol-peptones were prepared from peptones A, B and C.

*Carbonate free alkali.* Solutions of sodium hydroxide for use in titrations were freed from carbonate by the addition of barium chloride and filtration before standardisation with oxalic acid (phenolphthalein indicator).

*Chromotropic acid reagent*[2]. 0·2 g. chromotropic acid + 20 ml. water was filtered and 80 ml. sulphuric acid solution (2 volumes sulphuric acid A.R. + 1 volume water) added to the filtrate; it was stored in a stoppered bottle protected from light and was prepared freshly each week.

*Vorländer's Reagent*[4] (*dimedone solution*). 0·2 g. dimedone in 100 ml. of McIlavaine buffer (22·7 ml., 0·2M sodium phosphate + 2·3 ml., 0·1M citric acid) was prepared freshly each week.

## Methods

*Neutralising titration values* (*N.T.Vs.*) *and formol titration values* (*F.T.Vs.*). 10 ml. of peptone solution, containing approximately 100 mg. peptone, was titrated with 0·1N NaOH to a pink colour with phenolphthalein to obtain the neutralising titre. 10 ml. of neutral HCHO solution, equal parts of formalin and water neutralised to phenolphthalein, was then added and the mixture again titrated with 0·1N NaOH to a pink colour to obtain the formol titration titre. The titres were used to calculate the number of ml. of 0·1N NaOH required by 1 g. of the original peptone for neutralisation before (N.T.V.) and after addition of the HCHO (F.T.V.). These values were used by Bullock and Sen in their work on papain[5] and the tryptic activity of pancreatin[6].

*Determination of formaldehyde by the chromotropic acid reagent*[2,7]. 1 ml. of test solution, containing up to 12 $\mu$g. HCHO, was mixed with 9 ml. of chromotropic acid reagent, heated at boiling water bath temperature for 30 minutes and the resultant purple colour evaluated in a Spekker photoelectric absorptiometer using a combination of blue ($OB_2$) and orange ($OY_2$) filters and as blank a solution obtained by repeating the above process using 1 ml. of water in place of 1 ml. HCHO solution. There was a practically linear relation between the quantity of HCHO

and the colour developed under the conditions. A calibration curve was used to convert Spekker readings into $\mu$g. of HCHO in subsequent assays.

*Determination of chemically free and chemically bound HCHO by Vorländer's Reagent*[4]. 2 ml. of test solution, containing up to 24 $\mu$g. total HCHO, was mixed with 2 ml. of Vörlander's reagent and held at 37° for 30 minutes[7]. HCHO was determined in 1 ml. of this mixture by chromotropic acid reagent as described above. The HCHO estimated in this way, that is that strongly enough bound to peptone to resist combination with Vorländer's but nevertheless capable of reacting with

TABLE I

PERCENTAGE OF HCHO, ADDED TO WATER AS FORMALIN, WHICH COMBINES WITH VÖRLANDER'S REAGENT

| Experiment number | HCHO added mg. | HCHO reacting with CA in presence of VR, mg. | HCHO combining with VR, mg. | HCHO combining with VR per cent |
|---|---|---|---|---|
| 1 | 52·0 | 1·4 | 50·6 | 97·3 |
| 2 | 49·1 | 0·9 | 48·2 | 98·2 |
| 3 | 99·2 | 3·6 | 95·6 | 96·4 |
| 4 | 227·2 | 6·4 | 220·8 | 97·2 |
| 5 | 164·0 | 3·1 | 160·9 | 98·1 |

CA = Chromotropic acid reagent.
VR = Vörlander's reagent.

chromotropic acid reagent, will be referred to below as bound HCHO (V.R.) Free HCHO (V.R.) was determined as the difference between the figures for HCHO by chromotropic acid reagent in the presence and in the absence of Vorländer's reagent. Macfadyen[7] found that only 98·5 per cent of free HCHO is fixed by dimedone. Table I shows that under the conditions used in this work when a dilution of formalin A.R. is examined by the Vorländer reaction about 97·4 per cent is returned as "free" and 2·6 per cent as "bound". Making the assumption that such a dilution contains only free HCHO all experimental figures were adjusted to allow for this finding before being recorded as free HCHO (V.R.).

## RESULTS

The reactions between peptone and formaldehyde have been found to be complex. Not only is there a difference in the ways in which formaldehyde reacts with the peptone when, on the one hand both are in solution, or, on the other hand, when the peptone is as powder and HCHO as it occurs in the vapour phase over formalin, but even under constant environmental conditions the changes are progressive and dependent on the degree of access of moisture vapour or water. Three types of changes have been followed. (1) Changes when (*a*) peptone powder is exposed to the vapour phase over formalin, and when (*b*) the products are dissolved in water. (2) Changes when the dried products of (1), i.e., formol-peptones are dissolved in water. (3) Changes in peptone solutions to which are added quantities of HCHO similar to those present in the formol-peptones.

(1) *Changes in neutralising and formol titration values when* (a) *peptone powder is exposed to the vapour phase over formalin, and* (b) *further changes in these values when the products are dissolved in water.* (*a*) The N.T.Vs. of peptones A, B and C were found to be 10·5, 14·2 and 3·3 respectively,

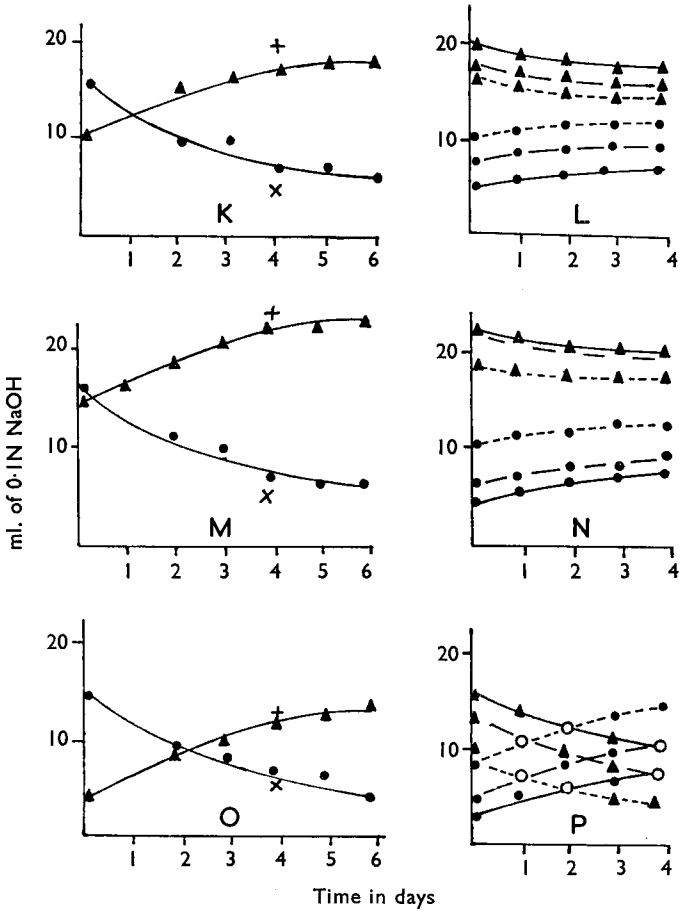



FIG. 1. Changes with time in the neutralising titration values (N.T.V. ▲ ▲) and formol titration values (F.T.V. ● ●) of (*a*) peptones A, B and C when exposed to the vapour phase over formalin (graphs K, M and O respectively) (*b*) solutions of the resultant pastes after exposure for 2 days (– – – – L, N and P) and for 4 days (— — L, N and P) (*c*) solutions of the corresponding formol-peptones (—— L, N and P).

In K, M and O, + represents the N.T.V. and × the F.T.V. of the corresponding formol-peptone.

the corresponding F.T.Vs. being 16·0, 15·4 and 14·9. Assuming that one equivalent of alkali corresponds to one equivalent of nitrogen these figures correspond to 2·24 per cent, 2·16 per cent and 2·09 per cent of amino nitrogen in the corresponding peptones.

Two gram quantities of peptone were, as described above, exposed in a desiccator to the vapour phase over formalin. The N.T.Vs. and F.T.Vs. of the resultant sticky masses were determined after two, three, four, five and six days' exposure in the desiccator. For comparative purposes and to avoid complications due to the moisture content of the peptones, the values were expressed per gram of original peptone. The results for peptones A, B and C are graphed in Figure 1, K, M and O.

(*b*) When the resultant sticky masses were dissolved in water the N.T.Vs. and F.T.Vs. of the solutions changed slowly over the first three or four days and then became constant for some time. These changes are shown in Figure 1, L, N and P.

(2) The N.T.Vs. and F.T.Vs. of formol-peptones A, B and C are shown marked × and + respectively in Figure 1, K, M and O. It was found that different batches of formol-peptone prepared from the same sample of peptone were of similar composition. It can be seen from the × and + marks in Figure 1 that the formol-peptone C differed considerably in composition from formol-peptones A and B (see also Fig. 2). The changes which occurred in the first four days when these formol-peptones were dissolved in water are graphed in Figure 1, L, N and P. In an experiment which lasted ten days the values became practically constant after four days.

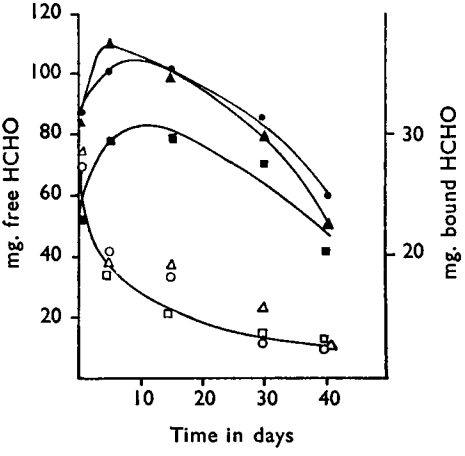



FIG. 2. Changes in bound HCHO and free HCHO on storage of solutions of formol-peptones.

| Free HCHO | | | Bound HCHO | | |
|---|---|---|---|---|---|
| Peptone | A | ▲ | Peptone | A | △ |
| ,, | B | ● | ,, | B | ○ |
| ,, | C | ■ | ,, | C | □ |

*Chemically bound and chemically free formaldehyde in solutions of formol-peptones.* There is a sense in which all the HCHO in formol-peptone could be considered to be bound either physically or chemically since the final step in the preparation was a six day storage *in vacuo*. There is also a sense in which the state of the HCHO in formol-peptones cannot be determined by the chemical methods described above, since, in such methods, aqueous solutions are used and, as shown below, as soon as formol-peptones are exposed to either water vapour or water, changes in the state of combination of the HCHO set in. However, for the present purposes, it will be considered that the state of chemical binding of the HCHO in a formol-peptone can be judged by examining a freshly prepared solution.

*Calculation of chemically bound formaldehyde from F.T.Vs.* When formaldehyde reacts with peptone it must react with some chemically active centres, generally considered to be free-$NH_2$ groups. If, therefore, a small quantity of HCHO is added to some peptone the F.T.V. of the latter will decrease in proportion to the number of receptors blocked and therefore it is reasonable to consider the drop in F.T.V. to be a measure of the chemically bound formaldehyde. If the F.T.V. of peptone in a solution containing HCHO increases, it indicates that some of the HCHO previously bound to the peptone has become free. If the F.T.V. decreases then conversely some of the HCHO has become bound to the peptone. By making these assumptions it is possible to calculate the bound HCHO in formol-peptones. Thus for peptone A:—

F.T.V. original peptone = 16·0
F.T.V. formol-peptone A = 4·3

Difference = 11·7 ml. 0·1N NaOH

but 1 ml. 0·1N NaOH in the formol titration is ≡ 3 mg. HCHO
∴ 11·7 ml. 0·1N NaOH ≡ 35·1 mg. HCHO

Thus a quantity of formol-peptone A equivalent to 1 g. of original peptone A contained 35·1 mg. bound HCHO. The corresponding quantities for formol-peptones B and C were 33·2 mg. and 25·2 mg. respectively.

In the following discussion bound formaldehyde determined in this way is referred to as bound HCHO (F.T.V.). Changes in the F.T.Vs. of solutions of formol-peptones can thus be expressed as changes in bound HCHO (F.T.V.). It was found that the values for bound HCHO (F.T.V.) were about 10 per cent and 20 per cent higher than bound HCHO (V.R.) for formol-peptones A and B respectively. The two values were about the same for formol-peptone C.

The changes in free HCHO (V.R.) and bound HCHO (V.R.) which occur when formol-peptone solutions are stored are shown in Figure 2.

*The fate of HCHO added as Formalin A.R. to* 1 *per cent peptone solutions.* It was decided that it would be useful to compare the difference in the mode of combination between HCHO and peptone when the former is (*a*) taken up from the vapour phase over formalin as in the preparation of formol-peptone and (*b*) added as formalin A.R. to 1 per cent peptone solution. To do this the above results with formol-peptone solutions were compared with similar results obtained by storing 1 per cent peptone solutions to which had been added quantities of HCHO corresponding to the quantities of total HCHO estimated to be present in the corresponding 1 per cent formol-peptone solutions. Thus, in 1 per cent solutions of peptones A and B were incorporated quantities of formalin A.R. corresponding to 0·1 per cent HCHO while to the 1 per cent solution of peptone C, 0·06 per cent HCHO was added.

The results of submitting such solutions, immediately on preparation and after storage, to the usual analytical procedure is shown in Figures 3 and 4. These should be compared with Figures 1 and 2.

*Manner of the uptake of HCHO by peptone from the vapour phase over formalin.* The increase in weight of the peptone on conversion to formol-peptone falls between that required for the simple addition of HCHO (M.Wt. = 30) and that required for this followed by elimination of 1 molecule of water (HCHO — $H_2O$ = 30 — 18 = 12). Using peptone

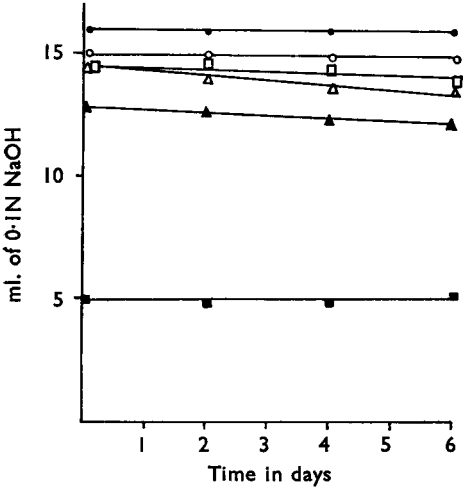



FIG. 3. Changes with time in N.T.Vs. and F.T.Vs. when to 1 per cent solutions of peptones A, B and C are added 0·1 per cent, 0·1 per cent and 0·06 per cent respectively of HCHO.

| Peptone | N.T.V. | F.T.V. |
|---|---|---|
| A | ▲ | △ |
| B | ● | ○ |
| C | ■ | □ |

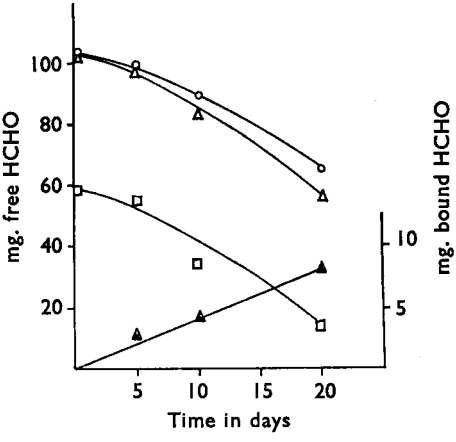



FIG. 4. Changes in free HCHO and bound HCHO in 1 per cent aqueous solutions of peptones A, B and C.

| | HCHO Added | Free | Bound |
|---|---|---|---|
| A | 0·1 per cent | △ | ▲ |
| B | 0·1 ,, | ○ | ▲ |
| C | 0·06 ,, | □ | ▲ |

A as an example, the following calculation illustrates the way in which the results shown in Table II were obtained.

1·9564 g. dry peptone gave 2·1022 g. formol-peptone showing an increase of 0·1458 g. due to uptake of HCHO. But by the chromotropic acid reagent method the formol-peptone contained the equivalent of 0·1879 g. HCHO. If therefore the HCHO had been taken up without loss of water the increase in weight would have been 0·1879 g. On the other hand if for every molecule of HCHO added 1 molecule of water had been lost the increase in weight would have been $0{\cdot}1879 \times \frac{12}{30} = 0{\cdot}07516$ g.

Increase in weight with no loss of water = 0·1879. Increase in weight with 100 per cent loss of water = 0·0752. Difference = 0·1127. Increase in weight with no loss of water = 0·1879. Increase in weight found = 0·1458. Difference = 0·0421. Therefore the percentage of the reactions occurring with loss of water = $100 \times \frac{0{\cdot}0421}{0{\cdot}1127} = 37$. Therefore the percentage of the reactions occurring without loss of water = 63.

*Storage of formol-peptone.* Two samples of formol-peptone powder A were stored for 45 days. The first in a desiccator over $P_2O_5$, lost only 3·7 per cent of its total HCHO content (chromotropic acid method). The powder remained dry and free flowing. The second sample which was stored in the laboratory in a bottle closed with a cotton wool plug lost 28 per cent of its total HCHO. The powder caked due to uptake of moisture. In an attempt to ascertain whether the decrease in HCHO content of the formol-peptone in the second sample was due to release and volatilisation of HCHO resulting from moisture uptake, a thin layer

TABLE II

DATA FOR CALCULATING THE PERCENTAGE OF THE REACTIONS BETWEEN HCHO AND PEPTONE INVOLVING ELIMINATION OF WATER

| Peptone | Weight taken | Weight after drying | Weight of resultant formol-peptone | Increase in weight due to uptake of HCHO | HCHO content by CA | Percentage of reactions not involving elimination of $H_2O$ |
|---|---|---|---|---|---|---|
| A | 2·0572 | 1·9564 | 2·1022 | 0·1458 | 0·1879 | 63 |
| B | 2·0062 | 2·0056 | 2·2056 | 0·2000 | 0·2456 | 69 |
| C | 2·0900 | 2·0758 | 2·2700 | 0·1942 | 0·2268 | 76 |

TABLE III

LOSS OF HCHO FROM FORMOL-PEPTONE POWDER WHEN STORED OVER: (*a*) 0·1N $K_2Cr_2O_7$ + $H_2SO_4$ AND (*b*) SOLID $K_2Cr_2O_7$ + $H_2SO_4$. FIGURES GIVE THE LOSS OF HCHO AS PER CENT OF THE TOTAL PRESENT (CA METHOD)

| (*a*) | | | (*b*) | | |
|---|---|---|---|---|---|
| Time of storage, days | Loss by CA | Loss by $K_2Cr_2O_7$ reduction | Time of storage, days | Loss by CA | Loss by $K_2Cr_2O_7$ reduction |
| 4 | 57 | 62 | 7 | 4·0 | 10·0 |
| 6 | 62 | 64 | 30 | 4·5 | 11·5 |

of formol-peptone was stored over (*a*) acidified 0·1N $K_2Cr_2O_7$ and (*b*) solid $K_2Cr_2O_7$ moistened with strong sulphuric acid. The results are shown in the Table III.

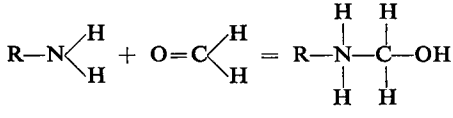
## DISCUSSION

It is apparent from Figure 1, K, M and O that in addition to water, peptone powder absorbed HCHO when exposed in a thin layer to the vapour of formalin; the N.T.V. rose and the F.T.V. fell. The sum of these two values remained approximately constant. Presumably the HCHO in the vapour phase in the presence of the moisture reacted with the peptone in a manner comparable to that in which addition takes place during a formol titration. The drop in F.T.V. was at first rapid then it slowed but was not complete after six days. The products became increasingly less soluble in water during the six days. When the resultant sticky masses were dissolved in water there was an initial slight reversal of the changes which had been progressing. As shown in Figure 1, L, N and P, the N.T.Vs. fell and the F.T.Vs. rose, these changes being progressive for four days when the values remained approximately constant for the next six days. Taking all these facts into consideration it was decided

to prepare the formol-peptones by drying the peptone pastes after four days exposure to the vapour phase over formalin. One would have expected that when the pastes were removed from the formalin vapour and placed over $P_2O_5$ under reduced pressure the uptake of HCHO would have ceased or even been reversed. However, from Figure 1, K, M and O it can be seen that the N.T.V. (marked +) has increased and the F.T.V. (marked ×) has decreased beyond the values to be expected from exposure for four days. The pastes presumably contained free HCHO which continued to react with the peptone. It should be mentioned that when the pastes were exposed to reduced pressure they frothed to a considerable extent due to the vapourisation of the water and free HCHO. This considerably facilitated the drying process and resulted in a porous, easily powdered, solid residue being obtained. In spite of these facts and in spite of the fact that the dried and powdered formol-peptones were left over $P_2O_5$ under reduced pressure for six days, when the formol peptone powders were dissolved in water to give a 1 per cent solution the N.T.Vs. fell and the F.T.Vs. rose in a manner which paralleled the changes occurring when the corresponding pastes were similarly dissolved in water. Presumably the equilibrium between HCHO and peptone is different in pastes and in the 1 per cent solutions of the peptone. When 1 per cent formol-peptone solutions were stored, as shown in Figure 2, the bound HCHO (V.R.) fell, at first rapidly, then with increasing slowness. There was, during the first six days, a rise in free HCHO (V.R.) which was followed by a fall which became increasingly rapid. The fall was probably due to loss of HCHO by volatilisation from the solution. It was observed that the solutions, after one month became turbid due to multiplication of adventitious micro-organisms. Initially the free HCHO rose to a greater extent than the bound HCHO fell. This was due to the fact that the total HCHO as judged by the chromotropic acid method rose. During the first six days the rise in total HCHO calculated per gram of original peptone was 15·9 mg., 7·7 mg. and 18·0 mg. respectively for formol-peptones A, B and C. Presumably in formol-peptone some HCHO is bound so firmly that it no longer reacts with chromotropic acid; some of this HCHO becomes reactive again in the 1 per cent solution.

A comparison of Figures 3 and 4 with Figures 1 and 2 shows that in solutions of identical composition the strength of binding between HCHO and peptone may vary. In 1 per cent formol-peptone solution the HCHO is largely bound, in a 1 per cent solution of the same peptone a corresponding quantity of HCHO added as formalin A.R. is mainly free and remains mainly in the free state until it can no longer be detected—the free HCHO falling from the beginning and continuing to fall with increasing rapidity.

Nitschmann and Hadorn[8] suggested that when formaldehyde vapour was taken up by casein the HCHO was first bound as "methylol radical".



The methylol radicals then condensed with adjacent reactive groups with the elimination of water forming methylene bridges.

An attempt was made to ascertain whether similar reactions occurred between peptone and HCHO. It appeared (Table II) that while in some cases water is eliminated, in the majority of cases the HCHO and peptone react without loss of water. It must, however, be emphasised that the methods used in the determinations on which the calculations are based are liable to considerable error.

Even when formol-peptone powder is stored over concentrated $H_2SO_4$ and solid $K_2Cr_2O_7$ reducing vapours (HCHO) are evolved to a slight extent (Table III). When stored over 0·1N $K_2Cr_2O_7$ acidified with sulphuric acid, the powders take up water and evolve HCHO to a considerable extent. It is obvious that in all experiments where the disinfectant effects of HCHO are being studied the presence or absence of water must be taken into account.

Perhaps the most interesting conclusion which can be drawn from the present work is that the interactions between HCHO and peptone are complex and do not readily reach equilibrium.

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