XXXVIII. THE DETERMINATION OF AMINO-ACIDS IN WHEAT FLOUR.

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THE work here reported was undertaken as a preliminary to the investigation of the changes in the amino-acid content of flour dough during fermentation. In order to study these changes it is necessary to stop the enzyme action at different times before extracting and estimating the amino-acid content, unless the extract can be prepared in a time negligibly short in comparison with the time that fermentation has proceeded, and provided that there is no production of amino-acid in the aqueous extract. A disadvantage of the freezing method employed by Brownlee and Bailey [1930] to inhibit enzyme action is that the enzymes are not destroyed, and may become active during the extraction if this is carried out at normal temperatures, so that observed results may not necessarily be due to fermentation in the dough.

Denham and Scott Blair [1927] found that the variations in amino-acid content of flour-water suspensions for the first few hours after mixing, which were described by Swanson and Tague [1916; 1917], also occurred in the clear extract of the flour and concluded that the proteolytic activity of a flour could be measured by the increase in amino-acid content of the extract. The method of estimation used by Denham and Scott Blair, however, is open to criticism, as shown later. Swanson and Tague [1916] found that the amino-acid content of a flour extract increased on standing, but as their estimations were made weekly they are not comparable with those of Denham and Scott Blair [1927]. Herd [1931] reported that "the change in amino-nitrogen content of the flour extracts is negligible in all cases over a period of 118 hours," but the change in titre reported by Denham and Scott Blair [1927] would not cause a significant change in the titres reported by Herd [1931], and differences in technique render the two sets of results not strictly comparable.

In view of these apparently conflicting statements it seemed advisable to investigate the change in amino-acid content of a flour extract on standing, particularly for the period immediately after the separation of the flour.

Methods based on the formaldehyde titration of Sørensen [1907, 1] and the alkalimetric titration of Foreman [1920] have been used in flour work, and the Sørensen method has always been found the most suitable, though in the data published by Cairns and Bailey [1928] on a comparison of the two methods, the Foreman titration was carried out in 50 % alcohol, and its rejection was therefore not entirely justified since in 50 % alcohol the polypeptides would titrate but only a part of the amino-acids [see Willstätter and Waldschmidt-Leitz, 1921].

EXPERIMENTAL.

Although the procedures described by Swanson and Tague [1916] (for the Sørensen titration), by Foreman [1920] and by Harris [1923] (for a modified Foreman titration) all gave results accurate to within a few per cent. of the theoretical figures with pure amino-acids, these methods were

found to be unsuitable for flour extracts, the Foreman and Harris methods because they involve the dilution of the extract with nine volumes of 95 % alcohol (and the amino-acid content of the extract is originally very small) and the Swanson and Tague method because the slight opalescence of the flour extract renders the final end-point with phenolphthalein or thymolphthalein somewhat indefinite. The technique recommended by Brown [1923] for the formaldehyde titration of bacteriological media was therefore tried and was found very satisfactory.

To 10 ml. of flour extract was added 0.2 ml. of phenol red solution and the solution titrated to p_H 8.0 with $N/14$ NaOH, using a micro-burette calibrated to 0.01 ml. The end-point was obtained by matching with a buffer solution of p_H 8.0 in a comparator block. This titration (stage 1) represents the titratable acidity of the extract. To the sample were then added 8 ml. of commercial 40 % formaldehyde, 0.18 ml. of indicator solution, and the mixture was again titrated to p_H 8.0. (0.18 ml. of indicator was added because the blank titration on the formaldehyde is about 1 ml.). This second stage titration represents the alkali required both to neutralise the amino-acid in solution and to bring the formaldehyde to p_H 8.0. In each case suitable screens of extract and of diluted extract were used in the buffer side of the comparator block. This second stage titration was corrected by subtracting from the titre a blank titration to p_H 8.0 of 8 ml. of formaldehyde diluted with 10 ml. of distilled water instead of bringing the formaldehyde to p_H 8.0 before adding to the flour extract. No change in the blank titration was ever observed during the few hours occupied by the experiments to be described.

The end-points obtained by this method were very sharp and distinct and were sensitive to less than 0.01 ml. of $N/14$ NaOH. An endeavour to increase the sensitivity of the method by using $N/100$ alkali was not very successful owing to lack of distinctness of the end-point. Other indicators offered no advantage.

It is recognised that, under the conditions of this work, titrating the test solution to a colour match with the standard buffer solution does not necessarily mean that the p_H of the test solution and the buffer solution are the same [see Halton and Fisher, 1928], but the conditions are at least reproducible. Moreover, the electrometric determination of the p_H of a solution containing 16 % of formaldehyde would introduce difficulties in the error of the junction potential with the formaldehyde solution.

Table I. *Amino-nitrogen in flour extract at various times.*

Titration	Time (mins.)	Stage 1, ml. $N/14$ alkali per 2 g. flour	Stage 2, mg. amino-N per 2 g. flour
Flour (a)			
1	0	0.21	0.30
2	10	0.21	0.30
3	20	0.21	0.30
4	60	0.22	0.30
5	90	0.22	0.29
6	120	0.21	0.30
7	165	0.21	0.31
8	225	0.21	0.30
9	345	0.22	0.30
10	495	0.21	0.31
Flour (b)			
1	0	0.24	0.30
2	10	0.24	0.30
3	25	0.23	0.31
4	40	0.22	0.30
5	75	0.23	0.30
6	120	0.22	0.30
Flour (c)			
1	0	0.31	0.41
2	20	0.32	0.42
3	40	0.32	0.41
4	70	0.32	0.41
5	120	0.33	0.41
6	180	0.32	0.41

For the preparation of the extract the method described by Denham and Scott Blair was used. Flour, equivalent to 40 g. dry flour, was extracted by shaking for five minutes with 200 ml. of distilled water to which toluene had been added, the mixture centrifuged and the slightly opalescent extract decanted through a filter. The filtrate was titrated immediately and after various intervals of time. Representative results, given in Table I, for three different flours, (a) from a typical mill mixture, (b) from English wheat, (c) a 30 % low grade flour, show that there was no change in the amino-acid content of the extract on standing in the presence of toluene, nor was there any change in titratable acidity.

Further experiments in the absence of toluene gave the same results, thus confirming the finding of Denham and Scott Blair that for short period experiments toluene did not affect the results. Similar results were obtained when the accuracy of the method was increased five-fold by titrating 50 ml. of solution instead of 10 ml., so that the failure to observe an increase in amino-acid content with time was not due to the insensitivity of the method. Experiments were conducted in which the extract was maintained at 25° and 37° respectively, but no increase in amino-acid titration was obtained.

That the flours used exhibited the phenomenon of increase in amino-acid content with increasing time of extraction is shown in Table II. This table also shows the increase in titratable acidity (stage 1) with increasing time of extraction. This increase is due, at least in part, to an increase in the buffer value of the extract, caused by the production of inorganic phosphate from phytin by the action of phytase [Bailey and Peterson, 1921], but may also be due to the production of organic acids by fermentation processes. The low-grade flour (c) shows a much greater increase in amino-nitrogen than flours (a) or (b).

Table II. *Increase in amino-nitrogen with time of extraction.*

Time of extraction	Stage 1, ml. N/14 alkali per 2 g. flour	Stage 2, mg. amino-N per 2 g. flour
Flour (a)		
5	0.19	0.30
20	0.27	0.32
35	0.32	0.35
50	0.33	0.35
Flour (b)		
5	0.24	0.30
15	0.28	0.33
30	0.33	0.33
55	0.40	0.37
75	0.44	0.38
105	0.49	0.39
Flour (c)		
5	0.31	0.42
30	0.56	0.48
60	0.73	0.54
120	0.85	0.55

Since the results shown in Table I are directly opposed to the findings of Denham and Scott Blair, the work was repeated, using the method of estimation described by these authors (an acid titration of one portion to p_H 5.5 with methyl red and an alkali titration of a second portion to "a full rose colour" with phenolphthalein¹ in the presence of formaldehyde) both in the presence and absence of toluene. With the mill mixture and English flours no increase in amino-acid content was obtained either with or without toluene, but the extract of the low-grade flour showed an increase in the titration with time whether

¹ For this end-point a buffer solution of p_H 9.0 was used in the work here described.

Table III. *Amino-nitrogen content of extract of low-grade flour (no toluene).*

Time mins.	Denham and Scott Blair method			Brown method	
	ml. acid N/14	ml. alkali N/14	Total amino-N mg. per 2 g. flour	Stage 1	Stage 2, mg. amino-N per 2 g. flour
0	0.11	0.77	0.88	0.32	0.41
20	0.11	0.85	0.96	0.31	0.41
40	0.11	0.88	0.99	0.31	0.40
60	0.12	0.90	1.02	0.31	0.41
120	0.14	0.91	1.05	0.31	0.41

toluene were present or not. Some typical figures are given in Table III which show an increase in the amino-nitrogen content of the extract as estimated by the method of Denham and Scott Blair and no increase by the technique of Brown. Table IV shows the absence of any change in amino-nitrogen content of the "mill mixture" and the English flour and the increase in the case of the low-grade flour by the Denham and Scott Blair method irrespective of the presence or absence of toluene.

Table IV. *Amino-nitrogen content of flour extracts with and without toluene.*

Time mins.	Denham and Scott Blair method					
	Without toluene			With toluene		
	ml. acid N/14	ml. alkali N/14	Total amino-N mg. per 2 g. flour	ml. acid N/14	ml. alkali N/14	Total amino-N mg. per 2 g. flour
English flour						
0	0.06	0.63	0.69	0.06	0.63	0.69
30	0.06	0.62	0.68	0.06	0.63	0.69
60	0.06	0.64	0.70	0.06	0.63	0.69
120	0.06	0.63	0.69	0.06	0.63	0.69
240	0.06	0.63	0.69	0.06	0.63	0.69
Mill mixture						
0	0.07	0.60	0.67	0.07	0.61	0.68
30	0.07	0.60	0.67	0.07	0.60	0.67
60	0.07	0.61	0.68	0.07	0.61	0.68
120	0.07	0.61	0.68	0.07	0.61	0.68
Low grade flour						
0	0.10	0.79	0.89	0.11	0.80	0.91
30	0.11	0.88	0.99	0.11	0.89	1.00
60	0.11	0.92	1.03	0.12	0.92	1.04
120	0.12	0.94	1.06	0.13	0.94	1.07
180	0.13	0.96	1.09	0.13	0.95	1.08

The slight discrepancies between the amino-nitrogen content of the low grade extract with and without toluene are due to the technique of this experiment which was to prepare several hundred ml. of extract, divide this into two portions and add toluene to one portion. Each portion was then titrated in turn, so that before the initial titration the "toluene" extract had stood a slightly longer period than had the "no toluene" extract, and this is reflected in the slightly higher amino-nitrogen content of the "toluene" extract.

Table III also illustrates the great difference in the absolute magnitude of the amino-acid content as determined by the two methods; that obtained by the Denham and Scott Blair method (the sum of the two titrations) being some two to three times as great as that obtained by titrating to the same p_H before and after the addition of the formalin solution. This discrepancy is due to the titratable acidity and buffer capacity of the flour extract which are included as amino-acid by the Denham and Scott Blair method.

Though the main object of this investigation had been accomplished in showing that no measurable proteolysis occurred in the flour extracts for the few hours immediately subsequent to the separation of the flour, it was thought to be of interest to try and explain the anomalous result obtained with the low-grade flour. Extracts of this flour, prepared as described above, were analysed in the following manner after various periods of standing. 10 ml. of extract were titrated with $N/14$ HCl to p_H 5.5 as in the Denham and Scott Blair method. To another 10 ml. was added 0.2 ml. of thymol blue indicator and the solution titrated to match successively the colours in buffers of p_H 8.0, 8.5, 8.7 and 9.0; formaldehyde and further indicator solution were then added and the mixture again titrated to these end-points. Table V shows the results obtained in one such experiment when toluene water was used for the extraction. The amino-acid by the method of Denham and Scott Blair is the sum of the titration to p_H 5.5, the titratable acidity and the amino-acid by the method of Brown.

From Table V it is seen that there is no change in the buffer value of the solution as shown by the acid titration to p_H 5.5 and the alkali titrations to

Table V. *Determination of amino-acid at various times in the aqueous extract of a low-grade flour by the methods of Brown and of Denham and Scott Blair.*

Time in mins.		from first titration	0	20	40	60	120
		from mixing	20	40	60	80	140
Acid titration to p_H 5.5; ml. $N/14$ HCl			0.10	0.10	0.10	0.10	0.10
p_H 8.0	Titration before adding formaldehyde; ml. $N/14$ NaOH		0.31	0.31	0.31	0.31	0.31
	Amino-nitrogen mg. per 2 g. flour	Brown Denham and Scott Blair	0.42 0.83	0.41 0.82	0.42 0.83	0.42 0.83	0.41 0.82
p_H 8.5	Titration before adding formaldehyde; ml. $N/14$ NaOH		0.39	0.38	0.39	0.39	0.38
	Amino-nitrogen mg. per 2 g. flour	Brown Denham and Scott Blair	0.38 0.87	0.38 0.86	0.38 0.87	0.38 0.87	0.38 0.86
p_H 8.7	Titration before adding formaldehyde; ml. $N/14$ NaOH		0.41	0.40	0.41	0.42	0.41
	Amino-nitrogen mg. per 2 g. flour	Brown Denham and Scott Blair	0.36 0.87	0.35 0.85	0.35 0.86	0.34 0.86	0.35 0.86
p_H 9.0	Titration before adding formaldehyde; ml. $N/14$ NaOH		0.48	0.48	0.48	0.48	0.48
	Amino-nitrogen mg. per 2 g. flour	Brown Denham and Scott Blair	0.28 0.86	0.33 0.91	0.38 0.96	0.40 0.99	0.48 1.06

p_H 8.0, 8.5, 8.7 or 9.0 respectively. Also this latter titration indicates that there is no change in the titratable acidity of the extract on standing. The amino-acid by the method of Brown is less the higher the p_H to which the solution is titrated, in accordance with the theory of the titration. At p_H 8.0, 8.5 and 8.7 there is no increase in amino-acid as estimated by either method, but at p_H 9.0 there is an increase by both methods.

It was suspected that this increase at p_H 9.0 was due to the production by enzyme action of substances containing both carboxyl and amino-groups but which did not become titratable even under the action of formaldehyde until p_H 9.0 was reached. If so, such increase might be enhanced were the enzyme

allowed to act on the flour substrate instead of on extracted protein only. A suspension of 40 g. of "dry" flour in 200 ml. of distilled water was allowed to autolyse at 27°. From time to time samples were taken, centrifuged, filtered and the filtrate titrated to p_H 8.0, 8.5, 8.7 and 9.0 respectively as before. The results of one such experiment are shown graphically in Fig. 1, plotting amino-nitrogen against time.

Fig. 1. Amino-nitrogen in flour-water digest at various times.

Initially the amino-acid content is lower the higher the p_H used, as is to be expected, and all values increase with time. This increase is due to further extraction¹ of the amino-acids from the flour and also to the production of amino-acids from the proteins. The rapid increase for about an hour is due almost entirely to extraction, the subsequent slow increase is due to proteolysis, and the slope of this second portion of the graph measures the proteolytic activity of the flour. The graphs at p_H 8.0, 8.5 and 8.7 are practically parallel, but the graph for p_H 9.0 crosses the other graphs, indicating the more rapid production of substances titratable at p_H 9.0.

The addition of yeast to a flour-water suspension did not enhance this effect, because the amino-acid produced was used by the yeast in its metabolism, and this rendered difficult the interpretation of the results from the standpoint of the present work. The indications were, however, that at each estimation after the first a greater titration was obtained at p_H 9.0 than at p_H 8.0 after the addition of formaldehyde. There was also evidence that the titration at p_H 8.7 was increased in the presence of yeast.

Table VI shows the results obtained from a takadiastase digest of the gluten washed from this flour. The amino-nitrogen estimated at p_H 9.0 increases more

¹ It may be noted that extraction was complete in one hour, so that the method of estimating amino-acids in flour given in the Methods of Analysis of the Association of Official Agricultural Chemists, p. 168 (3rd edition, 1930), which requires extraction for a three-hour period, measures not only the amino-acid originally present, but also that produced by some two hours' proteolysis, unless the extraction be carried out at a low temperature.

Table VI. *Amino-nitrogen (mg. per 10 ml. of solution) formed in a gluten-takadiastase digest.*

Time hours	Titra- tion	p_H 8.0		p_H 8.5		p_H 8.7		p_H 9.0	
0	1	0.06		0.06		<0.07		<0.08	
	2		0.00		0.00		0.00		0.00
1	1	0.10		0.12		0.13		0.14	
	2		0.05		0.03		0.02		0.02
2	1	0.12		0.14		0.15		0.16	
	2		0.06		0.04		0.06		0.07
3	1	0.14		0.16		0.17		0.18	
	2		0.09		0.07		0.09		0.10
4	1	0.16		0.18		0.19		0.21	
	2		0.12		0.10		0.12		0.13
5	1	0.18		0.20		0.22		0.23	
	2		0.14		0.12		0.14		0.18

Titration 1: initial alkali titration.

Titration 2: alkali titration in presence of formaldehyde less the formaldehyde blank, i.e. amino-nitrogen.

rapidly than that at p_H 8.0. In all cases the amino-nitrogen at p_H 8.5 is less than that at p_H 8.0 for the corresponding time in accordance with theory, but it is clear that the substances affecting the estimation at p_H 9.0 are also exercising an influence at p_H 8.7, but to a lesser extent. The action of takadiastase on the gluten is thus comparable with the changes occurring in the flour extract and in the flour suspension.

No such abnormal increase at p_H 9.0 was obtained in pepsin digests of gluten, caseinogen or egg-albumin.

An acid hydrolysis of the moist gluten from this low-grade flour was followed by estimation of the amino-acid from time to time by titration to p_H 8.0, 8.5, 8.7 and 9.0 respectively as before. The results shown in Table VII indicate that hydrolysis was normal, and that the amino-acid estimated at p_H 9.0 is always less than that at p_H 8.0.

Table VII. *Amino-acid formed in acid hydrolysis of flour-gluten.*

Time hours	mg. of amino-nitrogen at			
	p_H 8.0	p_H 8.5	p_H 8.7	p_H 9.0
0	0.00	0.00	0.00	0.00
0.5	0.65	0.62	0.60	0.52
1.0	0.84	0.81	0.80	0.76
2.0	0.93	0.88	0.88	0.82
3.0	1.02	0.98	0.98	0.91
6.0	1.06	1.03	1.03	0.98
9.0	1.09	1.05	1.05	0.98

To determine, if possible, the class of substance responsible for the abnormal increase, various materials probably occurring in the flour-water suspension were titrated to p_H 8.0, 8.5, 8.7 and 9.0 respectively before and after the addition of formalin. Neither protein, peptone nor amino-acid solutions showed any increased titration at p_H 9.0. Indeed all showed the expected decrease in titration with increase in p_H value. These results are shown in Table VIII. All possible mixtures of these solutions were titrated similarly, and the values obtained were strictly additive and gave no evidence of an increased titration

at p_H 9.0 but showed the same reduction in titration as is illustrated in Table VIII. In each case the requisite amount of solution was taken, diluted to 10 ml. and then titrated.

Table VIII. *Amino-acid estimation at p_H 8.0, 8.5, 8.7 and 9.0 respectively.*

Titration ml. N/14 NaOH		Phenol red		Thymol blue			
		p_H 7.0	p_H 8.0	p_H 8.0	p_H 8.5	p_H 8.7	p_H 9.0
Egg-albumin	1	0.03	0.08	0.08	0.09	0.10	0.11
	2	0.21	0.25	0.25	0.24	0.23	0.20
Peptone	1	0.06	0.11	0.11	0.16	0.18	0.20
	2	0.18	0.18	0.18	0.15	0.14	0.09
Alanine (theory 0.91 ml.)	1	0.01	0.02	0.02	0.06	0.09	0.12
	2	0.74	0.89	0.89	0.87	0.86	0.81
Glutamic acid hydrochloride (theory 0.91 ml.)	1	1.82	1.83	1.83	1.89	1.92	2.01
	2	0.57	0.88	0.88	0.83	0.81	0.76

Titration 1: solution without formaldehyde.

Titration 2: difference between titrations before and after the addition of formaldehyde corrected for the blank titration, i.e. titration due to amino-acid.

Owing to the large titration to neutralise the solution of glutamic acid hydrochloride, extra indicator was added to obtain the same indicator concentration as in the buffer solution. Also the formaldehyde blank was determined on 8 ml. of formalin diluted with 12 ml. of distilled water.

DISCUSSION.

The results obtained in this study of the estimation of amino-nitrogen in wheat flour show that extraction is complete in about one hour, and that no further formation of amino-acid occurs in the extract after separation from the flour. In the case of one flour, a low-grade, there was an apparent increase in amino-nitrogen in the extract when the estimation was carried out at p_H 9.0, but no increase at the lower p_H values used.

This increase is not due to more complete neutralisation of the amino-acids at the higher p_H , for the more recently published titration curves for individual amino-acids in the presence of formaldehyde show that neutralisation is complete at p_H 8.0¹ [Brown, 1923; Harris, 1929]. In Table VIII the low results at p_H 7.0 are due to incomplete neutralisation of the amino-acid in the presence of formaldehyde, and the low results at $p_H > 8.0$ are due to partial neutralisation of the amino-acids before the addition of formaldehyde. That the maximum titration at p_H 8.0 is slightly less than the theoretical titration value of 0.91 ml. is also due to this latter factor.

Moreover, the facts that in the case of the flour extract, the flour suspension and the takadiastase-gluten digest, lower titrations were recorded at p_H 8.5 than at p_H 8.0, and that there is not a progressive increase in titration with increase in p_H indicate that completion of neutralisation of the amino-acids is not the explanation of the phenomenon.

If it be accepted that the formaldehyde titration estimates carboxyl groups that become titratable because of the action of formaldehyde on the amino-groups, then the observed increase at p_H 9.0 is due to an increase in the number

¹ Since the above was written a recent paper by Van Slyke and Kirk [1933] has been received which shows titration curves of amino-acids compiled from various authors. The titration curves in the presence of formaldehyde show that neutralisation is not complete before p_H 9.0. However, these curves were "estimated from end-points at p_H 9 to 10, given by Sørensen" [1907, 2], and are said to be "only approximately exact" [Van Slyke and Kirk, 1933, p. 657], so that the experimental curves of Brown [1923] and of Harris [1929] are the more reliable.

of carboxyl groups (or amino-groups affected by formaldehyde) which, even in the presence of formaldehyde, do not titrate until p_H 9.0 is reached. That such increase is not due to the production of amino-acids in the usual sense of this term is shown by the titration values at p_H 8.0. Nor is this increase due to the production of proteoses or peptones, as shown by a pepsin digestion, though it occurs in a takadiastase digest of gluten. It is therefore due to substances formed in the enzymic hydrolysis of the proteoses or peptones formed from gluten. A combination of carboxyl and amino-groups in a compound of very low acid dissociation constant may be expected in a substance intermediate between the polypeptides and the amino-acids. Such a substance, under the action of formaldehyde, would not affect the amino-acid estimation at p_H 8.0 because of its very low acid dissociation constant but would become titratable at p_H 9.0. Then in the flour extract, if the proteins present are broken down, not to their constituent amino-acids but to some slightly larger grouping of a few amino-acids, this would be estimated at p_H 9.0 and not at p_H 8.0 and would explain the experimental results.

Although only one of the four flours examined to date has shown this phenomenon of increase in amino-nitrogen at p_H 9.0 and not at p_H 8.0, it seems from the work of Denham and Scott Blair and of Halton (unpublished) that it may be of more common occurrence than this would indicate.

In considering the question of protein changes in a flour dough it is necessary to take into account any such change as is indicated in this paper, for the physical properties of the dough will be affected by any protein degradation whether the decomposition be carried as far as the amino-acid stage or not.

Perhaps it should be emphasised that titration to p_H 9.0 before and after the addition of formaldehyde is not advocated as a method of estimating amino-acids, but that in conjunction with an estimation at p_H 8.0 it may yield some information not afforded by the amino-acid estimation alone.

SUMMARY.

1. The claim that proteolysis occurs in flour extracts has been studied for the few hours immediately subsequent to separation of the extract.
2. The technique of Brown for the estimation of amino-acids by the Sørensen method has been applied to flour extracts.
3. Proteolysis, as measured by the production of amino-acids, did not occur in the flour extracts studied.
4. For one flour of the four studied (a low-grade) the amino-acid determinations made on the extract at varying p_H values showed an increase with time if the solution were titrated to p_H 9.0 before and after the addition of formaldehyde, but no increase if the titration were carried to p_H 8.0 only.
5. It is suggested that this is due to the enzymic production of substances containing carboxyl and amino-groups and having a low acid dissociation constant, which do not titrate even in the presence of formaldehyde until p_H 9.0 is reached.

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