UNIVERSITY OF CINCINNATI

May 22 1940

I hereby recommend that the thesis prepared under my supervision by ROBERT L. HEGMAN

entitled "THE REACTION OF FORMALDEHYDE WITH FEATHER KERATIN"

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

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Form 668—G. S. and Ed.—1M—7-37

THE REACTION OF FORMALDEHYDE WITH FEATHER KERATIN

A dissertation submitted to the

Graduate School

of the University of Cincinnati

in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

June 1940

by

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Ch. E. University of Cincinnati 1936

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Acknowledgement

The counsel of the entire staff of Tanners' Council Research Laboratory is gratefully acknowledged. I especially am indebted to Dr. J. H. Highberger for his advice and assistance in this problem.

The fellowship was made possible through the kindness and generosity of the P. R. Mitchell Co., Cincinnati Ohio, who also supplied the materials used in this investigation.

Foreword

An investigation was made into the nature and extent of the reaction of aqueous formaldehyde with feather and down keratin, with the interpretation of such reactions when applied to the practical tannage of feathers.

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List of Illustrations

Feathers and down of domestic and foreign fowl are valuable raw materials of the bedding industry. The processing of these raw materials is usually limited to washing, sterilizing and drying. However, further processing is needed to overcome some of the disadvantages of a protein material.

One of these disadvantages, is the susceptibility of the feathers to degradation, accompanied by the formation of objectionable odors. This laboratory has completed an extensive survey of this odor problem (1).

Despite the difficulty in correlating odor formation with chemical analysis, since many substances can be detected by odor in concentrations too small for the ordinary methods of chemical analysis, much of the evidence showed that secondary protein decomposition products, formed by bacterial action, were one source of objectionable odors. A musty odor, typical of damp cellars and potatoes, was found to be associated with the presence of a soil actinomycetes, which when isolated, was capable of growth in pure culture on raw feathers.

The production of odors takes place only under conditions of high humidities. This follows from the increase of moisture content of the protein under such conditions. Experiments performed here, show that the total water content increases along an S-shaped curve with increase in relative humidity to a maximum of 1 part of water to three of protein,

at 100 percent relative humidity, and 75° Fahrenheit. The free water content, which is in equilibrium with the bound water, finally attains a concentration suitable for sustaining growth of micro-organisms.

High bacterial counts, of 5-25 million bacteria per gram of protein, were found even at common humidities. Furthermore, feathers stored under severe test conditions of high humidity, yielded the odors characterized by protein decomposition, but when sterilized and maintained so, did not yield these offensive odors. The bacteria present were mainly spore formers, which were extremely resistant to the action of ultra-violet light.

The ability of the common mercurial and phenolic disinfectants to prevent active growth when applied to the feathers, is distinctly limited. Failure of these compounds may be due to their inactivation by adsorption or chemical reaction with the protein. Phenyl mercuric acetate, or the corresponding hydroxide, was quantitatively removed from solution by agitation with feathers, while sodium ortho phenyl phenate was taken up to the extent of approximately 80 percent.

On the other hand, treatment with formaldehyde inhibited the destruction of the protein, and little odor was found associated with proteins protected in this manner. As a direct consequence of these observations, the following

investigation into the formaldehyde-keratin reaction was undertaken.

Feathers and down are epidermal proteins of the group typified by hair, horn, and wool. This group is characterized by insolubility, resistance to enzymatic digestion, and a high sulfur content. These are only related proteins, since the amino acid analysis varies in each, but the cystine content, which accounts for practically all of the sulfur present, (2), is much higher than in other groups of proteins.

Block has attempted to classify the keratins on the basis of their basic amino acid content, stating that histidine, lysine, and arginine, occur in the proportions of 1:4:12 respectively, (3), but the results of this paper are not in agreement with that definition.

Goddard and Michaelis (4), have shown that the keratins represent a special type among the proteins. Fibrous proteins are considered to consist of a chain of amino acids, one molecule wide, and of great length. Weak salt linkages between the residual groups of the di-carboxylic and di-amino amino acids, and Van der Waal's forces, bind these molecular chains into the fundamental fiber. On the other hand the keratins are held together by these same forces, plus the strong chemical linkage of the di-sulfide bond, which arises from the presence of cystine, which contributes a carboxyl

and an amino group to each of two neighboring chains. On this basis, one can explain the dispersion of the keratins, when treated with agents capable of breaking this bond. This important fact is necessary to explain many of the phenomena witnessed in this investigation.

Much data has been presented on the combination of formaldehyde with gelatin and collagen, little on such a combination with the keratins in general, and none with respect to feather keratin in particular.

Much of the previous work, involving the reaction of formaldehyde and collagen, is conveniently summarized in the paper of Bowes and Pleass (5).

The excellent work of Highberger and his co-workers, (12), has shown that the reaction of formaldehyde with collagen is essentially dependent on the lysine and arginine contents of this protein.

The results of Bowes and Pleass with goat hair, collagen and silk are difficult to interpret in terms of the exact amino acid contents of these proteins. However, their experimental procedure is open to an objection, to be cited later in this paper.

Methods and Materials

The material used was prepared from prime domestic Long Island Pekin Duck down and feathers, supplied by the P. R. Mitchell Company. The preparation was as follows:

1. The feathers or down was ground in a semi-micro Wiley mill.

2. The ground protein was washed with water, for 4 hours continuously on a large filter.

3. Fats were extracted with chloroform for 16 hours, in a Soxhlet extractor.

4. An unidentified yellow residue was extracted with ethyl alcohol in the Soxhlet for 4 hours.

5. After rinsing with ethyl ether, the preparation was allowed to air dry for 1 week.

The yield from such a procedure was of the order of 92-95 percent. The moisture content was determined by evaporation at 100° C. under reduced pressure to constant weight, in order that all analyses might be reported on the weight of the bone-dry protein. Total nitrogen content of both the feather and down preparations was 16.0 percent.

The basic amino acid content was determined by Tristam's modification (6), of Block's method (7). The results were corrected for the loss of 1.5 mg. of arginine to the histidine fraction, and for the solubility of arginine silver, which was taken as 1.6 mg. per 100 ml., as Tristam showed. The results are listed in Table 1.

The molal ratios of histidine: lysine: arginine are 0:1:10 and 0:1:50 for the down and feather keratin respectively.

These results are in accordance with Schenk's (8) observation, that different types of feathers from the same fowl, and different structural parts of the same feather

Table 1

Basic Amino Acids of Down and Feather Keratin Preparations

Expressed as Percent of Dry Protein

No.	Time of Hydrolysis in hours	Arginine	Lysine	Histidine	
1.	23	5.72	0.50	0.0	Down Keratin
2.	23	5.78	----	---	" "
3.	37	5.76	0.30	0.0	" "
4.	37	2.76	0.04	0.0	Feather Keratin

Expressed as millimols of amino acid per gram of dry protein

No.	Arginine	Lysine	Histidine
1.	.329	.034	0.00
2.	.332	----	----
3.	.331	.021	0.00
4.	.159	.003	0.00

show variations in the content of individual amino acids. Embryonic feathers contain higher percentages of the basic amino acids than mature feathers.

Moreover, these results are substantiated by the interpretation of the formaldehyde-keratin reactions, to be presented later. They cannot be classified under Block's definition of a true keratin, but the latter's contention is not fully justified at present.

The methods of tanning and formaldehyde analysis are essentially those of Highberger and Retzsch (9). Two gram samples of the protein are treated with 100 ml. of a solution containing a definite percentage of formaldehyde, which solution is buffered with phosphoric acid and potassium hydroxide. These solutions are M/10 with respect to the phosphate radical. At the end of the tanning period, the protein was introduced into a Wilson-Kern extractor, the tanning solution filtered off and checked for final pH, and the protein washed in a method to be described later. Some experiments were performed by washing the protein in a Buchner funnel after filtering, and allowing the keratin to air dry 48 hours before the analysis for formaldehyde was attempted.

Analysis for the formaldehyde present in the protein is made by digesting the protein in 200 ml. of 25 percent sulphuric acid, and distilling off the formaldehyde. The distillation is stopped when fumes of sulphur di-oxide and tri-oxide are formed, at which point the concentration of the

acid has increased to approximately 70 percent. The formaldehyde is distilled over during the digestion and the distillate is received in a 1.2 percent solution of sodium bisulfite, whose amount must be so calculated that a definite excess exists over that necessary for combination with the formaldehyde. After allowing the formaldehyde to react with the bisulfite for at least one hour, the excess bisulfite is titrated with iodine; the bisulfite in combination with the formaldehyde is liberated by addition of sodium bicarbonate and titrated with standard iodine. From this last titration, the amount of formaldehyde can be calculated.

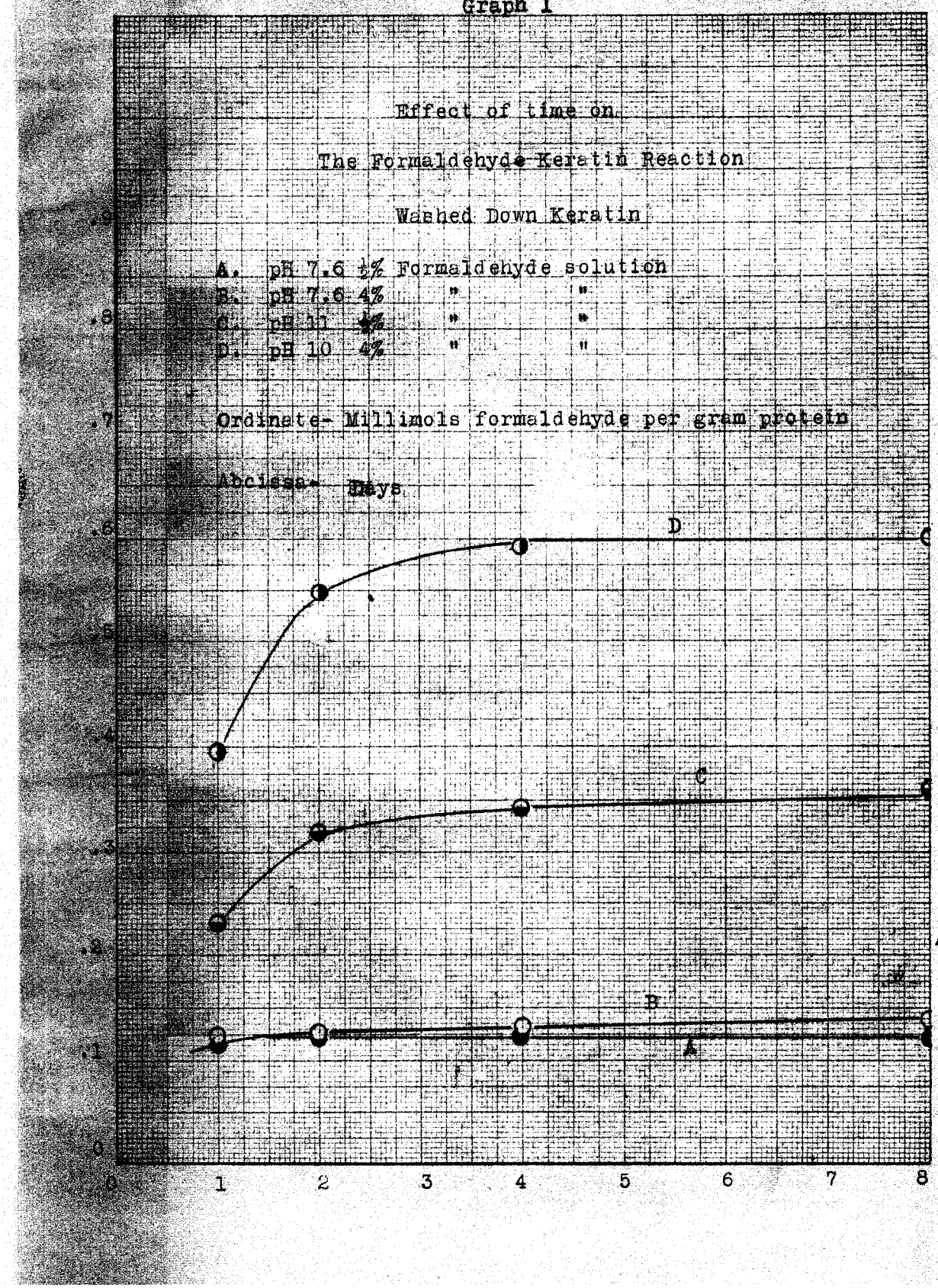
Time of Tanning

The results concerning the effect of time are best shown in the graph 1. The reaction is undoubtedly complete in 4 days, and practically so in two.

Washing

After tanning, the protein contains not only combined formaldehyde, but also a solution of free formaldehyde within the keratin fiber, which must be removed before analyzing for the amount fixed. Washing with water is a slow process since the formaldehyde does not diffuse readily when the concentration within the fiber has decreased to a low value. The amount of formaldehyde being washed out may be followed over the wash period, by adding bisulfite to the wash water, and titrating with iodine as before. Amounts of formaldehyde equivalent to one part in 10 million parts of

Graph I



wash water may be determined in this manner. The amount of formaldehyde present in the wash water, at the end of a one hour washing period, determines the rate of removal of formaldehyde per hour. On occasions where the determination could not be made every hour, such as overnight periods, the amount diffusing out over the long period was divided into portions which would correspond to the average of the two separate one hour periods, immediately preceding and following the long period. This corresponds to a process of smoothing the data by interpolation. This method of following the amount of formaldehyde washed out per hour, led to the development of the washing formula below.

Derivation of Washing Equation

Let F represent the amount of free formaldehyde within the fiber at any time t, and postulate that the amount removed per unit time, is proportional to F.

Therefore, $dF/dt = -aF$ which on integration becomes

$\ln F/F_0 = -at$ where F_0 is the amount of free formaldehyde present at t=0.

Therefore, $F = F_0 e^{-at}$

and, $-dF/dt = aF = aF_0 e^{-at}$

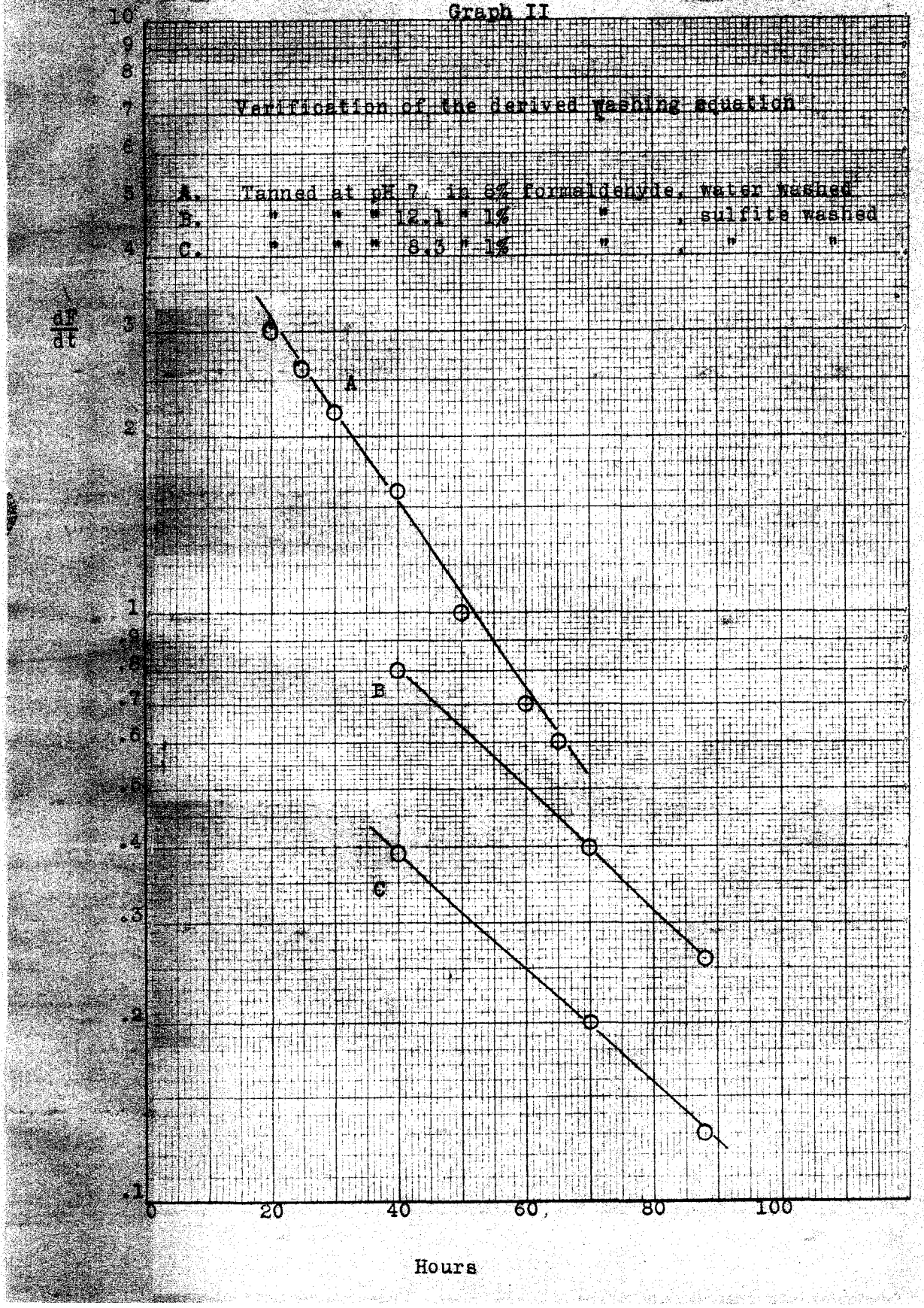
$\log (-dF/dt) = \log aF_0 - at \log e$

If we plot log (-dF/dt) against t, a straight line should be obtained, if the washing data follow the law above.

Graph II

Verification of the derived washing equation

A. Tanned at pH 7 in 6% formaldehyde, water washed
B. " " " 12.1 " 1% " " , sulfite washed
C. " " " 8.3 " 1% " " " "



The slope of the straight line obtained, will be equal to the quantity,

$$-a \log e = -.435\ a \quad .$$

Therefore, the amount of free formaldehyde at any time can be calculated from,

$$F_m = 1/a \quad (-dF/dt)_m \quad .$$

The postulate is verified on curve A of Graph II. The ordinate on this chart is (-dF/dt), expressed as ml. of N/100 Iodine per hour washing time for the two gram sample.

Because the formaldehyde concentration builds up in the wash water, the rate of removal varies with the length of time of the wash period. To overcome this difficulty sodium sulfite was added to the wash water, in order to keep the formaldehyde concentration fixed at a low value in the washing solution. When this is done the rate of removal is practically constant over short periods, and the method of interpolation resorted to at first is no longer necessary. For this reason too, the number of hours of washing are equal to the actual time washed and not to an equivalent time based on the interpolation process. The results of the sodium sulfite washings are presented in Table 2.

From curves B and C, a is equal to .025, and F at t=80 is equal to .03 and .015 millimols formaldehyde per gram of keratin for curve B, and curve C respectively.

All washings were carried to the point where the amount of free formaldehyde could be roughly calculated by

Table 2

Rate of Formaldehyde Removal at Various Times During Washing Down Keratin

Tanned in 1 Percent Formaldehyde Solution

Curve B-Graph 2 pH 12.1
Curve C-Graph 2 pH 8.3

Hours	(-dF/dt) B	C
40	.80	.39
70	.40	.20
88	.26	.13

Note: (-dF/dt) is expressed as ml. N/100 Iodine per hour for the 2 gram sample.

Table 3

Error in Analysis Due to Presence of Uncombined Formaldehyde

Percent Formaldehyde in the Tanning Solution	pH of Solution	Free Formaldehyde Content is Less Than:
4	11-12	.10 millimols
4	7-8	.04 per gram.
1	11-12	.04
1	7-8	.02

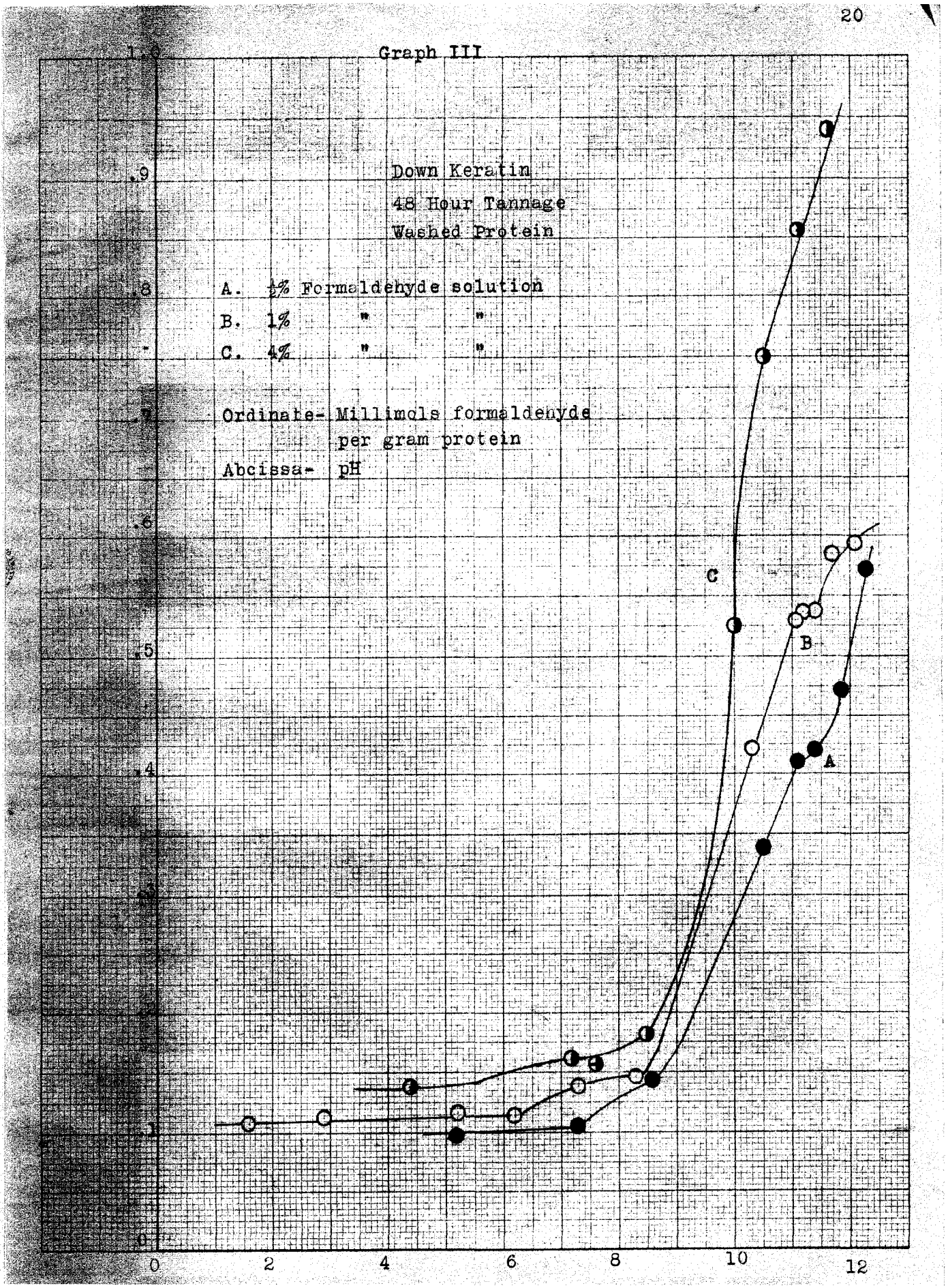
this method. A summary of the error due to the presence of free formaldehyde is presented in table 3.

For solutions of less than 1 percent, the error is even less, and for all solutions of 1 percent or less, of washed protein the amount of free formaldehyde left in the fibers, is less than the experimental error.

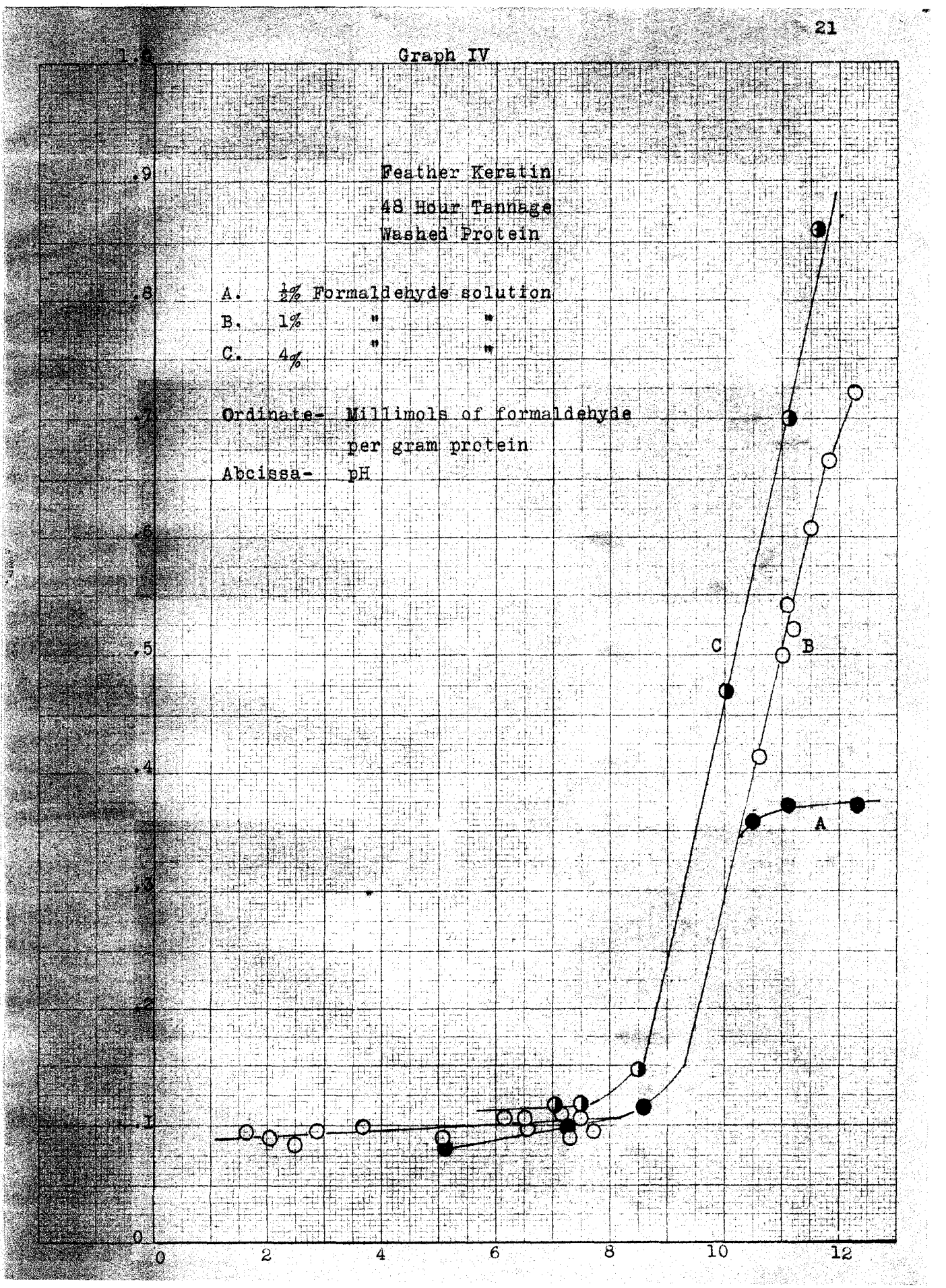
Washed Proteins

Graphs III and IV show the results obtained when the amount of fixed formaldehyde is determined on proteins washed by 1 percent sulphite solutions for approximately three days, at which time the above washing conditions are satisfied. The curves for down keratin in the region of pH 4 to 5 show the increase in free formaldehyde predicted by the washing data, i.e., if we assume there is no free formaldehyde left in the protein tanned in a ½ percent solution, the amount of free formaldehyde present in the protein tanned in a 1 percent solution is about .01 millimol per gram, and for 4 percent tanning concentration, about .035 millimol per gram. The amount of .10 millimol per gram existing below pH 5, is of no significance, because it represents the amount of aldehydes formed during the digestion of the protein, and blanks run on feathers which were not treated with formaldehyde, showed aldehyde formation of this order of magnitude. Rieser (10), has shown that definite amounts of acetaldehyde are obtained in the alkali cleavage of proteins, and that

Graph III



Graph IV



lesser amounts are obtained with proteins partially split with acids or enzymes. I have obtained bisulfite binding bodies from both the acid and alkali splitting of the keratins used. Undoubtedly, both aldehydes and ketones are distilled over during the digestion of the keratin. These amounts are relatively constant for constant hydrolytic conditions, and blanks which had been treated with solutions covering the whole range of pH used, from 2 to 12, yielded values which were not dependent upon the pH, but roughly upon the time required for the distillation to take place, and with the concentration of the acid in the digestion. As the concentration of the acid increased during the distillation, the rate of formation of bisulfite binding bodies also increased to a maximum at the end of the distillation. These values ranged from .05 - .10 millimols per gram dependent upon the above conditions. We may therefore take the flat portion of the curves below pH 5, to be equivalent to the value for the bisulfite binding bodies formed during the analysis plus the amount of free residual formaldehyde not removed from the fiber.

The region from pH 6-8, shows a characteristic jump in the case of the down keratin, which is noticeably absent in the feather keratin curves. This jump is shifted to the left with increasing concentration of formaldehyde, which follows the law of mass action when applied to the reaction of formaldehyde with undissociated amino groups, which

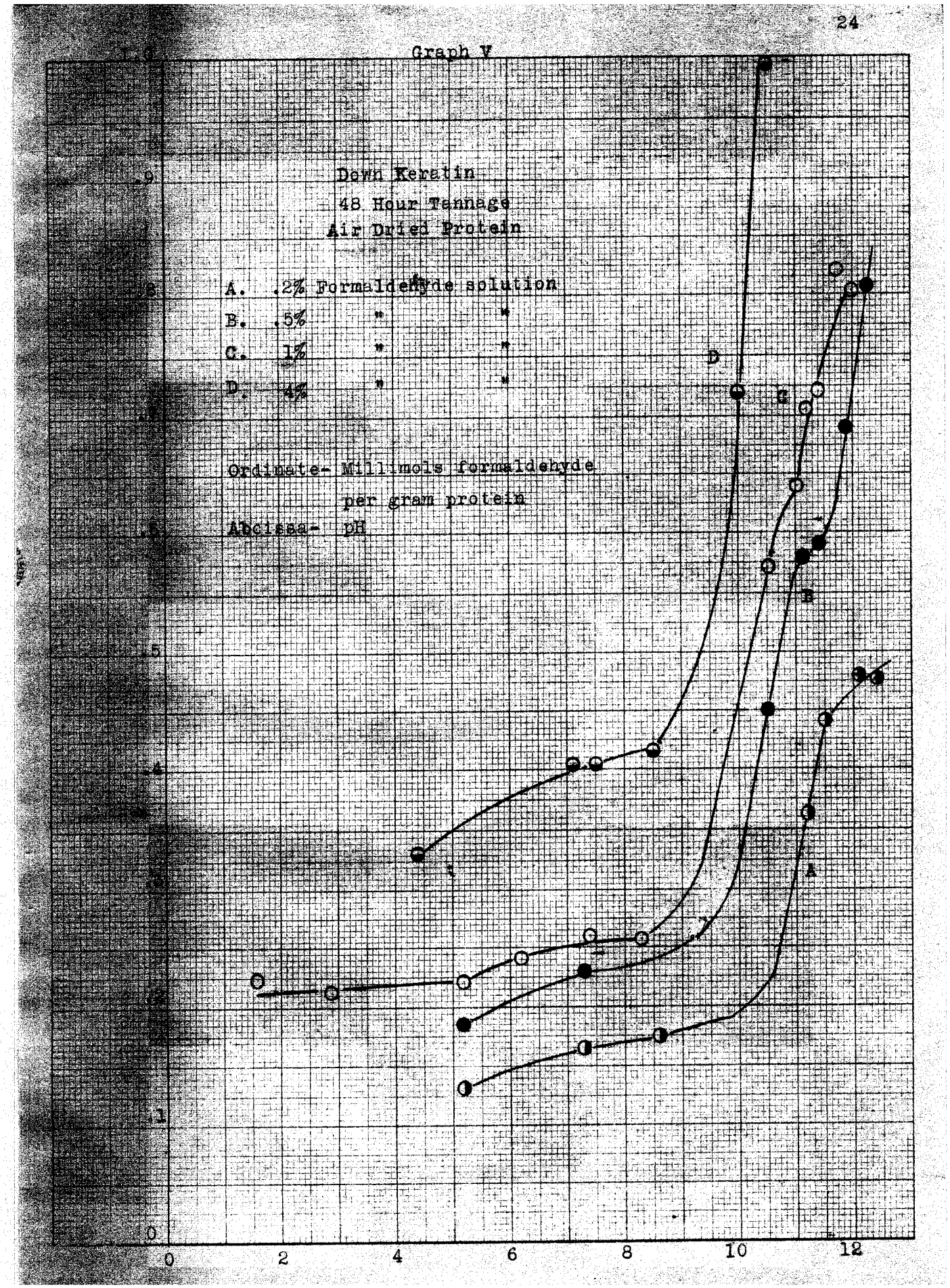
has been investigated by Salcedo (11) for collagen in this laboratory. The value of this jump (.035 millimols per gram), corresponds almost exactly with the amount of lysine isolated from the down keratin, and is support to Highberger's conclusion (12) that the reaction between formaldehyde and the free amino groups of lysine is confined to this region, in the nature of a 1:1 combination as proved by Salcedo (11).

The values of the jumps from the endpoint of the lysine reaction at pH 8 to pH 11 correspond to such a jump in the case of collagen where the reaction has been postulated to be occuring at the guanidine group of arginine (12). The value of this jump (.38 millimols per gram for the 1 percent formaldehyde--down keratin curve) corresponds closely to the arginine content as isolated. The value for feather keratin (.25) is higher than the amount of arginine actually isolated, but an interfering reaction at high alkalinities is more predominant for this protein than for the down keratin. This interfering reaction is accentuated at high pH, and high concentrations of formaldehyde, and is of such an extent, that it cannot be explained by the presence of large amounts of free formaldehyde, which I have shown to be of smaller magnitude. The explanation of this phenomena will be offered later in this paper.

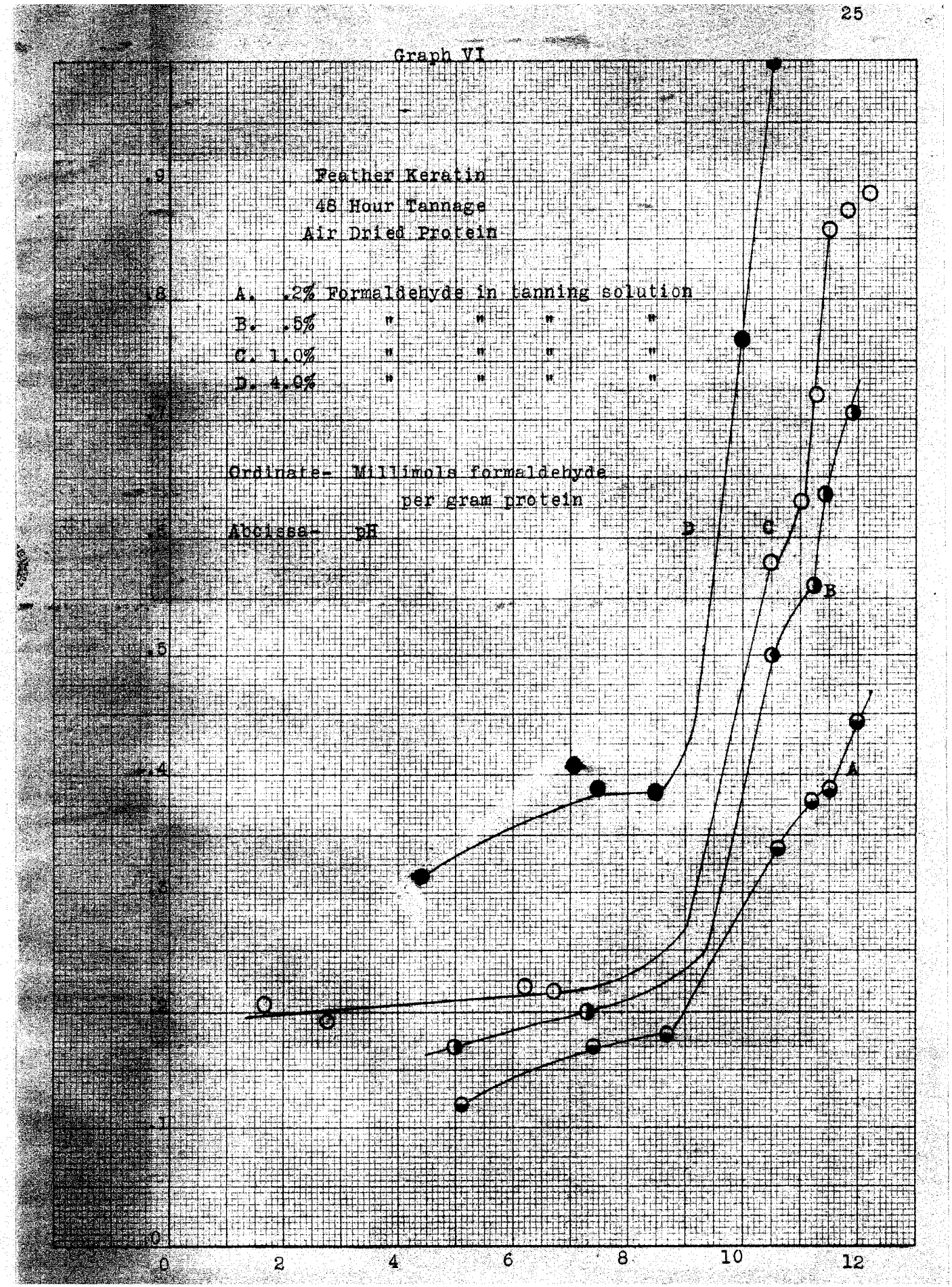
Air Dried Proteins

Graphs V and VI show the results obtained when the tanned proteins are superficially washed at the filter, and

Graph V



Graph VI



allowed to air dry for 48 hours. The amounts of free formaldehyde left in the fibers are directly proportional to the concentration of treating solution, and to the swelling of the protein in the tanned condition. Bowes and Pleass (5) tanned collagen, hair keratin and silk, in an .8 percent solution of formaldehyde, and determined the amount of formaldehyde left in the fibers on air-drying. Their results must be high when considered from this viewpoint. The proteins when dried weigh exactly the same as before the tanning process, showing that although the water is free to diffuse to and evaporate at the protein-air interface, there is a reduced tendency for the formaldehyde to do so.

The swelling was measured by filtering off the tanning solution, and weighing the protein before it was allowed to air dry. Both the down and feather keratin exhibited the same type of swelling, as shown in Table 4.

The swelling curve when plotted shows a sharp increase at pH 11. This is believed due to the hydrolytic rupture of the disulfide bonds at high alkalinities, and as such is needed to explain the nature of the reaction in this region.

Another observation was made in these determinations. Proteins tanned above a pH of 11.8, suffered a loss in weight amounting to about 10 percent, during the tanning process. Because of the analogy to the work of Michaelis (4) who dispersed the keratins by rupture of the disulfide bond with

Table 4

The Swelling of Formaldehyde-Tanned Keratin

Final pH of Tanning Solution	Swelling in Grams of Water per Gram of Keratin	
	Down	Feather
3.9	.72	.70
7.3	1.15	.80
8.5	1.2	.85
10.4	1.4	.9
11.1	1.5	1.2
11.6	1.7	1.5
12.0	2.0	2.0
12.4	2.4	2.6

alkalis and reducing agents, it appears as though a similar process is occuring in this region. Schoberl (13) states that the reduction is preceded by a hydrolysis to sulfhydryl compounds, and sulfinic acids. This explains too the digestion of wool and keratin by the larva of the common clothes moth, in whose middle intestine Linderstrom-Lang (14) found a pH of approximately 10.

In these two charts, the reactions with lysine and arginine in approximately 1:1 ratios with formaldehyde are evident at the lower concentrations, and the interfering reaction is also prominent at high pH.

The Interfering Reaction

It is obvious that at high alkalinities, the formation of a new compound with formaldehyde is taking place. The group involved is some group R. As R or the concentration of Formaldehyde, F, is increased, we find an increase in RF. Therefore the production of this group R, must be dependent upon pH, increasing with increase of pH. Since this phenomenon is not witnessed in the Collagen-Formaldehyde reactions, R must be some group not present in that protein, but one existing in keratin. The disulfide bond answers this requirement. This bond is split hydrolytically at high pH, and the resulting sulfhydryl group may react with formaldehyde in the manner postulated by Ratner and Clark (15) to form the group, Protein-S-CH_2OH. Working with cysteine, they found the reaction with formaldehyde to occur in two steps,

the first a combination of formaldehyde at the sulphur atom, and a subsequent ring closure, in which the remaining hydroxyl group of the formaldehyde combined with the amino group of cysteine. This ring closure probably does not take place in the reaction with the sulf-hydryl groups in proteins, since the amino group of cysteine is now combined in the peptide linkage. The reaction between cysteine and formaldehyde takes place over a wide range of pH. Since there is no evidence for the formation of RF at moderate alkalinities, R must be formed by one of two reactions occuring in the higher pH region. One of these reactions is the hydrolytic rupture of the disulfide bond. The other may be the production of the sulf-hydryl groups from the disulfide bond, or from the sulfinic acids formed by hydrolytic rupture, by reduction with formaldehyde.

The amount of cystine present is of the order of 10 percent, according to Wilson and Lewis (16). This amounts to 1 millimol per gram of sulfhydyl groups, when the protein is completely reduced, and is therefore of an amount capable of yielding the results obtained.

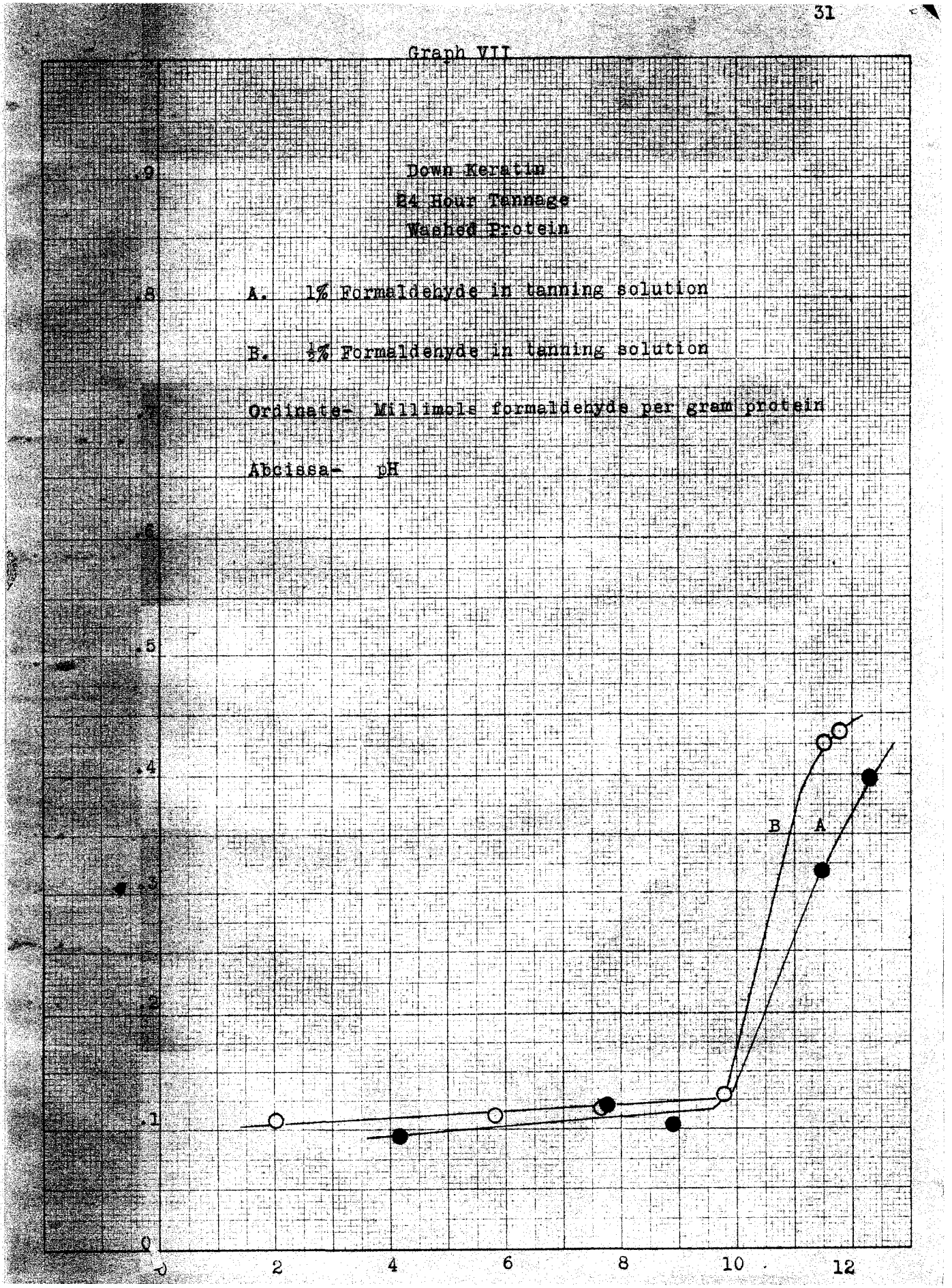
An attempt was made to put the down keratin completely in the sulf-hydryl form by reduction with thioglycollic acid, (4). The yield was small and the resulting protein was too soluble to be handled by our washing methods. However, such a protein tanned at pH 6.8 in a 1 percent solution of formaldehyde contained .50 millimols per gram when air dried,

compared to .25 millimols per gram for the original protein. Formaldehyde tannage in the presence of oxidizing agents which prevent the rupture of the disulfide bond, is impossible since the formaldehyde itself is oxidized under such conditions.

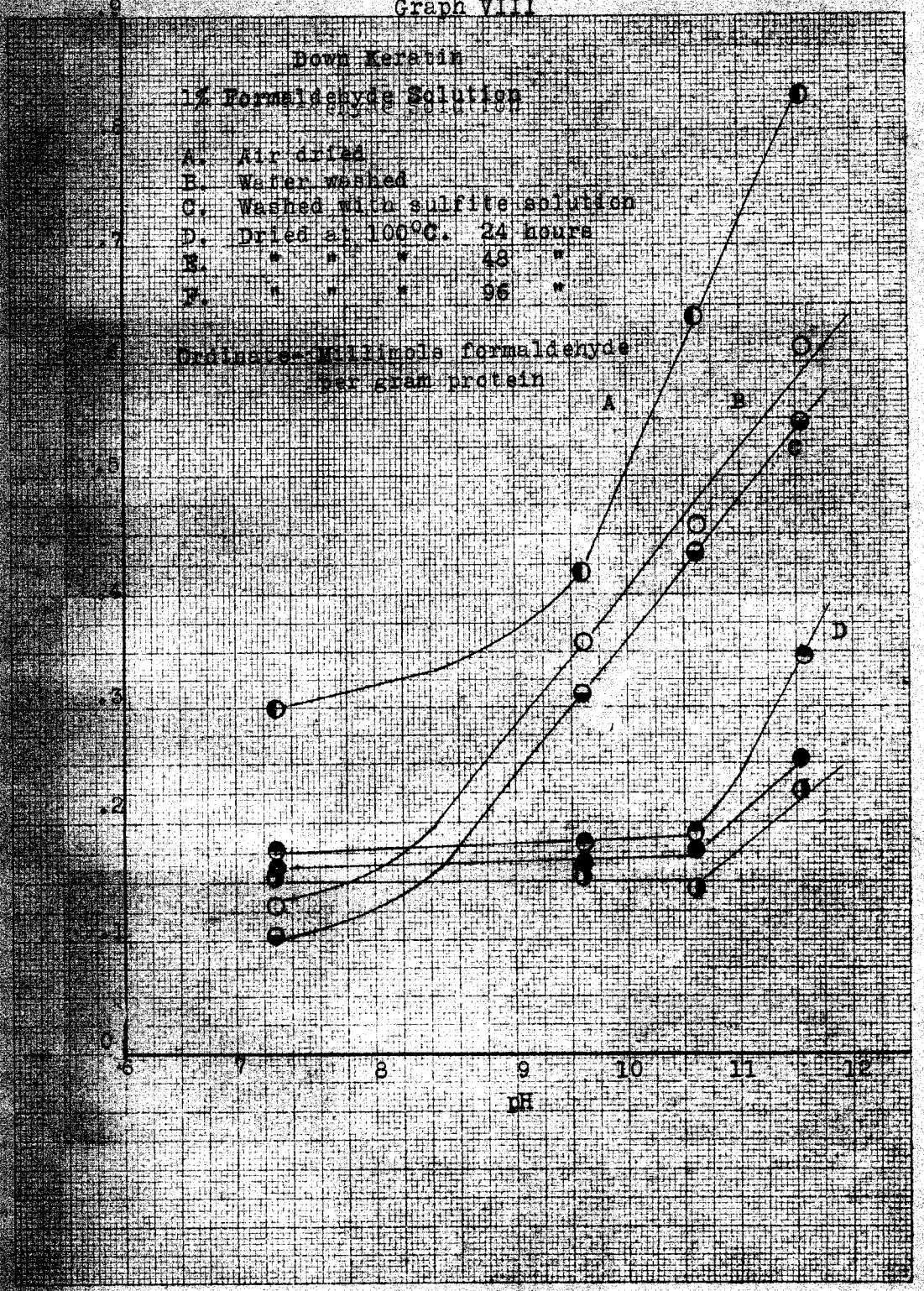
The extent of the interfering reaction is greatly reduced, when the concentration of formaldehyde within the fiber is low, and Graph VII shows the effect of short period tanning upon the reactions involved. In this case, the reaction was distinctly limited to the reaction with arginine.

All of these reactions between formaldehyde and lysine, arginine, and the sulf-hydryl group are equilibrium reactions, which may be reversed by strenuous removal of the free formaldehyde within the fiber. Graph VIII shows the results obtained, when the proteins are heated to 100°C., under reduced pressure for varying lengths of time, compared to air-drying and washing.

Graph VII



Graph VIII



Summary

The results of this thesis lead to the following conclusions:

1. At low concentrations the formaldehyde-keratin reaction is limited by the amount of lysine and arginine present.
2. This combination is in a simple 1:1 ratio of formaldehyde to the basic groups of these two amino acids.
3. These two amino acids may be approximately determined by this method.
4. Under conditions of high pH values, the disulfide bond is broken to yield sulfhydryl groups and sulfinic acids.
5. The disulphide bond is probably reduced by formaldehyde under alkaline conditions, to yield sulfhydryl groups.
6. Regardless of their methods of formation, the sulfhydryl groups react with formaldehyde at all values of pH.
7. The ratio of histidine: lysine: arginine was not found to be in the simple 1:4:12 ratio postulated by Block.
8. For this reason, it is suggested that the keratins be defined by their solubility when reduced under alkaline conditions, a specific reaction

for the disulfide bond, which is characteristic for the keratins.

9. Under the conditions used for treating feathers, to prevent odor formation; i.e. tanning at pH 6-8, the inhibitory effect gained is obtained by the retention of free formaldehyde within the fiber on air drying.

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