30. SOME APPLICATIONS OF³ PERIODIC ACID TO THE STUDY OF THE HYDROXYAMINO-ACIDS OF PROTEIN HYDROLYSATES

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LARGELY because of the lack of satisfactory analytical procedures, the hydroxy-amino-acids are at present the least well investigated category of protein constituents, in spite of the fact that the simplest hydroxyamino-acid, serine, was one of the earlier amino-acids to be discovered in proteins [Cramer, 1865].

Nicolet & Shinn [1939] showed that periodic acid, a reagent introduced into organic chemistry by Malaprade [1934] for the splitting of α -glycols, also splits serine, the products of oxidation being one molecule of formaldehyde and, probably, one molecule each of ammonia and glyoxylic acid. Van Slyke *et al.* [1940] have subsequently used this reagent for the study of the new base 'hydroxylysine', and have mentioned a simple method for the detection of amino-acids having an $-\text{OH}$ and an $-\text{NH}_2$ group in adjacent positions, by the liberation of NH_3 in the presence of periodate in strong K_2CO_3 solution.

Block & Bolling [1939] have made use of lead tetra-acetate, which reacts with α -glycols in the same way as periodic acid [Criegee *et al.* 1933], to determine threonine in protein hydrolysates. These workers determined the acetaldehyde liberated by a specific colorimetric method.

In the present work we have studied the reaction between periodic acid and hydroxyamino-acids both in solutions of known composition and in preparations derived from proteins.

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This has led to the development of analytical procedures which, though quantitatively not all that could be desired, are of high specificity, rapid, unlaborious and require only small amounts of material. They promise, therefore, to be of use in the study of proteins and preparations derived therefrom both for the analysis of complete hydrolysates and for the control of isolative procedures.

I. THE LIBERATION OF ACETALDEHYDE AND HIGHER ALDEHYDES BY PERIODIC ACID

[Cf. Martin & Synge, 1940]

On treatment with periodic acid, threonine yields acetaldehyde, and this may be separated from the formaldehyde resulting from serine, 'hydroxylysine' etc. by simple aeration, since formaldehyde, unlike its higher homologues, is not significantly volatile from aqueous solution [cf. Blair & Ledbury, 1925; Ledbury & Blair, 1925; Walker, 1940].

So far as we are aware, this difference between formaldehyde and its homologues has not previously been made use of for analytical purposes.

EXPERIMENTAL

In the present work, acetaldehyde was aerated from the reaction mixture in a 50 ml. washing tube directly into an absorption tower (separating funnel packed with glass beads and mounted in a Büchner flask, as shown in Fig. 1) containing 25 ml. of 0.3% aqueous NaHSO_3 . After rapid aeration for 30 min. the solution and washings from the tower were titrated in the Büchner flask with $N/300$ iodine solution [Friedemann & Kendall, 1929].

By placing a second absorption tower in series with the first, it was found that 95% absorption of acetaldehyde took place in the first tower. The figures for volatile aldehyde given subsequently in this paper are not corrected for this and refer to aldehyde as titrated in the single absorption tower.

On aeration of 5.35 $\mu\text{mol.}$ of acetaldehyde from 25 ml. of water in the aeration vessel, recoveries of 87–92% were obtained, and these were not affected by the presence of 8.75 $\mu\text{mol.}$ of formaldehyde in the acetaldehyde solution. Blank runs showed that the laboratory air was substantially free from aldehyde, and in no case was a 'reagent blank' found.

Yield of acetaldehyde from threonine

In all these tests synthetic *dl*-threonine was used. *dl*-*allo*-Threonine, prepared from *O*-methyl-*dl*-*allo*-threonine [cf. West & Carter, 1937], gave an identical yield, while samples of threonine of natural origin prepared from rabbit myosin by Sharp [1939] gave 70–72% of the yield given by the synthetic material.

Amounts of 0.5–1.5 mg. of threonine were treated at room temperature with 50 mg. of periodic acid (B.D.H.) in 25 ml. total volume of aqueous solution. The yield of acetaldehyde obtained in the absorption tower after 30 min. rapid aeration was found to be dependent on *pH*, as shown in Table 1.

A definite optimum yield at about *pH* 7 is to be noted. The reasons for the lower yields on either side of this optimum, and for the incomplete yield at the optimum, have not been investigated in detail; in acid solution neither periodic acid, iodic acid nor ammonia interferes with the removal of added acetaldehyde

Fig. 1. Apparatus for micro-separation of aldehydes.

Table 1. *Yield of acetaldehyde from dl-threonine treated with periodic acid in various solutions*

Solutions used	Yield of acetaldehyde %
Water	9-10
<i>M</i> /5 acetate buffer, pH 4.7	52
<i>M</i> /5 NaHCO ₃	66-72
<i>M</i> /5 phosphate buffer, pH 7.2	69
'Old' <i>M</i> /5 NaHCO ₃ , become more alkaline by loss of CO ₂	55
50% w/v K ₂ CO ₃ solution	45

from aqueous solution. It is therefore probable that, in acid solution, threonine is either only slowly attacked by periodic acid or that side reactions result in products other than acetaldehyde. On the alkaline side it seems possible that the simultaneously liberated ammonia interferes with the determination, or that acetaldehyde is slowly oxidized by the reagents.

All determinations were accordingly carried out in fresh *M*/5 NaHCO₃ solution, which, on addition of periodic acid and the sample for analysis (itself usually acid), maintained the pH of the reaction mixture at about 7 at least during the earlier stages of the aeration.

The aldehyde material obtained from *dl*-threonine was identified qualitatively as acetaldehyde by conversion into the 2:4-dinitrophenylhydrazone.

Yield of volatile aldehyde from other amino-acids

dl-Serine, under the same conditions, yielded no aldehyde in the absorption tower, nor did *dl*-alanine, *l*-cystine, *l*-methionine, *l*-hydroxyproline, *i*- β -hydroxy-glutamic acid, *l*-tyrosine, *l*-tryptophan, *l*-arginine, *dl*-lysine or *l*-histidine treated separately under the same conditions.

Yield of acetaldehyde from dl-threonine in the presence of other amino-acids

From a mixture of 0.78 mg. of *dl*-threonine and 2.9 mg. of *dl*-serine (Hofmann) 70% of the aldehyde calculated for the threonine present was obtained.

In order to control more rigidly the specificity of the determination before applying it to protein hydrolysates, a mixture of amino-acids was made up, as shown in Table 2, to simulate a wool hydrolysate. This mixture was refluxed

Table 2. *Mixture of amino-acids used in control experiments*

Amino-acid	N (mg.)	N as % of total N
Ammonia (as NH ₄ Cl)	26.4	5.3
<i>dl</i> -Alanine	32.4	6.4
<i>l</i> -Arginine (as hydrochloride)	96.9	19.4
<i>l</i> -Aspartic acid	11.7	2.3
<i>l</i> -Cystine	59.4	11.8
<i>l</i> -Glutamic acid	44.6	8.9
Glycine	83.0	16.5
<i>l</i> -Histidine (as hydrochloride)	9.0	1.8
<i>dl</i> -Leucine	51.8	10.3
<i>dl</i> -Lysine (as dihydrochloride)	22.3	4.4
<i>l</i> -Methionine	6.6	1.3
<i>dl</i> -Phenylalanine	12.8	2.6
<i>l</i> -Tryptophan	5.3	1.1
<i>l</i> -Tyrosine	16.2	3.2
<i>dl</i> -Valine	23.4	4.7
Total	501.8	100

for 24 hr. in 6*N* HCl, and then evaporated to dryness *in vacuo*; the residue was dissolved in water, and again evaporated to dryness. An aliquot of this material A, containing 40 mg. N, was treated with 200 mg. of periodic acid in excess of NaHCO₃ solution, and yielded only 0.44 μ mol. of volatile aldehyde, equivalent (1 mol. aldehyde = 1 atom N) to 0.014 % of the N in the reaction mixture.

When 10.1 mg. of *dl*-serine were added to the same mixture, no additional aldehyde was recovered. The addition of 9.5 mg. of *dl*-threonine gave volatile aldehyde equivalent to 70 % of the threonine added.

Volatile aldehyde from protein hydrolysates

In view of the satisfactory character of the above control experiments, it was decided to apply the determination to protein hydrolysates. The HCl hydrolysate of about 30 mg. of protein (after removal of excess HCl by repeated evaporation with water *in vacuo*) was treated with 50 mg. of periodic acid in 25 ml. *M*/5 NaHCO₃ solution in the usual manner. The volatile aldehyde obtained from a number of proteins is shown in Table 5 as N in percentage of the total N of the protein. In the cases of wool, casein, gelatin and wheat gluten, the recovery of additional aldehyde from threonine added to the reaction mixture with the protein hydrolysate was 65–70 %, substantially the same as in the absence of the hydrolysate. The above conditions were therefore adopted as standard for the determination.

Effect of concentration of reagents on yield of aldehyde

Increasing the concentration of periodic acid tenfold had no effect on the yield of volatile aldehyde, while similar increases of concentration of both protein hydrolysate and periodic acid (i.e. 300 mg. protein and 500 mg. periodic acid in 25 ml. *M*/5 NaHCO₃) in the case of some proteins, though not of others, lowered the percentage yield of aldehyde. The most extreme lowering observed (30 %) occurred in the case of wool.

DISCUSSION

Although successful in differentiating threonine from other hydroxyamino-acids such as serine, β -hydroxyglutamic acid, 'hydroxylysine' etc. the method outlined above cannot be expected to differentiate threonine from its immediate higher homologues. For this reason, in view particularly of the *ad hoc* character of the authentication of threonine as the result of a research on the nutritive properties of amino-acids [McCoy *et al.* 1935], as well as the reported, though unconfirmed, isolations of higher hydroxyamino-acids [cf. Vickery & Schmidt, 1931; Dunn, 1938], it seemed to us desirable to investigate the qualitative composition of the volatile aldehyde material which we obtained from the various protein hydrolysates. Employing the conventional 2:4-dinitrophenylhydrazone identification (aeration into a saturated solution of 2:4-dinitrophenylhydrazine in 2*N* HCl, followed by recrystallization of the resulting precipitate from alcohol), we obtained, in the cases of wool, casein and gelatin, only the dinitrophenylhydrazone of acetaldehyde, which was identified by melting point, mixed melting point and X-ray powder photographs. In the case of wheat gluten, however, a mixture of the usual yellow crystals of acetaldehyde dinitrophenylhydrazone with redder crystals resembling those of the propaldehyde derivative was obtained. The X-ray powder photographs given by this mixture differed from those given by the three other preparations, and were closely similar, as pointed out by Dr F. O. Bell in an Appendix to this paper, to the

photograph given by a mixture of about 3 parts of acetaldehyde dinitrophenylhydrazone to 1 part of the corresponding propaldehyde derivative. This conclusion was confirmed by the melting-point data, and in the preliminary note of this work [Martin & Synge, 1940] it was therefore suggested that a higher homologue of threonine, β -hydroxynorvaline, might be present in wheat gluten.

It seemed desirable to examine the matter quantitatively, and for this purpose we developed the method outlined below for the separation of acetaldehyde and propaldehyde on the micro scale.

MICRO-SEPARATION OF ACETALDEHYDE AND PROPALDEHYDE

The problem of separating successive members of a homologous series on the micro scale by physical means has not to our knowledge previously been attempted. It seemed to us that a simple method was available if it was possible to find a solvent of intermediate boiling point from which the substances to be separated could be differentiated by chemical means and for which Raoult's vapour pressure law was approximately obeyed. If a mixture of the two solutes were then allowed to reflux in excess of the solvent in an efficient fractionating column, the more volatile substance should be concentrated at the top of the column, while the less volatile should remain in the distilling vessel and the lower part of the column.

We accordingly set up the apparatus illustrated in Fig. 1, which incorporates a Lesesne & Lochte [1938] spinning band column, for which high efficiencies have been demonstrated by Baker *et al.* [1940]. The apparatus was made of pyrex glass; the spinning band was of aluminium and was driven through a pulley mounted on a Quickfit & Quartz bearing and gland with standard brass-glass conical joint. The column was about $1\frac{1}{2}$ m. long, and was lagged with wool. The material for analysis was aerated into the solvent in the distilling vessel, which was cooled with solid CO_2 , thus trapping the aldehydes. The distilling vessel was then placed in a water bath and the solvent was allowed to reflux with the band spinning at about 1000 r.p.m. while a current of air was drawn through the apparatus at a controlled rate of 120 bubbles per min. The air from the condenser passed to the absorption tower containing NaHSO_3 , the aldehyde trapped being determined as already described.

As the boiling points of acetaldehyde and propaldehyde are 21° and 49° respectively, ether (B.P. 37°) seemed the most suitable solvent to employ. However, commercial ether contains appreciable quantities of aldehyde, and ether freed from aldehyde by the procedure of Carey *et al.* [1933] (washing with 30% NaHSO_3 followed by 10% NaOH) was found to contain aldehyde again after standing in the laboratory for a few days.

In view of this we tried to use light petroleum which had been fractionated in an efficient distilling column (B.P. 34°). This did not give rise to aldehyde. With petroleum in the column, however, both acetaldehyde and propaldehyde were recovered quantitatively in the absorber, as both appear to form low-boiling azeotropes with petroleum.

As Hildebrand [1921] has shown thermodynamically, deviation from Raoult's law is to be expected whenever two liquids on mixing show volume or heat changes. Liquids are likely to obey Raoult's law when the ratios $\frac{\text{temperature coefficient of expansion}}{\text{compressibility}}$ are similar for both pure liquids and their mixtures. This ratio is a measure of the polarity of the liquids. Lecat [1930; 1932] has made an extensive empirical study of the phenomenon of azeotropy in binary mixtures, and has summarized the literature, but we have not been

able to find a treatment of the phenomenon in terms of molecular physics. It is clear, however, that azeotropy is to be expected in mixtures of liquids of unequal polarity and is related to the formation of separate liquid phases. The use of azeotropic distillation in the manufacture of absolute alcohol is well known. Recently Matuszak & Frey [1937] have used distillation with SO_2 for the separation of butane isomers from butene isomers; Schicktzan *et al.* [1939] have used CCl_4 for the separation of the lower aliphatic alcohols in fusel oil, and [1940] benzene and toluene for the separation of the lower fatty acids. It is evident that the extreme form of azeotropic distillation is the distillation of two liquid phases, as in the steam distillation of oils, and that azeotropic distillation depends not only on the volatility but also on the polarity of the substances to be separated, and thus offers some of the advantages of liquid extraction.

Owing to the azeotropy of the system propaldehyde-petroleum we were compelled to use ether as solvent; when this had been extracted with NaHSO_3 followed by NaOH it gave satisfactory blanks for aldehyde on distillation, although it proved impossible to estimate the propaldehyde remaining in the ether in the apparatus by direct extraction of this with NaHSO_3 solution, since, with ether, an aldehyde titration was always obtained on shaking with the solution in a separating funnel, and this increased as the extraction was prolonged. We were therefore forced to estimate propaldehyde etc. remaining in the apparatus by the difference between the aldehyde estimated by direct aeration and by aeration through the column. We obtained positive evidence for its presence by adding petroleum to the ether in the apparatus after the acetaldehyde had been removed. In the time employed about two-thirds of the propaldehyde present was in this way collected at the top of the column.

The results of the control experiments are shown in Table 3.

Table 3. *Aldehyde micro-separation. Control experiments*

Exp.	Aldehyde taken in $\mu\text{mol.}$		Aldehyde found in $\mu\text{mol.}$	
	Acetaldehyde	Propaldehyde	A	B
I	0.0	0.0	0.0	0.5
II	12.9	28.0	14.8	—
III	26.4	0.0	26.6	0.5
IV	0.0	29.5	0.8	17.0
V	26.0	1.3	29.6	1.6

In these experiments the aldehydes were first collected in 100 ml. of aldehyde-free ether cooled with solid CO_2 . The ether was then allowed to reflux in the column while a stream of air was drawn through the apparatus for 1 hr. at a rate of 120 bubbles per min. in the wash-bottle (Fig. 1), which contained water. At the end of this period the aldehyde collected in the absorber was titrated, giving the values shown in column A of Table 3. 100 ml. of aldehyde-free low-boiling petroleum were then added to the distilling vessel and aeration was continued for 2 hr. The aldehyde in the absorber was then again titrated, giving the values shown in column B. It will be seen from Exp. IV that about two-thirds of the propaldehyde present is carried up in the second stage of the distillation, and from comparing Exps. III and V that as little as 5% of propaldehyde in 1 mg. of acetaldehyde may readily be detected.

A control experiment with isovaleraldehyde showed that it did not reach the top of the column even after adding the petroleum; however, the autoxidation of aliphatic aldehydes higher than propaldehyde renders this analytical procedure unsuitable if a stream of air is employed.

The experiments with protein hydrolysates are shown in Table 4. Here the HCl hydrolysate of about 0.3 g. of protein was used, freed from excess of HCl by evaporation *in vacuo* and treated with 300 mg. of periodic acid in 10 ml. sat. aqueous NaHCO₃ solution. This mixture was aerated, and the volatile aldehyde was collected in cooled ether as already described. The experiments were then carried out exactly as the controls.

Table 4

Protein	Plant	Natural order	Vol. aldehyde as N in % of total N (cf. Table 5)	μmol. aldehyde found	
				A	B
Gluten	Wheat (<i>Triticum vulgare</i>)	Gramineae	1.2	26.2	0.7
Zein	Maize (<i>Zea Mays</i>)	Gramineae	1.2	26.9	0.9
Leaf protein	Cocksfoot (<i>Dactylis glomerata</i>)	Gramineae	2.2	47.6	0.8
Seed globulin	Peanut (<i>Arachis hypogaea</i>)	Leguminosae	1.1	36.0	1.0
Seed globulin (Edestin)	Hemp (<i>Cannabis sativa</i>)	Urticaceae	1.7	30.2	0.6
Seed globulin	Castor bean (<i>Ricinus communis</i>)	Euphorbiaceae	1.5	35.9	0.7

The results make it unlikely that in any case more than 3% of the aldehyde material was higher aldehyde than acetaldehyde. In each case rather more aldehyde was obtained as in column A than by direct aeration from the reaction mixture into the absorption vessel. There is thus no evidence for the occurrence in these proteins of higher homologues of threonine.

Discussion

Our earlier report of propaldehyde was based on melting-point and X-ray data on the dinitrophenylhydrazone material derived from wheat gluten. It subsequently came to our notice that acetaldehyde 2:4-dinitrophenylhydrazone is dimorphic [cf. Bryant, 1933]: this has been questioned by Campbell [1936]. The metastable form, obtained from the melt or by rapid evaporation from solution, bears a close resemblance to propaldehyde dinitrophenylhydrazone being decidedly more deeply coloured (red) than the stable form (yellow). In the light of this, we found that if authentic acetaldehyde and dinitrophenylhydrazine were allowed to react in HCl solution through which air from the reaction mixture with periodic acid of a wheat gluten hydrolysate had been drawn for sufficiently long to remove completely all acetaldehyde, propaldehyde etc. the resulting dinitrophenylhydrazone had a tendency to crystallize from alcohol in the metastable form even when crystallization was slow. It seems therefore that a trace of some slightly volatile contaminant promotes, in the case of the material from wheat gluten (not with casein, wool or gelatin), crystallization of the acetaldehyde derivative in the metastable form.

The dinitrophenylhydrazone material derived from wheat gluten, on chromatographic analysis in petroleum solution on MgO, gave two fractions very readily—a small proportion of red or brown material being very strongly adsorbed. The material obtained after the removal of this component showed, however, the same tendency to crystallize in the metastable form. The development of the chromatogram was not carried sufficiently far to permit of the separation of the acetaldehyde and propaldehyde derivatives, though control experiments suggested that this separation can be effected chromatographically; this requires long development, and the bands become too diffuse for the satisfactory visual detection of a small proportion of one component.

The various dinitrophenylhydrazone materials have been investigated by Dr F. O. Bell by the X-ray powder method; she presents her findings in an Appendix to this paper.

We conclude: (1) that the amount of propaldehyde obtained in this way from wheat gluten is at most small; (2) that acetaldehyde 2:4-dinitrophenylhydrazone, a compound much used by biochemists for the formal identification of acetaldehyde, has disadvantages for this purpose because of its dimorphism, which is now thoroughly substantiated by the X-ray work.

Criticism of the new procedure

The method for the determination of threonine, outlined above, is compared here with the method of Block & Bolling [1939].

(1) It requires the use of rather more material, though it remains on the micro scale.

(2) It is more rapid and less laborious. In particular it does not necessitate the transfer of material to an anhydrous solvent; the reaction is carried out in aqueous solution.

(3) The method has been shown to have a high specificity, threonine being the only known protein constituent to give volatile aldehyde under the conditions used for the determination. Block & Bolling report with their method interference by alanine and serine.

(4) It seems probable that higher homologues of threonine, if present, would be estimated by the procedure described here, though not by the procedure of Block & Bolling. To detect the presence of these we have developed the micro-separation technique described above, and the results suggest that higher homologues of threonine are absent from a number of proteins. We should like to emphasize the applicability of the type of separation described here for aldehydes to micro-separations in other homologous series. In its simplest form, a carrier of intermediate boiling point is required, from which the substances under analysis may be separated and determined by chemical means. It is necessary that at least one, and preferably both, of the substances to be separated should not show positive azeotropy with the solvent. (For separating substances of *different* chemical classes on the micro scale by distillation, 'carrier' solvents showing azeotropy with one class but not with the other may greatly facilitate separation, as has been shown by Matuszak & Frey [1937], and in this case it is not always necessary for the solvent to be of intermediate boiling point.)

(5) The yield of acetaldehyde obtained from threonine by our procedure seems to be about 70 % of the theoretical and to be largely independent of the presence of other amino-acids. It is impossible to deduce from Block & Bolling's paper what yield of acetaldehyde was obtained by them, since they used lithium lactate for checking the method; they found lithium lactate to give, under the same conditions as threonine, the same yield of acetaldehyde. Macara & Plimmer [1940] have recently reported experiments using the method of Block & Bolling. They found certain modifications of the procedure to be necessary, and failed to obtain very consistent results.

2. DETECTION AND ISOLATION OF FORMALDEHYDE LIBERATED BY PERIODIC ACID

We have not so far found a satisfactory method for determining the formaldehyde resulting from the action of periodic acid on serine etc.

Nicolet & Shinn [1939] obtained a good yield of formaldehyde through the dimedone derivative [Vorländer, 1929], after acting on serine with periodic acid, and, as shown below (Exp. I), we can confirm this result. The yield, however, is substantially reduced in the presence of a second amino-acid, such as tyrosine, which does not react with periodate, as shown in Exp. II, while in a complex mixture of amino-acids of known composition (Exp. III) no precipitation of formaldehyde-dimedone takes place. This can be ascribed to competition between the dimedone and the amino-acids for combination with the formaldehyde; in view of Exp. III it is not surprising that a wool hydrolysate, after treatment with periodate and removal of acetaldehyde by aeration, gave no precipitate on treatment with dimedone.

Nevertheless, though the dimedone method seems valueless for determining serine etc. in complete protein hydrolysates, it has proved of some value, as shown in Sections 4 and 5 of this paper, in setting lower limits to the purity of preparations derived from protein containing a high proportion of those amino-acids that yield formaldehyde on treatment with periodic acid.

EXPERIMENTAL

Exp. I. 10.8 mg. of *dl*-serine were treated with 50 mg. of periodic acid in 25 ml. of *M/5* NaHCO_3 at room temperature. After a few minutes, 60 mg. of dimedone (B.D.H.) were added. When the dimedone had dissolved, the mixture was acidified with acetic acid, and after an hour the resulting precipitate was filtered off, washed and dried. It weighed 26.9 mg., corresponding to a yield of 90 % of 1 mol. of formaldehyde per mol. of serine.

Exp. II. 10.1 mg. of *dl*-serine were treated exactly as in Exp. I except that 24.5 mg. of *l*-tyrosine were also present in the reaction mixture. The resulting precipitate weighed 9.4 mg., corresponding to a yield of 33 % of 1 mol. of formaldehyde per mol. of serine.

Exp. III. A portion of *material A* (cf. Section 1) containing altogether 40 mg. N was added to 10.1 mg. of *dl*-serine and was treated with 200 mg. of periodic acid in the presence of excess of NaHCO_3 solution. (Volume of reaction mixture, 27 ml.) On adding dimedone and acetic acid as in Exps. I and II, no precipitate resulted.

3. AMMONIA SPLIT FROM HYDROXYAMINO-ACIDS BY PERIODIC ACID

Van Slyke *et al.* [1940] reported that 1 mol. of NH_3 is instantaneously set free from serine, threonine, β -hydroxyglutamic acid and 'hydroxylysine' by periodic acid in strong aqueous K_2CO_3 solution. They determined the NH_3 liberated by the aeration method of Van Slyke & Cullen [cf. Peters & Van Slyke, 1932]. Macara & Plimmer [1940] have recently employed this method for the determination of amino-acids of this type in hydrolysates of fractions derived from casein. They too report quantitative recovery of NH_3 , although details of the experimental conditions are not given. Since the present work was carried out, we have received from Dr. Van Slyke, in a private communication, details of his procedure; this involves the use in the reaction mixture of NaOH as well as K_2CO_3 ; a reagent is also added to prevent, by competition, condensation of NH_3 with the aldehydes also formed in the reaction. Nevertheless, while the experi-

ments described here are quantitatively imperfect, they serve to demonstrate the specificity of the method, and the results obtained with protein hydrolysates are at least of comparative significance.

EXPERIMENTAL

In these experiments we used simple aeration tubes (as shown in Fig. 1) without the perforated bubblers of Van Slyke & Cullen. Absorption of ammonia in the standard acid was found to be 92%; all the results obtained below have been corrected for this loss. This cruder apparatus seems to have an accuracy of about $\pm 5\%$, but in view of the low yields of NH_3 we were content with this.

Exp. I. 5.92 mg. of *dl*-serine in 10 ml. of 50% w/v aqueous K_2CO_3 solution with 50 mg. of periodic acid gave on aeration at room temperature 88% of 1 mol. of NH_3 . Under the same conditions, equivalent quantities of the following amino-acids gave the yields of NH_3 stated: threonine, 83%; *dl*-allothreonine, 86%; *i*- β -hydroxyglutamic acid, 71%.

Exp. II. An amount of *material A* (cf. Section 1) containing 7.6 mg. N was added (as 0.5 ml. of aqueous solution) to 8 ml. of 62% w/v aqueous K_2CO_3 . The ammonia set free was removed by aeration, and was found to be 102% of the amount calculated on the basis of the NH_4Cl used in making up this mixture.

100 mg. of periodic acid were then added, and the aeration was continued. No further ammonia was detected in the acid of the absorption vessel.

Exp. III. *Exp. II* was repeated, adding known quantities of hydroxyamino-acids to the mixture before carrying out the determination. On adding 5.92 mg. of *dl*-serine, 72% of 1 mol. of NH_3 was recovered on treatment with periodic acid; on adding 7.6 mg. of threonine, 77% of 1 mol. of N_3 was recovered.

Table 5. *Hydroxyamino-acid determinations on various protein hydrolysates*

Protein	N % of protein	As % of total N of protein		
		'Amide-N'	NH_3 -N lib. by periodate	'Threonine-N' (uncorrected)
Wool: Merino 64's I	16.7	8.2	9.2	3.3
Merino 64's II	—	—	—	3.5
Cotswold*	16.55	8.7	9.2	3.0
Commercial pure wool flannel	16.6	8.3	9.9	2.8
Silk fibroin	18.3	2.2	9.4	0.4
Myosin (68)†	16.8	7.5	4.3	1.9
Myosin (KB)†	16.8	7.9	4.5	2.0
Myosin (X)†	16.8	7.4	4.1	1.9
Myogen (C) ₂ †	16.8	7.0	5.4	2.4
Myogen (D)†	16.8	5.7	5.3	1.9
Gelatin (Coignet Gold Label)	—	—	3.4	1.0
Casein B.D.H.	13.4	10.4	6.3	2.1
Egg albumin	15.8	7.9	7.2	1.6
Wheat gluten B.D.H.	13.5	23.3	5.3	1.2
Zein	16.3	19.7	5.9	1.2
Leaf protein: <i>Dactylis glomerata</i>	13.1‡	7.5	5.4	2.1
Seed globulin: <i>Arachis hypogaea</i>	—	12.3	5.0	1.1
<i>Cannabis sativa</i>	18.55	11.0	5.4	1.7
<i>Ricinus communis</i>	—	11.1	5.3	1.5

* Before analysis cystine had been removed from the hydrolysate by Cu_2O .

† These preparations of rabbit muscle proteins were provided by Dr J. G. Sharp of the Low Temperature Research Station, Cambridge. Myosin KB is a sample of myosin reprecipitated six times.

‡ On ash-free basis: the preparation contained 2.4% of ash.

On the basis of these experiments, we have adopted the following routine method for the estimation of the hydroxyamino-acid content of complete protein hydrolysates:

An amount of an HCl hydrolysate (from which excess of HCl has been removed by repeated evaporation with water *in vacuo*), corresponding to 60–70 mg. of protein and not exceeding 2 ml. in volume, is placed in the aeration tube with 8 ml. 62 % w/v aqueous K_2CO_3 solution, and the 'amide-N' is determined by aeration into standard acid. 100 mg. of periodic acid are then added, and the aeration is repeated. The ammonia thus set free is taken as a measure of the hydroxyamino-acids of this type present. In most cases rapid aeration was not interfered with by frothing of the reaction mixture.

In Table 5 are set out parallel determinations of 'ammonia-N', 'N liberated by periodate' and 'threonine-N' as percentages of the total N of the proteins, as determined by the Kjeldahl procedure.

Discussion

All the indications are that the 'threonine' values set out in Table 5 are 30–35 % low (cf. Section 1) and the 'N liberated by periodate' 20–30 % low. The results are, at least, of comparative significance, and set lower limits to the amount of the amino-acids in question that are present.

In the cases of wool, casein, gelatin, gluten, cocksfoot leaf protein and the seed globulins of *Arachis*, *Cannabis* and *Ricinus*, there is good evidence that the volatile aldehyde set free is very largely acetaldehyde, and therefore probably derived from threonine (cf. Section 1).

The high content of hydroxyamino-acids in wool and silk fibroin, compared with the other proteins studied, is to be noted. In the case of wool, about one-third of this quantity appears to be threonine: silk fibroin, however, appears to have the lowest threonine content of all the proteins investigated. In each case, the difference between these values is probably mostly due to serine—in which silk fibroin is known to be comparatively rich [Fischer & Skita, 1902; Abderhalden, 1922]. In the present work (Section 4) it is shown that wool also contains considerable quantities of serine.

Decomposition of a proportion of this serine may be expected to account for part of the ammonia found in the acid hydrolysates of both these proteins [cf. Damodaran & Ramachandran, 1941].

From Table 5 zein would be expected to be fairly rich in serine. This is in agreement with the isolation from zein by Osborne & Jones [1910] of 1 % of serine.

4. THE HYDROXYAMINO-ACID FRACTION OF WOOL

In this section we report the results of an investigation, using the analytical procedures developed above, of the fraction obtained from a wool hydrolysate by the acetylation-benzoylation procedure of Synge [1939]. In that paper, a fraction from wool was reported containing 5.3 % of the N of the wool (96 % of it amino-N). In a subsequent paper [Martin & Synge, 1941] we described a similar preparation (made after removal of the higher monoamino-acids from the hydrolysate by acetylation and chloroform extraction) containing 5.9 % of the N of the wool (95 % of it amino-N). This latter material, obtained as *N*-acetyl derivatives in the form of a nearly colourless glass, was employed in the present investigation.

EXPERIMENTAL

Some of the *N*-acetylated material was hydrolysed by refluxing for 3 hr. in 15 ml. of 2*N* H₂SO₄. This contained 122.5 mg. N, and all values given below refer to this original quantity, being corrected for aliquots removed for analysis.

SO₄⁼ was then exactly removed with baryta, and the filtrate and washings from BaSO₄ were made up to standard volume. A threonine determination (cf. Section 1) on an aliquot of this material gave the threonine-N (uncorrected) as 9.2 % of the N of the fraction, i.e. 0.54 % of the total N of the wool.

The rest of the solution was concentrated by evaporation *in vacuo* and deposited crops of insoluble crystalline material, which were removed by filtration (tyrosine). This material amounted in all to 323 mg., equivalent to 25.0 mg. N, i.e. 20 % of the N of the fraction and 1.2 % of the N of the original wool.

The aqueous filtrate from tyrosine on addition of alcohol gave a syrup which slowly crystallized. These crystals were recrystallized from water-alcohol several times; after two crystallizations the threonine content (uncorrected) of the crystalline material had fallen to about 1 %, at which value it remained on further recrystallization.

The final product (*material S*) weighed 330 mg.

Analytical data on material S

Elementary analysis. Found: C, 31.8; H, 6.35; N, 12.4; ash, 6.6 %. The product therefore contained 40.8 mg. N, i.e. 33 % of the N of the hydroxyamino-acid fraction and 1.95 % of the total N of the wool. 'Corrected' for ash content, the analytical figures become: C, 34.1; H, 6.81; N, 13.3 % (calc. for serine, C₃H₇O₃N: C, 34.3; H, 6.66; N, 13.3 %).

Optical rotation. *Material S* had $[\alpha]_D^{17.5} - 8.06^\circ$ (water, *c* = 5). Fischer & Jacobs [1906] record *L*-serine as having $[\alpha]_D^{20} - 6.83^\circ$ (water, *c* = 10).

Formaldehyde on treatment with periodate. On treating 13.5 mg. of *S* with periodate and dimedone, as described in Section 2, 25.4 mg. of formaldehyde dimedone were obtained. On recrystallization this material had m.p. 188–189°, not depressed on admixture of authentic formaldehyde dimedone, for which Vorländer [1929] records m.p. 189°. (Found: C, 70.5; H, 8.33 %. Calc. for C₁₇H₂₄O₄: C, 69.9; H, 8.23.)

The yield of formaldehyde was thus equivalent to 73 % of the N of the material.

Ammonia on treatment with periodate. 7.2 mg. of *material S* gave, on treatment with K₂CO₃-periodate as in Section 3, 88 % of its N as NH₃—the same yield as that obtained from pure serine under the same conditions.

Material S gave a negative orcin-H₂SO₄ test for carbohydrate.

Discussion

From the above analytical data we conclude that *material S* consists largely of serine, contaminated by inorganic material. The fact that its optical rotation is significantly higher than that reported for *L*-serine by Fischer & Jacobs may be ascribed either to a contaminant of high laevorotation or to a raising of the rotation produced by the inorganic material present. It seems probable, nevertheless, that the acetylation-benzoylation procedure affords a method for isolating serine from proteins in the optically active state. This has not often been reported since Fischer [1907] obtained his active product from silk [cf. Posternak & Posternak, 1927; Lipmann & Levene, 1932].

The results of the present work on the hydroxyamino-acid fraction of wool are summarized in Table 6.

Table 6. *Composition of the hydroxyamino-acid fraction prepared from wool*

	N as % of N of fraction	N as % of N of wool
Identified as: serine	33	1.95
threonine (uncorrected)	9.2	0.54
tyrosine	20	1.2
Unidentified	37.8	2.2
Total	100	5.9

It seems likely that a large proportion of the unidentified N is serine, as unavoidable losses were encountered in working up *material S*.

By making use of the figures given in Table 5 for the original wool hydrolysate (Merino 64's I) it is possible to form an estimate of the yields obtained by the acetylation-benzoylation procedure.

The threonine content of the original hydrolysate (corrected—assuming the figure in Table 5 to be low by 30 %) is 4.7 % of total N. The threonine present in the hydroxyamino-acid fraction (from Table 6, corrected in the same way) is only 0.77 % of the total N of the wool. This represents a recovery, in the hydroxyamino-acid fraction, of 16 % of the threonine present in the hydrolysate.

The recovery of serine may be estimated as follows: assuming that the 'N liberated by periodate' as given in Table 3 is 25 % low (cf. Section 3), we have 'N liberated by periodate' as 12.2 % of the total N of wool. Subtracting the 'corrected' value for threonine-N of 4.7 %, this leaves a maximum value for serine-N in the original hydrolysate of 7.5 % of the total N. In the hydroxyamino-acid fraction, the upper limit for serine is (total N—threonine N (corrected)—tyrosine N)=4.0 % of total N of wool (cf. Table 6). On this basis, a recovery of about 50 % of the serine of the original hydrolysate is obtained in the hydroxyamino-acid fraction.

This difference between the behaviours of serine and threonine may perhaps be ascribed to a more ready benzoylation of the primary alcoholic group of serine than of the secondary alcoholic group of threonine, in the course of preparing the hydroxyamino-acid fraction.

Nevertheless, in hydroxyproline, as shown by Synge [1939], the secondary alcoholic group is readily benzoylated, as is the phenolic—OH group of tyrosine.

5. 'HYDROXYLYSINE'

Van Slyke & Hiller [1921] reported the occurrence of a base of unknown constitution in the lysine fraction of a gelatin hydrolysate. Schryver *et al.* [1925] reported the isolation of a new base from isinglass, of which they prepared a tribenzoyl derivative. They suggested that it was a hydroxy derivative of lysine, and tentatively assigned the —OH group to the β -position, by analogy with serine and β -hydroxyglutamic acid. Van Slyke *et al.* [1938] reported the isolation of a base from the lysine fraction of a gelatin hydrolysate by fractional crystallization of the picrates; it had the composition of hydroxylysine and its picrate had m.p. 225°. More recently Van Slyke *et al.* [1940] have analysed the electro-metric titration curve of the new base, and have shown that the compound is

split by periodic acid to give one molecule of formaldehyde and one molecule of ammonia. They suggest that the new base is either δ -hydroxy- $\alpha\epsilon$ -diaminohexanoic acid or ϵ -hydroxy- $\alpha\delta$ -diaminohexanoic acid.

Shortly after the 1938 note of Van Slyke *et al.* one of us (R. L. M. S.) applied the acetylation-benzoylation procedure of Synge [1939] to the lysine fractions of gelatin and isinglass hydrolysates and in each case isolated a picrate corresponding to the description given by Van Slyke *et al.* [1938].

EXPERIMENTAL

Approximately 150 g. of gelatin (Coignet Gold Label) containing 21.2 g. N were hydrolysed by refluxing with 1.5 l. of 6N H_2SO_4 for 6 hr. The hydrolysate was cooled and treated with 300 g. of phosphotungstic acid (B.D.H.) at a final volume of 5 l. After keeping overnight in the ice-chest, the precipitate was filtered off and decomposed with baryta as usual. The filtrate and washings from the decomposition were concentrated *in vacuo* to remove NH_3 , acidified to thymol blue with H_2SO_4 and treated with Ag_2SO_4 at 100° until excess had been added (brown precipitate on testing a portion with baryta). The solution was cooled, saturated with $\text{Ba}(\text{OH})_2$ and filtered, and the filtrate, after acidification to thymol blue with H_2SO_4 (to remove Ba), was treated with H_2S to remove Ag. The filtrate from Ag_2S and BaSO_4 was freed from H_2S by concentration *in vacuo* and was then treated with 20 ml. of H_2SO_4 and 100 g. of phosphotungstic acid at a volume of 1.5 l. After keeping overnight in the ice-chest, the precipitate was filtered off and decomposed with baryta as usual. The filtrate was acidified with H_2SO_4 to precipitate Ba, and the filtrate from BaSO_4 , the 'lysine fraction', was found to contain 4.3 % of the original N of the gelatin in the form of amino-N ($\frac{1}{2}$ hr. Van Slyke determination). This solution was then treated with two-thirds of the theoretical quantity of picric acid, and the resulting lysine picrate, after recrystallization from water, corresponded to 1.93 % of the total N of the original gelatin. The combined mother liquors and washings from this were freed from picric acid by acidification with H_2SO_4 and repeated extraction with chloroform. The aqueous residue was then brought to pH 8.5 with NaOH, concentrated to small volume *in vacuo* and subjected to the acetylation-benzoylation procedure [Synge, 1939], employing for the acetylation 200 ml. 2N NaOH and 20 ml. of acetic anhydride and for the benzoylation 185 ml. 2N NaOH and 23 ml. of benzoyl chloride. The resulting *N*-acetylated material was hydrolysed in 108 ml. 2N H_2SO_4 by refluxing for 3 hr., after which the solution was found to contain 57.5 mg. amino-N ($\frac{1}{2}$ hr. Van Slyke determination) equivalent to 0.27 % of the total N of the gelatin. 20 g. of phosphotungstic acid were added in 60 ml. of water. Precipitation of the resulting phosphotungstate, a fluffy, crystalline material (which did not readily settle, but filled the body of the solution), was slow, but had ended after keeping overnight in the ice-chest. The precipitate was filtered off and decomposed in the usual manner with baryta; the amino-N obtained was found ($\frac{1}{2}$ hr. Van Slyke determination) to correspond to 0.13 % of the total N of the gelatin.

This material yielded with picric acid 121 mg. of a crystalline picrate, which on recrystallization from water had explosion point $226-227^\circ$ and amino-N ($\frac{1}{2}$ hr. Van Slyke determination) 7.38 %. ($\text{C}_6\text{H}_{14}\text{O}_3\text{N}_2$. $\text{C}_6\text{H}_3\text{O}_7\text{N}_3$ requires amino-N 7.17 %.)

A picrate having the same properties was obtained in the same way from isinglass (Boots 'Besyet'); the product resulting from decomposition of the final phosphotungstic acid precipitate contained amino-N equivalent to 0.15 % of the original N of the isinglass.

Several preparations obtained in this way were combined, and after the appearance of the 1940 note of Van Slyke *et al.* it seemed to us desirable to test the material for formaldehyde and ammonia on treatment with periodate.

A control experiment with synthetic *dl*-lysine [cf. Eck & Marvel, 1939] showed that on treatment with periodate lysine produces neither of these substances.

Formaldehyde liberated by periodate from the base. 57.3 mg. of the combined picrate preparations were freed from picric acid by extraction with chloroform from 10 ml. of 0.15*N* HCl. The aqueous residue was freed from chloroform by aeration, and treated with 1 g. of NaHCO₃ and 120 mg. of periodic acid at a volume of 30 ml.; 120 mg. of dimedone were then added, and when it was dissolved the mixture was acidified with acetic acid. After keeping 1.5 hr. the resulting precipitate was filtered off, washed and dried. It weighed 28 mg., corresponding to a yield of 65 % of 1 mol. of formaldehyde per mol. of 'hydroxylysine'. On recrystallization from alcohol the material had m.p. 187–188°, which was not depressed on admixture of authentic formaldehyde-dimedone. (Found: C, 69.6; H, 8.14 %. Calc. for C₁₇H₂₄O₄: C, 69.9; H, 8.23 %.)

Ammonia liberated by periodate from the base. After removal of picric acid as above, followed by removal of chloroform by aeration, an amount of the base corresponding to 16.8 mg. of picrate gave on treatment with K₂CO₃ and periodic acid as in Section 3 0.19 mg. of NH₃-N. This is equivalent to the liberation of 31 % of a mol. of NH₃ per mol. of 'hydroxylysine'.

Discussion

It will be seen that the above experiments confirm the discovery of Van Slyke and co-workers although the yields of formaldehyde and ammonia fall short both of the 'theoretical' and of those obtained with serine and threonine under the same conditions. The method employed for the isolation of the base is in itself an indication that the base possesses a free hydroxyl group.

It seemed of interest to obtain an estimate of the amount of the base possibly present in gelatin. A sample of the 'lysine fraction' obtained as described above from Coignet Gold Label gelatin was treated with K₂CO₃ and periodic acid as in Section 3. 0.20 % of the total N of the gelatin was obtained as ammonia, suggesting that at least 0.4 % of the total N of the gelatin is in the form of 'hydroxylysine'. In view of the low yield of NH₃ obtained in this way from our purest preparation of the base, the amount actually present may be considerably greater.

It appears therefore that the yield of 'hydroxylysine' isolated by the acetylation-benzoylation procedure from the 'lysine fraction' is not high (< 33 %), in spite of the enormous excesses of reagents which it was possible to employ. This, by analogy with the behaviour of serine and threonine (cf. Section 4) is perhaps an indication in favour of the alcoholic group being secondary, i.e. favours the view that 'hydroxylysine' is δ -hydroxy- $\alpha\epsilon$ -diaminohexonic acid rather than ϵ -hydroxy- $\alpha\delta$ -diaminohexonic acid.

A parallel experiment on the 'lysine fraction' of wool (Merino 64's I) prepared in exactly the same manner gave < 0.01 % of the total N of the wool in the form of NH₃ liberated by K₂CO₃ periodate.

In a private communication Dr Van Slyke informs us that the hydroxylysine-N in gelatin and wool amounts respectively to 0.9 and 0.11 % of the total N (minimal figures based on N liberated by periodate from recrystallized phosphotungstic acid precipitates).

The formula suggested for hydroxylysine by Van Slyke and co-workers may be said to have a biological justification. The idea that proline may have the same biological origin as the basic amino-acids was held by its discoverer [Fischer, 1903]. Wada's [1933] demonstration of the conversion of citrulline into proline on boiling with acid lends support to this hypothesis (cf. also Sørensen's [1905] conversion of δ -hydroxy- α -aminovaleric acid into proline). It is not likely that this reaction occurs to any great extent during the acid hydrolysis of proteins, although since the work of Wada [1933] and Fearon [1939] citrulline is to be regarded as a probable constituent of proteins, and its breakdown during acid hydrolysis would account for the small amounts of CO_2 that are set free [cf. Dunn, 1925] as well as for some NH_3 . The balance of evidence suggests that proline residues exist pre-formed in proteins. If, however, their biological precursor is to be regarded as an ornithine derivative, then the precursor of hydroxyproline would be a derivative of γ -hydroxyornithine, which is homologous with δ -hydroxylysine. On this view it is to be expected that the asymmetry of the γ -C atom of hydroxyproline is the same as that of the δ -C atom of hydroxylysine. Neither configuration has yet been ascertained. In this connexion it is of interest that Felix & Mager [1937] report the occurrence of small amounts of hydroxyproline in clupein, together with still smaller amounts of a base having the elementary composition of hydroxyarginine. In the present work it is shown that hydroxyproline and hydroxylysine, both present in a gelatin hydrolysate, can only be present in small quantity in wool.

SUMMARY

1. Threonine on treatment with periodate at pH 7 yields 70% of 1 mol. of acetaldehyde. Acetaldehyde and higher homologues, unlike formaldehyde, may be removed from aqueous solution by simple aeration; this provides the basis of a micro-method for the determination of threonine and higher homologues in complete protein hydrolysates and other material of biological interest. The determination is not interfered with by serine, 'hydroxylysine' or other known naturally occurring amino-acids.

2. The aldehyde material obtained in this way from a number of proteins has been shown to consist almost entirely of acetaldehyde. We have therefore no evidence for the occurrence of higher homologues of threonine in these proteins.

3. To demonstrate this, we have developed a micro-method for the separation of acetaldehyde from its higher homologues, which depends on 'carrier' distillation using ether as a solvent. By this means as little as 5% of propaldehyde can be detected in 1 mg. of acetaldehyde. The principles of this type of separation, and its applicability to micro-separations in other homologous series, are discussed.

4. The explanation of our earlier report [Martin & Syngé, 1940] of propaldehyde in the aldehyde material from wheat gluten appears to lie in the dimorphism of acetaldehyde dinitrophenylhydrazones. In an appendix, Dr Bell discusses the relevant X-ray data.

5. The conditions for precipitation by dimedone of the formaldehyde set free from serine etc. by periodic acid have been studied. The presence of other amino-acids seriously lowers the yield obtained.

6. The production of NH_3 by periodate from such hydroxyamino-acids as serine, threonine, β -hydroxyglutamic acid and hydroxylysine in the presence of K_2CO_3 [cf. Van Slyke *et al.* 1940] has been shown by control experiments to be

applicable to the estimation of the aggregate of these substances in complete protein hydrolysates.

7. The results of threonine and aggregate hydroxyamino-acid estimations on a number of proteins are tabulated and discussed.

8. Using the methods here developed, the hydroxyamino-acid fraction obtained from a wool hydrolysate by the acetylation-benzoylation procedure of Synge [1939] has been investigated. This procedure appears to give low recoveries of threonine. Nearly 2 % of the N of wool was isolated in the form of fairly pure serine showing optical activity.

9. A preparation agreeing in properties with the 'hydroxylysine' of Van Slyke *et al.* has been obtained from the lysine fractions of gelatin and isinglass hydrolysates by the acetylation-benzoylation procedure. This material, like Van Slyke's, yields ammonia and formaldehyde on treatment with periodic acid.

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APPENDIX

X-RAY INVESTIGATION OF THE 2:4-DINITROPHENYLHYDRAZINE COMPOUNDS WITH VARIOUS ALDEHYDES AND KETONES AND WITH PRODUCTS LIBERATED BY PERIODIC ACID FROM PROTEIN HYDROLYSATES

By FLORENCE O. BELL

The 2:4-dinitrophenylhydrazone of the compound obtained from wheat gluten by the action of periodic acid (see above) is a mixture of yellow and orange-red crystals; acetaldehyde 2:4-dinitrophenylhydrazone as usually prepared by crystallization from solvents is yellow, and the propaldehyde derivative (however prepared) is red. The melting point of the gluten derivative is lower than that of the yellow acetaldehyde derivative, and it is therefore possible that the gluten compound is formed from acetaldehyde (derived from threonine) with small amounts of propaldehyde (derived from a higher homologue of threonine). In an attempt to determine the nature of the gluten compound, X-ray powder photographs were taken of the gluten 2:4-dinitrophenylhydrazine derivative, of the hydrazones formed with acetaldehyde, propaldehyde and acetone respectively, and of various mixtures of these derivatives.

Direct comparison photographs of specimens were taken on two halves of one and the same photographic film, the specimens being exactly interchangeable so that specimen-to-film distance, development conditions etc. were the same for both photographs.

Crystals of the gluten aldehyde derivative ground to a powder were compared with the powdered derivatives of acetaldehyde, propaldehyde and acetone

respectively, but the numbers, distribution and intensities of the lines in the gluten photograph were not the same as those of the other three compounds, although certain strong lines appeared in approximately the same positions in all four photographs: the gluten derivative was certainly not identical with the yellow crystals of the acetaldehyde derivative, whereas aldehydes derived similarly from casein, gelatin, wool and gliadin were in all cases identified, in the form of the 2:4-dinitrophenylhydrazone, as acetaldehyde. On the assumption, then, that in addition to acetaldehyde another aldehyde or ketone was present in the gluten preparation, the latter was compared with various mixtures of aldehyde and ketone derivatives.

It was noticed at this stage, however, that fused yellow crystals of acetaldehyde 2:4-dinitrophenylhydrazone give on cooling *red* crystals, similar to those of the gluten and propaldehyde derivatives, and that there are pronounced differences between the X-ray photographs (see Pl. 2, figs. 1, 2) of these yellow and red dimorphs. Further, we found that this dimorphism of acetaldehyde 2:4-dinitrophenylhydrazone had already been noted and studied crystallographically and optically by Bryant [1933].

In spite of this promising clue, the X-ray photograph of the gluten compound (also fused in this case) still did not correspond exactly with that of the red metastable acetaldehyde derivative (see Pl. 2, fig. 3), although it had similar intensities and distribution of lines. Small quantities of propaldehyde or acetone added to the acetaldehyde produced marked effects on the X-ray photograph of the 2:4-dinitrophenylhydrazone (see Pl. 2, figs. 4, 5), as would be expected from Bryant's results: he observed that very small amounts of propaldehyde mixed with acetaldehyde in the 2:4-dinitrophenylhydrazone modified the optical constants of the crystals considerably.

It seems clear, therefore, from these investigations that the aldehyde liberated from gluten hydrolysates by means of periodic acid is at any rate not pure acetaldehyde, since the photograph of its 2:4-dinitrophenylhydrazone does not agree strictly either with that of the yellow or with that of the red form of acetaldehyde 2:4-dinitrophenylhydrazone. No artificial mixture of aldehydes or ketones has so far been found which exactly corresponds to the gluten compound. A mixture of three parts of the acetaldehyde derivative and one part of the propaldehyde derivative crystallized out together from alcohol (see Pl. 2, fig. 6), and also a fused mixture of the acetaldehyde derivative with 2% of the propaldehyde derivative, both gave X-ray photographs closely corresponding to that of the gluten compound, crystallized and fused respectively, but again neither was really identical with it. The presence of small quantities of acetone in the gluten compound appears definitely unlikely from the X-ray photographs. There is some contaminant, nevertheless, and moreover a contaminant that predisposes the acetaldehyde derivative to crystallize in the red form just as though it had been prepared from the melt; but what this contaminant is it must be confessed is still an open question. It is tempting to assume that a small proportion of propaldehyde or of some higher aldehyde is present, but the problem is so complicated by dimorphism and mixed crystallization effects that it would require much more time to investigate satisfactorily than is immediately available, so it is better to suspend judgment for the moment.

I am much indebted to Dr K. M. Rudall for taking a number of the X-ray photographs, and to Dr W. T. Astbury for the benefit of valuable discussion.

EXPLANATION OF PLATE 2

Fig. 1. Stable yellow acetaldehyde 2:4-dinitrophenylhydrazone.

Fig. 2. Comparison of metastable red acetaldehyde 2:4-dinitrophenylhydrazone (left) and propaldehyde 2:4-dinitrophenylhydrazone (right).

Fig. 3. Comparison of red acetaldehyde 2:4-dinitrophenylhydrazone (left) and fused gluten compound 2:4-dinitrophenylhydrazone (right).

Fig. 4. Comparison of red acetaldehyde 2:4-dinitrophenylhydrazone (left) and acetaldehyde 2:4-dinitrophenylhydrazone plus 2% propaldehyde 2:4-dinitrophenylhydrazone fused (right).

Fig. 5. Comparison of red acetaldehyde 2:4-dinitrophenylhydrazone (left) and acetaldehyde 2:4-dinitrophenylhydrazone plus 2% acetone 2:4-dinitrophenylhydrazone fused (right).

Fig. 6. Comparison of gluten compound 2:4-dinitrophenylhydrazone (left) and acetaldehyde 2:4-dinitrophenylhydrazone plus 25% propaldehyde 2:4-dinitrophenylhydrazone crystals (right).

All X-ray photographs taken with Cu K_α radiation, and specimen to film distance approximately 3 cm.

Figs. 1-6.