rancid phosphatides, whose amount is small in relation to the total phospholipid.

3. Degraded phosphatides were present in the fraction expected to contain phosphatidic acid. It was therefore considered impossible either to exclude or confirm the presence of naturally occurring phosphatidic acid.

4. The bearing of these facts on the isolation of phosphatidic acid reported by Hübscher & Clark (1960) is discussed.

5. Linoleic acid (70%) and linolenic acid (12–15%) were the main fatty acids in ox-liver cardiolipin.

6. Phosphatidylinositol isolated in yields of 64–70% of the total inositide contained stearic acid as virtually the only saturated acid (45–55%) and polyenoic acids in varying proportion as the main unsaturated components.

7. Successive fractions of liver lecithin with decreasing iodine values obtained by chromatography on silicic acid contained similar proportions of saturated acids and decreasing proportions of polyenoic acids.

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The Amide Nitrogen Content of Gelatins

By J. E. EASTOE, JOAN E. LONG AND ALICE L. D. WILLAN

The British Gelatine and Glue Research Association, 2A Dalmeny Avenue, London, N. 7

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It has long been known that the isoionic points of gelatins prepared from alkali-treated collagens are in general considerably lower than those of gelatins extracted after acid pretreatment. Ames (1952) has shown that this fall in the isoionic point of alkali-processed gelatins is associated with a loss of ammonia during pretreatment. The ammonia has been assumed to be derived mainly from hydrolysis of amide side chains which thus give rise to new free carboxyl groups in the intact molecule of the gelatin precursor. Even acid pretreatment may result in some loss of amide groups, if conditions are sufficiently severe (Ames, 1957). A picture has gradually emerged of a series of

possible gelatins, ranging from those with an isoionic point above pH 9, with substantially all the original amide groups still intact, to those prepared after a prolonged lime soak with a limiting minimum isoionic point at approximately pH 4.80 and presumably zero amide content. The exact relationship between the isoionic point and the amide content has not, however, been experimentally investigated. A survey of these two characteristics for a representative selection from a wide range of gelatins available in this Laboratory is described in this paper. For this purpose a rapid and accurate method for the determination of amide nitrogen in gelatin was developed.

EXPERIMENTAL

Gelatins. These were selected from about 150 samples to represent, as far as possible, a continuous range of isoionic points (pI) between 4.8 and 9.5. One sample only was available in the range pI 5.5–7.5. To remove ammonium salts, possibly present in some gelatins, 10% solutions were deionized with mixed-bed ion-exchange resins (Janus, Kenchington & Ward, 1951). The solutions were allowed to set and the gels dried in a current of air at approximately 20°.

Methods

Moisture. The loss of weight on drying a thin film of gelatin for 18 hr. at 105° was used to calculate moisture content (B.S. 757: 1959 and Eastoe & Williams, 1959).

Ash. The gelatin was slowly incinerated in a platinum dish over a small Bunsen flame and the residue was heated for 2 hr. at 550° in an electric muffle furnace. The small fluffy pellets of ash were transferred with a needle to a piece of foil and weighed on a microbalance.

Isoionic point. This was determined by the deionization method of Janus, Kenchington & Ward (1951) with a glass electrode and Cambridge pH meter.

Determination of amide nitrogen

0.05M-Sodium tetraborate-0.15N-sodium hydroxide. Sodium tetraborate (19.1 g. of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, A.R.) and NaOH (6 g., A.R.) were dissolved in 1 l. of water, previously boiled to remove CO_2 .

1% (w/v) Boric acid with indicator. Boric acid (10 g. of H_3BO_3 , A.R.) was dissolved in 1 l. of boiled water and 8 ml. of mixed indicator solution was added. The cold solution was adjusted to a neutral-grey colour by addition of approximately 0.7 ml. of 0.1N-NaOH. This reagent kept well in borosilicate glass in the dark.

Mixed indicator. Equal volumes of ethanolic 0.033% methylene blue and 0.05% methyl red solutions were mixed.

Procedure. Approximately 2.5 g. of gelatin was accurately weighed into a 500 ml. round-bottom flask and dissolved in 100 ml. of warm 2N-HCl. A few alundum chips (no. 14 grit, Norton Abrasives Ltd.) were added and the solution was boiled under reflux for 1 hr. Sufficient mixed indicator was added to the cold solution to enable the colour to be easily seen. Some gelatins gave rise to dark-brown hydrolysates and for these it was necessary to use external indicator paper. The flask was stoppered and 2N-NaOH was added initially in approximately 10 ml. portions and later in smaller amounts, the stopper being removed only during the additions. The contents were mixed and kept cool by partially immersing the flask in cold water. The pH of the solution was adjusted to the end point of the indicator (approx. pH 5.2) and the solution diluted to 250 ml.

The steam-distillation apparatus of Yuen & Pollard (1953) was used for separation of ammonia. Water containing 0.1% of syrupy phosphoric acid was added to the steam-generator flask and approximately 100 ml. was boiled off to remove CO_2 before a series of distillations. A portion (20 ml.) of the neutralized hydrolysate was added to the distillation apparatus, followed by 20 ml. of 0.05M-sodium tetraborate-0.15N-NaOH solution. The distillate was collected in 10 ml. of the 1% boric acid solution, distillation being carried out until approximately

40 ml. had collected (approximately 5 min.). When necessary, frothing was prevented by the initial addition of 2 drops of silicone M.S. experimental antifoam emulsion (Hopkin and Williams Ltd.). The distillate was titrated with 0.01N-HCl to the first faint permanent pink colour. Blanks (without gelatin) were carried through the whole procedure and the blank titration was subtracted from the experimental values. The amide-nitrogen value was calculated for the dry, ash-free protein.

Factors affecting the accuracy of the amide determination

Solution for absorbing ammonia. Plain aqueous 1% boric acid, widely used for ammonia absorption in determinations of total N, is normally slightly on the acid side of the achromatic end point of screened methyl red indicator. The requirements for ammonia absorption and titration are identical in amide-N and total-N determinations, except that, under some conditions CO_2 may distil over in amide determinations, owing to the lower pH of the boiling solution. In blank determinations the amount of CO_2 may exceed the quantity of ammonia distilling. If the solution is initially on the acid side of the end point, distillation of CO_2 does not result in a colour change and may not be noticed. A convenient reagent is obtained by adding a definite concentration of indicator to the boric acid solution in bulk and then adjusting it to the end point.

Conditions for distillation of ammonia. In the present investigation acid hydrolysates were made alkaline in two stages. 2N-NaOH solution was first added to bring the solution to approximately pH 5.0. This step was not critical in producing variations in results, if carried out as described. A portion of the diluted hydrolysate at pH 5.0 was then mixed with an equal volume of an alkaline solution in the distillation apparatus. The use of 0.05M-sodium tetraborate, 0.05M-sodium tetraborate-0.15N-NaOH and N-NaOH gave final solutions of pH 8.6, 9.95 and > 13 respectively at 22°.

N-NaOH solution was unsuitable because it brought about continuous loss of ammonia from boiling gelatin hydrolysates, presumably owing to decomposition of amino acids at the high pH. Neither 0.05M-sodium tetraborate nor 0.05M-sodium tetraborate-0.15N-NaOH did this. Experiments showed that distillation of ammonia from a standard (approx. 0.004M) NH_4Cl solution was complete when the first 20 ml. (of the specified 50 ml.) of distillate had collected, irrespective of whether 0.05M-sodium tetraborate or N-NaOH was added.

Experiments to investigate apparent recovery of ammonia from the standard NH_4Cl solution showed that it was variable and up to 1% lower with 0.05M-sodium tetraborate than with N-NaOH, which gave the theoretical recovery. The end point was also less sharp with sodium tetraborate. These effects were traced to distillation of CO_2 at the lower pH. The higher pH value reached with the 0.05M-sodium tetraborate-0.15N-NaOH reagent greatly reduced the quantity of CO_2 distilling over and largely overcame the difficulty.

Distillates from 20 ml. portions of NH_4Cl solution, to which equal volumes of the various alkaline reagents had been added, were titrated to a faint-pink colour, boiled for 30 sec. to remove CO_2 and quickly cooled. Where CO_2 was present the solution became green again and more 0.01N-HCl was added to restore the pink colour. The initial and

Table 1. Mean titration values for distillates from 20 ml. of 0.00398M-ammonium chloride solution made alkaline with various reagents

7.95 ml. corresponds to a theoretically complete recovery.

Reagent		0.01 N-HCl added (ml.)	
		Initially	Total after boiling
0.05 M-Sodium tetraborate	Blank	0.00	0.13
	NH ₄ Cl	7.90	8.08
	Difference	7.90	7.95
0.05 M-Sodium tetraborate-0.15 N-NaOH	Blank	0.11	0.14
	NH ₄ Cl	8.04	8.09
	Difference	7.93	7.95
N-NaOH	Blank	0.16	0.16
	NH ₄ Cl	8.11	8.11
	Difference	7.95	7.95

Fig. 1. Ammonia N released from acid-processed gelatin after hydrolysis with hydrochloric acid for various periods. O, Boiling 6N-HCl; ●, boiling 2N-HCl; △, 12N-HCl at 37°. Time is expressed in hours for 2N- and 6N-HCl and in days for 12N-HCl.

Fig. 2. Ammonia N released from lime-processed gelatin after hydrolysis with hydrochloric acid for various periods. Details of symbols and time scale are the same as those given in Fig. 1.

total volumes of acid added are given in Table 1. Removal of CO₂ increased the titration by 0.13–0.18 ml. of 0.01 N-HCl with 0.05M-sodium tetraborate and by only 0.03–0.05 ml. with 0.05M-sodium tetraborate–0.15N-NaOH. The distillate from the solution with N-NaOH remained pink after boiling. The differences between the initial titrations for NH₄Cl solution and blank with 0.05M-sodium tetraborate and 0.05M-sodium tetraborate–0.15N-NaOH were respectively 0.05 and 0.02 ml. lower than with N-NaOH (Table 1). Boiling the distillate enabled identical results to be obtained with all three reagents but was not adopted

because it complicated the experimental procedure. Instead, the sodium tetraborate–NaOH reagent was used since it resulted in titration values only 0.3% below theoretical and gave much sharper end points than those with sodium tetraborate alone.

Hydrolysis of the amide groups of gelatin by hydrochloric acid. The release of ammonia from two gelatins (one an acid-processed sample of high amide content and the other lime-processed and of fairly low amide content) by boiling 2N-HCl and boiling 6N-HCl for periods from 15 min. to 24 hr. and by 12N-HCl at 37° for 3–20 days was studied. The ammonia was determined in the 2N-HCl hydrolysates as described above. The 6N-HCl hydrolysates were initially

neutralized with 6N-NaOH and the bulk of the acid from the 12N-HCl hydrolysates was removed by evaporation on a warm plate at 35° before evaporation of the last portion *in vacuo* at room temperature. The ammonia was then determined.

The quantities of ammonia N released in these experiments are summarized in Figs. 1 and 2. With 12N-HCl, a very slight increase for the amide value of the acid-processed gelatin was evident over the period 3–20 days. The scatter of individual values, however, was of the same order as this increase. No such increase was evident for the lime-processed gelatin. Mean values of 0.624 and 0.187% respectively were calculated and are represented by the horizontal lines in Figs. 1 and 2.

Both boiling 2N-HCl and 6N-HCl showed substantial increases in the quantity of ammonia liberated in periods up to 24 hr. Almost equal amounts of excess of nitrogen were liberated in the period from 15 min. to 24 hr. from both types of gelatin, 0.032% by 2N-HCl and 0.078% by 6N-HCl. The quantity of ammonia N released during short periods of heating ($\frac{1}{4}$ –1 hr.) were all closely similar for both acid concentrations and consequently did not differ from values extrapolated to zero time by more than the experimental error. For the acid-processed gelatin, extrapolation gave values of 0.624% with both 2N-HCl and 6N-HCl, which was identical with the mean value with 12N-HCl. For the lime-processed gelatin, identical values of 0.182% were obtained by extrapolation, slightly lower than the mean value of 0.187% obtained with 12N-HCl at 37°.

Since all three methods of hydrolysis gave closely similar results for both types of gelatin studied, 2N-HCl was selected for routine use, being more convenient than 12N-HCl and having a lower rate of release of additional ammonia than 6N-HCl. Extrapolation to zero time did not result in values significantly different from those obtained with heating periods up to 1 hr. with 2N-HCl. A period of 1 hr. was selected, since it provides a safety margin.

RESULTS

The amide-nitrogen contents of a series of deionized gelatins are summarized in Table 2 in order of increasing isoionic points. The amide

values were determined by the method described in the Experimental section and also after hydrolysis with 6N-HCl for 2 hr., a small correction, based on the average of the initial slopes of the upper curves in Figs. 1 and 2, being applied for additional non-amide nitrogen set free during this period. Values obtained by the two methods showed good agreement, being slightly higher with 6N-HCl.

DISCUSSION

The method for amide nitrogen described has an accuracy approaching that of the lengthier procedure of Gordon, Martin & Synge (1941). Errors due to distillation of carbon dioxide have been reduced to the same order as those associated with the normal titration end point. The findings of Leach & Parkhill (1955) that identical values for the amide content of wool are obtained after hydrolysis with either hot 2N-hydrochloric acid for 1 hr. or 12N-hydrochloric acid at 37° for 10 days have been confirmed for gelatin. Tristram (1949) mentioned that Rees (1946) also found that hydrolysis with dilute boiling acid for 2 hr. invariably gave the same results as 10N-hydrochloric acid at 37° for 10 days; it seems likely that the present method would be valid for all proteins that dissolve readily in 2N-hydrochloric acid. For those not easily soluble, a preliminary treatment with more concentrated acid, followed by dilution before boiling, is suggested.

It has been found that approximately 0.078% of ammonia nitrogen is slowly liberated from gelatin on boiling with 6N-hydrochloric acid for 24 hr., after the rapid initial release of ammonia from amide groups. This experimental value is in good agreement with the value (0.0764%) calculated from the serine and threonine contents of gelatin (Eastoe, 1955) and the data of Rees (1946) for the

Table 2. *Amide-nitrogen contents and isoionic points of deionized gelatins*

Designation	Type	Moisture (%)	Ash (%)	pI	Amide N (g./100 g. of protein)	
					2N-HCl (1 hr.)	6N-HCl (2 hr.)
133	Limed	15.6	0.064	4.80	0.109	0.132
E.K.	Calf skin, limed	13.5	0.03	4.82	0.018	0.027
70	Sinew, lime-NaOH	16.3	0.083	4.82	0.054	0.063
90	Hide, limed	15.1	0.032	4.90	0.095	0.103
1	Hide, limed	15.8	0.092	4.94	0.111	0.118
150	Hide, limed	—	—	4.96	0.178	—
19	Calf skin, lime-NaOH	14.7	0.015	5.15	0.200	0.212
126	Bone glue	15.4	0.13	5.47	0.493	0.508
8790	—	16.1	0.073	6.5	0.442	0.451
A.S.	Ox sinew, acid	16.6	0.34	7.5	0.613	0.634
142	Ossein, acid	16.9	0.037	7.8	0.498	0.511
136	Pig skin, acid	16.9	0.021	8.4	0.543	0.555
96	Pig skin, acid	16.5	0.018	9.4	0.624	0.628
149	Pig skin, acid	16.6	0.016	9.4	—	0.615

decomposition of these amino acids in boiling 6*N*-hydrochloric acid.

The value of 0.62 % of amide nitrogen found for the two pig-skin gelatins of *pI* 9.4 is identical with that determined previously for a similar gelatin by resin chromatography (Eastoe, 1955). Bowes, Elliott & Moss (1955) found 0.52 and 0.66 % respectively for citrate-soluble collagen and native ox-hide collagen. The intermediate value for acid-processed pig-skin gelatin suggests that practically all the amide groups have remained intact during pretreatment and extraction.

The points plotted in Fig. 3, with two conspicuous exceptions, lie close to the smooth curve represented by the continuous line. The exceptional samples are the acid sinew gelatin (A.S.) and the bone glue (126). These samples and a long-limited gelatin of European origin differed from all the other gelatins in that their solutions in both 2*N*-hydrochloric acid and 6*N*-hydrochloric acid rapidly darkened on heating and deposited a brown precipitate, the hydrolysates developing a marked caramel odour. This behaviour was attributed to the presence of polysaccharide impurities, the component sugars of which were released by hydrolysis and reacted with amino compounds from the gelatin to produce brown products and free ammonia (browning reaction, see Blake & Plaster, 1950). Similar amounts of ammonia were produced with both concentrations of acid.

The smooth curve on which the remaining points

Fig. 3. Relation between amide-N content and isoionic point for a range of gelatins compared with the titration curve for an alkali-processed gelatin. Amide N-*pI* curve (full line); titration curve for gelatin of *pI* 4.92 (Kenington & Ward, 1954) (broken line); titration curve moved upwards by 0.04 m-equiv./g. (dotted line). Designation of gelatins is as given in Table 2.

lay was similar in shape to the titration curve of an alkali-processed gelatin of *pI* 4.92 (Kenington & Ward, 1954), which is shown by a broken line in Fig. 3. The titration curve runs approximately parallel to that relating amide content to isoionic point. The dotted line in Fig. 3 was obtained by displacing the titration curve upwards by approximately 0.04 m-equiv./g., so that it crosses the *X*-axis at *pI* 4.80. This derived curve is intended to correspond to that of a gelatin the amide groups of which have been completely removed by alkaline pretreatment. It is similar in shape and position to the amide-*pI* curve, except that it is slightly less steep below pH 5 and rises much more steeply above pH 8. The deviation of the curves at high pH has not been explained. The amide-*pI* curve depends upon values for only two gelatins of high isoionic point, which, however, agree closely with one another.

The relative shapes of the portions of the curves below pH 8 may be theoretically justified by considering the gradual replacement of carboxyl groups in an isoionic gelatin of zero amide content by amide groups. Wherever a given number of charged carboxyl groups are replaced by electrically neutral amide groups, there will be a shift in isoionic point equal to the shift in pH produced by removing the same number of protons from the gelatin molecule during titration. Amide groups replacing uncharged carboxyl groups, however, will not affect the isoionic point. There will be a preponderance of charged over uncharged carboxyl groups in the pH range shown in Fig. 3, and the proportion of uncharged carboxyl groups will fall rapidly with increase in pH above pH 4.8. Thus, near pH 4.8, a significant but not predominant proportion of amide groups introduced would replace uncharged carboxyl groups and so would not contribute to a change in isoionic point. Because of the extra number of amide groups needed to replace uncharged carboxyl groups the amide-*pI* curve would be expected to be steeper than the titration curve in this region. At higher pH values the number of amide groups replacing uncharged carboxyl groups will tend towards zero so that the two curves will have the same shape.

SUMMARY

1. A rapid and convenient method for the determination of amide nitrogen in gelatin, based on preliminary hydrolysis with 2*N*-hydrochloric acid, is described.

2. No significant decomposition, resulting in release of non-amide ammonia, occurs during the distillation stage. Errors resulting from the distillation of carbon dioxide have been decreased to a low level. The method of hydrolysis gives

results very close to those obtained with 12N-hydrochloric acid at 37°.

3. The amide contents of a series of deionized gelatins, ranging from pI 4.80 to 9.4, are related to their isoionic points by a curve of similar shape to the titration curve.

4. Certain gelatins and glues that show evidence of containing polysaccharide impurities give rise to larger quantities of ammonia on hydrolysis than would be expected from their isoionic points.

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Formation of Labelled Amino Acids by Exchange Transamination

By R. G. HILLER AND D. A. WALKER
Queen Mary College (University of London), London, E. 1

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Glutamate-aspartate transaminase, which catalyses reaction (1)

is known to catalyse also the exchange of an amino group (reaction 2) between glutamate and its keto acid analogue (Nisonoff, Barnes, Enns & Schuching 1954; Jenkins, Yphantis & Sizer, 1959).

To the best of our knowledge the possible catalysis of the corresponding reaction (3), between aspartate and its keto acid analogue,

has not previously been investigated.

If such reactions occurred *in vivo* they would have a bearing on the interpretation of the results of $^{14}\text{CO}_2$ -feeding experiments. Accumulation of label in glutamate or aspartate would then not necessarily be indicative of synthesis in the usual sense, since these acids could be formed (in reactions 2 and 3) at the expense of their unlabelled counterparts. Clearly, however, much would depend upon the relative rates of the normal transamination and what we shall call 'exchange transamination' (reactions 2 and 3). The following experiments were

therefore primarily carried out to determine (a) whether or not $^{14}\text{CO}_2$ could be introduced into

aspartate in a model system by carboxylation of phosphopyruvate followed by exchange transamination of the resulting oxaloacetate, and (b) if the rate of incorporation was of the same order as that

of the corresponding system in which glutamate serves as the amino donor in the normal transamination.

MATERIALS AND METHODS

Phosphoenolpyruvate was from the Sigma Chemical Co., St Louis, Mo., U.S.A. L- ^{14}C Glutamic acid and sodium ^{14}C bicarbonate were obtained from The Radiochemical Centre, Amersham, Bucks. Castor beans were kindly given by the Baker Castor Oil Co., San Diego, Calif., U.S.A.

Enzymes. Mitochondria were prepared from the endosperm of 50 4-day castor-oil-bean seedlings (*Ricinus communis* var. Cimarron) according to the method of Beevers & Walker (1956) and washed three times in cold 0.5M-sucrose-0.1M-phosphate (NaH_2PO_4) adjusted to pH 7.1 by the addition of potassium hydroxide. They were then sus-