CCLI. THE ISOLATION OF AMINO-ACIDS IN THE FORM OF THE CORRESPONDING CARBAMIDO-ACIDS AND HYDANTOINS.

I. THE DERIVATIVES OF THE MONO-AMINO-MONOCARBOXYLIC ACIDS.

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LIPPICH [1906] called attention to the insolubility of α -carbamido-isobutylic acid, prepared by the action of potassium cyanate or urea on leucine, and suggested the use of this derivative for the isolation and characterisation of the amino-acid. Later [1908] he described the carbamido-acids corresponding with glycine, alanine, α -amino-isobutyric acid, valine, leucine, aspartic and glutamic acids, tyrosine and taurine. He also described the anhydrides of some of these carbamido-acids. In a subsequent paper [1914, 1] he discussed further the reliability of the carbamido-reaction as a qualitative test for leucine. In the same year [1914, 2] he described the application of the reaction to the isolation of small quantities of leucine and other amino-acids added to urine and to blood-serum. Dakin [1910, 2] observed the production of carbamido-acids in urine concentrated by heat. Weiland [1912], Embden and Schmitz [1912] and Kondo [1912] found the reaction useful for the isolation of amino-acids from urine. However until lately, apparently no amino-acid had been isolated in this way from normal or pathological urine unless it had first been fed in quantity to the animal or introduced directly into the blood-stream or the urine itself. Hoppe-Seyler [1933] has obtained a carbamido-hydantoin derivative of lysine from the urine of a cystinuric patient, using a silver precipitation.

Lippich [1914, 1] and Dakin [1918] called attention to the usefulness of the hydantoins derived from the amino-acids for the isolation and characterisation of the latter. Dakin remarks that the ordinary hydantoins are more useful in this respect than the phenylhydantoins or the thiohydantoins. He described methods [1918; 1920] for the purification of proline and hydroxyproline respectively which involve the isolation of these substances as their hydantoin derivatives.

It does not appear from the literature that anyone has converted protein hydrolysates as a whole into the carbamido-acids or hydantoins with a view to the separation of the derivatives of the various amino-acids. In this preliminary paper experiments are described which show that it is easy to isolate a number of amino-acids from protein hydrolysates as the corresponding carbamido-acids in yields similar to those obtained by other methods. The method is applicable to relatively small samples of protein, 25 g. being a suitable quantity.

Perhaps the greatest advantage consists in the certainty of the characterisation of the amino-acids isolated in this way. In particular the melting-points of the carbamido-acids and hydantoins are more characteristic than those of the amino-acids themselves.

In general the hydantoins are less soluble in water than the amino-acids from which they are derived. One part of leucine dissolves in 44 parts of water at 20° whereas one part of the hydantoin requires 400 parts. There is also a general parallelism between the solubilities of the amino-acids and those of the corresponding hydantoins.

The possibility of evolving a scheme for the separation of the α -carbamido-acids and hydantoins from each other is favoured by the solubility of many of these substances in organic solvents.

Various proteins, including caseinogen, cod-muscle protein, gelatin, edestin and haemoglobin were hydrolysed by boiling with hydrochloric acid. The hydrolysate in each case was converted into a mixture of carbamido-acids by removal of the acid as far as possible by concentrating *in vacuo*, neutralising and boiling the mixture of amino-acids with potassium cyanate or urea. After careful acidification of the cold solution to the turning point of Congo red, a precipitate separated out. On recrystallisation of this material, after decoloration of its aqueous solution with charcoal, it was found to consist of a mixture of the carbamido-acids derived from leucine, *isoleucine* (if present in the protein) and phenylalanine. Means were found whereby these were separated from each other in the form of the hydantoins.

The carbamido-acids remaining in solution were separated into two fractions, *viz.* those soluble in alcohol at p_H 4, and those insoluble. The carbamido-acids derived from the monoamino-monocarboxylic acids are soluble in alcohol at this p_H , whereas the greater part at least of the remaining material is relatively insoluble. In some experiments this separation by means of alcohol was carried out before precipitation of the water-insoluble carbamido-acids.

An alternative method of removing the derivatives of some of the monoamino-monocarboxylic acids depends on the solubility of their hydantoins in ether or chloroform. The hydantoins obtained from glycine, serine, cystine, cysteine and tyrosine are however insoluble in these solvents.

By separating the carbamido-acids by means of alcohol at p_H 4, and converting them into hydantoins in the usual way, the derivatives of leucine, *isoleucine*, valine, phenylalanine, alanine, proline and hydroxyproline can be removed by means of ether or chloroform and that of tyrosine obtained in pure crystalline form from the residue insoluble in ether.

In the case of cod-muscle protein the amount of tyrosine so isolated was greater, in the case of caseinogen less, than the values previously recorded. It was found, however, that the extraction of α -carbamido- β -(*p*-hydroxyphenyl) propionic acid at p_H 4 by means of alcohol was not complete since the insoluble fraction still gave a strong Millon's reaction. This may be explicable by the resemblance of the carbamido-acid to the dibasic derivatives since it possesses a phenolic group. Undoubtedly the technique can be improved to give a more nearly quantitative separation of the tyrosine derivative.

Means were sought for the separation of the various hydantoins derived from the monoamino-monocarboxylic acids. To this end the approximate solubilities of the individual hydantoins in various solvents were determined. It is hoped to publish the data obtained at an early date. Peculiarities of solubility which have been found useful are discussed below.

*iso*Butylhydantoin (from leucine) can be extracted, along with most of its

homologues and other substances already mentioned, by means of ether. From the mixture so obtained the leucine, *isoleucine* and phenylalanine derivatives can be separated together by utilising their insolubility in water. Of these three, that of leucine has the lowest solubility in chloroform and small quantities of the other two as well as the derivatives of valine and proline can be removed from larger amounts of leucine by the use of this solvent. Benzene gives a more complete, if more tedious, separation, however, since the hydantoin from leucine is only soluble to the extent of one part in about 25,000 parts of benzene at 20°, the other substances concerned being many times more soluble. A litre of benzene at 20° removes only 0.5 g. of benzylhydantoin but at higher temperatures the *isobutyl*hydantoin becomes more soluble and the separation less complete. The active and racemic varieties of *isobutyl*hydantoin do not show marked differences in solubilities, melting-points or crystalline forms.

*sec*Butylhydantoin (from *isoleucine*) is much more soluble in water, ether, chloroform and benzene than are *isobutyl*- and benzyl-hydantoins. *sec*Butyl- and benzyl-hydantoins can be separated from *isobutyl*hydantoin most completely by means of benzene as mentioned above. Whereas α -carbamido-*sec*butylacetic acid is less soluble in water than α -carbamido- β -phenylpropionic acid, *sec*butylhydantoin is much more soluble than benzylhydantoin. By utilising these two solubility relationships alternately, a fairly good separation of racemic *isoleucine* from phenylalanine is obtainable.

Active *sec*butylhydantoin crystallises from water with H_2O and this property can be used for its precipitation from benzene. A few drops of water added to a solution of active *sec*butyl- and benzyl-hydantoins in benzene, precipitate the hydrate of the former almost completely. Active *isopropyl*hydantoin separates from benzene in the same form as active *sec*butylhydantoin. The separation of these two substances from one another (whether active or racemic) depends on the greater insolubility of the *sec*butylhydantoin in water. Alternatively the mixture of the two may be converted into the carbamido-acids and the less soluble α -carbamido-*sec*butylacetic acid separated by crystallisation from water. Active *sec*butylhydantoin can sometimes be made to crystallise from water in large flattened needles which may be 2 cm. in length. These can be picked out with forceps and wiped clean. A specimen can thus be obtained pure without using benzene. It is also possible to crystallise a mixture of the leucine and *isoleucine* derivatives from water in such a way that a crop of the former separates out first and the mother-liquor after filtration deposits needles of the latter free from the isomeride. As pointed out by Dakin and Dudley [1914] progressive racemisation of the hydantoins occurs when they are heated with mineral acids but more slowly than in alkaline solution. If the conversion of the carbamido-acids into the hydantoins be carried out with due care little racemisation occurs, but the possibility that both the active and racemic forms may be present must be considered. Of course racemisation may occur under certain conditions of hydrolysis of the protein and also in the conversion of the amino-acids into carbamido-acids by boiling the hydrolysate with urea under alkaline conditions.

Benzylhydantoin (from phenylalanine) can be separated from *isobutyl*hydantoin by means of benzene as described. Its low solubility in water (1 : 500) provides a convenient means of separating it from the other ether-soluble hydantoins.

p-Hydroxybenzylhydantoin (from tyrosine) is almost insoluble in cold water, ether and chloroform. It is fairly soluble in alcohol and in acetone and to a small extent in ethyl acetate. On acetylation of the phenolic group a substance soluble in ether is obtained.

Methylhydantoin (from alanine) is very soluble in water and in alcohol, moderately soluble in ether. It can be obtained in quantity from silk fibroin by hydrolysis of the protein, conversion of the amino-acids into hydantoins and extraction of the methylhydantoin with ether in a continuous extractor.

Ordinary hydantoin is insoluble in ether and only moderately soluble in alcohol. It can be precipitated in quantity from the mixture of hydantoins obtained as above from silk fibroin by adding alcohol to the concentrated solution. It can then be partially purified from the salts, which are also precipitated by the alcohol, by extraction with hot glacial acetic acid from which the hydantoin crystallises on cooling. A further purification can be effected by extraction with acetone in a Soxhlet apparatus. A separation of glycine and alanine from each other is thus available which should find use as an adjunct to the ester method in which these two amino-acids may be obtained in the same fraction. Hydantoic acid is less soluble in alcohol than hydantoin, and this property has also been found useful.

The hydantoin from proline has a similar solubility in water to that from valine but it is much more soluble in chloroform and in benzene than the latter. Dakin [1918] described the purification of proline by extraction of the carbamido-acids by ether, that derived from proline being insoluble, followed by conversion of the proline derivative into the hydantoin and extraction of the latter by ether. This process succeeded no doubt under the conditions obtaining, but it is necessary to point out that α -carbamido-isobutylacetic acid is only soluble to the extent of one part in 50,000 parts of wet ether [Lippich, 1914, 1] and so could not be removed as such from the proline derivative by that solvent. It is suggested here that in Dakin's experiments no leucine was present, or the acidity of the solution was such that the hydantoin was slowly formed in the case of the leucine derivative, while the proline derivative remained unaltered. Lippich [1914, 1] criticises the statements of Weiland [1912] and Embden and Schmitz [1912] that the carbamido-acids are soluble in ether. He gives the ether-solubility of the phenylalanine derivative as 1:4000 and that of the tyrosine derivative as 1:3000.

The hydantoin from hydroxyproline is more soluble in water than that from proline and much less soluble in benzene and in chloroform than the latter, benzene being particularly useful for the separation of the two. The high solubility of the hydroxyproline derivative renders it unsuitable for final isolation and weighing of the amino-acid in this form. Dakin [1920] extracted the hydantoin from hydroxyproline with ether and reconverted it into the amino-acid.

Hydroxymethylhydantoin (from serine) has a similar solubility in water to the hydantoin from proline, but it is insoluble in ether and in chloroform. On acetylation a substance soluble in ether is formed.

β -Methylmercaptoethylhydantoin (from methionine) is very soluble in water, and also in chloroform. It is fairly soluble in ether and slightly soluble in cold benzene.

Dithiomethyldihydantoin (from cystine) is insoluble in water, alcohol, ether, chloroform and benzene. The tyrosine derivative, being soluble in alcohol, can be separated from it by that solvent.

Thiolmethylhydantoin (from cysteine) has not been obtained crystalline. It appears to be very soluble in water and in alcohol, but only slightly soluble in ether, in chloroform and in benzene.

It will be observed that a means of fractionating the sulphur of proteins is available owing to the differences in the properties of these hydantoins.

The hydantoin of the dicarboxylic amino-acids are insoluble in alcohol at p_H 6-7. If ammonia be added to an alcoholic solution of hydantoinpropionic acid the ammonium salt is precipitated above p_H 6 but redissolves as the p_H value of the solution is raised. A precipitate again forms (other than ammonium chloride) on bringing the p_H of the solution back to the region of neutrality. Since the hydantoin derived from the monoaminomonocarboxylic acids are soluble in alcohol at neutrality a method of separating the two groups is available. Unfortunately the presence of the derivatives of histidine and of arginine introduces complications. These substances have basic groups which do not react with potassium cyanate, and so they are capable of forming salts with the hydantoin derivatives of the dicarboxylic amino-acids. It appears that salts of this kind may be soluble in alcohol.

Hydantoinacetic acid (from aspartic acid) is very insoluble in water but crystallises slowly; the glutamic acid derivative is much more soluble. Both are insoluble in ether, chloroform and benzene. The hydantoin of pyrrolidone-carboxylic acid is slightly soluble in these three solvents at 20°.

δ -Carbamido-*n*-butylhydantoin (from lysine) has been studied lately by Hoppe-Seyler [1933]. It is moderately soluble in water, sparingly soluble in alcohol and insoluble in ether.

The arginine derivative is soluble in water, but insoluble in alcohol and in ether. The hydrochloride has similar properties.

Glyoxaline-4 (or 5)-methylhydantoin (from histidine) is very soluble in water and in alcohol and slightly soluble in wet ether. The hydrochloride behaves similarly. The hydantoin has not been prepared in the pure condition; an acetyl derivative has however been obtained.

Besides these substances the hydantoin derived from *nor*leucine, *nor*valine, α -aminobutyric acid, α -aminoisobutyric acid, tryptophan and other amino-acids have been studied to some extent.

EXPERIMENTAL.

Preparation of carbamido-acids and hydantoin from amino-acids and determination of their solubilities.

Various carbamido-acids and hydantoin were prepared from pure amino-acids for the determination of melting-points and solubility data.

The amino-acid was heated in solution with $1\frac{1}{2}$ equivalents of potassium cyanate for 30 minutes. The solution was then cooled and carefully acidified with dilute hydrochloric acid to the turning point of Congo red. If the carbamido-acid were sufficiently insoluble in water it was filtered off and recrystallised from water.

If the carbamido-acid could not be obtained readily in crystalline form, excess of hydrochloric acid was added and the solution was evaporated to dryness on the water-bath. The residue was extracted with alcohol or in some cases with ether, chloroform or hot benzene. The solution so obtained was evaporated to dryness, and the hydantoin was crystallised twice from water. In some cases the separated carbamido-acid was used for preparation of the hydantoin.

Solubility determinations were carried out as follows. A hot saturated solution of the derivative was cooled and kept at 20° for 24 hours. In cases where a copious precipitate was deposited at once when the hot solution was cooled, this time was shortened to a few hours. The separated material was filtered off and a weighed or measured quantity of the liquid (between 1 and 5 cc.) was evaporated to dryness in a tared dish. The residue was then dried and weighed. Throughout this work nitrogen determinations were carried out by the micro-Kjeldahl method and the melting-points given are uncorrected.

The isolation of the derivatives of leucine, isoleucine, phenylalanine and tyrosine from cod-muscle protein.

Cod-muscle protein was prepared as described for halibut-muscle protein by Osborne and Heyl [1908]. The protein (25 g. calculated on the dry ash-free basis) was hydrolysed by boiling with 250 cc. of 22 % hydrochloric acid for 40 hours. The acid was then removed as far as possible by evaporation *in vacuo*, and the residue was dissolved in 250 cc. of water. The solution was filtered and neutralised to litmus with sodium hydroxide solution. 30 g. of potassium cyanate were added, and the solution was boiled gently for 2 hours and then concentrated to a volume of approximately 100 cc. Sulphuric acid (25 %) was added to the cooled liquid until the excess of cyanate had been destroyed and the liquid was neutral to Congo red. On addition of 400 cc. of 95 % alcohol with stirring, much material dissolved in the alcohol whilst a pasty mass remained undissolved. The alcohol was decanted off and a fresh quantity added with vigorous stirring. The mass of salts and carbamido-acids was in this way extracted with about 1500 cc. of alcohol in five portions which were all decanted off and united. The alcohol deposited a little insoluble syrupy material on standing and was decanted from this next day.

A little water was added to the alcohol and the solution concentrated *in vacuo* to 100 cc. A precipitate separated out and was filtered off next day and recrystallised from water. It yielded 2.9 g. of material melting at 203° and containing 15.83 % of nitrogen. A further quantity of 0.7 g. of material was obtained in two crops melting at 197° and 180° respectively. The substances crystallised in needles quite insoluble in ether.

α -Carbamido-*isobutyl*acetic acid prepared from commercial *l*-leucine (found: N, 10.71 %, calc. 10.69 %) melted at 201–202° both in an open and closed capillary and contained 16.05 % N (calc. 16.09 %). The derivative of purchased *dl*-leucine (10.75 % N) melted at 195° both in an open and in a closed capillary and contained 16.01 % N. Lippich [1908; 1914, 1] found the melting-point of the active carbamido-acid to be 189–190° in a closed capillary and 200–210° in an open capillary. Dakin [1910, 1] found it to be 205–206°. Pinner and Spilker [1889] found 200° for the melting-point of the *dl*-carbamido-acid.

The isolated material was dissolved in hot alcohol, 20 cc. of 5 % hydrochloric acid were added, and the solution was evaporated to dryness on the water-bath. The residue was extracted repeatedly with small quantities of chloroform, and the insoluble portion was crystallised from water. Thereafter it weighed 1.8 g., melted at 209° and contained 17.92 % N. Under the microscope it appeared as thin plates, and macroscopically it was a light flaky powder like leucine itself.

The hydantoin prepared from pure *l*-leucine was similar in appearance. It melted at 212° in an open capillary and contained 17.98 % N (calc. 17.95 %). A mixture of the two substances melted at 210°. The hydantoin prepared from *dl*-leucine melted at 209°. Dakin [1910, 1] gives 212° and Pinner and Lifschütz [1887] 209–210° for the melting-points of the active and racemic hydantoins respectively. The hydantoin relatively insoluble in chloroform was therefore the derivative of ordinary leucine. The properties of the derivatives of *isoleucine* and *norleucine* are described below.

The chloroform solution was evaporated to dryness and the residue crystallised from water. A crop of needles separated and was filtered off. It weighed 0.25 g. The mother-liquor was concentrated and a further crop of large needles crystallised along with a little *isobutyl*hydantoin. Many of these needles, which were over 1 cm. in length, were separated by means of forceps and wiped clean. When dried in the desiccator they weighed 0.6 g. The residue was evaporated to dryness together with the aqueous mother-liquor from the crystallisation of the material insoluble in chloroform. The dry residue was treated with hot benzene and the suspension filtered when cold. The insoluble matter was recrystallised from water. It melted at 206° and the melting-point was unaltered when the substance was mixed with *isobutyl*hydantoin. It weighed 0.09 g. On evaporation of the benzene and crystallisation of the residue from water a mixture of needles and hexagonal plates was obtained, which after being dried in the desiccator weighed 0.3 g.

The substance which crystallised in needles agreed in all respects with the hydantoin prepared from active *isoleucine* and described below. It melted at 95° when heated rapidly. If the finely powdered substance were heated slowly it lost water of crystallisation without melting completely and then melted between 120° and 130°. The hydrated compound contained 16.12 % N (calc. 16.09 %).

α -Carbamido-*sec*butylacetic acid has not been described hitherto. 0.3 g. of commercial *d*-isoleucine (10.71 % N) was boiled with excess of potassium cyanate in 5 cc. of water for 15 minutes. When the cooled liquid was acidified carefully with dilute sulphuric acid the carbamido-acid was precipitated. This was filtered off and recrystallised from water. It crystallised in cubes and long flattened prisms and melted at 205°. It contained 15.95 % N (calc. 16.09 %). Its solubility in water at 20° was 1:700 approximately, whereas that of α -carbamido-*isobutyl*acetic acid is given as 1:1700 by Lippich [1908]. A mixture of equal parts of the carbamido-acids from leucine and isoleucine melted at 201° in an open capillary.

*sec*Butylhydantoin has not been described hitherto, but Lippich [1908] probably obtained it without being aware since he observes that *isobutyl*hydantoin crystallises from ether in needles. The presence of *isoleucine* in the leucine used by him for the preparation of carbamido-*isobutyl*acetic acid accounts for various discrepancies in melting-points and solubilities recorded by that author.

The hydantoin prepared from commercial *d*-isoleucine crystallised in long needles which gave up water of crystallisation on fusion. The hydrated product melted at 95° when the capillary containing the substance was suddenly immersed in a bath at that temperature or when a small crystal was placed in the capillary instead of the finely powdered material and the temperature raised in the usual way. When the powdered substance was heated slowly it melted between 120° and 130°. After careful fusion a melting-point of 150° was found with slight softening at 145°. Amongst the needle-like crystals which composed the bulk of the preparation a few hexagonal and rhombic plates were visible under the microscope. When the hydantoin was dissolved in dilute alkali and the solution warmed for some time, the hydantoin recovered by acidifying with excess of hydrochloric acid and boiling crystallised almost entirely in these plates. Dakin [1910, 1] has shown that the active hydantoins are readily racemised in alkaline solution. *iso*-Propyl- and benzyl-hydantoins show a similar change in crystalline form, when the optically active substances are kept in alkaline solution for some hours. Racemisation also occurs gradually when the active hydantoins are heated with concentrated hydrochloric acid.

The needle-like crystals of hydrated active *sec*butylhydantoin contained 15.93 % N (calc. 16.09 %). The substance crystallised from hot dry benzene in plates. If a few drops of water be added to a solution of active *sec*butylhydantoin in cold benzene a flocculent precipitate of the hydrated compound is thrown down in the form of extremely fine filaments. These evidently lose water readily since some of this material dried at 40° contained 17.66 % N. The same material dried at 150° for 3 hours contained 17.50 % N. Probably slight decomposition occurs at this temperature with loss of nitrogen.

In order to remove any suspicion that the *nor*leucine derivative might be present in the insoluble carbamido-acid fraction the carbamido-acid and hydantoin derived from that amino-acid were prepared by synthesis from *n*-hexoic acid. The acid was brominated by means of bromine and amorphous phosphorus and the product was poured into water and washed repeatedly with water. The α -bromo-*n*-hexoic acid was heated with large excess of concentrated ammonia in a closed vessel at 100° for 4 hours. In this way 30 g. of *n*-hexoic acid yielded 19.5 g. of *nor*leucine containing 10.87 % N (calc. 10.69 %). The phenyl *isocyanate* derivative melted at 137° and the phenylhydantoin at 110°. The phenyl *isocyanate* of *l*-*nor*leucine melts at 133° and the phenylhydantoin at 110–111° [Abderhalden and Heyns, 1933].

The carbamido-acid melted at 176° and contained 15.86 % N (calc. 16.09 %). Kondo [1912] found the melting-point of the inactive α -carbamido-*n*-hexoic acid to be 165°. One part of the carbamido-acid dissolved in approximately 375 parts of water at 20°. α -Carbamido-*n*-hexoic acid can therefore be separated from the leucine derivative and less completely from that of *isoleucine* by crystallisation from water. It would not appear in the insoluble carbamido-acid fraction unless present in considerable quantity in the protein.

The hydantoin melted at 139° and contained 17.78 % N (calc. 17.95 %). One part of *n*-butylhydantoin dissolves in approximately 200 parts of water at 20°.

The quantities of α -carbamidobutylacetic acids, *isobutyl*- and *secbutyl*-hydantoins isolated correspond to 10.2 g. of leucine-*isoleucine* or 6.1 g. of leucine and 3.6 g. of *isoleucine* per 100 g. of protein.

The mother-liquors from which the insoluble carbamido-acids from leucine and *isoleucine*

were first separated and that from which they were recrystallised were united, acidified with excess of concentrated hydrochloric acid and concentrated on the water-bath to a syrup. This syrup was stirred repeatedly with fresh quantities of ether until very little material was being removed each time. The ether was distilled off and the residue crystallised from water. In this way a crystalline fraction and a syrup were obtained. The former might be expected to consist chiefly of hydantoins from phenylalanine, leucine, *isoleucine*, valine and proline and the latter of those from valine, proline, hydroxyproline and alanine in so far as the related amino-acids were present in the protein.

The crystalline ether-soluble hydantoins were recrystallised from water. A relatively insoluble product was obtained which melted at 190–191°, contained 15.53 % N, and weighed 0.55 g. The greater part of this material was in short thick prisms which were transparent at first but became opaque as they dried in a desiccator. When the substance was strongly heated in a dry test-tube it emitted the odour of phenylacetaldehyde, and on ignition it burned with a smoky luminous flame. From these observations together with solubility considerations it was evidently a mixture of benzyl- and *isobutyl*-hydantoins. 0.2 g. of the dry material was therefore suspended in 500 cc. of benzene. The benzene suspension was warmed for some time, cooled and kept at room temperature overnight. The insoluble matter was then filtered off, washed with cold benzene, dried and weighed. It weighed 0.05 g. and melted at 197–202°. After recrystallisation from water it melted at 206°. A mixture with pure *isobutyl*hydantoin melted at the same temperature. The benzene filtrate was shaken repeatedly with ammoniacal water and the aqueous solution was concentrated to small volume. As the concentrated solution cooled a substance crystallised out in rectangular platelets. This material melted at 189–190° and contained 14.79 % N (calc. for benzylhydantoin 14.73 %). Wheeler and Hoffman [1911] give the melting-point of *dl*-benzylhydantoin as 190° and Dakin and Dudley [1914] give 195–196°. The active variety melts at 181–183° [Dakin and Dudley, 1914]. Concentration of the ammoniacal solution causes racemisation of the hydantoin.

The amount of benzylhydantoin in the mixture corresponds to a yield of 1.4 g. of phenylalanine from 100 g. of protein, and the additional *isobutyl*hydantoin raises the yield of leucine to 6.6 g. from 100 g. of protein.

The syrupy residue from which the benzylhydantoin and other hydantoins had been extracted by means of ether was diluted to about 100 cc. and kept for some days. It gradually deposited large spherical aggregates of a substance which was very insoluble in water and in ether, gave Millon's reaction strongly and after recrystallisation from hot water melted at 234° and contained 13.80 % N (calc. for $C_{10}H_{10}O_3N_2$, N 13.59 %). *p*-Hydroxybenzylhydantoin melts at 242–245° in a closed capillary [Lippich, 1908]. Wheeler and Hoffman [1911] give the melting-point of *dl-p*-hydroxybenzylhydantoin as 257–258°. The amount of material isolated was 0.85 g. corresponding to 3.0 g. of tyrosine from 100 g. of the protein.

The yield of tyrosine obtained by crystallising the amino-acid itself from a hydrolysate of 150 g. of cod-muscle protein was 2.3 g. per 100 g. of protein, a figure which agrees with that of Osborne and Heyl [1908] for halibut-muscle protein. However, Millon's reaction showed the presence of much tyrosine in the mother-liquors.

The melting-point of the isolated material was raised to 247° by recrystallisation from alcohol.

*The isolation of the derivatives of leucine, isoleucine, valine,
phenylalanine and tyrosine from caseinogen.*

As in the case of cod-muscle protein 25 g. of the protein (commercial caseinogen), calculated on the dry ash-free basis, were used. The method differed from that described above in that the water-insoluble carbamido-acids were precipitated and filtered off before extraction of the derivatives of the monoaminomonocarboxylic acids by means of alcohol at p_H 4.

The precipitate of insoluble carbamido-acids was decolorised by means of animal charcoal and recrystallised from hot water. Two different experiments gave almost the same yields of a mixture of α -carbamido-*isobutyl*acetic and α -carbamido-*secbutyl*acetic acids corresponding to 9.3 g. of leucine-*isoleucine* per 100 g. of protein. The mixture melted at 197° and contained 16.04 % N.

0.75 g. of this material was dissolved in dilute alcohol. A few cc. of concentrated hydrochloric acid were added, and the solution was evaporated to dryness on the water-bath to effect con-

version into the hydantoin. The residue was dried at 100° and then treated with 500 cc. of hot benzene. The benzene suspension was cooled and filtered after some hours. The insoluble material was dissolved in hot alcohol, the solution filtered, diluted with water and concentrated. 0.48 g. of hydantoin were obtained by crystallisation. This melted at 208° and contained 18.02 % N. It was therefore pure *isobutylhydantoin*.

The benzene filtrate was shaken repeatedly with fresh quantities of ammonia water and the aqueous extracts were evaporated to dryness. The residue weighed 0.15 g. It was dissolved in a little hot benzene, the solution was filtered and the filter-paper was washed with hot benzene. The precipitate which separated as the benzene cooled was filtered off and dried for 3 days at 90°. It weighed 0.12 g. and melted at 145–150° and contained 17.50 % N. It crystallised from water partly in platelets, partly in needles and was evidently a mixture of active and racemic *secbutylhydantoin*. The slightly low N content is due to the difficulty of expelling the last traces of water from the active material.

A further 0.04 g. of active *secbutylhydantoin* was obtained from the aqueous mother-liquor from which the *isobutylhydantoin* had been recrystallised by precipitation from benzene on addition of a drop of water. This melted at 100° when rapidly heated in the manner of hydrated *secbutylhydantoin*.

The proportion of leucine to *isoleucine* derivative in the insoluble carbamido-acids was 4:1 corresponding to 7.6 g. and 1.7 g. of leucine and *isoleucine* respectively per 100 g. of protein. Approximately this proportion of *isoleucine* has been found in caseinogen by various workers.

The ether-soluble hydantoin, obtained as described for cod-muscle protein, were crystallised and recrystallised from water. A relatively insoluble fraction was obtained which contained 15.47 % N. This was separated into *isobutyl*- and *benzylhydantoin*s by means of benzene as described for cod-muscle protein. The weight of the mixture was 0.85 g. and the proportion *isobutylhydantoin*:*benzylhydantoin* was 1:3, corresponding to 0.7 g. (8.3 g. altogether) of leucine, and 2.2 g. of phenylalanine per 100 g. of protein.

A second fraction of the ether-soluble hydantoin, more soluble in water than the first, was apparently a mixture of the derivatives of *isoleucine* (active and racemic), phenylalanine and leucine, but was not fully investigated.

The remainder of the ether-soluble material was concentrated to a sticky consistency and was rubbed up with 500 cc. of hot benzene. When the benzene was filtered and allowed to stand it deposited a precipitate which consisted of a felt of fine needles. This was filtered off and recrystallised from benzene. After being kept in a desiccator for some days it was found to contain 18.9 % N. The melting-point of the finely divided material was indefinite and appeared to vary from 80° to 130° according to the time of drying and the rate of heating. It crystallised from water in long needles which melted at 112°. One part dissolved in 40 parts of water at 20°.

dl-Valine was synthesised from *isovaleric acid* in the manner already described for *norleucine*. The valine contained 11.75 % N (calc. 11.96 %). The carbamido-acid contained 17.26 % N (calc. 17.50 %). The hydantoin contained 19.77 % N (calc. 19.71 %). The carbamido-acid melted at 187°. Lippich [1908] found the melting-point to be 176° in a closed capillary. The hydantoin melted at 143°. The hydantoin prepared from commercial *dl*-valine melted at 145°. Lippich [1914, 1] gives the melting-point of the hydantoin in a closed capillary as 132°.

The racemic hydantoin crystallised from water in hexagonal and rhombic plates similar to those of *dl-secbutylhydantoin*. One part of the hydantoin dissolved in approximately 23 parts of water at 20°, 130 parts of ether, 130 parts of chloroform and 2000 parts of benzene.

Some *L*-valine was purchased. It contained 12.15 % N. The hydantoin prepared from it was soluble in hot benzene from which it separated in a felt of fine needles. These melted indefinitely up to 143° according to the time of drying and the rate of heating in determining the melting-point. Nitrogen determinations carried out on this substance were lower (18.74 %) than the formula $C_7H_{10}O_2N_2$ requires (19.71 %). After racemisation, however, the nitrogen content of material crystallised from benzene and dried at ordinary temperature was 19.89 %, and the melting-point was 145°. One part of active *isopropylhydantoin* dissolves in approximately 32 parts of water at 20°.

The amount of *isopropylhydantoin* separated from caseinogen as described was 1.2 g. corresponding to a yield of about 4 g. of valine per 100 g. of protein.

That fraction of the hydantoins which was insoluble in ether but derived from carbamido-acids soluble in alcohol at $p_H 4$ deposited a precipitate of *p*-hydroxybenzylhydantoin in microscopic needles. When this was filtered off and recrystallised from dilute alcohol it melted at 248° and contained 13.79 % N. It weighed 0.80 g. corresponding to a yield of 2.9 g. of tyrosine per 100 g. of protein.

The isolation of the derivatives of leucine, phenylalanine and proline from gelatin.

Some proteins present an easier problem than others. In the case of gelatin the absence of isoleucine and valine in considerable quantities permits a comparatively easy separation of the hydantoin of proline.

A gelatin hydrolysate was made slightly alkaline with sodium hydroxide solution and boiled with urea equal in weight to the gelatin used. The solution was then made strongly acid by addition of concentrated hydrochloric acid and was boiled for 3 hours. (In the presence of urea and salts the formation of the hydantoins from the carbamido-acids takes place more slowly than in the case of pure carbamido-acids.) The solution of hydantoins was extracted with ether in a continuous extractor for some days. A mixture of hydantoins was obtained from which the derivatives of leucine, phenylalanine and proline were obtained pure by recrystallisation from water and the use of benzene as already described. The proline derivative contained 20.09 % N (calc. 20.0 %), and melted at 157° . When the isolated compound was mixed with a preparation made from pure proline picrate (m.p. 148°) and melting at 165° , the melting-point of the mixture was 161° .

There is no doubt that in the case of gelatin the yield of these three derivatives could be made practically quantitative, but in view of the desirability of proceeding with other parts of the work the experiment was discontinued.

The isolation of the derivatives of tyrosine, glycine and alanine from silk fibroin.

The protein (50 g. of silk waste calculated on the dry ash-free basis) was converted into a mixture of hydantoins in the manner described for gelatin. Potassium cyanate was however used instead of urea.

On concentration of the solution to 300 cc. about 3.5 g. of dark-coloured *p*-hydroxybenzylhydantoin separated and were filtered off. It gave an intense Millon reaction. The filtrate was concentrated to a syrup and treated with 300 cc. of absolute alcohol. A mixture of salts and organic matter was precipitated and was filtered off after several hours. The new filtrate was concentrated *in vacuo* and the residue was again treated with 300 cc. of absolute alcohol. More organic material separated, crystallising on the side of the beaker where it had been scratched. This was also removed by filtration. A third crop was obtained in like manner. The separated material was treated with 350 cc. of hot glacial acetic acid, and the liquid was filtered hot. The insoluble salts were washed repeatedly with hot glacial acetic acid, and the washings were added to the rest of the acetic acid solution. As the solution cooled a crystalline substance separated and was filtered off. A further quantity was obtained on evaporation of the acetic acid. The crude product was boiled in solution with dilute hydrochloric acid in order to reverse any acetylation which might have occurred. The solution was then evaporated to dryness, and the residue was extracted with acetone in a Soxhlet apparatus. After crystallisation from water the extracted material melted at 222° and contained 27.81 % N (calc. for hydantoin 28.0 %). A mixture with pure ordinary hydantoin (m.p. 220°) melted at the same temperature. The weight of hydantoin thus isolated was 14 g. corresponding to 21 g. of glycine per 100 g. of protein.

An unsuccessful attempt was made to separate the rest of the glycine and alanine from the mother-liquor as the carbamido-acids. For this purpose the solution of hydantoins was boiled with dilute alkali. This line of attack was abandoned, however, and the hydantoins were reformed in the racemic condition.

The mother-liquors were concentrated and extracted with ether in a continuous extractor. Small amounts of benzyl- and *sec*butyl-hydantoins were removed in the first few hours; thereafter a hydantoin very soluble in water collected in the receiver. The extraction was slow, occupying a period of many days, and the experiment was discontinued when about 6 g. of substance had been collected. This was crystallised from water, from which it separated, with water of crystallisation, in microscopic dumb-bell or kidney-shaped aggregates. It melted at 143° and contained

24.45 % N after being dried at 100°. Methylhydantoin requires 24.56 % N. Königs and Mylo [1908] found that the racemic hydantoin melted at 146.5°.

The amount of methylhydantoin isolated corresponded to 9 g. of alanine per 100 g. of protein, but considerably more could have been obtained if the extraction had been continued.

DISCUSSION OF RESULTS.

Whether it be considered by itself, or in conjunction with previously existing methods of protein analysis, the carbamido-method shows promise of considerable utility.

It should prevent the possibility of mixtures of commonly occurring amino-acids being mistaken for hitherto unknown substances.

Mixtures of leucine, *isoleucine* and valine, of tyrosine and cystine, and of glycine and alanine have been found difficult to resolve. The methods described or indicated here provide solutions of these problems.

The use of urea instead of potassium cyanate in conjunction with these methods of separation provides a cheap process for the preparation of certain amino-acids in the pure condition. The hydrolysis of the hydantoins, however, yields only racemic amino-acids.

SUMMARY.

1. The properties of the carbamido-acids and hydantoins corresponding to various amino-acids present in protein hydrolysates have been studied with reference to methods of characterisation and isolation of these amino-acids.

2. Methods of isolating leucine, *isoleucine*, valine, phenylalanine, tyrosine, proline, alanine and glycine from protein hydrolysates have been described.

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