THE HISTORY OF THE DISCOVERY OF THE AMINO ACIDS*

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INTRODUCTION

The observation that proteins, when they are subjected to the hydrolytic action of boiling acid, are decomposed into relatively simple crystalline substances, was made more than a century ago. At first only a few of these were distinguished but as time went on more of them were isolated, until today no less than twenty-one different, but allied, substances have been positively identified as products of the hydrolysis of proteins. No one protein is known to yield all of these but most proteins do yield some seventeen or eighteen. These substances are all, with two exceptions, α-amino acids, that is, they are substances of the general formula $R—CHNH_2—COOH$; the exceptions are proline and oxyproline, which are cyclic compounds and possess an α-imino group. Many of the properties of an individual amino acid are therefore common to all, and it is this close similarity in behavior that has rendered the problem of isolation so difficult. The radicals of the several amino acids are, however, widely different from each other. Three amino acids possess strongly acidic radicals and therefore titrate like ordinary carboxylic acids; three others have basic radicals and, of these, two are strong bases of the amine type; the rest are essentially neutral or, rather, amphoteric substances, but the relative strength of their acidic and basic groups is modified by the nature of the radical so that, even in this group, there is a considerable diversity in acid and basic properties.[1]

The capacity of the amino acids to form salts with each other, and their tendency to separate from solution in the form of mixed crystals, have prevented a clear understanding of the exact composition of the mixture of products derived from any one protein. The difficulties of the quantitative analysis of these mixtures can hardly be overemphasized, but, gradually, as knowledge of the qualitative composition increased, more and more progress has been made in the solution of the fundamental problem of the amino acid composition of proteins.

[1] A survey of the data on the dissociation constants of the amino acids is given by Kirk and Schmidt: Univ. Calif. Pub. Physiol. 7, 57–69 (1929).

This paper is written with the object of showing in some detail how the advances in knowledge of the qualitative composition of proteins, with respect to the amino acids that they yield on hydrolysis, have come about. The term amino acids as used here is, of course, restricted to the substances of this class that have been found to result from the hydrolysis of proteins. The discovery of some of these has been the result of mere chance; others have been the fruit of a well-conceived hypothesis; many were found by investigators bold enough to depart from long-established custom and apply a new method or a new reagent. It will become clear, however, that the great rewards have come only to those who possessed intelligence, skill, and patience of the highest order; the discoverers of the amino acids are among the élite of science.

Proteins[2] have provided problems for investigation for centuries. The manufacture of cheese and the preparation of glue, the discovery that ammonia could be obtained by the distillation of horn, or of dung,[3] the use of egg-white or blood for the clarification of hot solutions, are all technical applications of protein chemistry and are the result of shrewd observations made, perhaps, many thousands of years ago. Modern protein chemistry dates, however, from 1820, when Braconnot prepared glycine from gelatin in the course of his attempt to see if proteins behave like starch and are decomposed by acids with the production of sugar. Progress at first was slow, but in recent years it has been rapid indeed; nevertheless the complete solution of the problem of protein composition has not yet been attained. Many improvements in the methods of amino acid analysis must be made, new amino acids must be sought for and isolated, and the theory of protein constitution must be brought to a far more highly developed state than it is at present, before we shall have much reason to be satisfied with our knowledge of these baffling substances.

[2] For a full discussion of the origin of the term protein see Vickery and Osborne: Physiol. Rev. **8**, 393–446 (1928).

[3] The name ammonia is derived from the deity Jupiter Ammon, near whose temple in Libya this substance was first prepared by the distillation of camel dung.

The amino acids that have been isolated from hydrolysates of proteins and so thoroughly investigated and described that no reasonable doubt of their presence in these hydrolysates can be entertained are shown in table 1. In forming this list two fundamental criteria have been adopted. In order that an amino acid shall be accepted as a definite product of the hydrolysis of proteins it must also have been isolated by some worker other than its discoverer and, further, its constitution must have been established by synthesis and by demonstration of identity between the synthetic product and the racemized natural product, or by actual resolution of the synthetic product and preparation of the optically active natural isomer. Although these criteria may appear somewhat arbitrary they are essential, unless one is prepared to accept a host of preparations that have been described, from time to time, as definite homogeneous products of the complete hydrolysis of proteins but for the exact nature of which convincing proof has not been presented. Too many errors have been committed to warrant any but a thoroughly conservative attitude towards newly discovered amino acids. Even the greatest leaders have made mistakes; Fischer himself described diaminotrioxydodecanic acid in 1904, but withdrew it in 1917.

Other criteria for acceptance are more or less obvious. The substance must be liberated by hydrolysis from a preparation of a protein of demonstrated purity and must be adequately characterized by analysis of salts and of typical derivatives. It is desirable, though not essential, that a synthetic peptide containing it should have been prepared and shown to be attacked by enzymes, and it should also have been demonstrated that the substance is oxidized in the animal body.

The amino acids are arranged on the left side of table 1 in the order of their discovery, either in nature or as the result of synthesis; the discoverer and date are also given. On the right side of the table the amino acids are arranged in the order of their discovery as products of the hydrolysis of proteins. In one or two cases there is some doubt as to who should have the credit for this demonstration. This is partly due to the difficulty of being sure of the identity of the preparations described in some

TABLE 1

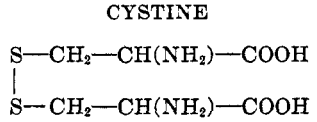
Amino acids that have been demonstrated to be products of the hydrolysis of proteins

	AMINO ACID	EARLIEST OBSERVATION OF THE AMINO ACID		AMINO ACID	EARLIEST OBSERVATION OF THE AMINO ACID AS A PRODUCT OF HYDROLYSIS OF PROTEINS	
1	Cystine	Wollaston	1810	Glycine	Braconnot	1820
2	Leucine	Proust	1819	Leucine	Braconnot	1820
3	Glycine	Braconnot	1820	Tyrosine	Bopp	1849
4	Aspartic acid	Plisson	1827	Serine	Cramer	1865
5	Tyrosine	Liebig	1846	Glutamic acid	Ritthausen	1866
6	Alanine	Strecker (synthesis)	1850	Aspartic acid	Ritthausen	1868
7	Valine	von Gorup-Besanez	1856	Phenylalanine	Schulze and Barbieri	1881
8	Serine	Cramer	1865	Alanine	Weyl (Schützenberger	1888 1879?)
9	Glutamic acid	Ritthausen	1866	Lysine	Drechsel	1889
10	Phenylalanine	Schulze	1879	Arginine	Hedin	1895
11	Arginine	Schulze	1886	Iodogorgoic acid	Drechsel	1896
12	Lysine	Drechsel	1889	Histidine	Kossel, Hedin	1896
13	Iodogorgoic acid	Drechsel	1896	Cystine	Mörner	1899
14	Histidine	Kossel, Hedin	1896	Valine	Fischer	1901
15	Proline	Willstätter (synthesis)	1900	Proline	Fischer	1901
16	Tryptophane	Hopkins and Cole	1901	Tryptophane	Hopkins and Cole	1901
17	Oxyproline	Fischer	1902	Oxyproline	Fischer	1902
18	Isoleucine	Ehrlich	1903	Isoleucine	Ehrlich	1903
19	Thyroxine	Kendall	1915	Thyroxine	Kendall	1915
20	Oxyglutamic acid	Dakin	1918	Oxyglutamic acid	Dakin	1918
21	Methionine	Mueller	1922	Methionine	Mueller	1922

of the early papers or of the purity of the preparation of protein employed, and also because in at least one case, that of histidine, simultaneous independent and equally meritorious discoveries were made. These points are discussed in detail in the following pages.

A word as to the methods that were used by the various investigators should perhaps be added. Leucine, glycine, alanine, tyrosine, phenylalanine, glutamic acid, and serine were more or less chance products of fractional crystallization. Aspartic acid was discovered by the application of a new experimental method, the precipitation of its barium or calcium salt by alcohol; this method was likewise instrumental in the discovery of oxyglutamic acid. The discovery of the basic amino acids was also the result of the introduction of a new precipitant, phosphotungstic acid, and of the skillful use of silver nitrate. Proline, oxyproline, and valine, Fischer's contributions to the list, were the products of another new method and of a new point of view. Isoleucine was found because Ehrlich believed that a difference of a few degrees in specific rotation between his preparations of leucine and those of others must have an explanation. Tryptophane was first isolated by methodically following a color reaction during a fractionation with a new reagent. Thyroxine and iodogorgoic acid were detected by analyses for iodine. Cystine was found in proteins by a man who was convinced that it must be there. Methionine was isolated as a result of an investigation of the nature of a substance in protein hydrolysates that stimulated the growth of certain bacteria. This search was side-tracked by the observation that sulfur was present, in the active fraction, in an unfamiliar type of combination.

CYSTINE



Although cystine was shown as recently as 1899 to be a product of the hydrolysis of proteins, it was the first amino acid to be discovered. In 1810 Wollaston (43) described a substance that

he had found in a new type of urinary calculus. It was soluble in both acids and alkalies and separated from alkaline solution in hexagonal plates on acidification with acetic acid. It burned with a characteristic unpleasant smell and a bluish flame; ammonia and carbon dioxide were present among the products of dry distillation, but he did not note the presence of sulfur. Wollaston wrote, "From the ready disposition of this substance to unite with both acids and alkalies, it would appear to be an oxide; and that it does, in fact, contain oxygen is proved by the formation of carbonic acid on distillation. . . . . I am therefore inclined to consider it as an oxide, and since both the calculi that have yet been observed have been taken from the bladder, it may be convenient to give it the name of *cystic oxide*, which will serve to distinguish it from other calculi."

Occasional references[4] to the occurrence of similar calculi are found in the early literature, Walchner's (42) identification of the substance in a calculus from a boy's urine being perhaps the most clearly substantiated. Lassaigne (25) claimed to have identified it in a calculus secured from a dog but his description is far from convincing. He described white transparent leaves secured by the evaporation of the solution in ammonia, but did not mention that the leaves were six-sided. The shape of cystine crystals is so striking that he could hardly have failed to refer to it if his substance had been in fact cystine. Furthermore the analytical figures he gave bear no relation whatever to the composition of cystine, and Berzelius (5) dismissed his identification as improbable.

The name cystine appears in Berzelius' Jahresbericht for 1832 in connection with a report on an observation by Venables of a urinary calculus (6). The exact date on which the new name was first used is not certain but, in the French edition of his textbook published in 1833 (7), Berzelius described the substance discovered by Wollaston and pointed out that, although cystic oxide resembled certain metallic oxides in regard to its solubility, the term oxide was inappropriately used as a designation for an

[4] An extensive bibliography of early observations of cystine stones is given by Liebig, Poggendorff and Wöhler (26), and also by Gmelin (20).

organic substance since most of these contain oxygen; "je me suis donc permis de changer le nom qu'avait proposé cet homme distingué." Berzelius is therefore responsible for the name cystine, and it is probable that he first used it when preparing the manuscript of the 1833 edition of his treatise.

The earliest recorded analysis of cystine was carried out by the distinguished English scientist William Prout (1785–1850), the discoverer of hydrochloric acid in gastric juice (37), and the man whose speculation that the atomic weights of the elements (35) are integral multiples of that of hydrogen has been so strikingly revived in recent years. Prout (36) analyzed a purified

TABLE 2

Analysis of cystine

		THEORY, ATOMIC PROPORTIONS	PERCENTAGE COMPOSITION	
			Found	Theoretical (modern atomic weights)
			per cent	*per cent*
Hydrogen	3 atoms	3.75	5.00	5.03
Carbon	3 "	22.50	30.00	29.96
Nitrogen	1/2 "	8.75	11.66	11.66
Oxygen	4 "	40.00	53.33	26.65
				26.70 (Sulfur)
		75.00	100.00	100.00

specimen of cystine secured from calculi collected by Marcet. His results were so extraordinary that they merit quotation in full and are given in table 2. Sulfur was not detected but its presence appeared to have had no effect upon the accuracy of his analysis!

Baudrimont and Malaguti (1) in 1837 announced that cystine contained sulfur. At Baudrimont's request Pelouze carried out analyses of a specimen of the same material for carbon, hydrogen, and nitrogen, and obtained results that confirmed Prout's. Pelouze informed Liebig of Baudrimont's discovery and also sent him a small sample of purified cystine. Liebig turned this material, together with a cystine calculus already in his possession, over to Thaulow for analysis. Thaulow conducted his final

analyses for carbon, hydrogen, nitrogen, and sulfur directly on the calculus without purification, and used Pelouze's purified material for practice on the method for nitrogen! His results (40), nevertheless, were in remarkably close agreement with the older analysis of Prout[5] and with the recent one of Pelouze, and the formula $C_6H_{12}N_2O_4S_2$[6] was suggested as being in closest agreement with the analytical results. Determinations of the nitrogen and the sulfur content of purified cystine were also made by Marchand in 1839. He found 11.88 per cent nitrogen and 25.55 per cent sulfur.

It is difficult to follow the changes in the formulation of organic compounds through the period when the atomic weight of carbon to one worker was 6, to another 12, when oxygen was taken sometimes as 8, sometimes as 16, and when there was no clear appreciation of the concept of molecular weight. Thaulow's correct formula was later written $C_6H_6NS_2O_4$ (O = 8, C = 6, S = 16) and, because the sum of the hydrogen and nitrogen atoms was odd, Gmelin (20), in his celebrated Handbuch, in 1852, arbitrarily changed this formula to $C_6H_7NS_2O_4$. Gerhardt (19) was more cautious and gave $C_{12}H_{12}N_2O_8S_4$ in his Lehrbuch published the following year; this is simply Thaulow's formula with the above mentioned atomic weights. Gerhardt affixed a question mark to this formulation, however. Grote (21) in 1864 carried out analyses for carbon, hydrogen, and sulfur on purified cystine and obtained figures in very close agreement with Gmelin's altered formula. He said, "Es ist hiernach unzweifelhaft dass bei der Analyse von Thaulow ein Verlust von Wasserstoff stattfand."

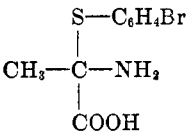
Dewar and Gamgee in 1871 (9) made the first attempt to

[5] In the French translation of Thaulow's paper Prout's name is misspelled Proust and this misspelling is repeated in Baudrimont and Malaguti's paper which follows Thaulow's. Evidently some confusion with the name of the former distinguished professor at Madrid was present in the mind of the proof reader.

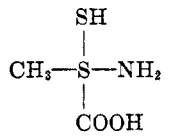
[6] Thaulow employed the following atomic weights: O = 100, C = 76.4, N = 88.5, H = 6, S = 201. These are equivalent to O = 16.0, C = 12.2, N = 14.16, H = 0.91, S = 32.06. Thaulow's symbols therefore had the same meaning to him that they do to us today; his formula is correct. Incidentally it may be interesting to recall that the use of unity for the atomic weight of oxygen was first suggested by Wollaston; the value 100 was used by Berzelius later.

ascribe a structural formula to cystine. They treated cystine with nitrous acid and isolated a silver compound which they thought to be silver pyruvate. They observed the formation of ammonia and free sulfur when cystine was heated to 150° with alkali, and the formation of hydrogen sulfide under the action of nascent hydrogen. The formula CH_2NH_2—CS—COOH appeared to express these reactions best, although they carried out no analyses to verify this composition.

Baumann and Preusse in 1881 (4) observed the elimination of bromophenylmercapturic acid by dogs to which bromobenzene had been administered. Hydrolysis of this yielded a substance that they regarded as bromophenylcystine, inasmuch as the formula they obtained by analysis could be accounted for by substituting C_6H_4Br for one of the hydrogen atoms of cystine, $C_3H_7SNO_2$. A study of the behavior of this substance, especially its decomposition to bromophenyl mercaptan, ammonia, and pyruvic acid under the action of alkalies led them to propose for it the formula,



and, as a logical deduction, the formula,



was proposed for cystine. In support of this formula they quoted three unpublished analyses by Hoppe-Seyler. One of these analyses gave a figure for hydrogen that agreed exactly with the requirements of theory, the other two were low. They pointed out that, if Dewar and Gamgee's formula were correct, methylamine should be a product of the decomposition of cystine; this, however, was not the case.

E. Külz was the next to take up the problem of the composition

of cystine. In 1884 (23) he reviewed all the previous analyses and pointed out that hydrogen determinations usually give somewhat high values. Two of Hoppe-Seyler's results, on the other hand, were low if the correct formula of cystine is $C_3H_7NSO_2$. Külz had available no less than 26 grams of cystic calculi as well as supplies of urine from a cystinuric patient. Purified preparations of cystine from this material were subjected to analysis. He wrote, "Für die Formel $C_3H_7NSO_2$ ist der Wasserstoff in sämmtlichen Analysen zu niedrig ausgefallen . . . . so stimmen sämmtliche Analysen gut, am besten speciell der Wasserstoff, ... zur Formel $C_3H_6NSO_2$, eine Formel, die freilich dem oben erwähnten Gesetze widersprechen würde. Ob sie die richtige ist, oder ob sie gar verdoppelt werden muss, wird erst die Synthese des Cystins endgültig entscheiden können."

It is indeed remarkable that the presence of cystine among the products of hydrolysis of proteins should have escaped notice for so many years. The presence of sulfur in proteins was recognized by Scheele and by Fourcroy in the eighteenth century, and attention was focused on this fact by the speculations of Mulder (41). Cramer, in 1865, suggested that there was an analogy between serine, his newly discovered amino acid from silk, and the cystine of urinary calculi, and it is clear that E. Külz, in 1886, suspected that cystine was present among the decomposition products of proteins.

A number of reasons may be advanced to account for the failure to find cystine in proteins. Up to 1873 sulfuric acid had been almost universally employed for the hydrolysis of proteins. The acid was usually removed as calcium sulfate and the cystine formed by the hydrolysis would, almost inevitably, be lost in the precipitate. Moreover, few hydrolyses of proteins of high cystine content, such as hair or horn, seem to have been attempted in the early days. The introduction of hydrochloric acid as a hydrolyzing agent by Hlasiwetz and Habermann (22) in 1873 would probably soon have brought cystine to light if stannous chloride had not almost invariably been added to the mixture in order to keep down humin formation during the hydrolysis. Cystine was thereby reduced to the much more soluble cysteine and thus es-

caped observation. Even Mörner, who hydrolyzed horn with hydrochloric acid and did not add tin, failed at first to hit upon the simple expedient of neutralizing the hydrolysate with sodium carbonate.

E. Külz of Marburg, in 1886, requested Richard Külz, his nephew and assistant, to investigate the problem "ob bei der Einwirkung des pankreatischen Saftes auf Eiweisskörper ausser den bis jetzt bekannten noch andere Spaltungsproducte entstehen, und besonders mit der Lösung der Frage, in welcher Form der Schwefel dabei auftritt." Richard Külz unfortunately died[7] before the work was completed and his observations remained unpublished until 1890, when E. Külz published a short paper (24) in which he stated that R. Külz "ist . . . . auf einen Befund gestossen, der mir schon jetzt der Mitteilung werth scheint." Külz had prepared a mixture of 290 grams of crude fibrin and 270 grams of minced pancreas which was protected from decomposition with salicylic acid and allowed to digest for about 48 hours, part of the time at room temperature. No hydrogen sulfide was evolved. The mixture was filtered and evaporated to half its volume, filtered, and allowed to stand. A white deposit separated that was insoluble in water, but when this was dissolved in ammonia and the solution was evaporated, a crust of well-formed, six-sided plates separated which were insoluble in acetic acid. A second crystallization from ammonia gave six-sided tablets. The material burned with a greenish-blue flame, contained both sulfur and nitrogen and was strongly levorotatory. E. Külz unhesitatingly identified it as cystine, a judgment for which his previous experience had thoroughly qualified him. He pointed out, however, that the experiment did not decide whether the cystine had been originally present in the pancreas employed, whether it arose from the action of the pancreas on the fibrin, or whether it had arisen from bacterial action. "Ja, die Möglichkeit, dass das Cystin dem angewandten Fibrin angehaftet habe, kann ohne Weiteres nicht absolut ausgeschlossen werden." Thus the first attempt to prepare cystine from protein ended in uncertainty.

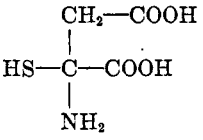
[7] Richard Külz disappeared during a solitary holiday expedition to the Beerberg in the Thüringer Wald; no trace of him was ever discovered in spite of the most careful search (personal communication from Prof. Fritz Külz, Kiel).

It is interesting to trace the evil fortune that pursued the investigators of cystine. Early workers lost any that may have separated from their hydrolysates in the precipitate of calcium sulfate which they filtered off and discarded. Later investigators inadvertently reduced the cystine to cysteine and so missed it. R. Külz died before he could complete an investigation that promised success. Emmerling (12), a bacteriologist, in 1894, observed cystine admixed with the tyrosine which he had prepared by the hydrolysis of horn, but he failed to follow up his observation with a positive identification. Suter (39) in 1895 discussed the whole problem of the presence of cystine in proteins in the light of Baumann's experiments on mercapturic acid elimination and of Cloetta's, Scherer's and Drechsel's observation of cystine in animal and human livers (8, 38, 10). Cystine was almost certainly an intermediary product of protein metabolism but "die Frage, ob das Cystin sich bei directer hydrolytischer Spaltung . . . . aus dem Eiweiss abspaltet, ist noch nicht gelöst." Baumann had suggested this problem to Suter but, by the grimmest mischance, had given him for investigation, not horn, nor hydrolysates of horn, but filtrates that remained over after work carried out years before on the isolation of tyrosine from horn! Very little cystine could have remained in them; most of the sirupy solutions he provided were acid, but a few had become alkaline and molds had formed on these.

The selection of horn was made because of Suter's own observation that this material contained unusually high proportions of sulfur in a form that yielded sulfide when the material was heated with alkaline lead acetate. Inasmuch as cystine behaved in an analogous fashion it seemed reasonable to suppose that horn should yield this amino acid in considerable amounts. Suter's attempts to isolate cystine were well conceived. He employed mercuric chloride as a reagent and found that a substance that contained both sulfur and nitrogen was precipitated. The substance likewise gave the sulfide reaction with alkaline lead acetate and, furthermore, gave an evanescent blue color with ferric chloride and a violet color with copper sulfate. He satisfied himself that cystine, after reduction to cysteine, gave somewhat

similar color reactions but failed in his attempts to isolate from the horn hydrolysates the material that was responsible for these reactions. He pointed out that both reactions are given by α-thiolactic acid and, in fact, isolated this substance from one of the tyrosine mother liquors that had molded. He concluded, somewhat ruefully, "vielleicht decken spätere Untersuchungen die Bedingungen auf, die erfüllt sein müssen, damit Cystin unter den Spaltungsproducten von Eiweisssubstanz auftritt."

Baumann added a few remarks to Suter's paper (3) in which he drew attention to the increasing number of sulfur compounds that had been observed to result either from metabolism, or from the decomposition of proteins, and to the interest in the precursor of these which must be present in the protein molecule. "Man könnte daran denken, dass es sich um eine geschwefelte Asparaginsäure handelt:



welche sehr wohl die Stammsubstanz des Cystins, des Cysteins, der Mercaptursäure, der Thiomilchsäure, und des Aethylsulfids sein könnte." This sums up the results of eighty-five years of study of cystine. Baumann was entirely correct only in his conviction that there was a mother substance in proteins from which all these products were derived.

But the ill-luck that had pursued the investigators of cystine could not, according to the law of probability, always reign supreme, although, as will presently appear, it did not immediately relax its influence. K. A. H. Mörner, in 1899 (March 8), reported to the Swedish Academy (28) that he had obtained cystine by the acid hydrolysis of horn. This announcement was followed by a paper in the Zeitschrift für physiologische Chemie (29) in which full details were given. Carefully purified horn shavings were heated with approximately 15 per cent hydrochloric acid at a temperature of 90–95° for one, or for two, weeks. The selection of these conditions of hydrolysis was made so as to avoid, as

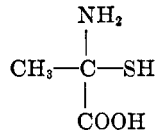
much as possible, the decomposition of the sulfur compounds and the production of hydrogen sulfide. After filtration and decolorization the acid was distilled off, the residue was dissolved in water, and neutralized with lead oxide. Alcohol was then added to complete the precipitation of the lead compounds. The precipitate was decomposed with oxalic acid and the acid solution was neutralized with ammonia, or else with calcium carbonate; it was then treated with an excess of ammonia, and filtered. The ammoniacal solution was evaporated at low pressure, whereupon cystine and tyrosine crystallized. These were separated by appropriate treatment with dilute ammonia. The presence of cystine in the different fractions was followed by application of the lead-blackening test and by the behavior towards Millon's reagent, which likewise gave information of the presence of tyrosine. The cystine was finally recrystallized from dilute ammonia by evaporation over sulfuric acid; Mörner says, "die Menge des Cystins war nicht gering. . . . . Insgesammt erhielt ich . . . . beinahe 2 1/2% der trockenen Hornsubstanz." The product was thoroughly identified by analysis and reactions.

This paper contains a vast amount of accurate information on the chemical behavior of cystine. Mörner observed the formation, when the hydrolysis of the protein was prolonged, of a more soluble and less strongly levorotatory variety of cystine which crystallized in needles; he found that residues of cystine in the mother liquors could be recovered by precipitation with copper acetate and by mercury salts and that these metal compounds, on decomposition with hydrogen sulfide, yielded much of their cystine in the form of cysteine. He discussed the meaning of the changes in optical activity of the cystine with their associated changes in solubility, but pointed out that the chemical properties of the two types of cystine were identical; "Alles spricht für die Annahme einer Stereoisomerie." He concluded, "Aus den oben mitgetheilten Untersuchungen geht hervor dass man durch hydrolytische Spaltung der Hornsubstanz bei der Einwirkung von Salzsäure Cystin in beträchtlicher Menge darstellen kann . . . . in der Hornsubstanz gewissermassen eine Cystingruppe präformirt vorfindet, oder jedenfalls eine Atomgruppe, welche leicht in Cystin übergeht."

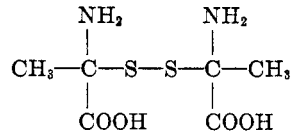
Shortly after the appearance of his first paper Mörner improved the method of preparing cystine from proteins. In a lecture given before the International Congress at Paris in 1900 he described the substitution of sodium hydroxide or ammonia for lead oxide in the neutralization of the hydrolysate and the great improvement in yield that resulted. He obtained 6.8 per cent of cystine from horn, 6.0 per cent from egg membrane, and 12.6 per cent from human hair. He had also obtained a 1 per cent yield from serum albumin. These were results that are difficult to match even today. In his audience was Gustav Embden, then a young assistant at Zürich. During the previous year Embden, without knowledge of Mörner's work, had hydrolyzed horn with hydrochloric acid and had isolated cystine! When this was brought to his attention Mörner at once recognized Embden's originality and generously permitted him to publish the essential points of the lecture as an introduction to the paper Embden was then preparing (11). Embden had boiled horn for 5 to 6 hours with concentrated hydrochloric acid; the hydrolysate was then neutralized in the cold with sodium hydroxide and, after standing for 24 hours, was filtered from the dark brown precipitate or "Melaninniederschlag." Embden unfortunately did not investigate this material; it must have contained much of the cystine. The filtrate was decolorized and evaporated and a number of successive crops of crystals were removed. By taking advantage of the greater solubility of the impurities in very dilute nitric acid Embden succeeded in isolating cystine from these fractions. He also isolated cystine from egg albumin and serum albumin, and obtained reactions for cystine in edestin. Cystine was thereby established as being a generally distributed amino acid rather than a product only of the insoluble keratins.

The investigations of Baumann and Preusse already mentioned had led to the conclusion that the sulfur and nitrogen atoms of cystine were both attached to the same carbon atom. In 1884 Baumann (2) obtained chemical evidence that Thaulow's original formulation of cystine ($C_6H_{12}N_2S_2O_4$) and Külz' idea of a double molecule were correct. When cystine was treated with tin and hydrochloric acid, it was converted into an easily oxidized sub-

stance of approximately the same ultimate composition but of entirely different properties. The relationships were obviously analogous to those between a mercaptan and a disulfide and the two substances were therefore formulated as follows:



Cysteine



Cystine

Baumann wrote, "Um die Beziehungen dieser Substanz zu dem Cystin zu bezeichnen, nenne ich diese Reduktionsprodukt des Cystins: Cystein." The mercapturic acids were obviously substitution products of cysteine rather than of cystine and their nomenclature was therefore corrected.

The formulation of cysteine as α-thio-α-aminopropionic acid did not go unchallenged. Neuberg (33), in 1902, pointed out that the production of pyruvic acid from cystine was no criterion for the position of the thiol group, as numerous cases were known in which a keto group resulted from conversions of hydroxyl compounds, probably with the intermediate formation of an ethylene oxide ring. An analogous reaction might occur in the case of cystine.

Neuberg wished to convert the labile thiol group into the more stable sulfonic acid group; to this end he subjected cysteine, prepared from cystine calculi, to the action of nitric acid. The product isolated turned out to be isethionic acid, $SO_3H—CH_2—CH_2—OH$, which could readily be accounted for by the action upon the amino group of the nitrous acid produced during the oxidation of the thiol group. It was evident that the thiol and the amino groups of cysteine, and therefore of cystine, were on different carbon atoms and only two possibilities for the formula of cysteine could be admitted. It might be α-thio-β-aminopropionic acid or α-amino-β-thiopropionic acid. Of these the second was more probable in view of the obvious relationship to serine. Neuberg suggested that, although this was true for cystine derived from calculi, the identity of this substance with cystine derived from proteins was not yet demonstrated.

While Neuberg's paper was still in proof an announcement was made by Friedmann (16) that he had converted protein cystine into taurine, $SO_3H—CH_2—CH_2—NH_2$, a demonstration that cystine from another source contained thiol and amino groups on different carbon atoms. The full description of this work came the following year (17). Friedmann likewise pointed out the weakness of Baumann's argument, and then described experimental work that thoroughly demonstrated cystine to be the disulfide α-amino-β-thiopropionic acid. Cystine, when treated in concentrated hydrochloric acid solution with sodium nitrite, was converted to dichlorodithiopropionic acid; reduction with zinc and hydrochloric acid removed the chlorine and gave a thiolactic acid. This was either α- or β-thiolactic acid. An attempt to identify this as its benzyl compound failed, and Friedmann therefore oxidized it with ferric chloride to the dithio acid; this was identical with the product obtained from β-iodopropionic acid with potassium hydrogen sulfide. The sulfur of cystine was therefore in the β position; the nitrogen might be either α or β. Friedmann regarded the former as more likely. He oxidized cystine with bromine and prepared the new substance cysteic acid,

$$\begin{array}{l} CH_2—SO_3H \\ | \\ CH—NH_2 \\ | \\ COOH \end{array}$$

thoroughly investigated it and its salts, proved that it was a sulfonic acid, and finally converted it[8] to taurine, $NH_2—CH_2—CH_2—SO_3H$, by heating it with water at 235°. The constitution of taurine was known; sulfur and nitrogen were on different carbon atoms. The sulfur in cystine was in the β position, hence the

[8] Gortner and Hoffman (Gortner, R. A. and Hoffman, W. F.: Sulfur in Proteins. J. Biol. Chem. **72**, 433–48 (1927)) have reported that they were unable to duplicate Friedmann's synthesis of taurine. Lewis and Lewis (Lewis, G. T. and Lewis, H. B.: The Metabolism of Sulfur. XI. Can Taurine Replace Cystine in the Diet of the Young White Rat? J. Biol. Chem. **69**, 589–97 (1926)) have also reported difficulty, but state that a successful preparation had been made by one of their associates. The matter is under investigation in the laboratory of one of the writers (S).

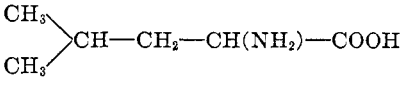
nitrogen must be in the α position and the formula was complete in every detail.

The accuracy of this formula was promptly confirmed by the synthesis of cystine by E. Erlenmeyer, Jr. (13, 14) from benzoyl serine. When treated with phosphorus pentasulfide a thiol group was introduced in place of the hydroxyl, and subsequent hydrolysis of the benzoyl group and oxidation gave inactive cystine.

During the early years of the present century a controversy arose as to the identity of the cystine from proteins with that from urinary calculi. Neuberg and Meyer (34) detailed a large number of points of difference between cystine from the two sources and Mörner's work (30, 31, 32), in which he obtained α-thiolactic acid from cystine, served to strengthen the view that stone cystine was the disulfide of α-thio-β-aminopropionic acid. Fischer and Suzuki (15), however, showed that Neuberg and Meyer's points of difference were all due to the presence of tyrosine in their preparation of stone cystine, and Gabriel's synthesis (18) of α-thio-β-aminopropionic acid, which differed in many ways from cysteine, finally settled the matter. Protein cystine and stone cystine are identical.

It required ninety-five years of investigation finally to settle the origin and constitution of cystine, but the problems presented by this fascinating amino acid are still far from solved. During the year 1930 a single journal, the Journal of Biological Chemistry, published no less than seventeen papers dealing directly with the chemistry or metabolism of cystine, and this represents but a small part of the total volume of work, for the most part of the highest quality, that was published on this substance during that year. Probably no other amino acid has so extensive a bibliography nor has attracted so much talent and, it is only fair to add, probably no other is so difficult to understand.

LEUCINE



In 1819 Proust (60) described a series of experiments, on different types of fermentation, that had been conducted in connection with a study of the principle to which different types of cheese owe their flavor. He had observed that gluten from wheat flour undergoes a spontaneous change which bears some analogies to that by which sweet substances become converted into new products and that, in addition to carbon dioxide, ammonia and acetic acid, two new substances were produced ". . . . premièrement, un acide particulier que j'ai cru pouvoir nommer *acide caséique*; puis un second produit que j'appelerai *oxide caséeux*." He suggested these names as convenient designations pending the outcome of further investigation which should decide whether or not the substances were indeed new.

Products of exactly the same nature were found among the products of fermentation of milk curds and he pointed out that, because of this, it would not be necessary to work with wheat gluten in order to obtain them. He commented at some length upon the good fortune that led him to study gluten before investigating milk curd, since otherwise he would never have observed the production of these substances by the fermentation of the former material. As he quaintly remarks, "Supposons, en effet, que j'eusse trouvé d'abord l'acide caséique dans le caillé, quel attrait d'utilité pouvait alors m'appeler vers la fermentation de la glutine? Aucun, quand j'y réfléchis, puisque la glutine fermentée n'est bonne à rien, puisque son fromage ne peut jamais entrer sur nos tables en concurrence avec celui de nos laitages."

"Oxide caséeux" was obtained from a water extract of fermented gluten. The extract was evaporated to a sirup and "sans retirer cette masse de la bassine, on la couvre d'alcool, on l'agite; elle se trouble, et il s'en sépare une poudre blanche abondante qu'on achève de laver sur le filtre avec de l'alcool, jusqu'à ce qu'on ne lui trouve plus de saveur fromageuse." By following a similar procedure, Proust was also able to isolate "oxide caséeux" from milk curds. The filtrate, after removal of his first crop of crystals, gave the product which he termed "l'acide caséique." As will appear from Braconnot's work, this was probably also leucine.

The chemical tests which Proust carried out were entirely of a qualitative nature. He observed that the substance was organic; he noted its solubility in various solvents, and its precipitability by salts of the heavy metals. It must have been evident to him that the substance contained nitrogen because on heating "acid caséique" with aqua regia "cet acide, chauffé dans une retorte, fournit les produits ordinaires aux matières animales, tels que carbonate d'ammoniaque, huile, hydrogène huileux et un charbon volumineux, sans aucune trace d'odeur prussique."

Although there is little doubt that the material isolated by Proust was essentially leucine, it is necessary to bear in mind that it was undoubtedly contaminated with other amino acids. The "leucine" of Proust, Braconnot and nearly all the subsequent investigators to the end of the nineteenth century was a white powdery substance which, under the microscope, was seen to consist of tiny nodules or balls of needles. Figures of this material may be found in many of the older textbooks and it is accurately described by Proust in the following terms: "D'abord, pour le purifier davantage, on le fait dissoudre dans l'eau bouillante, on filtre immédiatement, on évapore, et vers la fin on voit se former une nappe et des encroûtements qui s'accumulent sur les bords. Après le refroidissement, on jette tout sur un filtre afin de retenir dans l'eau mère des restes de caséate ammoniacal; enfin, on lave avec un peu d'eau froide, et l'on met a sécher. Notre produit a la légèreté, la blancheur et le spongieux de l'agaric blanc des drogueries. . . . . Trois dissolutions consécutives, des évaporations rapides ou spontanées n'ont rien changé à ses apparences. . . . . Les fragments en sont si légers qu'ils surnagent l'eau froide et l'eau bouillante; et c'est vers le 60^{me} degré qu'ils commencent à s'y dissoudre: l'eau ne semble pas les mouiller." And further, "l'oxide sous la forme de concrétions blanches globuleuses se présente à la vue, dans les fromages de glutine qui n'ont pu se dessécher."

A year after Proust isolated "oxide caséeux" from cheese, Henri Braconnot (45) obtained leucine by acid hydrolysis of muscle fibre and of wool. It was he who gave the name of leucine to the white crystalline substance which separated from the hydrolysate

on addition of alcohol. The crude protein was treated with twice its weight of concentrated sulfuric acid and allowed to stand for 24 hours. The mixture was then boiled for 5 hours, cooled, and saturated with calcium carbonate. The filtrate from the calcium sulfate was evaporated to a sirup. The final step in the isolation is best described in his own words: "On a fait bouillir, à plusieurs reprises, cet extrait avec de l'alcool à 34° Beaumé: les liqueurs réunies ont laissé déposer, par le refroidissement, environ un gramme d'une matière blanche particulière, que je désignerai provisoirement par le nom de leucine (λευκός blanc)." He made no analysis of the material nor did he realize that there was a relation between it and Proust's "oxide caséeux." In fact, no mention is made in Braconnot's paper of Proust's previous work.

In 1827, Braconnot (46) published a paper in which are given the results of a repetition of Proust's experiments on the fermentation of cheese. His opinion of Proust's work and his reasons for reinvestigating this subject are given in his opening paragraph. "Personne ne contestera à Proust les immenses services qu'il a rendus à la science; mais on est forcé de convenir que ses derniers travaux n'offrent pas toujours la précision et l'exactitude qu'on devait attendre d'un aussi habile chimiste. C'est après avoir fait quelques recherches sur l'hordeine, et m'être convaincu qu'elle n'est qu'un composé d'amidon, de matière animale et de fibre ligneuse, que je me suis déterminé a répéter les expériences du même chimiste sur la fermentation du caillé." To 750 grams of milk curds which had been drained, but not washed, he added a liter of water and permitted the mixture to stand at room temperature for a month. He then distilled part of the fluid to remove the putrid odor, filtered off a coagulum which had formed as the result of heating, and concentrated the fluid to a sirup. Then, "cette masse, délayée avec de l'alcool à 37°, a été partagée en deux matières, très-improprement appelées, la première *oxide caséeux*, et la seconde, retenue en dissolution dans l'alcool, *caséate d'ammoniaque*." The "oxide caséeux" was purified by recrystallizing from water. He noted its relative insolubility in water, that it left no residue on ignition, that when slowly heated an ammoniacal product was formed which turned red litmus paper

blue and effervesced when treated with acids, and that it was more soluble in hydrochloric acid than in water. He did not have facilities at his disposal for carrying out an analysis of the product and therefore was unable to determine its composition. It appeared to him that, inasmuch as "oxide caséeux" did not contain very much oxygen, the name was not appropriate and, "comme elle semble se former toutes les fois qu'on abandonne des substances animales à la putréfaction, je propose de la nommer *aposépédine* de *ἀποσῆπεδων*, resultant de la pourriture." Thus, without realizing any relationship, he gave another name to a product which seven years previously he had termed leucine.

Braconnot next examined the substance that remained in solution when alcohol was added to the sirupy hydrolysate and that had been named "caséate d'ammoniaque" by Proust. As the result of various experiments, Braconnot concluded that it was composed of "acide acétique libre; aposépédine; matière animale soluble dans l'eau et insoluble dans l'alcool rectifié (osmazome); matière animale soluble dans l'eau et dans l'alcool; huile jaune, fluide, très-âcre; résine brune, plus sapide; acétate de potasse; muriate de potasse; acétate d'ammoniaque, des traces."

The experiments of Braconnot are cited at some length because they illustrate the difficulties that confronted the early chemists who worked with proteins and amino acids and also show how slowly progress was made in this field.

In 1839, Mulder (59) found that the leucine Braconnot had obtained by acid hydrolysis of proteins could also be obtained by alkaline hydrolysis of similar substances. He noted its solubility and melting point and observed that, when heated at 108° with lead oxide, it lost no weight; it therefore contained no "chemisch gebundenes Wasser." Mulder alone of the early investigators appears to have isolated leucine in approximately pure form. He described one of his preparations as consisting of gleaming white plates that possessed a greasy feel and were difficult to moisten with water. He assigned to leucine the formula of $C_{12}H_{24}N_2O_4$ with a molecular weight of 1644.035[9] and, further, found that

[9] See atomic weights employed by Thaulow as given in footnote 6. The formula was doubled because the sum of the hydrogen and nitrogen atoms must be an even number.

100 parts of leucine combine with 27.6 parts of hydrochloric acid: save for a slight error in the estimation of hydrogen, this formula for leucine is double that accepted today.

Mulder (59) was the first to realize that Braconnot's "aposépédine" and leucine were one and the same substance. The following quotation leaves no doubt as to his ideas; "Es besitzt folgende Eigenschaften, wie ich sie an dem aus Leim, Fleisch und Eiweiss entweder vermittelst Schwefelsäure oder Kali bereiteten oder endlich dem aus verfaultem Käse abgeschiedenen Leucin beobachtete."

In 1846, Liebig (57) showed that leucine and another substance, which later proved to be tyrosine, were formed when casein was fused with potassium hydroxide.

Iljenko (55), in 1847, repeated the work of Braconnot but used casein that had been purified. Estimation of nitrogen in his "aposépédine" gave values nearly the same as those of Mulder; he believed therefore "dass Mulder mit Recht das Aposepedin bloss als unreines Leucin betrachtet." That leucine could be obtained from casein, either by bacterial putrefaction or by the action of alkali, was clearly seen by Iljenko. ". . . . So liefert auch das Casein das Leucin und die flüchtigen Säuren bei seiner Fäulniss und bei Einwirkung des Alkalis in höher Temperatur." He believed that in the process of putrefaction "Leucin ist durch Oxydation entstanden. . . . ."

Laurent and Gerhardt (56) analyzed leucine the following year. Their preparation was isolated from the putrefactive products of milk curds and the results of the analysis indicated the formula of leucine to be $C_6H_{13}NO_2$. This is the first correct formula of leucine to be published. They classified leucine in the series $C_nH_{2n+1}NO_2$; glycine constituted the second member of this series, sarcosine the third, and leucine the sixth. At this date only five of the amino acids had been isolated and the structure of none of these had been elucidated; consequently there is little wonder that the system of classification was arbitrary. However, the germ of a rational basis of classification was beginning to appear.

Liebig (57) had observed that valeric acid, together with am-

monia and hydrogen, were obtained by the fusion of leucine with caustic alkali; Laurent and Gerhardt demonstrated that, by a similar procedure, sarcosine yielded acetic acid and glycine yielded formic acid. Strecker (64), on treating leucine with fuming nitric acid, obtained oxycaproic acid. Shortly after the publication of Laurent and Gerhardt's paper, Cahours (47) took up the consideration of the relationship of leucine to "aposépédine" and showed from analyses that the two products were identical and could be represented by the formula $C_{12}H_{13}NO_4$ (equivalent to $C_6H_{13}NO_2$). His classification of leucine and glycocoll was the same as that previously given by Laurent and Gerhardt.

Cahours (48) in 1858, having shown the relationship of glycine to acetic acid (see glycine), followed the same line of reasoning and suggested that glycine, alanine, and leucine were amino acids of the fatty acid series analogous to the aminobenzoic acid series of compounds. Accordingly, glycine was aminoacetic acid, alanine was aminopropionic acid, and leucine was aminocaproic acid. The only error in this classification was due to the lack of knowledge of the correct structure of the carbon chain of leucine.

Further progress was made by Hüfner (53) who heated leucine with hydriodic acid in a sealed tube and obtained caproic acid and ammonium iodide. The question still remained as to whether the acid so obtained was identical with or was merely an isomeric form of the caproic acid found elsewhere in nature. To elucidate the question as to the relationship of caproic acid to leucine, Hüfner (54), in 1870, undertook the synthesis of leucine from commercial caproic acid. He prepared bromocaproic acid and treated this compound with ammonia. The solubility of the synthetic amino acid was about the same as that of the natural product. Another synthesis was carried out in accordance with Strecker's cyanohydrin method, a procedure by which, as early as 1855, Limpricht (58) had synthesized leucine from valeraldehyde ammonia and hydrocyanic acid. Hüfner now had three leucine preparations, one isolated from protein, the second prepared from caproic acid, and the third synthesized from valeric aldehyde. These differed slightly from each other; "Allerdings ist die Möglichkeit, dass diese geringen Differenzen ebenso durch

die Isomerie der in den dreien enthaltenen Amyle bedingt sein können, nicht völlig von der Hand zu weisen; allein nichts desto weniger möchte ich doch, eben um der Geringfügigkeit jener Differenzen willen, alle drei auf verschiedene Weise gebildeten Leucine lieber als identische Körper, wie als blosse isomere Verbindungen bezeichnen."

Although the work of Schützenberger (63) advanced our knowledge of the chemistry of amino acids very little, it is illustrative of the difficulties that confronted chemists in separating the constituents of a protein hydrolysate even as late as in 1879. He relied entirely upon fractional crystallization to separate the products of hydrolysis of proteins from each other, a method that has since been shown to be only partially effective. Compounds of the series $C_nH_{2n+1}NO_2$ were called leucines, compounds of the series $C_nH_{2n-1}NO_2$ were called leuceines; tyroleucine was a substance of the formula $C_7H_{11}NO_2$; alanine was termed *leucine propionique*.

The constitution of leucine was finally established about 1891 by Schulze and Likiernik (62). They set up the following criteria upon which to base a comparison of the synthetic product with leucine isolated from protein. (*a*) The solubility of the two products must be identical. Since synthetic leucine was racemic it was necessary to determine the solubility of the racemized natural product. Schulze and Bosshard (61) had shown that complete racemization occurred after heating leucine with barium hydroxide to 170°. (*b*) Identical products should be obtained by means of *Penicillium glaucum* from synthetic and from racemized natural leucine. (*c*) On treatment with nitrous acid, the synthetic and the natural leucine should yield the same hydroxy acid. It was found that synthetic α-aminoisobutylacetic acid satisfied these criteria, while leucine synthesized from caproic acid differed from racemic leucine prepared from protein.

Other syntheses have more recently been carried out by Erlenmeyer and Kunlin (49), Fischer and Schmitz (50), and Bouveault and Locquin (44). Fischer and Warburg (51) showed that it was possible to resolve racemic leucine into its optically active components by means of the formyl compounds. The separation

of leucine and tyrosine by taking advantage of the difference in the solubility of the two substances in glacial acetic acid was described by Habermann and Ehrenfeld in 1902 (52).

GLYCINE

$CH_2(NH_2)-COOH$

The discovery of glycine by Henri Braconnot (68) in 1820 is the first instance in which a pure amino acid was obtained from a protein by acid hydrolysis. Braconnot was interested in substances which, on acid hydrolysis, yielded sugar. He had already shown that sugar could be obtained from wood, bark, straw, and hemp by this procedure and it was natural that he should attempt to see if animal substances yield similar products. He therefore boiled gelatin with sulfuric acid for 5 hours, neutralized the acid with calcium carbonate, evaporated the filtrate from the calcium sulfate to a sirup and left it to stand for about a month. At the end of this time he noted that crystals, which adhered to the wall of the glass vessel, had formed. The crystals possessed a sweet taste. It appeared to him that the product was indeed a sugar and he therefore named it *sucre de gélatine.* This was translated into German as *Leimzucker.* The statement, "Nous allons examiner les propriétés de ce sucre, qui pourrait à la rigueur constituer un genre nouveau si l'on ne craignait de trop les multiplier" leaves no doubt as to Braconnot's idea of the new substance.

He observed that "sucre de gélatine" was more easily crystallizable than cane sugar, that it was about as sweet as glucose, that it was about as soluble in water as milk sugar and that it was not fermentable. On treating it with nitric acid he obtained a crystallizable product which he termed *acide nitrosaccharique.* He noted the physical properties of the new compound but carried out no further chemical work. He did not discover that it contained nitrogen. It appeared to him that "cette transformation est opérée par une soustraction d'hydrogène et d'azote dans les proportions nécessaires pour faire l'ammoniaque, et probablement par une absorption d'oxigène de l'acide sulfurique."

In 1838 Mulder (76) showed that both glycine and leucine could be obtained by hydrolyzing gelatin with potassium hydroxide, and that meat likewise yielded these two amino acids. Mulder analyzed his glycine with results that suggested the formula $C_8H_{18}N_4O_7$. The preparation, on being heated with lead oxide at 100°, lost 12.5 per cent of its weight, although in the absence of lead oxide it lost no weight when heated to 110°. He therefore concluded that it contained chemically bound water and, by allowing for this, the formula was reduced to $C_8H_{14}N_4O_5$ with a molecular weight of 1552.925. The compound was considered to contain two hydrogen atoms replaceable by base. Mulder's formula was gravely in error and the work of Boussingault (66, 67) at about the same time was even less accurate. In a later publication Mulder (77) stated that his original product, as well as those prepared by a number of other investigators, was probably contaminated with leucine. He now (1846) ascribed the composition $C_8H_{10}N_2O_8$ to glycine; translated into modern atomic weights this agrees with twice the present accepted formula. Mulder was not, however, the first to determine the correct formula of glycine.

Dessaignes (72) showed in 1845 that acid hydrolysis of the hippuric acid, which Ure had shown to be excreted in the urine when benzoic acid was ingested, yielded glycine. From the formulas of hippuric acid and of benzoic acid he predicted the formula of glycine, a prediction which had apparently also been made by Gerhardt. There was uncertainty, however, as to whether or not a molecule of water was lost in the cleavage of the hippuric acid. Dessaignes states, "Je serais plus porté a croire qu'au reste $C_4H_6N_2O_2$, il faut ajouter 2 équivalents d'eau, et que le véritable équivalent du sucre de gelatine est $C_4H_{10}N_2O_2$, comme l'a indiqué M. Gerhardt; mais je n'ai pas encore de preuve à apporter en faveur de cette manière de voir." But Dessaignes did not make an analysis of gycine!

Controversies were exceedingly spirited in the early days of chemistry and at times reached the height of sarcasm, as the following quotation from Gerhardt's (73) paper published in 1846 shows. "MM. Boussingault et Mulder avaient attribué au sucre

de gelatine des formules assez compliquées. Je rejette ces formules, les déclare erronées et y substitue les rapports $C_2H_5NO_2$. Nouvelle déception, nouvel arbitraire! M. Mulder reprend ses analyses, trouve qu'un mélange de leucine lui avait d'abord donné trop de carbone, et, par de nouvelles expériences, arrive exactement aux rapports proposés par moi, mais naturellement il ne me cite pas non plus."

The experimental data in support of the correct composition of glycine were supplied in 1846 from three different sources—Horsford (74), Laurent (75), and Mulder (77). Horsford's work was carried out in Liebig's laboratory in Giessen. It was he who suggested the term glycocoll in place of "Leimzucker." In a footnote to his article he writes "Für den wasserfreien Körper werde ich den schon vorgeschlagenen Namen *Glycocoll* gebrauchen. Wie unpassend der Name Zucker ist, hat schon Dessaignes angeführt, da er den süssen Geschmack mit vielen andern Körpern, die niemand Zucker nennt, theilt und nicht gährungsfähig ist." Two years later, Berzelius (65) suggested that this term be changed to glycine. "Dieser Name (Glycocoll) ist nicht wohlklingend und hat ausserdem den Fehler, dass er nicht mit den Namen der übrigen Basen harmonirt. Er ist zusammengesetzt aus γλυχύς, süss, und κόλλα, Leim. Da diese organische Base die einzige ist, welche süss schmeckt, so kann sie viel kürzer Glycin genannt werden, und diesen Namen werde ich anwenden."

Horsford's analysis of glycine prepared from hippuric acid gave results in accordance with the formula $C_4H_4NO_2HO$ which, in modern atomic weights, is equivalent to $C_2H_5NO_2$, the correct formula. He further showed that glycine combined both with acids and with bases and prepared a series of these compounds. The question which concerned him is expressed in his statement, "Es drängt sich nun von selbst die Frage auf, in welche der gewöhnlichen Abteilungen der Chemie soll man das Glycocoll stellen? Ist es eine Basis, eine Säure oder ein Salz?" His answer, "Man muss nach dem hier Vorgebrachten schliessen, das Glycocoll zu gleicher Zeit Säure, Basis und Salz sein kann, indem es alle Eigenschaften zeigt, wodurch jede dieser Klassen von den andern sich unterscheidet. Durch diesen Besitz so verschiedenartiger

Eigenschaften zeichnet sich dieser Körper vor allen anderen aus," clearly shows that his ideas agree with the present day conception of amphoteric electrolytes. This idea was, however, not entirely new. As early as 1810 Wollaston (79) had expressed similar views with respect to cystine.

The next step in the chemistry of glycine was the elucidation of its structure; Cahours (69) was probably the first to guess this correctly. He reasoned that, since aminobenzoic acid is formed from nitrobenzoic acid by reduction, nitroacetic acid on similar treatment should yield glycine; but nitroacetic acid had not been synthesized, so it was not possible to test this hypothesis experimentally. Later, however, he (70) showed that glycine was formed when monochloroacetic acid was treated with ammonia, and that, on treating glycine with nitrous acid, glycolic acid was produced. Cahours' experiments received further support when Perkin and Duppa (78), in the same year, synthesized glycocoll by treating monobromoacetic acid with ammonia. A number of other methods for the synthesis of glycine have been proposed; the most recent consists in hydrolyzing aminoacetonitrile with hydrobromic acid (71).

ASPARTIC ACID

$$\begin{array}{l} CH(NH_2)—COOH \\ | \\ CH_2—COOH \end{array}$$

The history of aspartic acid really begins with the discovery of asparagine by Vauquelin and Robiquet (118) in 1806. Vauquelin had, in this work, the coöperation of a young chemist, Robiquet, whom he describes as "jeune chimiste, qui réunit la solidité du raisonnement une grande habileté dans les expériences." Robiquet (111) had previously published a paper on the composition of the asparagus plant. The discovery of asparagine came about when a quantity of the juice of this plant, which had been concentrated by evaporation, was permitted to stand for some time. Vauquelin's own words best describe the discovery. "Ayant abandonné dans mon laboratoire, pendant un voyage qu'il (Robiquet) fit, une certaine quantité de suc d'asperges, concentré

par l'évaporation, j'y observai un assez grand nombre de cristaux, parmi lesquels deux me parurent appartenir à des substances nouvelles: comme ils avaient une forme, une transparence et une saveur différentes, il me fut facile de les séparer."

The chemical nature of this substance proved to be a puzzling problem to Vauquelin and Robiquet. They found that it left no ash on ignition and that, on treatment with nitric acid, it was decomposed with the liberation of nitrogen. They did not regard it as an acid, since it did not affect turmeric paper, nor was it a salt since it had no fixed base in combination. Without reporting an exact analysis they concluded that the substance contained hydrogen, oxygen, and carbon in definite proportions and probably also nitrogen. In their own words ". . . . il y existe un principe cristallisable comme les sels, et qui n'est cependant ni acide, ni sel neutre, et dont la solution dans l'eau n'est affectée par aucun des réactifs qui sont ordinairement employés pour reconnaître la présence et la nature des sels dissous dans l'eau; et un autre principe sucré qui parait avoir de l'analogie avec la manne." Vauquelin and Robiquet did not give a name to the new product in their original paper. Dulong (88) in 1826, in describing their work, refers, however, to the substance "qu'ils ont designée sous le nom d'asparagine." At this time the term was in general use; it is derived from ἀσπάραγος, asparagus.

Bacon (80) in 1826 found that when alcohol was added to the aqueous extract from the root of the marshmallow (*Althaea officinalis*) a crystalline product separated. This he considered to be a salt of malic acid with a vegetable base and he gave it the name *althèine*. In 1827, Plisson (106) repeated Bacon's experiments. He found that Bacon's product possessed essentially the same properties as the substance Vauquelin and Robiquet had found in asparagus and that had since received the name asparagine. "Le malate acide de M. Bacon n'est ni un sel, ni un acide, c'est une substance azotée particulière qui joint des propriétés de l'asparagine." When Plisson heated a solution of this substance with lead hydroxide, removed the lead with hydrogen sulfide and concentrated the filtrate, he obtained a crystalline acid which, after recrystallization three times from alcohol, possessed the

following properties: "Il est sous forme de petites plaques brillantes et assez semblables à celles de l'acide borique brisé; il a peu de saveur, il est beaucoup plus soluble dans l'eau chaude que dans l'eau froide qui n'en dissout que peu; il est encore moins soluble dans l'alcool et d'autant moins que celui-ci est plus concentré." Plisson gave the name aspartic acid to this substance. "Traitée par l'hydrate de plomb, cette substance azotée que je considère comme de l'asparagine, donne lieu principalement à de l'ammoniaque et à un acide nouveau que l'on pourrait nommer *asparagique*. . . . . Comme le mot *asparagique* pourrait donner à entendre que l'acide de ce nom se rencontre à l'état naturel, je crois qu'il serait mieux d'adopter celui *d'asparartique* qui rappellerait que cet acide est artificiel."

Tiedemann and Gmelin (117) gave the name *Gallenasparagin* to a substance which they isolated from ox bile by adding hydrochloric acid, concentrating the filtrate and precipitating with alcohol. Berzelius (81) commented as follows on their product: "Von dem Asparagin aus den Spargeln ist diese Substanz so wesentlich verschieden dass ich mich wundere, wie sie für dieselbe diesen Namen wählen konnten; denn die Asparaginkrystalle aus Spargeln haben, wie sie auch bemerken, andere Winkel und werden leicht von Salpetersäure zersetzt."

Blondeau and Plisson (82) demonstrated the presence of asparagine in the roots of the comfrey in 1827 and the following year Plisson (107) showed that the crystalline substance that Robiquet (112) had isolated from licorice root and named *l'agédoïte* was also asparagine.

In 1830 Plisson and Henry fils (108) published the first analyses of asparagine and of aspartic acid. The formula $C_{12}H_{12}N_8O_4$ was ascribed to asparagine and the formula $C_{14}H_{14}N_2O_7$ to aspartic acid. In order to see if the odor observed in the urines of individuals who had eaten asparagus was formed from asparagine, a large dose of this substance was ingested; the characteristic odor, however, did not appear.

The experiments of Plisson were repeated by Wittstock (119). He concluded that asparagine did not exist preformed in the roots of the marshmallow, but believed that it was merely the ammo-

nium salt of aspartic acid that was set free during the process of isolation.

Pelouze (103), in a letter to Liebig in 1833, gave the formula of asparagine, dried at 120°, as $C_8N_4H_{16}O_5$, and that of aspartic acid as $C_8N_2H_{14}O_7$. These formulas were very nearly correct; $C_8N_4H_{18}O_6$ and $C_8N_2H_{14}O_8$ are the correct formulas expressed in the atomic weights used by Pelouze. He further stated that by the action of acids or alkalis, or of steam under pressure, asparagine is converted into ammonium aspartate. Therefore ". . . . Asparagin ist spargelsäures Ammoniak weniger 1 Atom Wasser. Wir nennen deshalb das Asparagin Asparamid." He was inclined to believe that allantoin, cystic oxide (cystine) and caffeine were compounds analogous to asparagine.

These results were shortly afterwards reported in greater detail by Boutron-Charlard and Pelouze (83). They obtained asparagine by repeatedly extracting marshmallow roots with cold water, concentrating the fluid to a sirup and permitting crystallization to take place. For the preparation of aspartic acid asparagine was hydrolyzed with barium hydroxide, the barium was removed by means of sulfuric acid and the acid was permitted to crystallize. Their idea that the conversion of asparagine to ammonium aspartate represented an hydrolysis was quite clear and definite; they likened the reaction to the hydrolysis of urea to form ammonium carbonate, the hydrolysis of oxamide to yield ammonium oxalate and the hydrolysis of benzamide to form ammonium benzoate. Save for small errors in the oxygen their formulas for asparagine and for aspartic acid were correct. The products they worked with were apparently fairly pure since their aspartic acid did not possess the meat-like taste Plisson and Henry mentioned as characteristic. The experiments of Pelouze distinctly supported those of Plisson and disproved the chief contentions of Wittstock.

Shortly after the publication of this paper, Liebig (96), who had obtained his preparations from Pelouze, published an analysis of asparagine and of aspartic acid. His data indicated that the formula of asparagine is $C_8H_{16}N_4O_6$. In the crystalline state the product contained two molecules of water. To aspartic acid he

assigned the formula $C_8H_{10}N_2O_8$.[10] Liebig pointed out that, since the formula of asparagine was obtained by the addition of two formula weights of ammonia to one of aspartic acid, the conversion of asparagine to aspartic acid and ammonia should proceed without the entrance of water into the reaction. On the basis of this reasoning he concluded that "das Asparamid zu der Klasse von Amiden nicht gerechnet werden darf, sondern es gehört einer andern an, wo eine wasserfreie Sauerstoffsäure mit Ammoniak zu einem Körper verbunden ist, der mit Ammoniaksalzen keine Aehnlichkeit besitzt, obgleich er im crystallisirten Zustande genau die Menge Wasser enthält, welche dem Atomverhältniss des Wassers in den Ammoniaksalzen entspricht, die durch Sauerstoffsäuren gebildet werden, allein dieses Wasser kann durch Wärme daraus entfernt werden, ohne dass die Verbindung selbst geändert wird." Liebig was therefore led to question the view of Dumas that urea might be considered as an amide of carbon monoxide and suggested instead that it was formed from cyanic acid and ammonia and that its structure was entirely like that of asparagine. Liebig proposed that "man wird also vorläufig dem Asparamid seinen älteren Namen Asparagin wieder geben müssen."

This view, founded upon an erroneous analysis of aspartic acid and backed by the weight of Liebig's prestige, led, as will be seen, to some interesting developments. Under the date of May 30, 1833, Liebig (99) wrote to Berzelius, "Ich habe kürzlich die Asparaginsäure und das Asparamid analysirt, ich habe eine andere Zusammensetzung erhalten wie Pelouze aber sie ist so, dass die Theorie der Verwandlung des Asparamids in Asparaginsäures Ammoniak ganz so bleibt wie sie von Pelouze hingestellt worden ist. Asparamid ist $C_4N_2H_8O_3$, Asparaginsäure 2 $C_4NH_7O_4$, ich verbürge aber diese Resultate noch nicht ganz, ich habe sie Pelouze mitgetheilt um ihn zu veranlassen seine Arbeit zu wiederholen, er schreibt mir aber dass seine neuste Analyse mit seiner frühren übereinstimmt, und er will mir nun nochmals sehr reines Aspara-

[10] This formula was expressed in this doubled form so as to conform to the even number rule. The formula of asparagine was written so as to agree with that of aspartic acid.

gin und A. säure zur Analyse schicken." In 1838 Liebig (97) published an analysis of aspartic acid which he had obtained by hydrolyzing asparagine with potassium hydroxide. The data indicated that the formula of aspartic acid was $C_8H_{14}N_2O_8$. This, divided by two, represents the present accepted composition of aspartic acid.

The next step lay in the elucidation of the structure of asparagine and of aspartic acid. The first work on this subject was carried out by Piria (104) at the University of Pisa. He showed that asparagine could be obtained from a number of sources, particularly from vetch seedlings. On treating asparagine with copper oxide he obtained the crystalline copper salt. When subjected to bacterial fermentation asparagine was converted into ammonium succinate. Contrary to the statement of Liebig (98) that "l'acide aspartique lui-même, soumis a l'ébullition avec de l'acide chlorhydrique concentré, ou fondu avec de la potasse caustique, se transforme en ammoniaque et en un nouvel acide très-soluble dans l'eau et non étudié encore," Piria was able to show that asparagine, when boiled either with hydrochloric acid or with nitric acid free from nitrous acid, was converted into aspartic acid and that this substance was not decomposed by these acids. Alkaline fusion of asparagine led to the formation of acetic and oxalic acids. A very important observation made by Piria was that asparagine and aspartic acid, on treatment with nitrous acid, were converted into malic acid with loss of nitrogen. He concluded that "ces deux corps comme deux amides de l'acide malique correspondant à l'oxamide et l'acide oxamique, qui sont les amides de l'acide oxalique." Piria did not, however, realize the possibility that the two amides of malic acid were isomeric with aspartic acid and asparagine. Owing to his erroneous explanation of the transformation of the latter substances into malic acid, the chemistry of aspartic acid and asparagine was led astray.

Shortly after the publication of Piria's work, Dessaignes (87) announced that it was possible to convert the ammonium salts of malic, maleic, and fumaric acids into aspartic acid by heating. He showed further that aspartic acid was converted, by bacterial

action, into succinic acid. The work of Dessaignes attracted the attention of Pasteur (101), particularly since Dessaignes' synthesis of aspartic acid presented certain problems in the field of optical activity. Pasteur had shown that both malic acid and aspartic acid possessed the property of rotating the plane of polarized light, while fumaric acid did not. If Dessaignes' synthesis was correct it apparently involved the formation of optically active aspartic acid from inactive fumaric acid. But Dessaignes' synthetic aspartic acid proved to be optically inactive; this led Pasteur to a study of the chemical and optical properties of active and inactive aspartic and malic acids. He compared the malic acid that had been obtained from aspartic acid with the natural product with the result that "je m'assure que l'acide malique, ainsi obtenu, était en tout point identique, sous le triple point de vue chimique, cristallographique et optique, avec l'acide malique du sorbier, des pommes, des raisins, et du tabac."

In a subsequent communication Pasteur (102) made the very important observations that the diamide of malic acid, malamide, obtained by synthesis from malic acid ester and ammonia was isomeric but not identical with asparagine. It was also known that, whereas oxamide and oxaminic acid, when heated with alkali, yielded all of their nitrogen in the form of ammonia, asparagine, when similarly treated, yielded only one-half of its nitrogen in this form and aspartic acid yielded none at all. This at once reopened the question of the constitution of aspartic acid and of asparagine.

It was evident from the behavior toward alkali that the nitrogen in asparagine was present in two forms. Kolbe pointed out (94) that aspartic acid was an amino acid and a derivative of succinic acid, not of malic acid, while asparagine was the amide of aminosuccinic acid. He regarded aspartic acid as a dibasic acid, "dass sie von den Alkalien nicht zwei Atome zu sättigen vermag, ist bei ihren sehr schwach saueren Eigenschaften wenig befremdend. Gleichwie das Glycocoll, Alanin, Leucin und gar das Taurin, überhaupt die Amidosäuren von den primären Säuren dadurch in bemerkenswerther Weise sich unterscheiden, dass sie kaum noch als Säuren anzusprechen sind, und grösstentheils

sogar basische Eigenschaften haben, so ist auch bei der Asparaginsäure der sauere Character der Bernsteinsäure durch den Eintritt von Amid für Wasserstoff in dem Grade abgeschwächt, dass sie eben sowohl mit Säuren wie mit Basen chemische Verbindungen eingeht."

Although both Dessaignes (87) and Engel (90) had synthesized aspartic acid, their procedures did not throw any light upon the structure of this substance. It remained for Piutti (105) in 1887 to prove by synthesis the correct structure of asparagine. Ethyl oxalate and ethyl acetate, in the presence of sodium alcoholate, react to give the sodium salt of oxalacetic ester. This was converted into the oxime by treatment with hydroxylamine. On reduction with sodium amalgam the oxime was converted into aspartic acid. A second synthesis was carried out by Schmidt and Widmann (115). By treating acetylsuccinic acid ester with nitrous acid it was converted into a nitroso derivative which, on reduction, yielded aspartic acid.

The procedures of Schiff (114) or of Pachlopnik (100) are also employed for the preparation of aspartic acid; both methods use asparagine as the starting material. As early as 1876 Guareschi (93) studied the solubility of asparagine and aspartic acid and prepared certain uramino compounds. Fischer and Koenigs (91) synthesized a number of peptides containing aspartic acid.

Ritthausen (109) was the first to isolate aspartic acid from the products of protein hydrolysis. He worked with conglutin and legumin. After separating tyrosin and leucine he permitted the acidified solution to stand over sulfuric acid. A considerable quantity of white material separated which, when removed and recrystallized, yielded glutamic acid. The mother liquor deposited nothing further of a definite nature even when treated with alcohol and ether; its strongly acid reaction suggested, however, that some acidic substance was present and Ritthausen therefore added barium carbonate which dissolved with effervescence. After filtration from the excess of reagent the fluid was treated with alcohol. This step is of the greatest historical significance as will shortly appear. The result of this treatment

is best given in Ritthausen's own words. "Es entstand hierbei ein schmieriger seidenglänzender, bald zu zäher Masse erstarrender Niederschlag, der, wiederholt aufgelöst und gefällt, das Barytsalz, einer neuen stickstoffhaltigen, ohne Zweifel wohl einer Aminsäure, darstellt, und mit dem aus Conglutin der Lupinen in gleicher Weise erhaltenen Körper identisch ist." He was misled by the analysis as to the nature of the amino acid. He concluded "dass die Säure nicht Succinaminsäure ist sondern eine Aminsäure von der Zusammensetzung $C_8H_{14}N_2O_6$." He named the product *Legaminsäure* in the words ". . . . sollte späteren Untersuchungen sicher dargethan werden, dass die Säure eine eigenthümliche Substanz ist, würde ich ihr den Namen *Legaminsäure* beilegen."

In 1869 Ritthausen (110) published the results of further investigation of this substance. The material secured by the precipitation by means of alcohol of the calcium salts of the acids was found to be a mixture of two crystalline acids together with a noncrystalline sirup of acid reaction. The crystalline acids could be separated by means of 50–60 per cent alcohol in which a part was insoluble. This separated in rhombic prisms, or sometimes in leaflets, when recrystallized, and was found to be identical with aspartic acid. The more soluble crystalline acid was glutamic acid. The copper salt was found to be especially advantageous for the separation of small amounts of aspartic acid from the mother liquor, and this salt is still the most useful known compound of aspartic acid for isolation purposes. Aspartic and glutamic acids were recognized to be homologous compounds; the former yielded malic acid, the latter glutanic acid, on treatment with nitrous acid.

There are two points of the greatest significance in these papers. Ritthausen discovered aspartic acid as a result of precipitating its barium salt, and later its calcium salt, by means of alcohol. This method of dealing with the dibasic amino acids was independently rediscovered by Foreman (92) in 1914 and was later employed by Dakin (85). Neither Ritthausen nor the later investigators can, however, lay any claim to using the method

for the first time. It is at least as old as Scheele (113) and in the hands of this extraordinarily resourceful worker led to the discovery of malic acid. Curiously enough, however, the great value of the method was not emphasized either by Scheele nor, later, by Ritthausen and, save in some early work by Schulze (116) on the analysis of proteins, it was entirely overlooked until Foreman and Dakin developed it into a standard method of protein analysis.

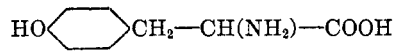
The second point is the nature of the acid in the sirupy mother liquor of the glutamic acid described by Ritthausen. Glutamic acid is largely converted into the very soluble pyrrolidonecarboxylic acid when its solutions are boiled with water. Ritthausen worked over his mother liquors for several months and secured about 8 grams of relatively pure glutamic acid from them. He recorded no suspicion that the glutamic acid might be undergoing change and there is every probability that an appreciable part of the glutamic acid present was, in fact, converted to pyrrolidonecarboxylic acid during his manipulations. But there is another highly significant possibility. Oxyglutamic acid is also extremely soluble and it is by no means improbable that this substance was likewise present. The properties of this acid are such, however, that there is little wonder Ritthausen failed to isolate it.

Ritthausen showed that aspartic acid was a constituent of vegetable proteins; Kreusler (95) extended the observations to animal proteins, and found it in casein and in egg proteins. It has since been observed to be widely if not universally distributed.

A number of syntheses of aspartic acid have been described. A recent one by Dunn and Smart (89) is of interest because of the novel conditions employed.

Hydroxyaspartic acid has been synthesized by Dakin (86). The product consists of inactive *para-* and *anti*-hydroxyaspartic acid but this substance has not yet been shown to occur in nature. Chibnall and Cannan (84) have extended Dakin's work by synthesizing hydroxy asparagine and measuring the dissociation constants. This product also has as yet not been found in nature.

TYROSINE



Tyrosine was discovered by Liebig (142) in 1846 in the course of an investigation of the nature of the products that proteins yield on decomposition with alkali. He fused crude casein with an equal weight of potassium hydroxide, dissolved the mass in hot water, acidified with acetic acid and permitted the solution to cool, ". . . . so scheidet sich eine Masse von sehr feinen Nadeln ab, welche in kaltem Wasser sehr schwer, in Alkohol und Aether unlöslich sind. Durch wiederholtes Auflösen in Wasser, dem man etwas kohlensaures Kali zusetzt und Fällung mit Essigsäure, erhält man diesen Körper rein weiss in seidenglänzenden Nadeln." Liebig's preliminary analysis suggested the formula $C_{16}NH_9O_5$ for the new substance but it is not certain that he regarded it as an amino acid, although some indication that the compound possessed amphoteric properties is given in the sentence "Der Körper, obwohl in Alkalien leicht löslich verbindet sich mit Säuren." He made no attempt to determine its structure. In a later paper (143) he mentioned that the same substance could be obtained from fibrin and from serum albumin, and gave the name tyrosine (*τυρός*, cheese) to it in the words ". . . . ein krystallinischer Körper, das Tyrosin (mit welchem Namen ich den Bd. LVII S. 127 der Annalen beschriebenen Körper belegt habe. . . . ."

de La Rue (129) obtained tyrosine in 1848 during an investigation on the composition of the cochineal insect. Carminic acid was removed from the aqueous extract with lead nitrate, and the filtrate, after removal of the lead, was concentrated to a sirup; a white crystalline product separated. This was recrystallized several times from hot water. An analysis indicated that the formula was $C_{18}H_{11}NO_6$ ($C_9H_{11}NO_3$ in modern atomic weights) which is the correct formula for tyrosine. Comparison of his product with a specimen of the substance Liebig had obtained from casein showed that they were identical. He answered the question whether tyrosine occurred in the free state, or was set

free in the course of the isolation, by the statement, "Man kann also annehmen, dass dieser Körper in dem getrockneten Insekte fertig gebildet enthalten ist."

Bopp (127) in 1849, in Liebig's laboratory, investigated the conditions under which tyrosine could best be obtained from casein, fibrin, and serum albumin. He found that it was very difficult so to control the conditions of the potassium hydroxide fusion as to obtain good yields of tyrosine. He noted, however, that both tyrosine and leucine were not destroyed by long continued boiling with hydrochloric or diluted sulfuric acid. Braconnot had obtained leucine from several proteins by boiling them with sulfuric acid but Mulder had stated that, if hydrochloric acid were used, only ammonia and the ammonium salt of humic acid were produced. This seemed to require verification. Bopp therefore treated casein with several times its weight of hot concentrated hydrochloric acid; the protein dissolved with the formation of an intense violet color that slowly turned brown as the heating was continued. After boiling the mixture for 6 to 8 hours the decomposition had proceeded far enough to permit the isolation of tyrosine and leucine. This experiment is of considerable historical importance; it is the first recorded successful hydrolysis of a protein by hydrochloric acid. Bopp used a mixture of sulfuric and hydrochloric acids in his main experiments. The reagents were removed by adding an excess of calcium carbonate and heating to expel ammonia; the precipitate was removed, a small excess of sulfuric acid was added to throw down the calcium, the excess of this and most of the chloride were removed by adding lead oxide; finally the excess of lead was precipitated by hydrogen sulfide. The filtrate, when evaporated, yielded leucine and tyrosine which were separated from the sirupy mother liquor by dilution with 80 per cent alcohol. Bopp did not regard it as necessary to identify his products by analysis; the crystalline form and behavior left him in no doubt of their identity and, indeed, his description of the tyrosine obtained by acid hydrolysis, and his comparison with the preparations secured from alkali fusion, leave no question that Bopp was the first to prepare tyrosine from acid hydrolysates. Hinterberger (139), also working in

Liebig's laboratory at about the same time, prepared tyrosine by sulfuric acid hydrolysis of horn; analysis of his product led to the correct formula $C_{18}H_{11}NO_6$ already given by de La Rue. His preparation was also identical with those of Liebig and of Bopp.

Müller (144), Leyer and Köller (141), Piria (147), Wicke (154), and Städeler (150, 151) during the next decade demonstrated the presence of tyrosine, together with leucine, in a wide variety of protein substances which included hair, feathers, gliadin, and silk fibroin. Their work definitely established the fact that tyrosine belonged to the group of substances which includes leucine and glycine. For example, Wicke wrote, "Das gleichzeitige Auftreten des Tyrosins und Leucins bei Zersetzung der Proteinsubstanzen, die einigermassen ähnlichen Formeln und leicht Löslichkeit beider in Säuren und Alkalien lassen vermuthen, dass das Tyrosin eine ähnliche Constitution wie das Leucin besitzt und vielleicht zur Reihe der aromatischen Säuren in demselben Verhältniss steht wie das Leucin zur Reihe der fetten Säuren."

The presence of tyrosine, and also of leucine, was demonstrated in tissues; Chevallier and Lassaigne (128) found wart-like, white round bodies of the size of poppy seeds in a cadaver. This substance was called by them *tubercules cystinoïdes* or *xanthoprotein*. No analysis was reported. They considered that the properties of this substance were intermediate between those of cystine and xanthine; the description, however, indicates that they were dealing with tyrosine. Frerichs and Städeler (135) were the first to report the presence of tyrosine and leucine in a diseased liver. Later (136) they demonstrated the presence of these amino acids in a number of other organs as well as in blood and urine. In 1860 Neukomm (145) reported the presence of both leucine and tyrosine in the organs of human cadavers in a variety of pathological conditions. The relation of tyrosine to homogentisic acid, a substance found in the urine in alcaptonuria, was first studied by Bödeker (126). It has been the subject of extensive study since that time (137).

Städeler (151) was the most thorough of the early workers who investigated the behavior of tyrosine. He prepared a series of compounds with bases, acids and heavy metals and a similar

series of compounds of mono- and dinitro-tyrosine as well. He showed that it was possible to convert tyrosine into chloranil ($C_6Cl_4O_2$), and recognized the importance of this reaction in throwing light upon the structure of tyrosine. "Von den mitgetheilten Zersetzungen des Tyrosins scheint vorläufig diese letztere allein geeignet zu sein, einiges Licht auf die Constitution desselben zu werfen." He emphasized the point that tyrosine belongs to the group of compounds that includes glycine and leucine. "Ohne Zweifel hat das Tyrosin eine ähnliche Constitution wie diese Körper; es verbindet sich ebenfalls nicht nur mit Basen, sondern auch mit stärkeren Säuren, und zwar nach Art des Ammoniaks." He noticed also that tyrosine is a dibasic acid ("tritt das Tyrosin als schwache zweibasische Säure auf").

Schmitt and Nasse in 1865 (149) concluded that tyrosine is related to salicylic acid. They based this view on these facts: (*a*) that tyrosine could be converted into chloranil; (*b*) that on dry distillation it yielded phenylalcohol; (*c*) that like salicylic acid it gave a colored compound with ferric chloride; (*d*) that it was a dibasic acid. They assumed that tyrosine was ethylaminosalicylic acid, and considered that it bore a relationship to salicylic acid similar to that which sarcosine bears to acetic acid. They were, however, unable to synthesize tyrosine by treating chloro- or iodo-salicylic acid with ethylamine. On heating aminosalicylic acid, carbon dioxide is split off and oxyphenylamine formed; Schmitt and Nasse therefore thought that, if tyrosine were ethylaminosalicylic acid, it should under similar conditions lose carbon dioxide and form ethyloxyphenylamine. Schmitt and Nasse did obtain a substance of the composition of the compound they expected; they did not, however, prove its structure nor consider the possibility that the carbon dioxide might be split off from a side chain.

The assumption of Schmitt and Nasse was disproved by Barth (122) when he showed that, on alkaline fusion, tyrosine yields acetic acid and *p*-oxybenzoic acid. From this evidence Barth assumed that tyrosine was the ethylamino derivative of *p*-oxybenzoic acid. In a later communication, Barth (123) corrected this assumption by showing that such substances as aminobenzoic

acid, amino-*p*-oxybenzoic acid, aminohydrocinnamic acid and aminosalicylic acid, on alkaline fusion, do not yield *p*-oxybenzoic acid but give non-crystalline products. This showed that the amino group was not replaced by hydrogen as should be the case if *p*-oxybenzoic acid were formed. Confronted with the fact that alkaline fusion of tyrosine gave almost quantitative yields of *p*-oxybenzoic acid, Barth made a second guess as to the structure of tyrosine and this later proved to be correct, ". . . . so erscheint es am wahrscheinlichsten, dass das Tyrosin als eine Oxyphenylamidopropionsäure zu betrachten ist." This view was in harmony with the observation that ammonia is set free when tyrosine is treated with hydriodic acid. Barth, moreover, showed that the product obtained by Schmitt and Nasse on carefully heating tyrosine was neither amidophlorol nor ethylaminooxybenzoic acid as the Schmitt and Nasse hypothesis would require, but a compound in which the amino group was contained in the side chain.

Ost (146) was able to confirm Barth's experiments on the alkali fusion of tyrosine and related compounds, but his attempt (124) to synthesize tyrosine by preparing oxyphenylchloroacetic acid and treating this with ammonia failed. Further proof of the structure of tyrosine as suggested by Barth was furnished by Baumann (125) when he showed that tyrosine yields *p*-oxyphenylpropionic acid on bacterial decomposition.

The synthesis of tyrosine was first accomplished by Erlenmeyer and Lipp (133) who treated *p*-aminophenylalanine with nitrous acid. A second synthesis was carried out by Erlenmeyer, Jr. and Halsey (132). They condensed hippuric acid with *p*-oxybenzaldehyde in the presence of acetic anhydride and sodium acetate to form the lactimid. On alkaline hydrolysis, *p*-oxy-α-benzoylaminocinnamic acid was formed which, on reduction with sodium amalgam, yielded benzoyltyrosine; hydrolysis of this yielded tyrosine. This synthesis is essentially the same as that used by Erlenmeyer, Jr. (131) for the preparation of phenylalanine. Wheeler and Hoffman (153) synthesized tyrosine by boiling anisalhydantoin with hydriodic acid and red phosphorus. In this reaction reduction of the double bond takes place, ammo-

nium iodide is set free, the hydantoin ring is opened and the urea grouping undergoes hydrolysis all in one operation. The yield of tyrosine is practically quantitative. Sasaki's (148) method consists in condensing glycine anhydride with anisaldehyde to form the diketopiperazine. On boiling with hydriodic acid and red phosphorus, reduction and hydrolysis take place yielding tyrosine to the extent of about 90 per cent.

The separation of leucine from tyrosine proved to be a stumbling block to many of the earlier workers; this was accomplished by Habermann and Ehrenfeld (138) by the use of glacial acetic acid, which dissolves leucine but not tyrosine. Suzuki (152) separated these amino acid by fractional recrystallization of the hydrochlorides.

Emil Fischer (134) was the first to separate the optical isomers of synthetic tyrosine. This was done by crystallization of the brucine or cinchonine salts of benzoyltyrosine. Abderhalden and Sickel (121) obtained *d*-tyrosine by the action of pancreatic juice on the racemic ethyl ester of tyrosine. They resolved the racemic mixture by means of the brucine salts of the formyl derivative. Finally, Ehrlich (130) accomplished the same thing by means of yeast in the presence of sugar.

On decarboxylation, tyrosine yields tyramine, a substance which physiologically is very potent. This decarboxylation was accomplished in the laboratory by Johnson and Daschavsky (140) by heating tyrosine with a mixture of diphenylmethane and diphenylamine. Abderhalden and Gebelein (120) later used diphenylamine alone for this purpose.

ALANINE

$$CH_3—CH(NH_2)—COOH$$

Alanine is one of two protein amino acids obtained by synthesis before being shown to be products of the hydrolysis of proteins; its discovery by Strecker (157) in 1850 is the basis of the now widely used cyanohydrin reaction. Liebig had shown that lactic acid, on oxidation, yields acetaldehyde. Strecker thought that it should be possible to synthesize lactic acid from aldehyde and formic acid. His reason for advancing this hypothesis was that

mandelic acid, on oxidation, yields benzaldehyde. Mandelic acid is formed by treating benzaldehyde with a mixture of hydrocyanic and hydrochloric acids, and it was supposed that the reaction involved the action of nascent formic acid on benzaldehyde. In reality, however, benzaldehyde cyanohydrin is formed which, on hydrolysis, yields mandelic acid.

Strecker obtained lactic acid but not in the expected way. He treated aldehyde ammonia with hydrocyanic acid in the presence of an excess of hydrochloric acid; on concentrating the solution ammonium chloride separated. Strecker's own words best describe his discovery, ". . . . und es bleibt eine stark saure, dicke Mutterlauge, welche die salzsäure Verbindung eines neuen Körpers enthält, den ich *Alanin* nennen will." Hydrochloric acid was removed as lead chloride and the excess of lead by means of hydrogen sulfide; the new substance crystallized when this solution was concentrated. In coining the word alanine Strecker used the first syllable of the word aldehyde in order to denote its origin.

Strecker noted that alanine combines both with acids and with alkalies and forms salts with the heavy metals. He classified it in a series of homologous compounds that included glycine as its first and leucine as its fifth member. On treating alanine with nitrous acid, lactic acid was obtained.

In 1875 Schützenberger and Bourgeois (156) subjected silk to the action of barium hydroxide in an autoclave at 150–200°. Fractional crystallization of the resulting amino acids yielded, according to their statement, 10 per cent of tyrosine, 60 per cent of a mixture of equal parts of glycine and alanine, 10 per cent of aminobutyric acid and 20 per cent of an amino acid of the acrylic acid series. None of the products was rigidly identified and no analyses were given. A few years later Schützenberger (155) published a long paper on the products of hydrolysis of egg albumin under similar experimental conditions. He separated the various substances by fractional crystallization and followed the composition of the fractions by carbon, hydrogen, and nitrogen determinations. An analysis of one of the fractions gave figures that agreed, within the conventional 0.2 per cent, with the theo-

retical composition of alanine. No other tests were described nor were any characteristic salts prepared. Although he does not refer to Strecker's synthetic alanine, the inference is that he was acquainted with it. This is borne out by his statement, "Parmi les composés amidés homologues de la leucine, j'ai encore rencontré, mais en petites quantités seulement, *l'alanine* $C_3H_7AzO_2$, ou leucine propionique; elle a été caractérisée par l'apparance de ses cristaux et par sa composition centisimale."

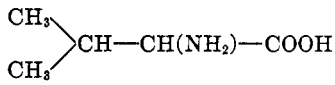
Schützenberger's claim to the discovery of alanine among the products of hydrolysis of proteins rests, then, upon a single analysis of a crystalline fraction but is unsupported by any other evidence. He may equally well be regarded as the discoverer of aminobutyric acid, although no subsequent worker has yet succeeded in identifying this amino acid among the products of hydrolysis of proteins.

In 1888 Weyl (158) isolated alanine from the hydrolytic cleavage products of silk fibroin. After removal of the tyrosine and evaporation of the solution a quantity of substance equivalent to 15 per cent of the protein crystallized out in a manner recalling the behavior of "leucine," that is to say, it separated in nodular masses. Cramer, in 1865, had recorded a similar experience, but unfortunately omitted to purify the material. Weyl recrystallized his substance from dilute alcohol with the aid of a little ammonia, and secured it finally in large rhombic plates which gave results, on analysis, very close to the theoretical requirements of alanine. Furthermore the behavior on heating was precisely like that of synthetic alanine and analysis of the copper salt confirmed the identification. Weyl stated that he could find no leucine in silk, and in fact the best modern analyses indicate that less than 2 per cent is present. He suggested that alanine acts as a substitute for leucine in this unusual protein. An excerpt from his article shows that he clearly recognized alanine as α-aminopropionic acid. "Die Analyse . . . . ergab Werthe welche mit Alanin (Amidopropionsäure) . . . . stimmen. Das Alanin der Seide ist hiernach höchst wahrscheinlich α-alanine." Weyl apparently did not consider that the presence of alanine in the protein molecule had been adequately established by Schützen-

berger and Bourgeois and, with some justice, claims the credit for this for himself in the statement, "Durch vorstehende Untersuchung ist Alanin zum ersten Male mit Sicherheit als Zersetzungsproduct eines Proteïds nachgewiesen worden."

It remained for Emil Fischer and his associates to show that alanine was a widely distributed constituent of proteins.

VALINE



In 1856, von Gorup-Besanez (163) published a paper on the chemical constituents of certain gland extracts. Among the substances in which he was interested, and which he sought, were the amino acids, particularly leucine and tyrosine. His method was to mince the glands, extract them with water, free the fluid from coagulable protein by heating, treat with barium hydroxide to precipitate sulfates and phosphates, and finally evaporate the fluid to a sirup for crystallization. He demonstrated the presence of leucine in the thymus, thyroid, spleen, liver, and pancreas, but only in the last was he able to demonstrate the presence of tyrosine. In the pancreas, von Gorup-Besanez found a substance that was very like leucine in its behavior, yet could be separated from the latter because of its lesser solubility in boiling alcohol. "Der in kochendem Weingeist schwieriger lösliche Theil besteht im Wesentlichen aus einem dem Leucin homologen und ihm in vielen Puncten ähnlichen Körper." An analysis of the recrystallized product showed that its composition could be expressed by the formula $C_{10}H_{11}NO_4 (= C_5H_{11}NO_2)$; this corresponds to the composition of valine. It was characterized by von Gorup-Besanez in the following words: "Weisse glänzende, mit freiem Auge erkennbare prismatische Krystalle, die für sich und in Flüssigkeiten betrachtet, durchsichtig sind, aber trocken und in grosserer Menge undurchsichtig erscheinen, indess keineswegs jenes blendend weisse kreideähnliche Aussehen zeigen, wie das Leucin." He classified the new substance as one of the homologous series of compounds which included glycine, alanine, and leucine, but carried out no further work on it.

Twenty-three years after the publication of von Gorup-Besanez' paper, Schützenberger (170) in an extensive investigation on the constituents of albumin, reported the presence of aminovaleric acid among the cleavage products. "Nous verrons plus loin que l'acide amidovalérique ou butalanine existe dans les cristallisations A; sa présence expliquerait pourquoi le carbone a été trouvé souvent intermédiaire entre 5 et 6 pour 1 atome d'azote, mais elle ne rend pas compte de l'abaissement de l'hydrogène su-dessous du rapport C^nH^{2n+1}." The formula $C_5H_{11}AzO_2$ was assigned to his butalanine and there is little doubt that he was dealing with valine.

Schulze and Barbieri (169) showed in 1883 that aminovaleric acid was present, along with other amino acids, in the sprouts of *Lupinus luteus*. Their product was isolated as the copper salt after removal of phenylalanine and leucine. Later Schulze (168) also found it in the sprouts of *Vicia sativa*.

von Gorup-Besanez (163) had indicated that the substance he had isolated from the pancreas was the fifth member of the series of compounds which included glycine, alanine, and leucine, and Schützenberger (170) without definite structural proof, considered his butalanine to be aminovaleric acid. The empirical formula $C_5H_{11}NO_2$ offered several possibilities; the substance might be either amino-*n*-valeric acid, aminoisovaleric acid, or ethylmethylaminoacetic acid. To elucidate the structure of von Gorup-Besanez' compound, Clark and Fittig (160) in 1866 undertook the synthesis of aminovaleric acid. Cahours (159) had previously stated that aminovaleric acid (Valeraminsäure) could be synthesized by treating bromovaleric acid with ammonia but, as he did not describe the properties of his product, it is doubtful that the synthesis was actually carried out. Clark and Fittig (160) synthesized aminovaleric acid by treating bromovaleric acid with aqueous ammonia at 100° for 24 hours. They made a comparison between their synthetic product and that isolated by von Gorup-Besanez. The solubilities of the two products were similar, but there was some difference in the melting points. Despite this, Clark and Fittig were inclined to regard the products as identical and attributed the differences noted to possible im-

purities in von Gorup-Besanez' product. In a note that appeared shortly after the publication of Clark and Fittig's paper, von Gorup-Besanez (164) accepted their conclusions as to the probable identity of the synthetic and the natural products. None of the early workers, however, considered the possibility that some of the differences between their products might be due to the difference in optical activity between the synthetic product and the natural; neither did the possibility that von Gorup-Besanez' product was aminoisovaleric acid occur to them.

Schmidt and Sachtleben (167) in 1878 pointed out that the aminoisobutylformic acid (aminoisovaleric acid) synthesized by them was identical with the aminovaleric acid synthesized by Clark and Fittig, and thus showed that the product synthesized by the latter was aminoisovaleric acid and not aminovaleric acid. This view received support from Lipp (165) who synthesized α-aminoisovaleric acid from aminoisovaleronitrile and showed that it was identical with Clark and Fittig's product.

Two years later Lipp (166) took up the problem of the structure of valine. He considered that, since Clark and Fittig's product was aminoisovaleric acid and did not exhibit properties identical to von Gorup-Besanez' product, it must be either amino-*n*-valeric acid or ethylmethylaminoacetic acid. He synthesized the former from *n*-butylaldehyde ammonia. A comparison between the products of von Gorup-Besanez, of Clark and Fittig, of Schützenberger, and his own amino-*n*-valeric acid was now made. This showed that von Gorup-Besanez' amino acid was not identical with the two synthetic amino acids. The difficulty lay in the fact that the comparison was made from the description of his product furnished by von Gorup-Besanez and not by direct observation of the actual preparation. Moreover, the question of the effect of the optical activity on the properties of the amino acid was again not taken into account.

In 1902, Slimmer (171) published the results of his syntheses of α-aminoisovaleric acid, α-amino-*n*-valeric acid, α-aminomethylethylacetic acid, together with such derivatives of each as the phenylisocyanate, esters and hydantoins. The properties of each compound were carefully determined.

Previous to 1901 valine was known only as a substance occasionally found in gland or plant extracts. Its relationship to the amino acids that result from the hydrolysis of proteins was obvious and had been noted by its discoverer, but the essential step of isolation from a protein hydrolysate became possible only after the development of the ester distillation method. Fischer found α-aminovaleric acid in the ester fractions that distilled at 40–80° at 10 mm. and identified the substance by analysis of the free acid and of its copper salt (161).

Fischer (162) later established the fact that valine is α-aminoisovaleric acid. He prepared the formyl derivative of synthetic *dl*-valine and resolved this into its optically active components by means of brucine. He showed that the optical rotation of his *d*-valine was essentially the same as that reported by Schulze for valine isolated from *Lupinus luteus*, and also found that, like the two optically isomeric leucines, the *d*- and *l*-valine can be distinguished by taste. *d*-Valine is only slightly sweet and at the same time somewhat bitter, while *l*-valine is decidedly sweet. Fischer is responsible for the suggestion that the term valine be used to designate α-aminoisovaleric acid. "Im Einverständniss mit Hrn. E. Schulze schlage ich dafür das Wort "Valin" vor woraus sich für das Radical $(CH_3)_2CH\cdot CH(NH_2)CO$, das in den Polypeptiden enthalten ist, die Bezeichnung "Valyl" ergiebt."

SERINE

$$CH_2(OH)—CH(NH_2)—COOH$$

Although serine is one of the most difficult of all the amino acids to isolate from proteins and is even yet an exceedingly rare substance, it was one of the early amino acids to be discovered. In 1865 Cramer (172) carried out a thorough investigation of raw silk. He isolated the gelatin-like protein found on the surface of the fibroin, named this protein sericine and subjected a purified specimen of 6 grams of it to hydrolysis with sulfuric acid. Concentration of the hydrolysate yielded a crop of tyrosine; "später erschienen Drusen von Erbsengrösse, die aus kleinen harten, etwas süsslich schmeckenden Krystallen zusammengesetzt

waren." A yield of approximately 10 per cent of this material was obtained. Recrystallization gave a preparation that he at first considered to be glycine; the copper content of the copper salt was, however, much too low. "In der That ergab auch die weitere Untersuchung eine durchaus abweichende Zusammensetzung. Ich werde den in Frage stehenden Körper unter dem Namen Serin beschrieben."

Larger quantities of crude sericine were hydrolyzed and more of the substance was secured. A very accurate analysis led to the correct formula $C_6H_7NO_6$, or in modern atomic weights $C_3H_7NO_3$. The new acid therefore differed from alanine by one oxygen equivalent but had many chemical properties in common with Strecker's synthetic substance. It formed soluble salts with mineral acids and with bases and the hydrochloride, nitrate, and sulfate were prepared in crystalline form.

The relation of synthetic alanine to lactic acid was known; according to its formula serine should be similarly related to glyceric acid. This substance was, in fact, obtained by Cramer by the action of nitrous acid on serine and was analyzed as its calcium salt. Cramer further drew the deduction that it should be possible to convert serine to alanine by reduction although he did not attempt the reaction; most significant of all he pointed out that cystine, then known only as a constituent of certain rare urinary calculi, was closely related to the new acid, inasmuch as cystine contains one atom of sulfur in place of one of the oxygen atoms of serine. Cramer's paper is one of the great classics of protein chemistry; for brilliance of conception and execution, for the far-reaching quality of its generalizations and for lucidity of expression it is hardly excelled.

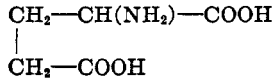
Serine was not encountered again for more than thirty years. Fischer and Skita (181) obtained a fraction, from the amino acid esters of high boiling point derived from the hydrolysis of silk, that was extremely difficult to purify. The results of analysis suggested that oxyacids were present and they thought serine to be a probable constituent. Later (182) they found that the difficulties arose largely from the decomposition of the esters during distillation; this could be largely avoided by conducting the later

stages of the distillation at very low pressure. Under these circumstances they were able to isolate 1.6 per cent of racemic serine from silk fibroin. They also confirmed Cramer's discovery of serine in sericine. Somewhat later Fischer and Dörpinghaus (178) obtained the same substance from horn, thereby showing that serine is not a unique component of silk proteins. Kossel and Dakin (183) obtained it from protamines, and within a few years it became clear that serine is a widely distributed amino acid component of proteins. In 1907 Fischer (177) demonstrated that the serine which results from the hydrolysis of silk is the levorotatory optical isomer and that the inactive products that had hitherto been obtained had been racemized during the process of isolation.

Serine was first synthesized by Fischer and Leuchs in 1902 (180) from glycolic aldehyde by the Strecker cyanohydrin method. A synthesis by the hippuric acid method has been described by E. Erlenmeyer, Jr. (176) and a synthesis from chloracetal, in which the chlorine atom is replaced by an ethoxy group by means of sodium ethylate, and the resulting acetal is hydrolyzed and employed in a cyanohydrin reaction, has been given by Leuchs and Geiger (184). The resolution of racemic serine was accomplished by Fischer and Jacobs (179) in 1906 by chemical methods and later by Ehrlich (174) by means of yeast. Serine was found in human sweat by Embden and Tachau in 1910 (175) and was isolated from an extract from green alfalfa leaves by Vickery in 1925 (185).

Daft and Coghill (173) have recently shown that serine is unstable when heated in strongly alkaline solutions, and is decomposed with the production of ammonia, glycine, alanine, oxalic acid, and lactic acid; pyruvic acid is an intermediate product of the highly complex reaction. This observation has an important bearing on the indirect methods for the analysis of amino acid mixtures.

GLUTAMIC ACID



In 1866 Werther, professor of mineralogy at Königsberg and one of the editors of the Journal für praktische Chemie, reported (192) that Professor Ritthausen had given him, for crystallographic measurement, a specimen of beautifully crystalline material together with the information that the substance had been obtained from the gluten of wheat flour. It was a monobasic nitrogenous acid, "deren Formel nach Analyse der freien Säure, des Baryt- und Kupfersalzes ist $C_{10}H_9NO_8$. Ich nenne sie Glutaminsäure mit Rucksicht auf das Material aus dem sie gewonnen ist."[11] In modern atomic weights this formula is $C_5H_9NO_4$ and is correct.

Ritthausen's paper (190) which soon followed, described the method by which the new acid had been obtained. According to Ritthausen, wheat gluten contains three alcohol-soluble proteins, gliadin, gluten-fibrin and mucedin, which differ in their solubility in alcohol-water mixtures. The later investigations of Osborne showed that gluten-fibrin and mucedin were probably merely fractions of the single alcohol-soluble protein gliadin that is present in wheat. So-called gluten-fibrin, however, was the source of the first glutamic acid. The protein was hydrolyzed with sulfuric acid and the reagent was removed by means of calcium hydroxide. Ritthausen observed that an acid sufficiently strong to decompose calcium carbonate was present. The excess of calcium was therefore precipitated with oxalic acid and the excess of this by means of lead carbonate. The solution was now found to contain a soluble lead salt. The lead was therefore removed as sulfide and the solution was concentrated. Crystals of tyrosine, mixed with another more soluble substance, separated. This was dissolved away from the tyrosine by careful treatment with hot water and, when cooled, this solution deposited gleaming rhombic crystals. When gliadin was treated in the same way a

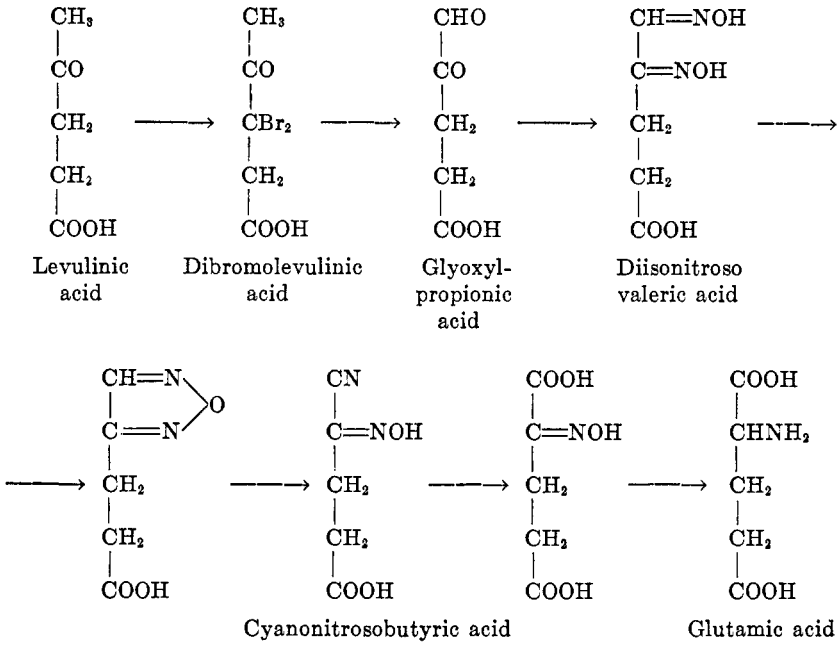
[11] Although this quotation is from Werther's paper it is clear from the context that he is quoting the words of a communication from Ritthausen; the "ich" therefore refers, not to Werther, but to Ritthausen. Proof of this is found in Ritthausen's first paper (190) in which the following words occur, ". . . . ist bereits bekannt dass ich durch Kochen des Klebers mit Schwefelsäure eine neue stickstoffhaltige Säure erhalten habe welcher ich den Namen Glutaminsäure gab."

yield of 30 per cent of the new acid was secured. Salts of barium, copper, and silver were prepared and analyzed; the nitrogen was entirely removed from the new acid by treatment with nitrous acid, and the product, glutanic acid, was recognized as being allied to malic acid. In subsequent publications Ritthausen (191) showed that glutamic acid is present in many other vegetable proteins.

Hlasiwetz and Habermann in 1873 (188) introduced the method of hydrolyzing proteins with hydrochloric acid in the presence of stannous chloride, added to prevent the formation of humin, and showed that glutamic acid can be conveniently isolated as its hydrochloride. They prepared this from casein, the first protein of animal origin shown to yield glutamic acid. As a result of their experiments they drew the deduction that casein yields exclusively leucine, tyrosine, glutamic and aspartic acids, and ammonia, and pointed out that the ammonia, which is an invariable product of protein hydrolysis, probably "von jener, im Casein primär enthaltenen Verbindungen abstammt, welche gleichzeitig Asparaginsäure und Glutaminsäure liefern." They suggested that a close analogy existed between the ammonia derived from proteins and that derived from asparagine and glutamine. "Verbindungen dieser Art welche beim Erhitzen mit Säuren oder Alkalien unter Wasseraufnahme Ammoniak verlieren und diese Säuren liefern, müssen im Casein und den Proteinstoffen überhaupt präexistirend angenommen werden."

The product of the action of nitrous acid on glutamic acid, described by Ritthausen as glutanic acid, was reduced with hydriodic acid by Dittmar (186) to a substance that Markownikoff (189) showed to be identical with the product of hydrolysis of trimethylene cyanide $CNCH_2—CH_2—CH_2CN$. Glutanic acid was therefore an hydroxyglutaric acid and, since it was different from the already known β-hydroxyglutaric acid, it could only be α-hydroxyglutaric acid. Glutamic acid was therefore α-aminoglutaric acid, $HOOC—CH_2—CH_2—CH(NH_2)COOH$. Final proof was secured by Wolff (193) who synthesized glutamic acid from levulinic acid by a most unusual method. Levulinic acid was brominated yielding a product which, when boiled with

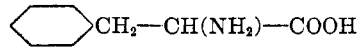
water, yielded glyoxylpropionic acid. This was treated with hydroxylamine, and the product was converted to a furazane derivative by sulfuric acid. This, in turn, was converted to cyanonitrosobutyric acid by sodium hydroxide hydrolysis; further hydrolysis and reduction of this gave glutamic acid.



Curiously enough there appears to be no other recorded synthesis of glutamic acid.

It is noteworthy that glutamic acid appears to be the only amino acid which has found commercial use. The monosodium salt is widely used in the Orient as a condiment (187).

PHENYLALANINE



If the synthesis of phenylaminopropionic acid from bromohydrocinnamic acid and ammonia by Posen (205) in 1879 can be accepted without question, the discovery of phenylalanine must

be simultaneously credited to both Posen and to Schulze and Barbieri (210). Posen obtained a product which, on analysis, yielded figures in agreement with those to be expected from phenylaminopropionic acid. It was slightly soluble in cold water, more so in hot water, and its behavior towards acids and alkalies showed that it possessed amphoteric properties. He stated, however, that it was soluble in alcohol and melted at 120–121°, which suggests benzoic acid rather than phenylalanine. It is quite possible that Posen by mistake carried out his melting point estimation on benzoic acid and not phenylalanine. On account of these discrepancies it is difficult to accept Posen's work although his claim cannot be wholly rejected.

A preliminary note, by Schulze and Barbieri, of the discovery of a new amino acid in the etiolated sprouts of *Lupinus luteus* was published in 1879. They extracted the sprouts with alcohol, added lead acetate, filtered off the precipitate, freed the filtrate from lead and concentrated it. A small amount of leucine, traces of tyrosine, and a considerable amount of asparagine and the new amino acid were obtained. This was partially purified by means of the copper salt. Analysis showed that a substance was present that contained about 10 per cent more carbon than does leucine, but they made no statement as to the possible structure of the new substance.

Two years later Schulze and Barbieri (211) reported the results of more extended investigations of lupine seedlings. An extract was prepared as before, the crystalline mass secured by evaporation was treated with alcohol that contained a little ammonia; asparagine remained undissolved and the alcoholic solution yielded crystals on evaporation. These were recrystallized several times, a crystalline copper salt was prepared, decomposed with hydrogen sulfide and the free acid was again secured. This material was taken through the copper salt stage a second time; the final product then crystallized in plates or leaves, or from dilute solution in hydrated needles. The analysis led to the correct formula $C_9H_{11}NO_2$ and oxidation yielded benzoic acid. Dry distillation gave a product that appeared to be phenylethylamine. "Unsere Amidosäure ist demnach als eine Phenylamidopropion-

säure anzusehen. . . . ." Tiemann (216) had already pointed out the possible existence of aromatic amino acids in the native protein molecule. "Die Bildung von Benzöesäure und Benzaldehyd bei der Oxydation der Eiweisskörper . . . . deuten darauf hin, dass in den Proteinsubstanzen auch Reste von Monosubstitutionsproducten des Benzols vorkommen."

There are four possible isomeric phenylamidopropionic acids which can be represented by the composition of the new substance. The product secured by Schulze and Barbieri melted at 250° which, in view of Posen's findings, excluded the possibility that it might be α-aminophenylpropionic acid. Similarly the possibility that it might be aminohydratropic acid, which melted at 169°, was also excluded. Schulze and Barbieri were inclined to favor the view that their amino acid was a homologue of phenylaminoacetic acid but made no definite commitment at this time.

Schulze and Barbieri observed that, like asparagine, little or no phenylalanine is found in the free state in the seeds of *Lupinus luteus* before germination. "Denn wir wissen, dass während der bei Lichtabschluss stattfindenden Keimung ein beträchtlicher Theil von den Eiweissstoffen der Samen zersetzt wird; das Asparagin, welches in Lupinenkeimlingen in so bedeutender Quantität auftritt, kann nur aus Eiweissstoffen entstanden sein; denn nicht eiweissartige Stickstoffverbindungen finden sich in den ungekeimten Lupinensamen nur in so geringer Menge vor, dass sie nicht entfernt hinreichen, um die im Asparagin sich vorfindende Stickstoffmenge zu liefern; die Annahme, dass auch die neben Asparagin sich vorfindenden Amidosäuren dem Zerfall von Eiweiss entstammen, dürfte also wohl eine sehr wahrscheinliche sein."

The new acid was therefore sought among the products of hydrolysis of lupine seed proteins. Extensive fractional crystallization did, indeed, give fractions that were mixtures of leucine with a substance that yielded benzoic acid on oxidation and the previously found volatile base on dry distillation; there was little doubt, therefore, that the phenylaminopropionic acid originated from the protein of the seed.

Less definitely characterized than the products of Posen and

of Schulze and Barbieri was the tyroleucine obtained by Schützenberger (214) in 1879. He regarded tyroleucine as being a combination of aminovaleric acid and a substance of the formula $C_9H_{11}NO_2$, which is that of phenylalanine. The indefiniteness of Schützenberger's work, however, lays it open to question. Schulze (208) believed that Schützenberger's leucëines may very possibly have included phenylalanine.

A year after the appearance of Schulze and Barbieri's paper, Erlenmeyer and Lipp (198) reported the synthesis of phenyl-α-aminopropionic acid, a term which they shortened to phenylalanine. Phenylacetaldehyde was treated with hydrocyanic acid and ammonia in accordance with the Strecker method and the nitrile was saponified to give phenylalanine.

Schulze and Barbieri (212) were quick to see the possible relationship of the amino acid they had isolated from *Lupinus luteus* to the synthetic product of Erlenmeyer and Lipp. They concluded ". . . . dass sie höchst wahrscheinlich identisch mit der von E. Erlenmeyer und A. Lipp vor kurzem synthetisch dargestellten Phenyl-α-Amido-propionsäure (Phenylalanin) ist. . . . ." In a subsequent publication Schulze and Barbieri (213) were able to isolate phenylalanine from the proteins of squash seed after acid hydrolysis as well as after hydrolysis with barium hydroxide (208). This definitely proved that phenylalanine is a product of the hydrolysis of the protein molecule. They also showed that, on heating phenylalanine, phenyl-lactimide and phenylethylamine were formed. The latter substance was the same as that which Erlenmeyer had found on heating his synthetic phenylalanine. This established the structural identity of the synthetic phenylalanine and the phenylalanine isolated from natural sources. Schulze (209) also showed that phenylalanine together with asparagine, glutamine, leucine, valine, tyrosine, guanidine, choline, and betaine, were present in etiolated vetch sprouts.

The subsequent work on phenylalanine has been chiefly concerned with its synthesis. No other amino acid has received as much attention from this standpoint and no other amino acid has been synthesized in so many different ways as has phenyl-

alanine. For this reason, the various synthetic reactions are given in detail. Erlenmeyer, Jr., (196) converted phenylpyruvic acid into the oxime which, on reduction, yielded phenylalanine. Shortly afterwards, he published a second method (197); benzaldehyde was condensed with hippuric acid in the presence of acetic anhydride. The lactimide was converted into benzoylaminocinnamic acid by hydrolysis. Reduction and subsequent hydrolysis of this yielded phenylalanine. Knoop and Hoessli (204) confirmed this synthesis. They used aluminum amalgam for the reduction. Fischer's (199) method consists in treating benzylmalonic ester with bromine, converting the resultant benzylbromomalonic acid into phenyl-α-bromopropionic acid by heating, and finally treating with ammonia. By treating cinnamic acid with hydroxylamine, Posner (206) obtained α-oxyamino-β-phenylpropionic acid which, on reduction with ammoniacal silver solution, gave phenylalanine. Sörensen (215) used his phthalimide method for the synthesis of phenylalanine. Bromomalonic ester is treated with potassium phthalimide to form phthalimidomalonic ester which is converted, by treatment with sodium alcoholate, into the sodium salt. This compound is converted, by benzyl chloride, into benzylphthalimidomalonic ester; saponification of the ester with sodium hydroxide yields the sodium salt of phthalimidobenzylmalonic acid which, by the action of hydrochloric acid, is split into phenylalanine, phthalic acid and carbon dioxide. Wheeler and Hoffman's (217) synthesis consists in condensing benzaldehyde with hydantoin to form benzalhydantoin; reduction with hydriodic acid and red phosphorus yields the hydantoin of phenylalanine and hydrolysis with barium hydroxide gives phenylalanine.

Wheeler and Hoffman's method was improved by Johnson and O'Brien (203). They treated hippuric acid with potassium thiocyanate to form 2-thio-3-benzoylhydantoin; on condensation with benzaldehyde, 3-benzoyl-2-thio-4-benzalhydantoin was obtained. This compound was hydrolyzed with warm hydrochloric acid which yielded benzalthiohydantoin; on reduction with tin and hydrochloric acid, the ring was broken and phenylalanine obtained.

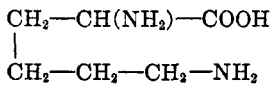
Sasaki (207) has proposed a method which can be used for the synthesis of both phenylalanine and tyrosine. The yield is about 83 per cent. Glycine anhydride and benzaldehyde are condensed in the presence of acetic anhydride and sodium acetate to give 3,6-dibenzal-2,5-diketopiperazine. On reduction with hydriodic acid and red phosphorus, the diketopiperazine ring is split and phenylalanine is obtained. The procedure of Curtius and Sieber (194) is somewhat longer. The potassium salt of benzylmalonic acid is treated with hydrazine to form the hydrazide of benzylmalonic acid. The latter substance is treated with sodium nitrite in the cold; this forms the azide of benzylmalonic acid. On heating the latter compound, nitrogen is split off and the anhydride of phenylalanine-*N*-carbonic acid is formed. On heating with hydrochloric acid, hydrolysis with simultaneous loss of carbon dioxide takes place, yielding phenylalanine. Harington and McCartney (202) have recently improved the method of Erlenmeyer, Jr. (197).

Fischer and Mouneyrat (200) were the first to resolve racemic phenylalanine into its optically active components. This was accomplished by means of the cinchonine salts of the benzoyl derivatives. They showed that the magnitude of the specific rotation of *d*-phenylalanine to the right was essentially the same as Schulze and his co-workers (213) found for the naturally occurring phenylalanine which rotates the plane of polarized light to the left. Later Fischer and Schoeller (201) carried out the resolution by means of the brucine salts of the formyl derivatives. They were also able to convert the ethyl ester of *l*-phenylalanine into the ethyl ester of *d*-α-bromohydrocinnamic acid. This was one of the first examples of the Walden inversion in the field of amino acids. Ehrlich (195) was able to resolve racemic phenylalanine with the aid of yeast, which does not destroy the dextro form. Fischer (199) and Fischer and Schoeller (201) were the first to prepare a series of peptides of phenylalanine and glycine.

The isolation from proteins of a second amino acid that contained an aromatic nucleus was a matter of the greatest importance and the interest that was aroused by the discovery of phenylalanine is reflected in the extent of the work on the synthe-

sis of this substance. It may also be mentioned that, previous to 1901, synthesis was almost the only sure way in which a specimen of phenylalanine could be secured. Schulze's isolation by direct crystallization methods furnishes an impressive example of the great technical skill of this investigator and it is doubtful if pure specimens of this substance were again isolated from natural sources until Fischer developed the ester distillation method of protein analysis. It was Fischer who demonstrated that phenylalanine, far from being an extremely rare substance, is widely distributed in nature.

LYSINE



Hlasiwetz and Habermann (227) in 1873 showed that proteins could be advantageously hydrolyzed by means of hydrochloric acid in the presence of tin. They believed that the decomposition took place smoothly and that the products were leucine, tyrosine, glutamic and aspartic acids and ammonia, with only a small residue of unknown material. Schützenberger (231) in 1879 decomposed proteins by heating them at 150° with barium hydroxide and likewise obtained aspartic and glutamic acids, tyrosine, and leucine, and in addition, a series of substances that he regarded as homologues of leucine. Other substances were found in small amounts but these seemed to be the chief products. Schulze (230) in 1885 pointed out the improbability that only four substances resulted from the hydrolysis of proteins and showed that the newly discovered phenylalanine was likewise a product.

The whole problem of the completeness with which a protein could be accounted for in terms of its products of hydrolysis was discussed by Drechsel (219) in 1889. Drechsel was particularly impressed by some work of Horbaczewski (228) who had accounted for only about 30 per cent of the keratins of horn as definite substances. Furthermore he was intrigued by the carbon dioxide observed by Schützenberger when proteins were heated

with alkali. He satisfied himself that little, if any, carbon dioxide was produced by acid hydrolysis and drew the conclusion, "dass bei der Spaltung der Eiweisskörper durch concentrierte Salzsäure noch andere, bisher noch nicht aufgefundene Produkte entstehen, welche sich in den Mutterlaugen der genannten Amidokörper finden, und wenigstens zum Theil beim Erhitzen mit Barythydrat Kohlensäure liefern müssen." Drechsel therefore repeated the experiment of Hlasiwetz and Habermann; he hydrolyzed casein by long-continued boiling with hydrochloric acid in the presence of stannous chloride, removed the tin, crystallized out as much crude glutaminic acid hydrochloride as possible and then diluted the sirupy mother liquor and treated it with phosphotungstic acid, a reagent long employed for the precipitation of alkaloids and recently used with remarkable success (see arginine) by Schulze in the investigation of plant extracts. The heavy precipitate was removed, washed, and decomposed with barium hydroxide, the excess of this reagent was carefully precipitated and the solution was acidified with hydrochloric acid and concentrated to a sirup; on standing a crystalline substance separated. This was redissolved, the solution was diluted with alcohol, and ether was added. The oil which separated crystallized in part on standing and the crystals were removed, pressed out dry on a porous tile and washed with absolute alcohol. The substance was recognized as the hydrochloride of a strong base since its solution, on treatment with silver carbonate to remove the chloride, was strongly alkaline. The chloroplatinate was therefore prepared, and was found to crystallize from dilute alcohol in long prisms. Analysis of this led to the formula $C_7H_{14}N_2O_2PtCl_6 + 4H_2O$. The mother liquors of the chloride yielded a chloroplatinate different in color from the first and of a composition that agreed with the formula $C_8H_{16}N_2O_2Cl_2 \cdot PtCl_4 + H_2O$. This substance was thought to be homologous with the first base. It is to be noted, however, that Drechsel did considerable violence to the analytical figures in ascribing this formula to it. The most important property of these substances that was recorded in the preliminary paper was the decomposition by means of alkali. The crude chloride was stable when heated

to 150° with acids but, when heated with barium hydroxide to 120°, decomposition took place with the liberation of barium carbonate. This behavior gave a clue to the observations of Schützenberger: "diese Basen sind die oder eine Quelle der Kohlensäure, welche Schützenberger fand."

Drechsel in the following year continued his study of the basic substance derived from proteins (220). In addition to the substance that formed a crystalline chloroplatinate a second base was isolated, as an acid double salt of silver nitrate, of the composition $C_6H_{13}N_3O_2 \cdot HNO_3AgNO_3$. He thought that the base contained one molecule of water of crystallization; it should therefore have the composition $C_6H_{11}N_3O$ which suggested that the new substance was homologous with creatine $C_4H_9N_3O_2$ and creatinine $C_4H_7N_3O$ "und die Vermuthung lag deshalb nahe, dass diese Basen, welche Lysatin bezw. Lysatinin heissen mögen, auch wirklich die Constitution des Kreatins bez. Kreatinins besitzen möchten." As a demonstration that this view was correct, Drechsel subjected the new substance to a short hydrolysis with barium hydroxide and then isolated urea from the products of the decomposition. Although urea and guanidine had previously been obtained from proteins in small amounts by oxidation, this was the first time that urea had been obtained from a protein by purely hydrolytic processes. The name lysatine (λύσις, loosing), applied by Drechsel to his new substance, illustrates the importance he attached to this fact.

The meaning of these preliminary observations did not become clear for some time. At least two different basic substances were present and one of these had been isolated in pure form, but the properties of the other substance were so confusing that several years elapsed before a full explanation was forthcoming. As will appear, the development of the chemistry of the basic amino acids depended entirely on the selection of the right reagent to employ at each stage. Drechsel made an immense advance when he first thought of applying phosphotungstic acid to the investigation of the products of protein hydrolysis, but the separation of the mixture of bases precipitated by this reagent required years of study.

The next publication from Drechsel's laboratory appeared early in 1891 (232). Drechsel had suggested to Siegfried that he should investigate other proteins than casein to see if the new basic substances were to be found in them. Conglutin, gluten-fibrin (probably gliadin), "hemiprotein," "oxyprotsulfonic acid," and egg albumin were therefore studied. These were commercial preparations probably secured from the firm of Grübler. In the course of the investigation of conglutin Siegfried made an extremely important observation which was later, in the hands of Kossel, to serve as the key to the whole problem of separating the basic amino acids. He said, "Ohne vorherige Fällung mit Phosphorwolframsäure würde man durch Silbernitrat aus den Zersetzungsproducten der Eiweisskörper durch Salzsäure nicht lediglich Asparaginsäure fällen, da, wie ich weiter unten zeigen werde, sich stets eine durch Phosphorwolframsäure fällbare Base vorfindet, welche ebenfalls ein in Wasser unlösliches amorphes Silbersalz bildet." He did not, however, work on this amorphous silver compound but studied the substances precipitated by phosphotungstic acid. His use of silver nitrate was, however, a direct outcome of Drechsel's observation that the base lysatine formed a crystalline double salt with this reagent; Drechsel was therefore the first to employ silver as a reagent in this field.

Siegfried isolated the base that formed a crystalline chloroplatinate and showed that it had the composition $C_8H_{22}N_2O_3Cl_6Pt$. When this salt was decomposed with hydrogen sulfide and the resulting hydrochloride was boiled with barium hydroxide, neither carbonate, acetic acid, oxalic acid, nor alcohol was produced; the preparation was therefore free from lysatine. After removal of the barium, the hydrochloride was obtained in crystals of the composition $C_6H_{14}N_2O_2 \cdot 2HCl$ and this preparation, treated with chloroplatinic acid yielded a substance identical with the initial chloroplatinate. It was obvious therefore that the two extra carbon atoms in this salt represented alcohol of crystallization and that its composition should be expressed $C_6H_{14}N_2O_2 \cdot C_2H_5OH \cdot H_2PtCl_6$. That this was the case was shown by a demonstration of alcohol in the crystalline salt. Siegfried secured specimens of the same salt from each of the

materials he investigated and it became clear that the new base was widely distributed in nature.

Drechsel and his assistants continued the investigation in a long paper (221), the greater part of which appeared under Drechsel's name, but sections of which were furnished by Siegfried, by Ernst Fischer, and by S. G. Hedin. A large quantity (10 kilograms) of casein was hydrolyzed and the basic substances were precipitated by phosphotungstic acid. The volatile base in the precipitate was shown to be ammonia, and the solution of the fixed bases was neutralized with hydrochloric acid. From this an impure chloride was obtained which did not give a satisfactory analysis; it was therefore recrystallized from concentrated hydrochloric acid, whereby a dichloride was obtained that analyzed sharply for a diaminocaproic acid. The monochloride was found to be only slightly acid to litmus. The formula $C_6H_{14}N_2O_2$ was deduced for the free base, and Drechsel pointed out that this was homologous with ornithine and that the substance itself had certain properties in common with ornithine. The name lysine is first employed as a designation for this base in the section of the paper by Ernst Fischer. He refers in the last paragraph to "die Base $C_6H_{14}N_2O_2$, welche mit dem Namen Lysin bezeichnet werden mag," and goes on to point out that it can be obtained by alkaline hydrolysis of gelatin under pressure and must therefore have been present in the solutions investigated by Schützenberger.

The last section of the paper was written by S. G. Hedin and describes the isolation of lysine chloroplatinate from a pancreatic digest of fibrin. Hedin showed that some of the same substance was formed by autolysis of the pancreas powder he employed, but that the much larger quantity isolated from the digest established the fact that lysine is a product of the digestion of fibrin. He could not, however, obtain more than traces of the second base, lysatine.

A final paper on lysine from Drechsel (222) recorded his attempt to crystallize the free base; the solid material secured was, however, a carbonate. Crystalline lysine was first prepared by Vickery and Leavenworth (234) in 1928. Drechsel's paper con-

tains the suggestion that lysine is a derivative of pentamethylenediamine; this was demonstrated to be the case, in 1899, when Ellinger (223, 224) showed that pentamethylenediamine could be obtained from lysine by anaerobic putrefaction. He suggested that the most probable constitution was expressed by the formula

$$NH_2CH_2—CH_2—CH_2—CH_2—CH(NH_2)—COOH$$

although the position of the carboxyl was not certainly established. Henderson (226) pointed out that the carboxyl could not be on the middle carbon atom because of the optical activity, and his demonstration that acetic and propionic acids were formed by alkali fusion strongly supported the view that the carboxyl was at the end of a straight chain. The synthesis of lysine by Fischer and Weigert (225) from γ-cyanopropylmalonic ester by treatment with nitrous acid followed by reduction finally settled the matter. Other syntheses have since been described by Sörensen (233) and by von Braun (218).

The importance of lysine in animal nutrition was first demonstrated in 1914 by Osborne and Mendel (229), who found that this amino acid behaves as a limiting factor on growth. Animals did not grow when the lysine-deficient gliadin formed the chief protein of the diet. When small doses of lysine were added to the diet, however, normal growth occurred. Without this addition animals could be maintained for long periods at approximately constant weight.

ARGININE

$$\begin{array}{l} CH_2—CH(NH_2)—COOH \\ \quad | \\ CH_2—CH_2—NH—C(NH_2)=NH \end{array}$$

In 1886 Schulze and Steiger announced their discovery of a new basic substance in extracts from etiolated lupine seedlings in a paper (244) that is a model of what such papers should be; concise and clear, each statement supported by exact experimental work and competent analysis that leaves no doubt as to the accuracy of the findings.

Schulze had long known that phosphotungstic acid gave white precipitates when added to plant extracts and also that these precipitates contained nitrogen. Among the substances in them he had recognized what he called peptones, since they gave a precipitate with tannic acid and also responded to the biuret test. These early observations had not been followed up owing to the pressure of other problems, and also because Schulze had felt that it would be unusually difficult to isolate definite substances from these precipitates.

Schulze and Steiger prepared a water extract of etiolated lupine seedlings, treated this successively with tannic acid and with lead acetate, removed the precipitates and, after acidifying with sulfuric acid and removing the lead sulfate, added an excess of phosphotungstic acid. The precipitate was decomposed with calcium hydroxide and the excess of this was removed as carbonate. The alkaline solution was neutralized with nitric acid and when evaporated to a thin sirup, "so krystallisirt aus derselben das salpetersaure Salz einer Base, welcher wir den Namen Arginin beilegen wollen." The nitrate had the composition $C_6H_{14}N_4O_2 \cdot HNO_3 \cdot 1/2H_2O$ and this formula was supported by analyses of the hydrochloride and of the insoluble copper nitrate double salt. The new base could be precipitated at neutral or alkaline reaction by mercury salts and the addition of sodium carbonate was especially advantageous. The yield of arginine obtained amounted to between 3 and 4 per cent of dry weight of the cotyledons. The substance could not be identified with any previously known but, in some points, it resembled creatinine.

A second paper (245), in which a description of a number of other compounds of arginine was given, soon followed. The new base was stable when heated with acids—even hydriodic acid did not affect it—but it was slowly decomposed by alkalies with the formation of carbon dioxide and ammonia and of a strongly basic substance which yielded a crystalline chloride and sulfate.[12]

Arginine contained about one-third of its nitrogen in a form

[12] This substance was identified in a later paper (246) as ornithine by means of its dibenzoyl compound, ornithuric acid, discovered many years before by Jaffé (239).

readily removed by nitrous acid; it contained neither sulfur nor phosphorus; it yielded precipitates with most of the so-called alkaloid reagents; it was present in the sprouts of squash seeds. Schulze was of the opinion that the new base arose from the decomposition of the protein of the seed during germination, and Drechsel's discovery of basic substances among the products of acid hydrolysis of proteins caused him to return to the study. The amount of arginine produced during the sprouting of lupine seeds was shown to exceed (242) the equivalent of the non-protein nitrogen of the seed. Protein must therefore have been converted, in part at least, into arginine during the germination process. This observation at once associated the new basic substance with proteins.

The next step was the demonstration that arginine could be decomposed by alkali with the production of urea. Schulze and Likiernik (243) pointed out that Drechsel's discovery of the two new bases, lysine and lysatine, among the products of hydrolysis of proteins greatly increased the significance of the relationships of basic substances to the chemistry of natural processes, and their observation that arginine, like lysatine, yielded urea when heated with alkali was a matter of the greatest importance. "Da Lysatin und Lysin in den argininhaltigen Keimpflanzen nicht enthalten zu sein scheinen, so darf es wohl für wahrscheinlich gelten, dass diejenigen Atomgruppen im Eiweissmolekül welche beim Kochen der Eiweissstoffe mit Salzsäure die eben genannten Basen liefern, bei dem im Keimpflanzen erfolgenden Eiweisszerfall zur Bildung des Arginins verwendet werden." Thus, as early as 1891, Schulze associated arginine with Drechsel's new base.

The discovery of new amino acids has again and again been a matter of learning to use a reagent in a new way; the isolation of arginine from proteins is an excellent example of this. Siegfried's paper (249) on the isolation of lysine from conglutin, which appeared just before the above mentioned papers of Schulze, contains a number of references to the behavior of protein hydrolysates, and the basic fractions therefrom, when silver nitrate is added. It will be recalled that lysatine was isolated by Drechsel

and by Siegfried as an acid silver nitrate double salt. It will be interesting to see how narrowly, in 1891, the Leipsic group missed adding the discovery of arginine and histidine to their brilliant discovery of lysine. Arginine forms an amorphous silver compound that is soluble in acid but is entirely insoluble in strongly alkaline solutions. Siegfried added silver nitrate to the solution of the free bases obtained from protein hydrolysates by precipitation with phosphotungstic acid; this solution was, of course, strongly alkaline. A voluminous precipitate formed and the reagent was added until no further material separated; the precipitate was removed and was found to contain no nitric acid. Nitric acid equivalent to the silver in the precipitate had therefore been added to the filtrate. The filtrate was then evaporated to a sirup; on standing a sticky, partially crystalline precipitate, which contained lysine, separated. The filtrate was then treated with alcohol when the *acid* silver nitrate double salt of lysatine, $C_6H_{13}N_3O_2 \cdot HNO_3AgNO_3$ began to separate. "Sobald sich einige dieser Krystalle bilden, giesst man die Lösung ab und spült das Gefäss und den am Boden festsitzenden schmierigen Niederschlag mit absolutem Alkohol ab. Durch Zusatz von Aether bewirkt man das vollständige Auskrystallisiren des Silberdoppelsalzes." This technique was hardly calculated to produce a pure substance yet the material was regarded, after a second precipitation in the same way, as pure lysatine.

The amorphous silver precipitate that separated first was apparently neglected; Siegfried carried out some analyses and obtained figures that suggested the formula $C_{11}H_{17}N_6O_6Ag_3$. A quantity of it secured from egg albumin was decomposed with hydrochloric acid and the resulting solution, after evaporation and treatment with alcohol and ether, yielded prismatic crystals that analyzed for $C_{11}H_{20}N_6O_6 \cdot 2HCl$. As will appear later this was almost certainly impure histidine, while the solution from which he separated his lysatine would have yielded crystalline arginine silver nitrate if he had hit upon the correct way to treat it.

Another member of the Leipsic group, Ernst Fischer (235), had prepared lysatine from gelatin, had heated the product with baryta until the urea that it produced was decomposed, and had

then isolated lysine from the resulting solution as a crystalline chloroplatinate. Unfortunately, however, instead of crystalline lysatine he had used a crude mixture for this experiment, and the logical conclusion that lysatine either contained, or yielded, lysine escaped him.

The third junior member of the group, S. G. Hedin, was familiar with these experiments. He had been assigned the problem of isolating lysine and lysatine from tryptic digests of fibrin. He had succeeded in preparing lysine but had failed to obtain lysatine. His preparation, after three crystallizations from alcohol and ether, separated in white needles, the appearance, behavior, and manner of preparation of which appeared to indicate that the compound must be the silver nitrate double salt of lysatine. "Indessen gaben die Analysen keine mit den von der Formel verlangten völlig übereinstimmenden Werthe. Zur weitere Reinigung des Salzes fehlte mir vorläufig an Material, doch gedenke ich die Versuche baldmöglichst wieder aufzunehmen und diesen Punkt klarzustellen."

This failure evidently disturbed him and, after returning to Lund, he repeated the work. In June 1894 he presented a paper (236) on a hydrolysate of horn in which he showed that a compound of the composition of lysatine silver nitrate could be obtained. It separated as crystals mixed with considerable oily material. The oil was separated, freed from silver and treated with chloroplatinic acid. Nothing but oil could be brought to separate. The new oil was removed, freed from platinum and the solution of the chloride, after evaporation to a sirup, was treated with alcohol and ether. The oily precipitate was removed and again treated with chloroplatinic acid. This time it crystallized and the product was immediately recognized as lysine chloroplatinate. Crude lysatine therefore contained lysine.

This gave Hedin an idea. He repeated the work on a larger scale and added silver nitrate to the base fraction as before, removed the amorphous silver precipitate and concentrated the solution to a thin sirup, *but did not add alcohol,* evidently feeling that alcohol precipitated too much. A crystalline substance slowly separated which was recrystallized from water and an-

alyzed. It gave figures for $C_6H_{14}N_4O_2 \cdot AgNO_3 \cdot 1/2H_2O$. This was new; no compound hitherto secured from proteins contained as many as four nitrogen atoms to six carbon atoms. He carried the salt back through the phosphotungstate to free base, prepared the same silver salt from this again, and also observed that the mother liquor contained a more soluble salt that crystallized in needles and had the composition $C_6H_{14}N_4O_2 \cdot AgNO_3 \cdot HNO_3$. On removal of silver, the nitrate $C_6H_{14}N_4O_2HNO_3 \cdot 1/2\ H_2O$ was obtained. From this a double salt with copper nitrate that crystallized with three molecules of water of constitution was secured. This clinched the identity; the substance was Schulze's arginine. Analyses and properties of copper salt and nitrate were identical.

The following year Hedin reported (237) the results of a vast amount of work. He showed that arginine formed two salts with nitric acid, a mono- and a di-nitrate; he prepared the monochloride but could not make the dichloride crystallize. A specimen of arginine, secured from Schulze, was shown to yield these salts as well as his two new silver double salts. Furthermore, he demonstrated that arginine from protein yielded carbon dioxide when heated with barium hydroxide, although he did not succeed in isolating enough urea from the reaction mixture for positive identification through analysis.

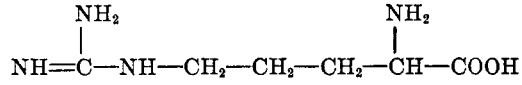
He showed that when silver nitrate is added to the alkaline solution of the bases, inasmuch as this always contained carbonate, silver carbonate was precipitated, thereby liberating nitric acid. Furthermore, the amorphous silver precipitate contained "andere anwesenden, nicht näher bekannten Basen als Silberverbindungen," but no nitric acid; the removal of silver by the precipitation of this material thereby set free still more nitric acid. Consequently the crystallization of the double silver salt of arginine could only proceed incompletely. By neutralization of the liberated nitric acid, however, a much more complete crystallization could be secured. Hedin investigated a number of proteins and found arginine in them all. In each case he observed that the mother liquor of the insoluble silver double salt contained the silver salt of another base.

A few months later his investigation of this was complete and

an explanation of the whole lysatine problem was reached (238). The solution of the bases derived from casein was treated with silver nitrate, the amorphous silver precipitate was removed and the filtrate was treated with barium hydroxide until brown silver oxide began to precipitate. Carbon dioxide was then passed in, the precipitate was removed and the filtrate was concentrated. Barium nitrate crystallized first from this alkaline solution and was followed by the silver nitrate double salt of arginine in small amount. Further evaporation brought about the separation of a mass of white crystals which, however, could not be satisfactorily purified by recrystallization. The addition of alcohol did not help. Hedin had observed that arginine forms two compounds with silver nitrate, a relatively insoluble double salt that crystallized with one-half molecule of water from somewhat alkaline solutions, and a much more soluble acid salt that crystallized with one molecule of nitric acid. The mother liquors of the arginine silver nitrate obviously contained another base which forms a silver nitrate double salt. In order to see if it also formed an acid silver nitrate double salt he added enough nitric acid to give a weakly acid reaction and then added alcohol. A crystalline substance immediately separated which, after recrystallization from dilute alcohol with the aid of ether, turned out to be the acid silver nitrate double salt of lysine. The same salt was readily prepared from lysine chloroplatinate. This cleared up the whole situation. Both arginine and lysine form two silver salts. The silver nitrate double salt of arginine is relatively insoluble and is easily prepared. The acid silver nitrate double salt of arginine is much more soluble. The acid silver nitrate double salt of lysine is a decidedly soluble salt but its neutral, or rather alkaline, silver nitrate double salt is very soluble. The pairs of compounds are analogous but the solubilities are in reversed order. When both bases are present in a solution together with silver nitrate, and the solution is treated with alcohol and ether as had been the custom, a mixture of the two less soluble salts separated, namely arginine silver nitrate and acid lysine silver nitrate. The product that had been obtained by Drechsel, Siegfried, Fischer, and himself and

recognized as lysatine silver nitrate had a composition almost exactly half-way between that of the arginine and lysine silver nitrates. "Wohl geht es aus meinen Versuchen nicht unzweideutig hervor, dass das Lysatinin als chemisches Individuum nicht existirt, aber so viel scheint doch bewiesen zu sein, dass das Lysatininsalz, nach üblichen Methoden dargestellt, beträchtliche Mengen Arginin und Lysin enthalten muss." Thus Hedin gently buried the mistake of his distinguished teacher.

Arginine rapidly assumed importance in protein chemistry. Kossel demonstrated (240) that it was unusually plentiful in the basic protein-like substances he had prepared from fish sperm. In 1897 Schulze and Winterstein (246, 247) found that arginine, when heated with alkalies, yielded ornithine in addition to urea. This was isolated as its dibenzoyl compound, the ornithuric acid (*ὄρνιθος*, bird; *urina*, urine) of Jaffé (239). They therefore suggested that arginine probably had the constitution,



and that a substance of this constitution could probably be obtained by the action of cyanamide on ornithine. That this was the case was shown in 1899 (248), although the position of the guanidino group was not established by this synthesis. It was merely assumed that this group was attached to the nitrogen atom in the δ-position.

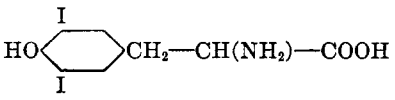
Definite proof of the structure of arginine was secured only in 1910. Sörensen (250) found that ornithuric acid, when hydrolyzed by acid, gave δ-benzoylornithine, but when hydrolyzed by alkalies it gave α-benzoylornithine. The condensation of cyanamide with these isomeric substances, followed by hydrolysis of the benzoyl group with strong acid, gave two isomeric basic substances of which the product from α-benzoylornithine was identical with racemic arginine.

The story of arginine would be incomplete without reference to Kossel's return to the subject a few years before his death. In 1924 Kossel and Gross (241) made the brilliant observation that

arginine forms a highly insoluble and beautifully crystalline compound with 2,4-dinitro-α-naphthol-7-sulfonic acid, or flavianic acid, as they suggested that this compound might more conveniently be called. This observation has led to a complete revision of Kossel's earlier methods for the isolation and estimation of arginine and has rendered it possible to obtain figures of a hitherto unapproached accuracy for the proportions of arginine yielded by proteins.

Kossel was led to investigate the possibilities of flavianic acid as a reagent for bases by some observations he had made as a young man on the capacity of naphthol yellow-S, the sodium salt of flavianic acid, to stain the nuclei of the sperm cells of fish. The chemical investigation of these sperm cells provided him with a full life-time of valuable work, but as an old man he returned to the study of the staining reaction; the result was a fitting climax to a distinguished career.

IODOGORGOIC ACID (3,5-DIIODOTYROSINE)



Iodine has been known, at least since 1848 (264),[13] to be a constituent of certain marine organisms, but its significance in physiology has been appreciated only since the winter of 1895–6 when the reports of two fundamental observations were published. Baumann (251) discovered iodine in the thyroid gland of animals, and Drechsel (253) isolated a new iodine-containing amino acid from the products of alkaline hydrolysis of the horny skeleton of the coral *Gorgonia Cavolinii*. These discoveries led to many investigations in two widely separated fields of biochemistry; recently, however, the isolation of Drechsel's substance from hog thyroids by Harington and Randall (255), and from partially purified thyreoglobulin by Foster (254), has brought these differ-

[13] It was known to Mulder in 1844, since a reference to some investigations of Crookewit on iodine in sponges occurs on p. 329 of Vol. I of Mulder's *Versuch einer allgemeinen physiologischen Chemie*, Braunschweig (1844), translated by H. Kolbe.

ent lines of investigation together and has enormously increased the importance of the curious substance first isolated from a somewhat rare Gorgonian coral.

Drechsel spent a short time in the autumn of 1894[14] at the Marine Zoological Station at Naples and occupied himself with the investigation of a small coral. The axial skeleton appeared to be of a horn-like nature, and he therefore subjected it to hydrolysis with hydrochloric acid, in which it soon dissolved. On continuing the heating, vapors of iodine were evolved from the solution in considerable quantities and in a manner that suggested the gradual decomposition of some unstable iodine-containing substance.

After having satisfied himself of the protein nature of the material by the isolation of lysine, tyrosine, and leucine from the acid hydrolysate, he proceeded to the investigation of the iodine compound. The dry crude protein contained 7.89 per cent of iodine and, since the proportion of iodine was greater than the proportion of ash, the iodine was known to be present in organic combination. No organic iodine compound was known to Drechsel that decomposed in a manner analogous to this substance and, in order to see if the iodine were present as a periodate which was decomposed in the presence of organic matter, he subjected the coral skeleton to hydrolysis with barium hydroxide in the hope that the barium iodate or periodate might be isolated. None could be found. The hydrolysate was therefore freed from barium and treated with silver nitrate. The precipitate which formed was flocculent and partially soluble in dilute nitric acid, and this solution, when boiled a few minutes with a drop or two of strong nitric acid, yielded a copious precipitate of silver

[14] Drechsel's paper (253) appeared in January, 1896, and must, therefore, have been written in 1895. Its first sentence is, "Während der Zeit von Mitte September bis Mitte October vorigen Jahres hatte ich Gelegenheit, im chemischen Laboratorium der zoologischen Station in Neapel einige Versuche anzustellen." The work was therefore done in 1894. Evidence of the correctness of this is found in Hundeshagen's paper (258) which was published August 15, 1895. In it he mentions a private communication from Drechsel that referred to the isolation from a sea animal of an iodine-containing amino fatty acid, the description of which was shortly to be published.

iodide. The organic iodine compound could therefore be precipitated by silver nitrate and was decomposed by hot nitric acid.

The isolation was effected by precipitation from the alkaline hydrolysate with an excess of silver nitrate; the precipitate was treated with cold nitric acid and filtered from the residue of silver sulfide and iodide and was then neutralized with ammonia. The grey precipitate was decomposed with the minimal necessary amount of hydrochloric acid and the dark solution, on evaporation, yielded an oily residue that slowly deposited crystals. By the careful addition of a little ether a white powdery mass was caused to separate that was filtered off, washed, and recrystallized from hot water. The substance was insoluble in acetic acid but soluble in alkalies and in acids; it formed a crystalline hydrochloride that was hydrolyzed by water; its silver compound was soluble in ammonia and in dilute nitric acid, but was decomposed with the liberation of silver iodide when boiled with excess of nitric acid. Carbon, hydrogen, and iodine determinations were made; these agreed, although very poorly, with the formula $C_4H_8NIO_2$. "Die Formel $C_4H_8NIO_2$ ist die einer Amidojodbuttersäure; bis ihre Constitution sicher erkannt ist, will ich die Substanz einstweilen als Jodgorgosäure bezeichnen."

This was the first organic iodine compound to be isolated from animal material. Drechsel considered it almost certain that the substance was a product of hydrolysis of what he called "ein jodirtes Albuminoid."

There was one important difference between the behavior of the isolated substance and the protein; whereas the latter yielded free iodine on boiling with hydrochloric acid, the crystalline substance was stable. Drechsel pointed out, however, that the instability of the protein might be associated with the presence of oxidizable organic substances during acid hydrolysis of the protein; "vielleicht entsteht ursprünglich aus dem Gorgonin eine Substanz, die der Jodoso oder Jodobenzöesäure V. Meyer's analog zusammengesetzt ist."

Drechsel refers in his paper to a private communication from Hundeshagen in which was described an investigation of the chemical nature of a number of sponges. Hundeshagen had

analyzed many species for iodine and had found several that contained more than 10 per cent of this element. He had observed the behavior of the sponge skeleton towards acids and alkalies, and had also found that the iodine compound was precipitated by silver nitrate from a neutral solution of the products of alkaline hydrolysis. He had not, however, been able to isolate it. Hundeshagen drew one very important deduction from his results. This was stated by Drechsel in the following words, "Seine silberhaltigen Lösungen zeigten das beschriebene Verhalten und gaben mit Millon's Reagens eine rothe Färbung; er hält es daher für möglich, dass die jodhaltige organische Verbindung Jodtyrosin ist, und die Substanz der Jodspongien ein jodirtes Spongin."

Hundeshagen published his observations (258) during the long interval that elapsed between Drechsel's investigation in 1894 and the appearance of his paper in 1896. He mentioned the receipt of a private communication from Drechsel regarding the isolation of iodogorgoic acid. From the standpoint of date of publication, therefore, Hundeshagen was the first to describe the chemical behavior of the iodine compound derived from the horny skeleton of certain marine animals; Drechsel was the first to isolate it.

It is curious that Drechsel did not follow up Hundeshagen's brilliant suggestion regarding the constitution of the iodine compound. The analysis of the crystalline preparation was hopelessly inaccurate but it should be remembered that he was a guest in a foreign laboratory and that he accomplished the isolation of cystine from the liver of a dolphin, the isolation of lysine, tyrosine, and leucine, and the discovery of a new amino acid, all in one month. There was probably little time to check the analysis and, moreover, he had only 0.34 gram of purified substance.

In 1903 Henze (256) took up the study of the protein of *Gorgonia Cavolinii*. Tyrosine, lysine, and arginine were isolated from this material after acid hydrolysis and its protein nature was placed beyond question. Henze had difficulty in repeating Drechsel's preparation of iodogorgoic acid but, by a slight modification, finally secured a small specimen. He pointed out that its formula as a butyric acid derivative was not supported by the analysis of Drechsel; moreover it gave a positive xanthoproteic

test which indicated that it had an aromatic structure; accurate nitrogen and iodine determinations set Drechsel's formula aside at once (nitrogen 3.78 per cent found, 6.11 calculated; iodine 57.3 per cent found, 55.46 calculated). Henze observed that the substance did not give Millon's reaction, but shrewdly noted that *ortho*-substituted tyrosine derivatives likewise fail to give this test.

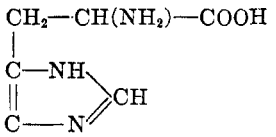
In 1905 Wheeler and Jamieson (265) synthesized 3,5-diiodotyrosine from *l*-tyrosine and arrived at the conclusion that their product was identical with those of Drechsel and of Henze. Henze in 1907 repeated their work (257) but could not reconcile the solubility and crystalline form of the synthetic substance with that of iodogorgoic acid derived from coral. It occurred to him, however, that iodogorgoic acid was prepared by alkaline hydrolysis and was optically inactive. He therefore racemized tyrosine, iodated it, and found that the product was identical with iodogorgoic acid in all respects. It is to be noted, however, that the natural substance is almost certainly optically active and derived from *l*-tyrosine; Wheeler and Jamieson's synthetic product, therefore, probably represents the real protein constituent.

Oswald demonstrated in 1909 (261) that the iodine of iodogorgoic acid is frequently extensively removed by enzymatic digestion and in this way resembles the iodine of the globulin of the thyroid gland. "Hiermit ist eine weitere Stütze dafür gewonnen dass das Jod im Schilddrüseneiweiss in ähnlicher Weise gebunden ist, wie im Dijodtyrosin, bezw. an Tyrosin gebunden, wenigstens teilweise, darin vorkommt." Later he succeeded (262) in isolating iodogorgoic acid from an alkaline hydrolysate of artificially iodinated protein thereby demonstrating that a part, at least, of the iodine that can be introduced into proteins attaches itself in the 3,5 position to the tyrosine.

Wheeler and Jamieson (265) had shown that synthetic diiodotyrosine does not give Millon's reaction. Oswald pointed out that this reaction is not given by diiodotyrosine solutions after a considerable part of the iodine has been set free by means of enzymes; he therefore suggested that the effect of the enzyme was to replace the iodine atoms by hydroxyl groups.

Wheeler and Mendel (266) in 1909 isolated iodogorgoic acid from the skeleton of the common sponge and Sugimoto (263), working in Mendel's laboratory, has recently prepared it from the American Gorgonian coral *Plexaura flexuosa*. There is little doubt that this amino acid is widely distributed in marine organisms; the extensive investigations of Hundeshagen (258), of Mendel (259), of Cook (252), and of Mörner (260) have shown that iodine is present in considerable proportions in a great many species. The demonstration that iodogorgoic acid occurs in the protein of mammalian thyroid glands shows that much more importance must now be attached to this amino acid than has formerly been customary.

HISTIDINE



In 1874 Miescher (274) observed that a large proportion of the dry weight of the sperm of the Rhine salmon consisted of a "nuclein" that was combined, in the sperm, "in einer unlöslichen, salzartigen Verbindungen mit einer organischen Base, dem Protamin." This basic substance could be secured from an acid extract of the material by precipitation with chloroplatinic acid or by mercuric nitrate. The new base contained a high proportion of nitrogen, the ratio of nitrogen to carbon atoms being approximately 5:9. Miescher was chiefly interested in the physiological aspects of the problem and he therefore invited Piccard to continue the investigation. Piccard (276) found that Miescher's original preparation of the chloroplatinate probably contained guanine and hypoxanthine; he also suggested improvements in the method to obtain protamine.

No further investigation of this substance was made until 1894, when Kossel observed that protamine yielded a precipitate with solutions of soluble proteins and that these precipitates had a number of properties in common with the histones. Kossel (272)

followed up this observation and, in the search for a more convenient source of protamine than the Rhine salmon, he investigated the sperm of the sturgeon. An analogous basic substance was found in this material; the meaning of Miescher's term protamine was therefore extended to cover the class of substances and the specific designations sturine and salmine were applied to the two individuals then known.

The characteristic property of these substances was that, after hydrolysis with strong acid, a large part of the products of hydrolysis could be precipitated by phosphotungstic acid. Protamines therefore yield a large proportion of strong organic bases. Kossel subjected sturine to hydrolysis with sulfuric acid, removed the reagent quantitatively with barium hydroxide, and added mercuric chloride to the strongly alkaline solution. The precipitate "enthält eine bisher unbekannte Base, für welche ich die Namen Histidin vorschlage" (ἱστός, tissue). The chloride of the new base, on analysis, gave results that agreed either with $C_{12}H_{20}N_6O_4 \cdot 2HCl \cdot 2H_2O$ or with $C_6H_9N_3O_2 \cdot HCl \cdot H_2O$. The determination of the molecular weight gave a figure intermediate between the requirements of these formulas. The free base crystallized readily from mixtures of water and alcohol, or even from water; its aqueous solution was alkaline. Measurements of the crystals of the chloride were carried out by Bauer and were communicated to Kossel in October 1895.

The results of this work were reported in the proceedings of the Prussian Academy of Science on April 9, 1896. On May 11, 1896 the editors of the Zeitschrift für physiologische Chemie[15] received a paper from S. G. Hedin (268) in which the isolation of a new base from protein hydrolysates was described. Hedin, in the course of his work on arginine, had accumulated a considerable amount of the amorphous precipitate which formed when silver nitrate was added to a solution of the free bases derived from proteins. This precipitate was decomposed by hydrogen sulfide and a solution was secured that yielded a non-crystallizing sirup when concentrated. It contained, as an impurity, a substance that

[15] Baumann and Kossel. Baumann died November 5, 1896.

liberated hydrogen sulfide when heated with alkali. The lead-blackening sulfur compound could be removed by precipitation with lead acetate and ammonia, and the filtrate, after removal of lead, was treated with silver nitrate. This solution was, of course, acid with acetic acid derived from the lead acetate and no precipitate separated. Careful addition of ammonia, however, brought down a voluminous amorphous precipitate which was soluble in excess of this reagent.

It is to be noted that Hedin employed at the very first the method which has since been shown to be by far the best for the isolation of histidine. The silver precipitate was decomposed by the minimal amount of hydrochloric acid, silver chloride was removed and, when the solution was decolorized and evaporated, a crystalline chloride separated which gave an analysis in excellent agreement with the formula $C_6H_9N_3O_2 \cdot HCl \cdot H_2O$. The free base was secured by the use of silver sulfate and was crystallized from aqueous alcohol in plates and needles that dissolved in water to form a very faintly alkaline solution. The results of an analysis agreed with the formula $C_6H_9N_3O_2$ and a determination of the molecular weight by the freezing point method gave 155.4 (theory, 155). The material was obviously purer than that of Kossel, who reported that the aqueous solution of the new base was strongly alkaline. This discovery was a logical outgrowth of Hedin's previous work on arginine and it was the merest chance that Kossel's brilliant discovery should have preceded his by only a few weeks. Hedin pointed out that the new base corresponded very closely in composition with a preparation secured by Siegfried (279) when he first encountered the amorphous silver precipitate. Siegfried had prepared a chloride from this material that gave figures, on analysis, corresponding to $C_{11}H_{20}N_6O_6 \cdot 2HCl$. If this formula were written $C_{12}H_{22}N_6O_6 \cdot 2HCl$, as it might have been without doing too much violence to the analytical results, and then halved to $C_6H_{11}N_3O_3 \cdot HCl$, a formula is obtained that differs very little from the correct one suggested by Hedin.

The new base was obviously closely related to the base histidine described by Kossel and, in a paragraph at the end of his paper, Hedin referred to the close similarity of the measurements of the

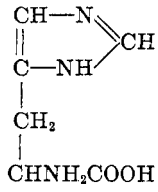
crystal angles of the chlorides of the two preparations. There were small differences, however, in the ultimate composition and in the behavior, which prevented complete identification of the two.

The simultaneous discovery of histidine in two widely separated laboratories in the course of work that originated from two totally different lines of research is of great interest. Hedin hit upon a method of isolation better than that of Kossel; his preparations were purer and his analytical work was superior. Moreover, the investigation was the logical development of observations on the behavior of protein hydrolysates with silver nitrate made originally by his fellow-worker Siegfried five years before and repeatedly encountered in his own work. Kossel on the other hand was investigating a wholly new field. This was his first paper on the subject with which his name is now most closely linked. That he should immediately apply severe hydrolysis for the decomposition of the new protamine was genius; his use of mercuric chloride was, however, largely conventional and, under the circumstances, he was extremely fortunate to secure material as pure as he did. Mercuric chloride is far from being a selective reagent in alkaline solutions, but the products of hydrolysis of a protamine contain so few amino acids in addition to arginine that the precipitate could have contained little except histidine. The final identification of the bases prepared by Kossel and by Hedin was made by Kossel and Kutscher (273).

Kossel and Hedin deserve equal credit as the independent discoverers of histidine; each so regarded himself. Kossel assumed the right to name his substance in 1896. Hedin (269) in 1898, in referring to the three bases derived from proteins wrote, "Da die ebene genannten Basen von Drechsel (Lysin) und von mir (Arginin und Histidin) unter den Spaltungsprodukten aller . . . . Proteinstoffe gefunden waren . . . ." etc. Perhaps the fairest assignment of credit is to regard Kossel as the discoverer of histidine in protamines and Hedin as the discoverer of histidine in proteins.

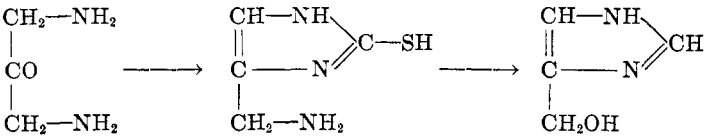
The wide distribution of histidine became apparent from the work of Kossel and his students and from that of Schulze (278).

Its constitution was not ascertained for some years. It was soon found that it was a diacid base and that it contained an asymmetric carbon atom. In 1903 Herzog (270) observed that histidine responds to the biuret test, that it contains no methyl or oxymethyl group, that it withstands oxidation in acid solution, although in alkaline solution it yields hydrocyanic acid, ammonia, and carbon dioxide, and that it can not be brominated. At about the same time Fränkel (267) showed that it contains a carboxyl and an amino group and gives Weidel's pyrimidine reaction. He suggested a constitution based on the pyrimidine ring but, since his formulation contained no asymmetric carbon atom, it was not accepted. Pauly in 1904 took up the problem at Kossel's request (275). He verified the presence of a carboxyl group by preparing a methyl ester. He showed that two equivalents of naphthalenesulfonyl chloride could be introduced, consequently histidine contained one secondary amine group in addition to the primary amine group; the third nitrogen atom must therefore be tertiary. The stability of histidine towards oxidation, and the presence of two hydrogen atoms replaceable by silver, suggested that a ring structure was present. Of the possible structures the imidazole ring seemed by far the most probable. Imidazole derivatives react with diazobenzenesulfonic acid to form highly colored products. Pauly found that histidine behaved in this way, and histidine, which had previously been written $(NH_2)\cdot C_5H_6N_2\cdot COOH$ was therefore formulated,



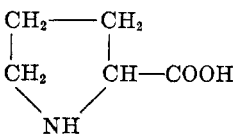
The formula contained an asymmetric carbon atom and, in fact, accounted for every known reaction of histidine. Knoop and Windaus (271) shortly afterwards confirmed this view by the demonstration that histidine, after treatment with nitrous acid to remove the α-amino group, could be reduced to β-imidazol-

propionic acid identical with the synthetic product. This conversion showed that Pauly was essentially correct, the only point remaining undetermined being the position of the primary amino group. This was finally proved to be an α-amino group by Pyman in 1911 (277) who synthesized racemic histidine from diaminoacetone. The imidazole ring was formed by condensation with potassium thiocyanate followed by oxidation with nitric acid.



The hydroxymethylglyoxaline was converted to the chloride with phosphorus pentachloride and this intermediate served for the synthesis of histidine by the sodium malonic ester method. Resolution of the racemic product was effected by means of *d*-tartaric acid.

PROLINE



Proline is the second amino acid to be obtained synthetically before its presence as a product of the hydrolysis of the protein molecule was recognized. It was first prepared in 1900 by Willstätter (292), who was interested in the position of the carboxyl group in hygric acid. This substance is obtained by oxidizing (289) hygrine and cuscohygrine, alkaloids found by Liebermann (287) in Peruvian cusco leaves. They are closely related to cocaine and tropa-cocaine. The question arose as to whether the carboxyl group in hygric acid (*N*-methylpyrrolidine-α-carboxylic acid) was in the α or the β position. From the fact that carbon dioxide was easily split off by dry distillation, Liebermann and Cybulski (288) were inclined to favor the view that it was in

the α position. The position of the carboxyl group would determine whether hygrine and cuscohygrine were related to the tropane group of alkaloids which are α_1 and α_2 substitution products of *N*-methylpyrrolidine.

Willstätter condensed sodium malonic ester with trimethylene-bromide and obtained bromopropylmalonic ester. By treating this product with bromine in the cold it was converted into α,δ-dibromopropylmalonic ester. This compound was then treated with ammoniacal methyl alcohol and was converted into an amide which, when saponified with barium hydroxide, yielded α-pyrrolidinecarboxylic acid. By treating dibromopropylmalonic ester with methylamine and subsequently saponifying, Willstätter obtained a small yield of *N*-methylpyrrolidinecarboxylic acid which proved to be identical with Liebermann's hygric acid. Thus, in an unexpected way, a protein amino acid was discovered.

A year later Fischer (280) published a synthesis of proline from phthalimide propylmalonic ester. This compound was treated with bromine and converted into phthalimide propylbromomalonic ester. By treating the latter substance with ammonia, Fischer had hoped to prepare a derivative of α,δ-diaminovaleric acid. Instead there was obtained a mixture of phthalimide and other products. On heating with hydrochloric acid at 100° he obtained α-pyrrolidinecarboxylic acid. Although this work appeared a year after Willstätter's publication, it had been begun without the knowledge of the latter's results. Fischer states, "Ich bemerke übrigens dass meine Versuche längst begonnen waren, bevor die Arbeit des Herrn Willstätter zu meiner Kenntniss kam."

More recently methods of synthesizing proline have been described by Sörensen and Andersen (291) and by Fischer and Zemplén (286).

Proline was first isolated from a protein by Fischer in 1901 (281). He hydrolyzed casein with hydrochloric acid, esterified the amino acids and distilled the esters. The fraction boiling at 65–80° was saponified and subjected to fractional crystallization. The mother liquor, resulting from one of these fractions, was

boiled with copper oxide and yielded the copper salt of racemic proline together with small amounts of other amino acids. This product was decomposed with hydrogen sulfide, extracted with alcohol, again converted into the copper salt and subsequently freed from copper. Analysis of the final product gave results which corresponded to pyrrolidinecarboxylic acid. Racemic proline copper crystallizes well from water but *l*-proline copper remains in the mother liquor and yields an amorphous mass when this is evaporated. Fischer devised the artifice of racemizing the free acid, prepared from this material, by treatment with barium hydroxide at 140–145°. The racemic copper salt could then be readily crystallized. Fischer prepared crystalline proline from alcohol and ether mixtures or from water with the aid of a little pyridine. His early preparations melted at 203–206° uncorrected; later, however, the pure acid of melting point 220° was obtained.

The most important problem in the early stage of the investigation was to obtain a demonstration that proline was a primary product of protein hydrolysis. There was a possibility that it might be formed by ring closure from some other amino acid. Fischer first attempted to get proline from arginine by subjecting the basic amino acid to the procedure used in preparing proline. Ornithine was likewise investigated without result. Together with Levene (281) he investigated a tryptic digest of casein and secured a small quantity of proline. In this case, however, the amino acid had been exposed to the action of acid during esterification and there was a possibility that this may have given rise to ring closure although Fischer regarded it as improbable. In 1902 Fischer reported (282) the isolation of proline from casein that had been hydrolyzed by alkali.

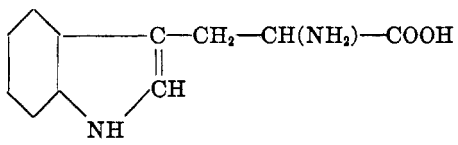
The final proof that proline is a primary product of protein hydrolysis was secured by Fischer and London (284), who isolated nearly as much proline from an enzymatic digest of gliadin as had been obtained by acid hydrolysis.

Proline is closely related to glutamic acid. If the latter acid is boiled with water ring closure occurs, and an equilibrium mixture of pyrrolidonecarboxylic acid and glutamic acid results. When this mixture is heated with acid the ring opens and glu-

tamic acid is again formed. The possibility that proline can be formed in nature by the reduction of the keto group in pyrrolidonecarboxylic acid is of great importance. Experimentally, however, this reduction is very difficult. It was accomplished by Fischer and Boehner in 1911 (283) although with small yields. A further extensive investigation of this subject was carried out in 1926 by McCay and Schmidt (290) with negative results.

Fischer (285) first suggested the use of the word proline to designate α-pyrrolidinecarboxylic acid in connection with his work on the synthesis of prolylalanine. An excerpt from his paper states his reason for the use of the term. "Für die Benennung derartiger Kombination ist das Wort α-Pyrrolidincarbonsäure zu lang. Wir halten es deshalb zweckmässig, das abgekürzte Wort 'Prolin', dessen Ableitung aus Pyrrolidin leicht verständlich ist, vorzuschlagen."

TRYPTOPHANE



The story of tryptophane, during the early years of protein chemistry, is the story of a color reaction. The literature contains innumerable references to more or less intense color reactions produced by the action of a wide variety of reagents on proteins or on their decomposition products. One of the earliest of these was described by Tiedemann and Gmelin (315)[16] in their

[16] The first edition of this extraordinary book appeared in 1826. It was an essay presented in 1825 to the French Academy in competition for a prize; the subject assigned by the Academy was "quel sont les phénomènes qui se succèdent dans les organes digestifs durant l'acte de la digestion. . . . . Les expériences devront être suivies dans les quatre classes d'animaux vertébrès." None of the essays was regarded by the Academy as worthy of a prize, but two were given honorable mention and an award of 1500 francs "à titre d'encouragement." The two essays selected were those submitted respectively by Leuret and Lassaigne and by Tiedemann and Gmelin. Tiedemann and Gmelin wrote to the Academy that their work "ne l'ayant pas trouvé digne du prix, nous ne pouvons accepter, ni la mention honorable, ni la recompense de 1500 francs. . . . . Nous ne tarderons pas, à soumettre nôtre travail au jugement impartial du monde savant."

elaborate investigation of the digestive processes of vertebrate animals. Pancreatic juice secured from a dog was evaporated to dryness and the residue was extracted with alcohol. The extract was in turn evaporated and the residue was dissolved in water and filtered. "Wenig wässriges Chlor färbte die Flüssigkeit lebhaft rosenroth, und schlug nach 12 Stunden zarte violette Flocken nieder, wobei sich die Flüssigkeit fast gänzlich entfärbte. Eine grössere Menge von Chlor zerstörte augenblicklich, ohne alle Trübung, die rothe Farbe, welche durch eine geringere Menge bewirkt war." A similar color test was obtained on the fluid found in the small intestine of a calf, of a hen, of a frog, and of a trout. In connection with the observation on the calf they wrote, "Ob diese Materie mit derjenigen einerlei ist, welche bei der Destillation des Inhaltes von verschiedenen Magen und andern Theilen des Darmkanals der Wiederkauer bei der Destillation übergieng, und beim Abdampfen derselben mit Salzsäure sich durch Röthung der Flüssigkeit zu erkennen gab, ist nicht ausgemacht, doch unwahrscheinlich."

Claude Bernard (295, p. 403–409) showed that a mascerate of the pancreas does not give the color reaction with chlorine water until putrefaction sets in. After putrefaction has been allowed to go on for some time the color reaction can no longer be obtained. Mascerates of liver, spleen, and certain other glands gave the reaction under similar circumstances. "Il semblerait que cette matière rouge existe dans les organes qui agissent chimiquement dans la vie de nutrition et non dans les appareils de la vie de relation . . . . . Enfin nous ajouterons que le tissu pancréatique, après avoir été bouilli, perd la propriété de donner une infusion susceptible de rougir par le chlore." Bernard made no attempt to account for the chemistry of this reaction but drew the following deduction (p. 406): "Nous dirons seulement que cette matière, qui a la propriété de se colorer, semble appartenir aux substances protéiques analogues à la caséine"; and again when referring to experiments on pure pancreatic juice (p. 433), "Cette matière colorante rouge, qui est la même qui se forme aussi dans le tissu pancréatique, se produit dans le suc pancréatique par suite de la décomposition de la matière organique coagulable contenue dans ce liquide."

Stadelmann (314), in quoting Bernard's paper in 1890, stated that Bernard had observed that the color reaction was given by a tryptic digest of casein. This statement appears, however, to be an error, although it has been widely quoted. Bernard's words are as quoted above; he may have tried the experiment but does not specifically say so. Curiously enough Bernard was not able to obtain the color reaction with either bromine or iodine (p. 434). Kühne, however, introduced bromine water as a reagent for the test (306), and it has now entirely replaced chlorine water for this purpose. Bernard was correct, however, with respect to iodine.

Kühne showed that indole is not produced during properly conducted tryptic digestions of protein; it is formed, however, if putrefaction occurs and this observation was confirmed by Nencki (309). The association of indole with the tryptophane reaction was due to the observation of Bernard that, after extensive putrefaction of pancreas, a substance is present that gives a violet color reaction with nitric acid; this substance he believed to be the same as that responsible for the reaction with chlorine water. Kühne identified the former substance as indole, and thus indole and tryptophane very early became associated with each other.

Neumeister (312) in 1890 found that "Das fragliche Chromogen lässt sich bei allen Processen, welche den tiefen Zerfall der Eiweisskörper herbeiführen, nachweisen und kann daher wohl als 'Tryptophan'[17] bezeichnet werden. Man beobachtet sein Auftreten ausser bei der Pankreasverdauung auch bei andauernder Fäulniss, beim Erhitzen der Eiweisskörper mit Barytlauge und beim Kochen derselben mit 5% Schwefelsäure." He found that the substance was stable in boiling water. Neumeister also made the observation, "Auch das Tryptophan selbst wird vom Amylalkohol aufgenommen und lässt sich hierdurch seinen Lösungen entziehen. Da Leucin und Tyrosin in Amylalkohol unlöslich sind, könnte das Tryptophan vielleicht auf diese Weise isoliert werden." In view of Dakin's introduction in 1918 of the use of butyl alcohol for the convenient preparation of tryptophane, this statement is extraordinarily interesting.

[17] The name is derived from *θρύπτομαι*, to be broken, and *φαίνω*, to bring to light.

A number of workers became interested in the tryptophane reaction about 1890 and the large tryptophane literature of this period indicates the importance that was attached to it. Stadelmann as well as Neumeister suggested a name for the color-producing substance (314): "Ich werde von der ursprünglichen Substanz, die also bei der tryptischen Verdauung z.B. direct entsteht, als dem Proteïnchromogen und von der Brom- resp. chlor-Verbindung die eben einen violetten rothen Körper darstellt als dem Proteinochrom sprechen." Gamgee (302) objected to this name because it implied that no other color-producing substance was present in the protein; the word was too inclusive in its implications. Neumeister's convenient and non-committal name was preferred by many and the investigations became centered around the constitution of the colored product of the action of halogens. Nencki (311) prepared the colored substance and observed the formation of indole and skatole from it after fusion with alkali. Beitler (294) attempted to isolate both colored substance and precursor but failed to obtain pure material. Kurajeff (307), however, obtained products that suggested the presence of an indole derivative that contained two nitrogen atoms. In Hopkins' opinion this worker had isolated a product which may have been a monobromo derivative of tryptophane.

Another striking color reaction of proteins was observed in 1874 by Adamkiewicz (293) when glacial acetic acid was mixed with a solution of albumin and the mixture was treated carefully with concentrated sulfuric acid. This reaction was studied by Hopkins and Cole in 1901 (303) who showed that the formation of the color was due to the presence of glyoxylic acid in the acetic acid employed. With this color test and the well-known tryptophane reaction as guides, they then took up the problem of isolating the chromogenic substance in protein digests (304).

Hopkins and Cole noticed that the glyoxylic acid reaction was given by an acid hydrolysate of proteins long after the biuret reaction had disappeared; it was evident that the chromogenic substance survived hydrolysis and it was therefore, probably, a relatively simple substance. They further observed that the

reaction was exceptionally intense when applied to the products of a tryptic digest that gave no biuret reaction. Enzymatic digestion, consequently, seemed a more suitable method for hydrolysis of the protein. Casein was therefore treated with an active preparation of pancreatin until the tryptophane reaction with bromine water attained maximal intensity.

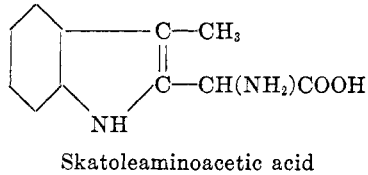
Beitler had shown that none of the more usual reagents could be successfully used for the precipitation of the chromogenic substances from protein digests. Hopkins and Cole found, however, that mercuric sulfate, a reagent that had previously been used but little for chemical separations, had a special selective action on the substance responsible for the tryptophane reaction, when added to a strongly acidified solution. "From the final products of proteid hydrolysis it throws down, in appreciable quantity, only cystine and the substance which forms the subject of this paper." The separation of cystine from the other product was easy, as it could be precipitated first by the addition of a small amount of reagent. The chromogenic substance could then be isolated by a second mercuric sulfate precipitation.

The final procedure adopted by Hopkins was relatively simple. The clear tryptic digest was acidified with 5 per cent by volume of sulfuric acid and the mercuric sulfate reagent was added in an amount of roughly 1 gram of mercuric sulfate per gram of protein digested. The yellow precipitate was removed, after from 12 to 24 hours, and washed free from tyrosine with 5 per cent sulfuric acid; it was then decomposed with hydrogen sulfide and the solution was again acidified by the addition of 5 per cent of sulfuric acid. The next step was the removal of cystine; mercuric sulfate was carefully added until a small permanent precipitate was produced. The solution was allowed to stand for half an hour and was then filtered; the precipitate so secured contained all but traces of the cystine. Excess of reagent was then added to the filtrate and the precipitate was removed after a few hours and decomposed with hydrogen sulfide. The sulfuric acid contained in this solution, derived from the decomposition of the mercuric sulfate compound, was exactly removed with baryta and the fil-

trate was mixed with alcohol. It is at this point that difficulty was encountered. It was found that the evaporation of the solution must be carried out with continued additions of alcohol as, otherwise, the product undergoes extensive decomposition. The alcohol concentration at the end should be over 60 per cent. After proper evaporation and cooling the solution deposited a magma of crystals that were filtered off and recrystallized from 75 per cent alcohol.

The new substance gave the tryptophane and glyoxylic acid reactions with great intensity. It responded to the pyrrole pine-splinter reaction and, when heated, gave off vapors of indole and skatole. The analysis agreed closely with the formula $C_{11}H_{12}N_2O_2$. The colored product of the action of bromine on the substance gave an absorption spectrum identical with that observed in the case of the tryptophane reaction. Hopkins and Cole therefore considered that it was desirable "that the new compound, which is the mother substance of the most characteristic coloured product, should continue to receive Neumeister's designation of tryptophane."

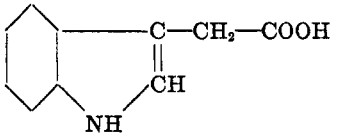
Nencki (308, 309, 310), Kühne (306), and also Salkowski (313) had found indole, skatole, skatolecarboxylic and skatoleacetic acid among the products of the putrefaction of proteins. Nencki in 1889 arrived at the conclusion that the mother substance of these substances might be skatoleaminoacetic acid.



Skatoleaminoacetic acid

The new substance of Hopkins and Cole likewise yielded these four products and they therefore regarded their new substance as a derivative of skatole. Ellinger (301, 296), however, found that tryptophane could behave as the precursor of indole in the intestine and the accuracy of Nencki's formula was therefore

called in doubt. Ellinger synthesized indole-3-acetic acid (297) and found that it was



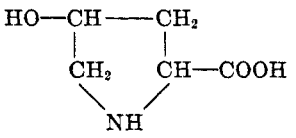
Indole-3-acetic acid

identical with the substance isolated by Salkowski. Tryptophane must therefore be an indole rather than a skatole derivative and must have one of the constitutions,

(I) I—CH_2—$CH(NH_2)COOH$
(II) I—$CH(NH_2)CH_2$—$COOH$
(III) I—$CH(COOH)CH_2$—NH_2
(IV) I—$C(NH_2)(CH_3)$—$COOH$

Formula III was at first regarded as most probable but the synthesis of indole-3-propionic acid (298) and the demonstration that the product was identical with Nencki's skatoleacetic acid showed that tryptophane must possess either formula I or II. The first was more probable, owing to the analogy with other amino acids. Hopkins and Cole (305) had obtained a substance C_9H_7NO, by the oxidation of tryptophane with ferric chloride, which Ellinger (299) showed to be β-indolealdehyde. Using this compound as a starting point Ellinger and Flamand in 1907 (300) synthesized tryptophane by the hippuric acid condensation method and showed that its constitution is represented by formula I.

OXYPROLINE



Thirty-seven years after the discovery of serine in silk gelatin by Cramer (316), Fischer and Skita (318) published a paper in

which they showed that serine was a constituent of fibroin. In referring to this discovery Fischer and Skita (318) wrote, "Wir glauben diese Beobachtung besonders hervorheben zu dürfen, da das Serin, welches zurzeit noch die einzige natürliche Oxyaminosäure der aliphatischen Reihe ist, bisher nur im Seidenleim aufgefunden wurde, und wir machen von neuem darauf aufmerksam, dass diese Oxyaminosäuren eine bis jetzt gar nicht gewürdigte grosse Bedeutung für das Studium der Proteïne haben." Four months later, Fischer (317) published a paper in which he described the isolation of oxyproline from gelatine. He stated, "Ich bin ferner überzeugt, dass kohlenstoffreichere Oxyaminosäuren noch in grösserer Zahl unter den Spaltungsproducten der Eiweisskörper vorhanden sind, denn es ist mir gelungen, eine derselben, von der Formel $C_5H_9O_3N$ aus dem Leim zu isoliren."

Gelatin was hydrolyzed and the esters were prepared and liberated from their acid salts in the usual manner. After extraction with ether there remained a residue which contained inorganic salts, and the basic amino acids, together with residues of the monoamino acids. The salts were eliminated by extracting the residue with acidified alcohol, which dissolved the amino acids, and these were again subjected to the esterification process. After a second extraction with acid alcohol the hydrochloric acid was removed with silver and the basic amino acids were precipitated with phosphotungstic acid. The excess of the reagent was removed with barium hydroxide in the usual manner and, after concentrating *in vacuo*, crystals of a new amino acid were obtained. By heating with hydriodic acid and phosphorus, the new substance was converted into proline. Although he had no absolute proof, Fischer surmised that the new amino acid was oxyproline. "Jedenfalls kann man aus dem Resultat des Versuches den recht wahrscheinlichen Schluss ziehen, dass die neue Aminosäure eine Oxypyrrolidine-α-carbonsäure ist. Leider liegt bisher keine Beobachtung vor welche ein Urteil über die Stellung des Hydroxyls gestattete."

The synthesis of oxypyrrolidinecarboxylic acid was first carried out by Leuchs (320) who, following the precedent of Fischer with

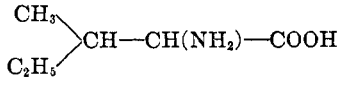
respect to proline, named the compound oxyproline. Epichlorhydrin and sodium malonic ester were condensed to α-chloro-β-oxypropylmalonic ester. By splitting off alcohol, this compound was converted into the lactone. The lactone was then converted into α-bromo-δ-chloro-γ-valerolactone by bromination. On treating with ammonia, *r*-oxyproline was formed; when treated with hydriodic acid, this was reduced to proline.

Leuchs and Felser (322) and Leuchs and Bormann (321) have attempted to place the position of the hydroxy group in the pyrrole ring. On heating natural oxyproline with barium hydroxide at 200°, it was only partially racemized, indicating that in oxyproline there are two asymmetric carbon atoms present. They believed that the oxyproline occurring in proteins is either γ- or β-oxyproline but their experiments do not differentiate as to which of these two possibilities is correct.

Hammarsten (319) has synthesized γ-oxyproline but furnished no comparison between his preparation and natural oxyproline. His preparation melted at the same temperature as that of Leuchs.

The difficulty of deciding the exact constitution of oxyproline arises from the presence of the second asymmetric carbon atom in the ring. This atom is apparently not racemized by ordinary treatment and, consequently, the decision will have to await the preparation of the oxyprolines derived from the four optically active isomeric intermediates. In view of the fact that at least eight optically active isomeric oxyprolines are possible it is perhaps not surprising that the synthesis has not yet been accomplished.

ISOLEUCINE



Emil Fischer in 1901, when investigating the separation of the individual amino acids of the leucine fraction secured by the ester distillation method (327), found that successive crops of crystals of different specific rotations were obtained. The least soluble

fraction had a lower, but the second and other fractions all had a higher rotation than that described by Schulze for *l*-leucine. He wrote, "solange dieser Wert nicht als zu niedrig erkannt ist, muss man annehmen, dass in der Fraction D und höchst wahrscheinlich auch in B und C eine gleich zusammengesetzte, aber stärker drehende Aminosäure enthalten ist." Attempts to separate this substance from leucine failed.

The explanation of this behavior came from a brilliant investigation of the nitrogenous substances in beet-sugar molasses carried out by Felix Ehrlich in 1903 (324). This material was customarily evaporated to a thick sirup, previous to its distillation for the recovery of trimethylamine and ammonia, and of the potassium salts. The sirup, on standing, deposited a crystalline mass, more than half of which frequently consisted of organic substances. Ehrlich filtered this material and extracted the residue with hot alcohol. The extract, when evaporated, deposited a material that showed the properties of a mixture of amino acids. He observed that the product was soluble in alcohol that contained a little ammonia; by the use of this reagent he succeeded in obtaining a fairly pure crude leucine in a yield of 1 to 2 grams per kilogram of sirup. This, when recrystallized yielded the gleaming plates typical of pure leucine and gave results, on analysis, that agreed with the formula of leucine. Its properties, however, were somewhat different from those described by Schulze for pure *l*-leucine. It was slightly less soluble in water, its specific rotation in 20 per cent hydrochloric acid was somewhat higher and the melting point was lower. The most striking difference lay in the solubility of the copper salt. Pure *l*-leucine copper is very insoluble in water or in alcohol, although when mixed with valine the mixed copper salts are much more soluble. Ehrlich's material, however, gave two copper salts, one insoluble and the other much more soluble in water. Furthermore the benzenesulfonyl chloride derivatives of the leucines obtained from the two copper salts differed in melting point. "Die weitere Untersuchung zeigte nun, dass die . . . . Substanz trotz ihrer grossen Aehnlichkeit mit dem *l*-Leucin nicht einheitlich war, sondern aus einem Gemisch von *r*-Leucin, *l*-Leucin und einem Isomeren des

Leucins, dem *d*-Isoleucin, bestand." This explanation was obtained by investigating the alcoholic mother liquors from which the preparation had been recrystallized. These yielded a preparation with the composition of leucine that had a specific rotation of $[\alpha]_D^2 = +28.1°$ (whereas *l*-leucine has $[\alpha]_D^{20} = +17.3°$) in 22 per cent hydrochloric acid and which was weakly dextrorotatory in water (*l*-leucine is levorotatory); it was soluble in only 30 parts of water at 19°. This material was obviously entirely different from Schulze's *l*-leucine. Similar preparations were secured from the more soluble fractions of the copper salt. Although no homogeneous product had been obtained it was evident that the material in hand was a mixture of *l*-leucine with a dextrorotatory substance of the same composition and therefore probably an isomer.

At this point Ehrlich made the brilliant observation that the copper salt of the isomeric leucine was soluble in methyl alcohol and could be partially extracted from the mixed copper salts with this solvent. He was therefore able to secure relatively pure specimens. Only a part of the isoleucine present could be secured by a single extraction; it was then necessary to decompose the insoluble copper salt, reconvert it to the copper salt and extract again, and further repetition of these operations was frequently necessary. The new substance, when crystallized from water and alcohol, usually separated in leaves indistinguishable from ordinary leucine. By slow evaporation of a solution that was only slightly supersaturated it was obtained in tablets and prisms. The specific rotation in water was +9.74° and in 20 per cent hydrochloric acid was +36.8°; the copper salt crystallized in leaves differing in appearance from *l*-leucine and, unlike this, was soluble in methyl and benzyl alcohol. The benzoyl and other similar derivatives differed in melting point from the compounds of *l*-leucine described by Fischer.

In order to settle the question of the origin of this substance, Ehrlich next prepared crude leucine from a pancreatic digest of fibrin. This material likewise gave a copper salt, part of which was soluble in methyl alcohol, and from this fraction he prepared isoleucine that corresponded in properties with the preparation

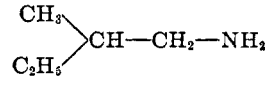
from beet molasses. It was therefore clear that isoleucine was a product of protein decomposition and not an artifact produced during the industrial processes through which beet-sugar molasses had passed. He also secured specimens from egg albumin, wheat gluten and beef muscle. These observations cleared up most of the discrepancies that had been observed in the behavior of leucine from proteins. Ehrlich later showed that the original isoleucine preparation obtained from fibrin contained traces of valine and pointed out the great difficulty of separating isoleucine from this substance. The success of the early preparations from beet molasses was due to the absence of valine from this material.

Ehrlich continued the investigation of isoleucine and in 1907 (325) obtained evidence of its constitution. Isoleucine and leucine crystallize together in mixed crystals and it is impossible to separate the two substances by any process of crystallization; furthermore the two amino acids are almost always found together in nature. Ehrlich pointed out that there was one other pair of well-known substances, isoamyl alcohol and *d*-amyl alcohol that likewise always occurred together and that all of their derivatives formed similar series of mixed crystals. "In der Tat liess sich nun zeigen, dass die so auffallend übereinstimmenden Eigenschaften dieser beiden Verbindungspaare keine zufälligen sind, sondern dass zwischen den beiden Leucinen und Amylalkoholen sehr nahe chemische Beziehungen bestehen und eine weitgehende Analogie, die besonders für die Frage der Entstehung des Fuselöls bedeutungsvoll geworden ist."

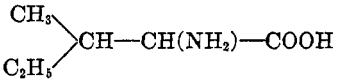
It had long been known that when isoleucine was heated to 200° isoamylamine of the constitution

$$(CH_3)_2{=}CH_2{-}CH_2{-}CH_2{-}NH_2$$

was produced. Isoleucine, when treated the same way, gave an optically active amylamine that was identical with the *d*-amylamine



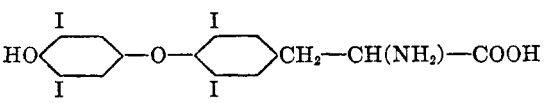
synthesized by Marckwald (328), except that the optical activity was slightly lower. This indicated that *d*-isoleucine must be one of the four possible optical isomers of the constitution



This view was supported by the result of fermenting isoleucine with yeast in the presence of sugar since a *d*-amyl alcohol was obtained. This was proved by oxidation of the alcohol to the dextrorotatory valeric acid identical with methylethylacetic acid. A synthesis was therefore attempted starting with *d*-amyl alcohol. This was oxidized to *d*-valeraldehyde and converted to the amino acid by Strecker's method. The product yielded a copper salt soluble in methyl alcohol, but the free acid was different in some respects from *d*-isoleucine. Isoleucine contains two optically active carbon atoms and, since the synthesis started with *d*-amyl alcohol, the product should be a mixture of the two isomers produced by the introduction of the second asymmetric carbon atom. One of these should be *d*-isoleucine, the other was named allo-isoleucine. A similar mixture of isomers was produced by heating *d*-isoleucine with barium hydroxide under pressure. No means of separating these substances was found, but pure allo-isoleucine was secured by fermentation of the mixture for this substance was not utilized by the yeast. The preparations of allo-isoleucine secured in this way from synthetic material and from isoleucine were identical.

A complete synthesis of isoleucine was carried out by Bouveault and Locquin in 1906 (323) starting with *secondary*-butyl acetoacetic ester and still another by Ehrlich (326) who started with *secondary*-butyl iodide and used the malonic ester method.

THYROXINE



Although the condition recognized as goitre has long been known, it was not until about 1883 that the relationship of this

disease to the activity of the thyroid gland was shown by Kocher (347). With the demonstration by Murray (349) in 1891 of the value of thyroid extract in the treatment of hypothyroidism, interest in the isolation of the active constituent of the gland was aroused, and further impetus was given when Roos (353), working in Baumann's laboratory in Freiburg, showed that the administration of desiccated thyroid gland led to an increase in nitrogen metabolism. Baumann was himself actively engaged in work on the thyroid problem. He was of the opinion that the gland contained a principle of the nature of an enzyme or of a protein and his early attempts at concentration of this principle were directed towards the investigation of its stability.

Roos had investigated the stability of the active substance towards acid. The glands were boiled with 10 per cent sulfuric acid for a day; the insoluble material was then removed and thoroughly extracted with diluted alcohol. This extract contained the active substance. After evaporation of the solution the residue was dissolved in dilute alkali and reprecipitated by acid. This product received the name *thyroiodin.*

Baumann (330) fused the material with sodium hydroxide and potassium nitrate, dissolved the melt in water and acidified with nitric acid. He noted that the fluid was yellow in color and on shaking with chloroform, a violet color, indicative of iodine, passed into the chloroform. Baumann's reaction to this discovery is best expressed in his own words, "Als ich diese Beobachtung zuerst machte, glaubte ich an alles Andere eher, als dass das Jod meiner Substanz angehöre. Indessen blieb ich darüber doch nicht lange im Zweifel, denn alle Reagentien erwiesen sich als völlig rein und frei von Jod." Baumann recognized that the iodine was not in the form of inorganic iodine; he thought that it was combined as a complex in a manner analogous to the form in which iron is contained in hemoglobin. This is clearly brought out in the statement, "Es handelt sich dabei offenbar nicht um eine Wirkung des freien Jods oder eines Jodsalzes, sondern um die Bildung derjenigen specifischen organischen Jodverbindung, welche wir in dem Thyrojodin soweit als möglich isolirt haben. Dieser Vorgang scheint ganz ähnlich demjenigen der Aufnahme

des Eisens zu sein, dessen Wirkung dem Organismus auch erst dann zu Statten kommt, wenn es in diejenige organische Eisenverbindung, aus welcher der Blutfarbstoff besteht, übergeführt ist."

Even prior to the work of Baumann, Kocher (348) had reasoned that certain analogies exist between the therapeutic action of iodine and the active principle of the thyroid gland. "Die Analogie der giftigen Wirkung von Jod und Schilddrüsensaft ist auch darin analog, dass in solchen Fällen ausser den nervösen Symptomen eine ganz bedeutende und rasche Abmagerung eintreten kann, die zum Aussetzen des Mittels nöthigt." At his suggestion Tschirch looked for iodine in the thyroid gland, but reported negative results; it was probably lost during the process of dry ashing.

Baumann's observation at once drew attention to the importance of iodine in animal physiology. Although the nature of the organic compound of iodine that occurs in the gland has only recently been established, it early became clear that the substance was in some way associated with the proteins of the tissue. Oswald (351), in 1900, prepared an active globulin-like material from a water extract of thyroid tissue; this received the name *Jodthyreoglobulin* and appeared to be a mixture of iodine-containing and iodine-free proteins.

Investigation of the physiological properties of iodogorgoic acid by Strouse and Voegtlin (355) showed that this substance did not possess the same activity as desiccated thyroid gland. Hofmeister (341), Oswald (352), Roos (354), and others prepared and studied the properties of iodinated proteins. None of these products had physiological properties like those of Baumann's "Thyroiodin" and it became evident that the iodine-containing complex of the globulin from the thyroid gland was a substance of a highly special nature.

Nürenberg (350) thoroughly investigated the "Jodthyreoglobulin" of Oswald and placed its protein nature beyond doubt; his attempt to isolate iodogorgoic acid from it failed, however, although he succeeded in demonstrating the presence of an organic iodine compound that could be precipitated by silver nitrate in

faintly acid solution. He observed that a commercial product from thyroid glands called "iodothyrine" gave a positive xanthoproteic reaction but negative Adamkiewicz, Millon, and Ehrlich tests. After heating the material in an autoclave a positive Millon reaction was obtained, and after reduction with sodium in alcohol solution, positive tests for tryptophane were secured. This led to the conclusion that iodine is combined with tyrosine and possibly also with tryptophane, in the physiologically active material and the view that one or both of these amino acids are in some way associated with this activity prevailed until the final proof of the structure of thyroxine was obtained.

Nürenberg subjected "Jodthyreoglobulin" to hydrolysis by barium hydroxide; at the end of 30 hours the solution was filtered from an insoluble residue which he considered to be barium carbonate and did not examine. There is little doubt that, had he done so, a clue to the preparation of thyroxine might have been obtained some years before Kendall's work was begun.

The isolation of the substance to which the therapeutic activity of thyroid gland tissue is due was first accomplished by Kendall in 1915 (343). It is unnecessary to describe the preliminary experimental work which led to the isolation; the problem appeared, at the outset, to be relatively simple but proved very difficult in practice. Thyroid tissue contains a physiologically active substance associated with and proportional to its iodine content, and also associated with the globulin fraction. Under certain conditions hydrolysis of this protein could be effected without serious loss of activity, although prolonged enzymatic or acid hydrolysis destroyed it. The active iodine compound appeared to be related in some way to tyrosine or to tryptophane although attempts to isolate the only known natural iodine compound of tyrosine—iodogorgoic acid—from thyroid glands had failed. Furthermore, synthetic substances such as tetraiodohistidine, triiodoimidazole, iodotryptophane, or even iodogorgoic acid itself or iodized proteins did not possess physiological properties in any way analogous to those of the thyroid gland (333). So much had been learned by previous workers.

Kendall found that severe hydrolysis was necessary to liberate

the active substance and, in view of the instability of the organic iodine complex in acids, he employed alkaline hydrolyzing reagents. It was found that 24 hour hydrolysis by 5 per cent sodium hydroxide gave a solution from which a precipitate separated, on acidification, that contained about one-quarter of the iodine and all of the physiological activity. This material was heated, with a mixture of sodium and barium hydroxides, for 18 hours at 100°. An insoluble, iodine-containing precipitate was removed and, when the solution was neutralized, a second precipitate that contained a relatively high proportion of iodine separated. The material was treated three times successively in the same way, the proportion of iodine in the acid precipitate rising finally to 47 per cent. The product was dissolved in alcohol and evaporated on the water bath; inadvertently it was allowed to go to dryness and was heated for about an hour. The addition of alcohol dissolved a part of the material and left a white insoluble residue that weighed 18.6 milligrams; this contained 60 per cent of iodine. When this was dissolved in sodium hydroxide and the solution was neutralized and boiled, the substance separated in crystalline form. Approximately 200 milligrams of the substance were then prepared in the same way, and this was soon identified as the physiologically active principle of the gland; it was named thyroxine (344).

It was two years before Kendall secured his next specimen. An attempt was made to work with large quantities, but the process that went relatively smoothly in glass apparatus on the laboratory scale was valueless when metal vessels were substituted. Finally, after enameled ware or nickel apparatus had been introduced, thyroxine was again secured and, by 1919, about 33 grams had been prepared from over 3 tons of fresh thyroid gland.

The analysis of a substance that contains 65.3 per cent of iodine is a difficult problem, and Kendall was misled, by a nitrogen determination that was slightly too high, into drawing the conclusion that the substance contained three atoms of iodine to one of nitrogen. He formulated the substance as $C_{11}H_{10}NO_3I_3$, and suggested that its structure was probably 4,5,6-trihydro-4,5,6-

triiodo-2-oxy-β-indolepropionic acid. Numerous compounds of thyroxine were prepared the analyses of which did not disagree too seriously with the requirements of this formula, and many of the peculiar properties of thyroxine were explained in terms of hypothetical structural changes.

The chief qualitative evidence brought forward by Kendall in favor of an indole nucleus as the foundation of the structure of thyroxine was the result of a pine-splinter test. Clinical investigation had shown that the substance was undoubtedly the chief active principle of the gland (331) and interest in its fundamental structure was therefore intense. The work of Hicks (340) on the ultraviolet absorption spectra of thyroxine, tryptophane and 2-hydroxyindole-3-propionic acid supported Kendall's views.

Kendall's formulations were, however, by no means generally accepted. The great difficulty of the investigation doubtless prevented many from undertaking to check his results; nevertheless Harington in 1926 published results that showed Kendall's fundamental assumptions of the structure of thyroxine to be incorrect (334). Harington pointed out that the pine-splinter reaction, as employed by Kendall, was not specific for the indole nucleus; furthermore, that the reduced indole structure that had been suggested was inherently improbable on purely chemical grounds. He wrote, "It is fully apparent, therefore, that without considerable further chemical evidence it is impossible to accept the formula proposed by Kendall and that the constitution of thyroxine must be regarded as not proven."

Harington first devised an improved method for the isolation of thyroxine whereby a larger yield of the product might be secured. He employed 10 per cent barium hydroxide for the preliminary hydrolysis of the tissue and the insoluble precipitate that remained, after neutralizing the preliminary hydrolysate, was further hydrolyzed by stronger barium hydroxide. The precipitate obtained by neutralizing this hydrolysate was dissolved in hot dilute sodium hydroxide, barium was removed by sodium sulfate and the filtrate from the barium sulfate was acidified and boiled until the precipitate that separated became granular. This material was dissolved in alkali, alcohol was added, and the

solution was acidified with acetic acid; thyroxine separated in crystalline form in a yield of 0.08 per cent of the dry gland tissue and was further purified by repetition of the final operations. Analysis of this substance, and also of a preparation obtained commercially by Kendall's original method, showed that the ratio of iodine to nitrogen atoms is 4:1 and that the empirical formula should be $C_{15}H_{11}O_4NI_4$. The theoretical requirements of this formula differ very little from those of Kendall's formula except in the case of nitrogen.

Harington next found (335) that the iodine could be quantitatively removed from thyroxine by hydrogen in the presence of a palladium catalyst. The product of this reaction, desiodothyroxine (later called thyronine), had the empirical formula $C_{15}H_{15}O_4N$. It responded positively to Millon's reaction, and gave a ninhydrin reaction; all of its nitrogen was in the amino form and the constitution was therefore that of an α-amino acid in which one oxygen was present as a phenolic group. The proportions of carbon and hydrogen suggested the presence of two benzene rings. Fusion with potassium hydroxide at 250° yielded *p*-hydroxybenzoic acid, a substance $C_{13}H_{12}O_2$, and a minute amount of quinol; fusion at 310° in an atmosphere of hydrogen gave *p*-hydroxybenzoic acid and quinol in good yields, together with ammonia and oxalic acid. The substance $C_{13}H_{12}O_2$ possessed one phenolic group; the other oxygen atom was inert. "These experiments in the first place reinforce the suggestion of the presence of two benzene rings, one at least of which has a phenolic or phenol ether group in the *para* position to a side chain from which a two carbon fragment is split off as oxalic acid."

Exhaustive methylation yielded a betaine which, when boiled with alkali, lost trimethylamine and produced an unsaturated acid $C_{16}H_{14}O_4$ that contained one methoxyl. Oxidation of this gave oxalic acid and a neutral substance $C_{14}H_{12}O_3$ which, in turn, yielded a semicarbazone and a phenylhydrazone. This was at first thought to be a ketone, but the action of phosphorus pentachloride on its oxime gave a nitrile instead of an anilide; the oxidation product was therefore an aldehyde and further oxidation of this gave the acid $C_{14}H_{12}O_4$.

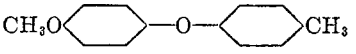
"Reviewing the results up to the present point then, we have

in the first place by the potash fusion demonstrated the probable presence in desiodothyroxine of two benzene rings. The behavior of the compound on exhaustive methylation proves almost with certainty that it is an amino acid; moreover, the presence of one methoxyl group in the unsaturated acid $C_{16}H_{14}O_4$ proves the presence of one phenolic group only in desiodothyroxine; finally the splitting off of oxalic acid by oxidation, with the formation of a residual stable acid is evidence of the presence of a three carbon side chain; on the experiments hitherto described the degradation may be represented thus:

$$HO\cdot C_{12}H_8O\cdot CH_2\cdot CHNH_2\cdot COOH \longrightarrow CH_3O\cdot C_{12}H_8O\cdot CH{=}CHCOOH$$
$$\longrightarrow CH_3O\cdot C_{12}H_8O\cdot CHO \longrightarrow CH_3O\cdot C_{12}H_8O\cdot COOH$$

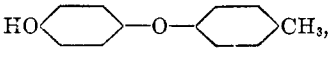
The remaining question is therefore the character of the two benzene ring group $-C_{12}H_8O-$. The two benzene rings composing this group cannot be linked through a carbon atom, since such a linkage would have led, on the above scheme of degradation, to the formation of a ketone (benzophenone derivative) on oxidation in place of the aldehyde actually obtained. They must therefore be linked either directly (diphenyl) or through the remaining oxygen atom which is as yet unaccounted for. This indifferent oxygen atom would be difficult to account for on the diphenyl hypothesis, so that the existence of an oxide linkage between the two benzene rings seemed the most probable supposition. At this point, therefore, it was decided to attempt to meet the degradation by synthesis, proceeding on the hypothesis that the group $-C_{12}H_8O-$ represented diphenyl ether minus two hydrogen atoms."

p-Bromoanisol was therefore condensed with the potassium salt of *p*-cresol to yield the compound,



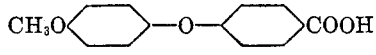
(I)

When boiled with hydriodic acid, this gave



(II)

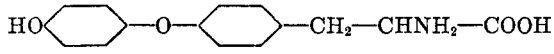
which was identical with the product of potash fusion of desiodothyroxine, $C_{13}H_{12}O_2$. Further, on oxidizing I with permanganate, the acid



(III)

was formed, and this was identical with the acid $C_{14}H_{12}O_2$ produced by the oxidation of desiodothyroxine.

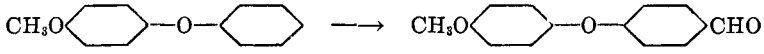
Desiodothyroxine must therefore have the formula,



(IV)

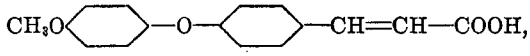
The synthesis of this substance was next attempted. *p*-Bromoanisol was condensed with potassium phenate and the product was converted to an aldehyde by Gattermann's hydrocyanic acid method.

(V)



(VI)

This was identical with the aldehyde $C_{14}H_{12}O_3$ produced during the degradation and was further identified by oxidation to III. The unsaturated acid,



(VII)

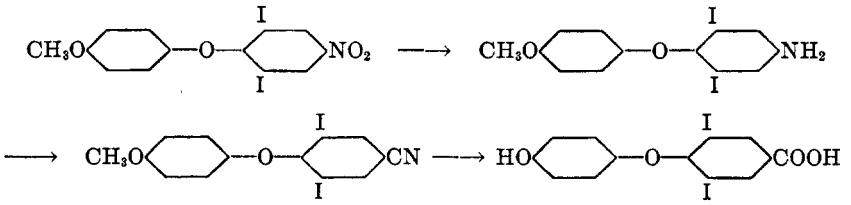
was also prepared by Perkins' method, and was found to be identical with the unsaturated acid $C_{16}H_{14}O_4$ obtained during the degradation.

Desiodothyroxine (IV) was prepared from this aldehyde by two methods; the diketopiperazine condensation method of Sasaki, and the hydantoin condensation method of Wheeler and Hoffmann; in both cases the product was identical with that secured from natural thyroxine.

Thyroxine itself must therefore be a tetraiodo substitution product of this nucleus; the most probable positions for these iodine atoms, were 3,5,3′,5′, because of the analogy with iodogorgoic acid. That this was the case was shown soon afterwards by Harington and Barger (337). On direct treatment of desiodothyroxine with iodine, only two iodine atoms could be introduced. It was therefore evident that the two iodine atoms in the 3,5 positions must be already in place before the phenyl ether synthesis is effected. The brilliant observation was made that the iodine atom in the 4 position in 3,4,5-triiodonitrobenzene is preferentially activated in such a way that condensation at this position can be effected with phenols. Quinol monomethyl ether was therefore condensed with 3,4,5-triiodonitrobenzene,

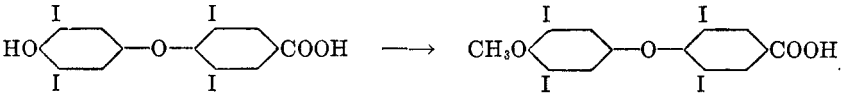
(VIII)

the nitro derivative was reduced to the amino compound, and this was converted to the nitrile. Hydrolysis then yielded the acid IX.



(IX)

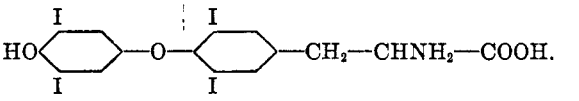
This compound was iodinated to the substance X which, on methylation, yielded XI,



(X) (XI)

a compound that was identical with the product secured from natural thyroxine that had been subjected to exhaustive methylation followed by oxidation of the side chain. This series of ex-

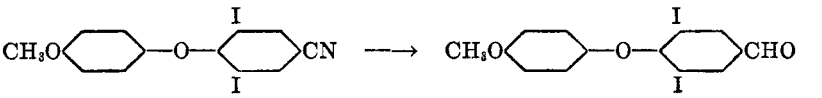
periments proved that the constitution of thyroxine is represented by formula XII,



(XII)

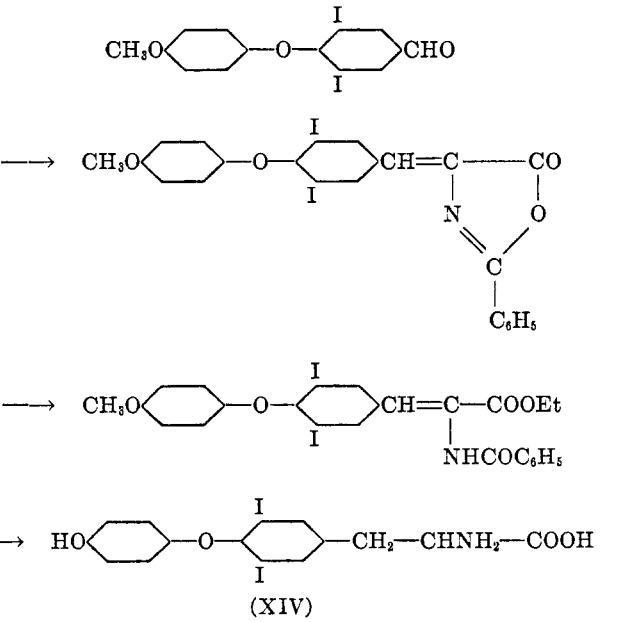
Thyroxine

The actual synthesis of thyroxine was carried out starting with the nitrile which was converted to the aldehyde XIII.



(XIII)

It was obvious that the steps that had led to the amino acid desiodothyroxine could hardly be expected to succeed in this case since alkaline reduction would remove the iodine atoms. Success was obtained however when the aldehyde was condensed with hippuric acid and the azlactone produced was reduced by hydriodic acid and red phosphorus.



(XIV)

The product XIV, when treated with iodine in ammoniacal solution, yielded thyroxine, XII, identical in all respects with the substance obtained from the thyroid gland.

It is one of the curious ways of science that two individuals should independently and almost simultaneously arrive at the same conclusions. In a footnote to their article, Harington and Barger state that Dakin had come substantially to the same conclusions as Harington regarding the constitution of thyroxine. On hearing that Harington had communicated a paper, Dakin generously withdrew his paper on the same subject from publication.

Thyroxine, as prepared from the thyroid gland, or by synthesis, is optically inactive owing to racemization during the alkaline hydrolysis by which it is liberated from the gland proteins. Harington therefore attempted the resolution (336) in order to see if the physiological activity of the two isomers is different. Owing to the insolubility of thyroxine and its salts the separation by means of alkaloids was not feasible. He therefore started with the formyl derivative of 3,5-diiodothyronine (the name thyronine was suggested to replace the name desiodothyroxine) and prepared the salts with the two isomeric α-phenylethylamines. An insoluble salt separated from the solution, but this could not be obtained optically pure. The mother liquor, however, yielded a soluble fraction that could be purified by recrystallization. The free acid was recovered from the salt, hydrolyzed and iodinated to thyroxine. *l*-Thyroxine was obtained from the *l*-α-phenylethylamine salt and had a specific rotation of $-3.2°$. The isomer was obtained from the *d*-α-phenylethylamine salt and had a specific rotation of $+2.97$. Physiological tests of these products indicated that the preparation of *l*-thyroxine was about three times as active as *d*-thyroxine. If it is assumed that the dextro isomer is physiologically inactive, it would indicate that the resolution had yielded preparations that were respectively 75 per cent pure. It was clear, however, that *l*-thyroxine is definitely much more active physiologically than its isomer, and this is therefore probably the isomer that occurs in nature.

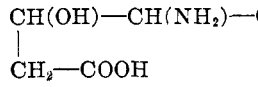
Ashley and Harington (329) in 1928 reported the preparation of a series of peptides containing thyroxine together with esters and other derivatives of thyroxine. A year later Harington and Randall (338) and Foster (332) almost simultaneously announced the isolation of diiodotyrosine from the thyroid gland. Some indications that such a compound might be present had already been given by Ingvaldsen and Cameron (342). This demonstration of the presence of diiodotyrosine in the thyroid gland add support to the view expressed by Harington and Barger that thyroxine is probably derived from tyrosine through the intermediate stage of diiodotyrosine, two molecules of which may undergo oxidative coupling with the loss of one side chain.

A year after the appearance of Harington's first paper on thyroxine, Kendall (345) announced that the greater yield of thyroxine which Harington had reported by the use of barium hydroxide instead of sodium hydroxide for hydrolysis could not be attributed to the hydrolyzing agent but rather to the fact that English thyroid glands contain a greater percentage of thyroxine. Kendall explained the discrepancy between the molecular weight he had found and that reported by Harington as possibly due to the addition of some substance during the preparation of his thyroxine derivatives. Kendall's molecular weight was based on the iodine content of the acetyl derivative, the ureide, and the sulfate of thyroxine. Kendall accepted Harington's proof for the constitution of thyroxine in the following words: "I congratulate Harington in bringing to a successful close the identification and synthesis of one of the most interesting substances known."

In a subsequent paper Kendall and Simonsen (346) showed, in confirmation of previous work by others, that there is a seasonal variation in the iodine content of the thyroid glands of American animals which may amount to 300 per cent.

In 1930 Harington and Salter (339) succeeded in preparing thyroxine from crude thyreoglobulin and from gland tissue that had been hydrolyzed by the successive action of pepsin and trypsin. The product was obtained in small yield but was identical with synthetic optically active thyroxine.

OXYGLUTAMIC ACID



In 1908 Osborne, Leavenworth and Brautlecht (363) investigated the relationship between the quantities of ammonia that could be obtained from proteins by hydrolysis and the quantities of aspartic and glutamic acid yielded by the same proteins. Hlasiwetz and Habermann (361) had suggested, as early as 1873, that these two acids were probably combined in the protein in the form of amides, and Osborne and his associates attempted to obtain quantitative evidence that this was the case. They calculated the quantity of ammonia that might be expected on this assumption and compared the theoretical with the actual amount. Striking agreement was found in many cases. "Marked exceptions, however, are shown by the proteins of the cereals, for which the amount calculated falls very much below that found analytically." They pointed out that small deficits might be due to "the uncertainties attending the isolation of these dibasic acids, but in the case of the cereal proteins these differences are so large that it does not seem possible to explain them in this way. . . . . It is therefore probable that the cereal proteins in some way differ in structure from all the others which have been examined, and that they may possibly contain some other dibasic acid not yet isolated from their decomposition products."

This prediction was fulfilled ten years later by the discovery of β-hydroxyglutamic acid by Dakin as a result of the application of a new method and of the reintroduction of an old and completely forgotten method, into the technique of amino acid analysis.

It has already been pointed out that Ritthausen's discovery of aspartic acid among the products of hydrolysis of proteins was due to his observation that the mother liquor from the crystallization of the glutamic acid contained a strongly acid substance. He had therefore neutralized it with barium carbonate and added alcohol; the barium salt of the acid thereupon separated. Later he employed the calcium salt, and similar methods were used by Schulze (364) for the separation of glutamic and aspartic acids

from protein hydrolysates. The possibilities of this method seem, however, to have been overlooked by all save one subsequent investigator[18] until 1914, when Foreman (360) became interested in the problem of improving the methods for the estimation of the dicarboxylic amino acids. He wrote:

"It seemed possible that a method might be based on some essential difference between the two types—dibasic and monobasic amino acids. As it is well known that the calcium salts of non-nitrogenous dibasic organic acids have a much higher degree of insolubility in water or alcohol than the calcium salts of non-nitrogenous monobasic acids, it was thought that the same principle might apply in the case of the dibasic and monobasic amino acids. Experiments were therefore made with a view to testing this matter.

"Calcium chloride solution was added to a solution of the amino acids obtained by the hydrolysis of caseinogen. No precipitate resulted, and on adding much alcohol only a small precipitate was obtained. When, however, another portion of the same solution was made alkaline with lime, a copious precipitate appeared on the addition of alcohol. The precipitate seemed to increase somewhat in quantity when the free ammonia was removed before the alcohol was added.

"Solutions of all the monoamino acids found in proteins, with the exception of oxyproline, were then treated separately with lime and alcohol. Glutaminic and aspartic acids, cystine and tyrosine all gave calcium salts insoluble in alcohol. The calcium salts of the other monoamino acids, however, were found to be very soluble in alcohol, and the solutions all remained perfectly clear when the alcohol was added. Pyrrolidonecarboxylic acid has since been tried in the same way, and a copious precipitate was obtained.

"Since these observations were made I have found that Abderhalden and Kautzsch (356) have made the calcium salts of aspartic, glutaminic,

[18] C. T. Mörner in 1913 (Zur Charakteristik des 3,5-Dibromtyrosins. Z. physiol. Chem. **88**, 124–37 (1913)) observed that dibromotyrosine, tyrosine, aspartic acid, and glutamic acid all yield precipitates when their solutions, in an excess of aqueous barium hydroxide, are treated with five volumes of alcohol. A number of other amino acids were tested but did not give precipitates. He made use of this property for the isolation of dibromotyrosine (see p. 165) from an hydrolysate of the horn-like skeleton of *Primnoa lepadifera*, and states that tyrosine, glutamic acid, and aspartic acid together with oxalic acid were also isolated in pure form from the fraction secured by precipitation with barium hydroxide and alcohol; no details of this work are given however.

and pyrrolidonecarboxylic acids separately from the pure substances in a similar way. They have not suggested, however, that any quantitative use could be made of these facts in reference to separations from hydrolytic products derived from proteins."

Foreman had obviously overlooked the early papers of Ritthausen and of Schulze, as well as Mörner's more recent work.

In working up the material precipitated from a casein hydrolysate by calcium hydroxide and alcohol, Foreman obtained the amino acids in dry form and extracted the mixture with glacial acetic acid. The extract, on evaporation, yielded a gum that was found to contain considerable pyrrolidonecarboxylic acid doubtless derived by the internal condensation of glutamic acid during the evaporations. Not all of the gum could be so accounted for and indirect evidence was secured that a substance soluble in glacial acetic acid was present which contained about 10 per cent of amino nitrogen. "The identity of this constituent of the gum has not yet been established." Foreman's investigations were interrupted by the outbreak of the war.

Dakin, in 1918 (357), reported the results of an extensive investigation of the methods of separation of the amino acids. He described the now well-known butyl alcohol method for the extraction of the monoamino acids and the isolation, from the aqueous solution that contained the non-extractable basic and dicarboxylic amino acids, of a new substance, β-hydroxyglutamic acid. This solution was an exceptionally favorable starting point for the investigation of the dicarboxylic acids as was shown by the results for the glutamic acid determination. Dakin obtained 21.6 per cent of this acid by direct crystallization of the hydrochloride from a solution derived from casein, a result much higher than those of previous investigators with the exception of Foreman who had obtained 21.8 per cent. The mother liquor from the glutamic acid hydrochloride was treated with calcium hydroxide and alcohol, as described by Foreman, and the aspartic acid was removed from the precipitated material as its lead salt. A small amount of basic substance was then removed with phosphotungstic acid. After freeing the solution from reagents it was found still to contain a strong organic acid. This was therefore

precipitated as its silver salt by the alternate addition of silver nitrate and sodium hydroxide. The silver compound was decomposed by hydrogen sulfide and the solution was concentrated at low temperature to a sirup. Thick prisms began to separate when this had stood in a vacuum desiccator for some time.

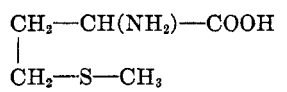
The new acid was optically active. It was extremely soluble in water and gave salts with metals that did not crystallize readily. It titrated as a monobasic acid in water, as a dibasic acid in the presence of formaldehyde. Its analysis and molecular weight corresponded to the formula $C_5H_9O_5N$, and its nitrogen, provided the preparation had not been heated unduly, was all amino nitrogen. Heated above 100° it lost water and the amino nitrogen was converted into imino nitrogen, a reaction clearly analogous to the formation of pyrrolidonecarboxylic acid from glutamic acid. A pyrrole reaction could be secured after heating the product with zinc dust. Glutamic acid was produced by reduction of the new acid with hydriodic acid. The acid did not give a lactone; substitution of an hydroxyl in the γ-position was therefore improbable. Oxidation with chloramine-T yielded an aldehyde which, in turn, gave an osazone of the composition $C_3H_3COOH(N \cdot NH \cdot C_6H_4NO_2)_2$ with *p*-nitrophenylhydrazine. The formation of this product indicated the presence of an hydroxyl group on the carbon atom adjacent to the α-amino group. Dakin wrote, "the above results can hardly be reconciled with any other structure for the acid than that of an α-amino-β-hydroxyglutaric acid, i.e. β-hydroxyglutamic acid, $COOH \cdot CH(NH_2) \cdot CH(OH) \cdot CH_2 \cdot COOH$." The accuracy of this formula was attested by the preparation and analysis of salts of silver, copper, lead, calcium, and barium and of the naphthalenesulfonyl chloride derivative. Furthermore a number of characteristic color reactions with phenols were described.

The presence of this acid among the products of hydrolysis of casein recalled the statement of Skraup (365) that hydroxyaspartic acid was one of the products of the hydrolysis of casein. "A search for tartaric and racemic acid among the products of the action of nitrous acid upon the dicarboxylic acids of caseinogen gave negative results, thus indicating the probable absence of hydroxyaspartic acid."

Dakin pointed out the relationship of the new acid to the material described by Foreman. He wrote, "While the writer's experiments originated from a totally different direction and were not influenced by Foreman's observations, there is little doubt that if the latter had been able to pursue his investigations uninterruptedly he would have isolated β-hydroxyglutamic acid."

A later paper by Dakin (358) described a synthesis of oxyglutamic acid. This proved to be extremely difficult but was finally accomplished; glutamic acid furnished the starting material. The position of the hydroxyl group was established by a synthesis of the osazone that had been obtained from the product of the oxidation of oxyglutamic acid with chloramine-T, and useful salts with various alkaloids were described. The new acid was also isolated from gliadin and glutenin. Dakin subsequently (359) obtained 2.5 per cent of it from zein and Jones and Wilson (362) have found as much as 7.7 per cent of it in gliadin. These observations are therefore verifications of the prediction of Osborne in 1908.

METHIONINE



In 1921 Mueller (367) published a short paper in which he recorded the observation that there was a substance present in meat infusion, as well as in hydrolysates of some proteins, that was essential for the growth of hemolytic streptococcus and of certain strains of pneumococcus. This substance could be removed from beef infusion by boiling with Norit for 15 minutes. The cocci would grow, however, if to such a culture medium 1 per cent of commercial peptone were added, although a 1 per cent peptone solution by itself, with suitable salts and sugars added, did not furnish an adequate medium for the streptococcus. A solution containing the products of sulfuric acid hydrolysis of casein was an equally effective adjuvant, and preparations were secured from hydrolyzed edestin or meat protein that were also valuable, although one prepared from egg white was only weakly active.

Hydrolysates of wheat gluten, gelatin, wool, and silk were ineffective. These observations indicated that the active substance was probably an amino acid of limited distribution, and published analyses of these proteins indicated that none of the known amino acids possessed a distribution similar to that of the hypothetical active substance.

Mueller therefore attempted a fractionation of casein hydrolysates by various reagents and found that mercuric sulfate precipitated the active substance in the presence of 5 per cent sulfuric acid. This led to tests upon cystine, tyrosine, tryptophane, and histidine, the amino acids known to be precipitated by this reagent, but all were inactive. Further tests indicated that the substance was not precipitated by silver and alkali in the histidine fraction, but was partly precipitated along with arginine; it was apparently destroyed by phosphotungstic acid.

Later (368) Mueller obtained the impression, from attempts at fractional precipitation by mercuric sulfate, that two factors might be present, both of which were necessary for the growth of the streptococcus. Further work showed that the filtrate from a silver precipitation at "moderately alkaline reaction to litmus" gave a precipitate with mercuric sulfate that was inactive, and a filtrate from which tyrosine separated on evaporation, after removal of reagents. The mother liquor then deposited nodular masses of needles which were quite soluble in water and in 70–80 per cent alcohol. These were obviously impure but were so active that 0.01 milligram added to 25 cubic centimeters of medium sufficed for the growth of the streptococcus. The material gave a moderate reaction with Folin's phenol reagent but no nitroprusside test. It contained 10.6 per cent of nitrogen and sulfur was also present although no lead-blackening sulfur test could be obtained. Cystine was therefore absent and the high proportion of nitrogen showed that little tyrosine was present in the product.

In 1922 (369) Mueller announced the discovery of a new sulfur-containing amino acid among the products of hydrolysis of casein. It is a curious fact that Mueller was led to this discovery by a biological test, namely an acceleration of the growth of streptococcus; when the substance was finally obtained in a

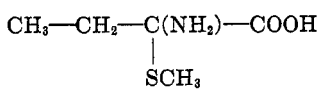
nearly pure form, however, it had no particular influence on the growth of the bacteria. After the discovery of the non-cystine sulfur his investigation had been continued purely from the chemical point of view. The procedure for the isolation of the new substance was laborious. The protein was hydrolyzed by sulfuric acid, neutralized by sodium carbonate and the solution was treated with mercuric sulfate. The precipitate was decomposed by hydrogen sulfide and the solution was treated again with mercuric sulfate. This removed much extraneous matter and the sulfur compound remained in the filtrate. More impurities were removed by a silver precipitation at alkaline reaction and the sulfur compound, in a yield of 10 grams from 30 pounds of casein, was secured by fractional crystallization from the filtrate. Analysis led to the formula $C_{11}H_{23}SN_2O_4$, but later work demonstrated that the preparation contained phenylalanine as an impurity. Mueller showed, however, that the nitrogen was all in the amino form, that the substance was a neutral amino acid and that the sulfur was extraordinarily stable.

In 1923 Mueller repeated the work (370) on a large scale and improved the method of preparation materially. The procedure now consisted of hydrolysis followed by neutralization of the hydrolysate and precipitation of the product by mercuric sulfate at a neutral reaction to litmus. The precipitate was thoroughly washed and then extracted by hot 2 per cent barium hydroxide solution four successive times. The extracts were freed from mercury by barium sulfide and then from barium. After concentration, mercuric chloride was added and the precipitate was removed, washed, and decomposed with barium sulfide. Reagents were removed from the filtrate which was then evaporated *in vacuo*. The crystals that separated were redissolved by heating and several volumes of alcohol were added; the product then separated in well-formed crystals. The material was, however, still impure. Further purification was effected, although with considerable loss, by a repetition of the precipitation with mercuric chloride. The yield varied from 0.2–0.4 per cent of the casein.

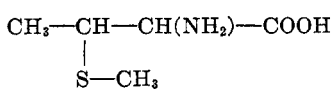
Analysis of this material and determination of the molecular

weight led to the formula $C_5H_{11}SNO_2$. It melted at 280–281° in a sealed tube and had $[\alpha]_D^{20} = -7.2°$. A copper salt and naphthylisocyanate derivative were prepared and analyzed. Mueller prepared the same substance after an alkali hydrolysis of casein and also after sulfuric acid hydrolysis in a procedure in which hydrogen selenide was employed for the decomposition of the mercury compounds in order to remove the possibility that sulfur had been introduced artificially. He likewise got it from egg albumin, edestin, and wool. A possible structure $C_2H_5—S—CH_2—CH(NH_2)COOH$, was eliminated by synthesis. Although many properties of the new substance corresponded with those of ethyl cysteine, the stability of the sulfur in the two compounds was different. When ethyl cysteine was treated with boiling dilute alkali ethyl mercaptan was split off; the new substance withstood this treatment. A biological test demonstrated (371) that the new amino acid was oxidized in the animal body.

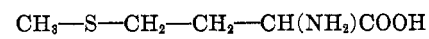
In 1925 Odake (372) isolated from yeast extracts a substance that possessed the properties of Mueller's new amino acid. Barger and Coyne (366) in 1928 became involved in a study of the non-cystine sulfur of proteins and prepared Mueller's substance. Inasmuch as Mueller had eliminated ethyl cysteine as a possibility and the methylthiol group is, like methoxyl, fairly common in nature, Barger and Coyne felt that the substance probably was a methylthiol derivative of one of the butyric acids. A test showed that Mueller's substance contained one methylthiol group, it was almost certainly an α-amino acid and consequently, its constitution was probably represented by one of the following formulas.



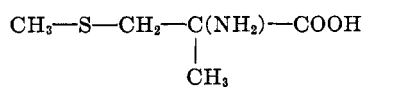
(I)



(II)



(III)



(IV)

Of these formula III was the most likely because of the analogy with cheirolin, a mustard oil present in wallflower seeds, which

has the constitution $CH_3—SO_2—CH_2—CH_2—CH_2—NCS$, and also because phenyl ethyl mustard oil is obviously allied to phenylalanine. "We therefore decided to synthesize the racemic substance (III), and indeed found it to be identical with Mueller's acid, except as regards optical activity. Later, Dr. Mueller informed us that he had himself arrived at the same constitution and had unsuccessfully attempted to prove it by synthesis. Since the amino acid has a good title to be regarded as a constituent of protein, a shorter name than γ-methylthiol-α-amino butyric acid seems desirable, and, after consultation with Dr. Mueller, we suggest for it the name methionine, in allusion to the characteristic grouping."

Barger and Coyne's synthesis started from β-methylthiolpropaldehyde, $CH_3—S—CH_2—CH_2CHO$, prepared from methyl mercaptan and sodium ethoxide by hydrolysis of the acetal. This product yielded the required amino acid, although in small yield (6 per cent), when the Strecker cyanohydrin method was applied.

A somewhat more satisfactory synthesis is that of Windus and Marvel (373) in which β-chloroethyl alcohol was allowed to react with sodium methyl mercaptide to form methylthiolethyl alcohol. This product was converted to the chloride with thionyl chloride and this served as the starting point of a conventional malonic ester synthesis.

OTHER PRODUCTS OF PROTEIN HYDROLYSIS

In addition to the twenty-one amino acids that have been clearly characterized as definite products of the hydrolysis of proteins there is one unique substance that has hitherto been obtained only by a single investigator from a single source. This is 3,5-dibromotyrosine, which was isolated in 1913 by C. T. Mörner from the horny skeleton of the coral *Primnoa lepadifera* (402). Mörner's identification of the product is so complete in every detail and there is so little chance for confusion with other compounds in the case of a substance that contains 47.2 per cent of bromine, that the exclusion of this interesting substance from the list of well established amino acid products of the hydrolysis of proteins is perhaps arbitrary. Nevertheless it has seemed wise to

adhere to the criteria adopted in the introduction of this paper and await the isolation of dibromotyrosine by some other investigator.

3,5-Dibromotyrosine was first synthesized by v. Gorup-Besanez (398) in 1863 by the action of bromine vapor on dry tyrosine. The identification of iodogorgoic acid from *Gorgonia Cavolinii* as 3,5-diiodotyrosine as a result of the work of Wheeler and Jamieson and of Henze, and the observation that bromine was present, in addition to iodine, in more than 50 different species of marine animals allied to the gorgonian corals, led Mörner, about 20 years ago (401), to search for dibromotyrosine in one of these. The axial skeleton was hydrolyzed by heating with barium hydroxide and, after cooling and removing the crystals of reagent, five volumes of alcohol were added. The precipitate was found to contain 78 per cent of the bromine; it was decomposed by carbon dioxide and the solution was treated with excess of basic lead acetate. After a reprecipitation with this reagent, the solution on evaporation yielded a crude crystalline product rich in bromine. More of the same substance was secured from the filtrates from the lead precipitates and, after extensive recrystallization, a product was finally obtained that was free from tyrosine and corresponded in all respects with synthetic 3,5-dibromo-*dl*-tyrosine. The yield was small, less than 0.2 per cent, and accounted for less than 3 per cent of the bromine of the original material.

Mörner's brilliant observation leaves no doubt whatever that dibromotyrosine, or bromogorgoic acid as he termed it, is a substance of physiological importance in at least one group of organisms. A further study of this substance would be highly desirable.

A method for the synthesis of dibromotyrosine much more convenient than that of v. Gorup-Besanez has been described by Zeynek (415).

Many other substances have been described by various investigators, from time to time, as definite products of the amino acid type derived by hydrolysis from proteins. None of these has been characterized in such a way as to comply with the criteria adopted

in the introduction of this paper; some of them, however, are substances that might logically be expected to be present among the products of hydrolysis of proteins, while others are almost certainly either mixtures of known amino acids or peptide-like compounds of these. Further investigation is required in each case before the substance in question can be safely accepted as a primary constituent of the protein. In the following paragraphs a number of these preparations are discussed, but no attempt has been made to present an exhaustive treatment of them.

In 1904, Skraup (410) described the isolation from casein of a number of amino acids; diaminoglutaric acid, $C_5H_{12}O_4N_2$, diaminoadipic acid, $C_6H_{14}O_4N_2$; oxyaminosuccinic (hydroxyaspartic) acid, $C_4H_7O_5N$; dioxydiaminosuberic acid, $C_8H_{16}N_2O_6$; a tribasic acid, "Caseansäure," $C_9H_{16}N_2O_6$; and a dibasic acid, caseinic acid, $C_{12}H_{16}N_2O_5$.

The amounts of oxyaminosuccinic acid, diaminoadipic acid, and dioxyaminosuberic acid Skraup obtained were very small and the preparations were of questionable purity; the constitution of none of them was established. With the exceptions of caseinic acid and possibly hydroxyaspartic acid, which has received a good deal of attention (386), none of these merits serious consideration at the present time. Skraup's caseinic acid was precipitable by phosphotungstic acid, and was isolated as the copper salt. On decomposing this he obtained two caseinic acids of the same composition but of different melting points. The first was optically active, the second was inactive.

Shortly after Skraup submitted his paper for publication, Fischer and Abderhalden (390) described the isolation of a compound from casein that was, in some respects, similar to Skraup's caseinic acid. Their product had a composition that could be represented by the formula $C_{12}H_{26}N_2O_5$; it was a saturated aliphatic oxyamino acid and received the name diaminotrioxydodecanic acid. This substance was found to occur in impure tyrosine preparations and could be separated by precipitation with phosphotungstic acid. The chemical constitution was not determined. In 1917 Fischer (389) stated that the individuality of this substance had become doubtful and it was therefore omitted from his list of established amino acids.

In 1927 Fränkel and Friedmann obtained (394), from a pancreatic digest of casein, a product that they regarded as possibly identical with Fischer and Abderhalden's substance. No proof of the constitution was presented, however, and the formula they ascribe to the anhydrous substance is so close to twice that of leucine as to suggest that they may have had an impure specimen of racemic leucine in hand.

Abderhalden and Kempe in 1907 (376) obtained, as a product of the hydrolysis of casein, a preparation that was considered to be oxytryptophane. In 1924 (378) Abderhalden and Sickel reëxamined this substance and concluded that it was 6-hydroxy-2,3,dihydroindolyl-3-alanine. Somewhat later (379), however, they prepared more of the material, purified it more thoroughly and arrived at the conclusion that it was a mixture that consisted chiefly of a peptide of tyrosine and proline. The original preparation probably contained tryptophane as well.

The possibility that a third isomeric leucine may occur in proteins has been frequently discussed but the difficulty of demonstrating this is so great that two investigators only have advanced definite claims in this connection. Thudichum, in 1901 (411), described experiments on the products of hydrolysis of neuroplastin. The crude leucine, secured by direct crystallization, was purified as the copper salt and a product was finally obtained that differed from ordinary leucine in solubility. Convincing evidence that this was *n*-leucine was, however, not provided.

Abderhalden and Weil, in 1912 (380), investigated the proteins of nerve tissue and isolated a substance, of the composition of leucine, which did not agree in optical properties with either leucine or isoleucine. Later (381) they presented evidence in support of the view that this substance was α-amino-*n*-caproic acid. They at first suggested the name "caprine" for this substance, but Abderhalden, Froehlich and Fuchs (375) substituted the name norleucine. Abderhalden and Weil (382) contrasted the melting points, rotations and other physical properties of their preparation with those of leucine and of isoleucine, and also compared the melting point of the naphthalenesulfonyl chloride derivative with that of synthetic norleucine; the agreement was

fairly close. They further drew attention to the differences in color of the preparations of the three copper salts as evidence of differences in structure.

Unfortunately Abderhalden and Weil give no precise description of the method by which their leucine isomer was secured. They apparently relied on fractional crystallization. In view of the difficulty of separating the two known isomeric leucines by this method, not to mention the difficulty of removing traces of valine, which might have important effects upon the physical properties of the resulting product, it would seem only conservative to require still more convincing evidence of the existence of norleucine among the products of hydrolysis of proteins than that given by Abderhalden and Weil.

Aminobutyric acid was stated to be a product of the alkaline hydrolysis of silk fibroin by Schützenberger and Bourgeois (409), but a reinvestigation by Fischer and Skita (392) showed that this conclusion was incorrect. Fischer and Mouneyrat (391) prepared synthetic aminobutyric acid and resolved it into the optical isomers; the properties of the active substance are therefore on record.

Foreman, in 1913 (393), during an investigation of a proline fraction secured from casein, obtained a preparation the composition of which corresponded to aminobutyric acid. The crude material was extracted with chloroform and then with cold absolute alcohol; the insoluble residue yielded the preparation in question. The evidence for its identity consisted solely in the ultimate analysis of the free acid and of the copper salt. No other derivatives were prepared and the melting point was much lower than that given by Fischer and Mouneyrat. Abderhalden and Weil, in 1913 (382), likewise claimed to have isolated aminobutyric acid from several proteins but provided no experimental evidence in support of this.

Van Slyke and Hiller (413) compared the histidine content of a number of proteins, as indicated by the Koessler and Hanke colorimetric method, with that determined from the non-amino nitrogen in the phosphotungstate precipitate after the removal of arginine. In the cases of casein, edestin, and fibrin the agree-

ment was good, but in the case of gelatin the values obtained from the non-amino nitrogen were much larger than those from the colorimetric method. In attempting to identify the substance responsible for this difference, Van Slyke and Hiller removed lysine, arginine, and histidine from the fraction precipitated by phosphotungstic acid; recrystallization of the material in the filtrate yielded a product in which the ratio of total to amino nitrogen was 2:1. Analysis of the copper salt prepared from this indicated the composition $(C_7H_9O_4N_2)_2Cu$. Van Slyke and Robson (414) later concluded, from the ratio of amino nitrogen to total nitrogen, and from the fact that the product gave a test for the pyrrol group, that the compound may be dihydroxypyrrolealanine. The structure has not been confirmed by synthesis nor has the free amino acid been prepared in a crystalline state.

Schryver and Buston (405) have reported the presence of hydroxyvaline and hydroxyaminobutyric acid among the soluble barium carbamates derived from the oat protein. This fraction was separated by means of the zinc salts into (*a*) a sub-fraction only slightly soluble in cold water; this contained leucine; (*b*) a sub-fraction easily soluble in cold water but insoluble in alcohol; this yielded alanine and valine; (*c*) a sub-fraction soluble in both cold water and alcohol; the two new amino acids occurred in this fraction. It was found that hydroxyaminobutyric acid yields a copper salt insoluble in methyl alcohol while the copper salt of hydroxyvaline is soluble in methyl alcohol. Analyses of the copper salt, the benzoyl, and the phenylisocyanate derivatives of the amino acid obtained from the copper salt insoluble in methyl alcohol, suggested that this substance was hydroxyaminobutyric acid. The positions of the hydroxyl and amino groups were not established nor was an attempt made to synthesize this amino acid.

Analyses of the copper salt, the benzoyl and the phenylisocyanate derivatives of the amino acid obtained from the copper salt soluble in methyl alcohol, indicated a composition corresponding to that of hydroxyvaline. The positions of the amino and hydroxy groups were not determined.

Gortner and Hoffmann (397) isolated a substance from the pro-

tein teozein, which they consider to be hydroxyaminobutyric acid, and the presence of the same substance in casein is suggested by the work of Rimington (404). Brazier (385) has reported the presence of hydroxyvaline in zein.

Schryver, Buston and Mukherjee (408) found that the glycine fraction, secured during the separation of the products of hydrolysis of fish gelatin by the carbamate method, contained a base that could be separated as its phosphotungstate and reprecipitated as a mercury salt. It was hygroscopic and absorbed carbon dioxide from the air and both the copper salt and the crystalline nitrate of this base were very deliquescent. The base was not precipitated by silver salts from solutions made alkaline with barium hydroxide and, consequently, it could be separated from arginine and histidine. Its composition corresponded to that of hydroxylysine. Schryver and Buston (407) believed that the hydroxy group is in the β-position although no direct evidence from synthesis was offered. Evidence for the presence of this basic amino acid in the proteins of oats, cabbage leaf, hemp seed, and gelatin was obtained.

Schryver and Buston (406) have also described a substance they isolated from oat protein and the protein of the castor bean, the composition of which corresponded to the formula $C_8H_{15}O_3N_3$. They gave it the name protoctine "to indicate a base with eight carbon atoms, derived from proteins." Its constitution was not determined. The product was soluble in water and in absolute alcohol, but insoluble in ether. It was precipitated from solution by mercuric chloride and barium hydroxide but not by silver nitrate and alkali. In alkaline solution it gave an orange-red color with diazobenzenesulfonic acid. The substance contained one amino, one carboxyl, and one hydroxyl group.

Fränkel and Monasterio (395) dialyzed a digest obtained from hemoglobin and treated the concentrated dialysate with methyl alcohol. A fraction was secured which, on analysis, had the composition $C_{22}H_{46}N_4O_7$. The preparation contained four amino groups and three carboxyls. Its structure was not elucidated but a name *Tetratrisäure* was given to it. No evidence that this is a homogeneous substance was presented.

Abderhalden and Bahn (374) have recently described indirect evidence for the presence of α-amino-*n*-valeric acid or norvaline among the products of hydrolysis of globin, and Abderhalden and Reich (377) have made similar observations upon products derived from casein. Their method is entirely new. The valine-leucine fraction secured by the ester distillation method was subjected to fractional crystallization and a valine fraction was isolated. This was treated with nitrosyl bromide whereby it was converted to α-bromovaleric acid; this was in turn treated with ammonia, and the rate at which bromide ion was split off was determined. This rate was compared with the rates at which bromide is removed from synthetic α-bromo-*n*-valeric acid and from the analogous compound secured from natural valine, α-bromoisovaleric acid. The curves for the products secured from globin and from casein corresponded with that of α-bromo-*n*-valeric acid. The conclusion was drawn that norvaline is one of the products of hydrolysis of these two proteins.

This review would be incomplete without a reference to two amino acids that have not yet been isolated from the products of hydrolysis of proteins but which further investigation may be expected to reveal; these are thiolhistidine and dihydroxyphenylalanine.

Evidence for the presence of ergothioneine, the betaine of thiolhistidine, in human and in animal blood has been obtained by Eagles and Johnson (387) and by Newton, Benedict, and Dakin (403). This substance, which was originally discovered in ergot of rye by Tanret, has been synthesized by Barger and Ewins (384). Eagles and Vars (388) investigated the physiology of ergothioneine and found that it was absent from the blood of pigs that had been restricted to a diet in which casein formed the source of protein, but appeared in the blood soon after the same animals were placed upon a diet that contained a large proportion of corn. This observation suggested that ergothioneine arises from a precursor in the corn diet and tests upon zein revealed that this protein, on hydrolysis, yields a solution that responds positively to Hunter's highly specific color test for the thiolimidazole ring. Weakly positive tests were obtained with a number of other pro-

teins but casein and gelatin do not yield the substance responsible for the test. It is probable that the substance that gives this test is thiolhistidine and searches for this substance have been conducted in several laboratories although hitherto without success. Experiments by Eagles and Vars in 1928 in the laboratory of one of the writers showed, however, that the reactive substance can be precipitated by silver salts at a reaction somewhat more acid than that necessary to precipitate histidine, and this observation has also been made by Ashley and Harington (383). The substance rapidly disappeared, however, during the attempts to recover it from the silver precipitate.

l-2-Thiolhistidine has been synthesized by Ashley and Harington.

Dihydroxyphenylalanine was first found by Torquati (412) in 1913, in aqueous extracts of the pods and sprouts of *Vicia faba*, but the identity of the substance was not recognized by him. Guggenheim (399), in the same year, pointed out that Torquati's substance was probably dihydroxyphenylalanine and proved this by repeating the preparation and definitely establishing the constitution and its identity with the synthetic substance prepared by Funk (396). Miller (400) has since identified it in an extract of the velvet bean (*Stizolobium deeringianum*) and has also shown that it is probably widely distributed in this genus.

No attempts to obtain dihydroxyphenylalanine from hydrolysates of proteins have come to our attention but Guggenheim pointed out that owing to the ease with which it is oxidized this substance may be in part responsible for the humin observed when proteins are hydrolyzed by acids. Further investigation will alone decide this.

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