a method which involved replacement of bromine with deuterium in the case of brominated anilines by means of Raney nickel–aluminum alloy in the presence of deuterium oxide and sodium deuteroxide was studied. *o*-Bromoaniline yielded aniline which was found to contain an amount of deuterium corresponding to the presence of 0.79 atom of deuterium for each aniline molecule. Furthermore, bromination of this sample of aniline gave rise to 2,4,6-tribromoaniline which contained only 0.03 atom of deuterium for each aromatic nucleus, and conversion of the deuterated aniline to *p*-bromoacetanilide yielded a product which showed a deuterium content equivalent to 0.80 atom for each aromatic nucleus. Thus, practically all of the deuterium was in the *ortho* position of the aniline, and the conversion of the bromoanilines to the corresponding deuteroanilines in the presence of Raney alloy appeared to be feasible. However, when this procedure was applied to *m*-bromoaniline and *p*-bromoaniline anomalous results were obtained. Instead of *m*-deuteroaniline and *p*-deuteroaniline, mixtures resulted and the compositions of these mixtures indicated a pronounced tendency for the formation of *o*-deuteroaniline. In order to obtain further information it was decided to ascertain whether aniline undergoes protium–deuterium exchange in the presence of deuterium oxide and sodium deuteroxide. Accordingly, ordinary aniline was refluxed for two hours with heavy water (*ca.* 99% D_2O) and sodium deuteroxide. After separation, the aniline was treated with about 20 times its own volume of methanol in order to transform N–D to N–H bonds. The methanol was removed by evaporation and the aniline was analyzed for deuterium. The mole fraction of deuterium [D/(D + H)] was found to be 0.0067. The mole fraction of deuterium in monodeuteroaniline is $^1/_7$ or 0.143. Therefore little or no protium–deuterium exchange occurs in the presence of base alone. Facile deuteration of aniline takes place, however, if Raney alloy is added. Aniline, after having been heated under reflux for 3.25 hours with deuterium oxide (*ca.* 95%), sodium deuteroxide and Raney alloy was separated, treated with methanol, as previously described, and distilled. Analysis[1] of this sample of aniline for deuterium was performed after 5.11-fold dilution with ordinary aniline. This dilution was made in order to avoid having to take into account the mass 4 peak on the mass spectrometer. In order to determine the distribution of the deuterium in the aromatic nucleus, the derivatives listed in Table I were also prepared and analyzed. In the case of sample II, the conditions were the same except that the aniline was heated under reflux for 24 hours.

TABLE I

Compound	$\frac{D}{D+H}$ for the undiluted sample I	II	No. of deuterium atoms per aromatic nucleus I	II
Aniline-d_x	0.144	0.313	1.02	2.19
Acetanilide-d_x	.117		1.05	
2,4,6-Tribromoaniline-d_x	.0276	.0680	0.11	0.27
p-Bromoacetanilide-d_x	.112	.235	0.89	1.88

(1) W. M. Lauer and W. E. Noland, THIS JOURNAL, **75**, 3689 (1953).

These results indicate the following percentage distribution of deuterium in the two samples.

	I	II
ortho	76.5	73.5
meta	10.8	12.3
para	12.7	14.2

It is evident that under the above-described conditions, exchange in the *ortho* position predominates. These results perhaps can be accounted for by assuming that the aniline molecule is anchored to the catalyst through the electron pair on the nitrogen; the rest of the molecule being less firmly attached. As a consequence, the *ortho* positions are in closest proximity to the catalyst and the probability of exchange in these positions is therefore the highest.

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The Amino Acid Composition of Human Salivary Amylase[1]

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The present study was undertaken in continuation of some work on the interaction of salivary amylase and chloride ions. Evidence for combination of chloride with the amylase, other than the well known activation of the enzyme, has been presented.[2] It seemed possible that some indication as to the nature of the structure responsible for the particular affinity of amylase for this anion might be revealed by amino acid analyses.

Salivary amylase was crystallized according to a previously described procedure.[2] Following hydrolysis of the protein its amino acid content was determined by chromatography on the ion-exchange resin Dowex-50, 8% cross-linked, according to the method of Moore and Stein.[3]

Experimental

Four times crystallized amylase was dissolved by adding 0.2 *N* sodium hydroxide dropwise to reach a *p*H of 10.4. The solution was then adjusted to *p*H 8.0 with 0.1 *N* acetic acid and the amylase was precipitated with three volumes of acetone at −14°. It was washed twice with acetone and dried *in vacuo* over sulfuric acid at room temperature. The amylase was used in this form for the amino acid analyses. Dry weight was determined on an aliquot by drying at 78° *in vacuo* over phosphorus pentoxide. The dry protein contained 0.78% ash and, on the basis of dry, ash-free protein, 17.04% nitrogen and 1.69% sulfur.[4] All the following results are reported on the basis of dry, ash-free protein.

Samples of the amylase, approximately 25 mg. in each portion, were hydrolyzed with 5 ml. of constant boiling hydrochloric acid in sealed tubes at 110° for 24 or 48 hours. The hydrochloric acid was removed *in vacuo*, the sample was made up to 5 ml. with water and 1 ml. containing approximately 5 mg. of amino acid mixture was applied to each column.

The Dowex-50 was a sample kindly provided by Drs.

(1) This work was carried out at the Rockefeller Institute for Medical Research. The generosity of Dr. Gertrude E. Perlmann in making the necessary laboratory space and equipment available is gratefully acknowledged.

(2) J. Muus, *Compt. rend. Lab. Carlsberg, Ser. Chim.*, **28**, 317 (1953).

(3) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(4) These determinations were done by Mr. S. Theodore Bella.

Stein and Moore from the product used by them.[3,5] A modified ninhydrin reagent was used.[6]

Table I shows the results of the amino acid determinations on the hydrolysates. The first column gives the values after 24 hr. hydrolysis and the second those after 48 hr. In most cases the two sets of results agree closely and the averages are given in the third column. Proline was overlooked in the 48 hr. sample. In the case of phenylalanine and tyrosine the 24 hr. values are used because considerably better separation was obtained. Serine and threonine are corrected for loss during hydrolysis. The correction was on the basis of the loss that occurred during hydrolysis of 24 to 48 hours. It was somewhat more for serine and less for threonine than that found by Rees.[7] A similar correction was applied to methionine, aspartic acid and glutamic acid. The difference between the 24 hours and 48 hours values seemed definitely outside of the experimental error. Losses of these amino acids during hydrolysis have been reported by Hirs[8] who also found slow release of isoleucine. Similar observations were made by Smith and collaborators[9,10] who in addition to serine, threonine, aspartic and glutamic acid also noticed some destruction of lysine on prolonged acid hydrolysis.

TABLE I

AMINO ACID COMPOSITION OF SALIVARY AMYLASE

Amino acid	Amino acid nitrogen, g. per 100 g. protein nitrogen Hydrolysis 24 hr.	48 hr.	Av. or cor.[a]	Amino acid[a] G. per 100 g. protein	Amino acid residue[a]
Alanine	4.08	4.12	4.10	4.43	3.53
Arginine	16.80	16.30	16.55	8.75	7.85
Aspartic acid[b]	11.39	10.65	12.0	19.3	16.7
1/2 Cystine[c]			(3.0)	(4.4)	(3.7)
Glutamic acid[b]	4.89	4.30	5.4	9.6	8.4
Glycine	7.56	7.43	7.50	6.82	5.18
Histidine	5.03	5.28	5.16	3.24	2.86
Isoleucine	3.57	3.73	3.65	5.80	5.01
Leucine	3.49	3.77	3.63	5.77	4.98
Lysine	7.36	6.94	7.15	6.33	5.56
Methionine[b]	1.22	1.06	1.3	2.4	2.1
Phenylalanine[d]	3.60	3.53	3.60	7.20	6.42
Proline	2.60		2.6	3.6	3.0
Serine[b]	5.09	3.92	6.1	7.8	6.4
Threonine[b]	3.01	2.91	3.1	4.5	3.8
Tryptophan[e]			(5.8)	7.2	6.6
Tyrosine[d]	2.51	2.37	2.51	5.51	4.96
Valine	4.81	4.89	4.85	6.89	5.83
Ammonia[f]	10.16	12.27	7.9		
Total without cys. and try.[g]	97.2	93.5	97.1		
Total with cys. and try.					102.9

[a] Average values except as indicated. [b] Corrected for loss during hydrolysis on the basis of difference between the 24 hr. and 48 hr. value. [c] On the assumption that total sulfur minus methionine sulfur is cystine sulfur. [d] 24 hr. values used because better separation was obtained. [e] On the basis of absorption in the range of 270–284 mμ with correction for tyrosine. [f] The 24 hr. value minus ammonia equivalent to corrections applied under (*b*). [g] Cystine and tryptophan are not included in the total nitrogen because it is assumed that part of the nitrogen from these amino acids appeared as ammonia.

Cystine was not recovered on the columns. A small peak was observed at the break-through point which might indicate oxidation to cysteic acid. If the assumption is made that the difference between the total sulfur and the methionine sulfur is all cystine sulfur, a value of 4.4 g. of cystine per 100 g. of protein is obtained.

Since acid hydrolysis was used tryptophan was not determined. It, therefore, was estimated from the absorption curve of the unhydrolyzed amylase in the range of 270 to 284 mμ with correction for the tyrosine. A shift of 4 mμ in the absorption bands toward longer wave length was assumed.[11]

Amide nitrogen was not determined. The ammonia value is corrected on the assumption that the nitrogen from the amino acids destroyed during hydrolysis, except for tryptophan, was transformed to ammonia. The ammonia value is obviously an approximation and, therefore, may account for the fact that the recovery of total nitrogen is somewhat high.

Discussion

The nitrogen content of 17.04% found here is in excellent agreement with the value of 16.94% obtained previously in this Laboratory on a similarly prepared sample of four times crystallized amylase dried in the same manner but without ash correction.[12] Bernfeld, *et al.*,[13] reported that their crystalline salivary amylase contained 15.8% nitrogen. They do not record the conditions of drying which may explain the discrepancy. However, it is more difficult to account for their failure to find sulfur.[14] Although no cystine was recovered in the experiments described here, the methionine peak was unmistakable and the method of preparation renders contamination with inorganic sulfur extremely unlikely. Moreover, the sulfur content of 1.69% exceeds that of the ash content.

The sum of the amino acid residues accounts for 103% of the dry weight of the amylase, a value within the experimental error especially in view of the possibility of overcorrection in some cases. If the 24 hr. value for aspartic and glutamic acid is used, the sum of the residues is 101.6%.

The most salient feature of the amino acid composition is the high aspartic acid content. There are 22.7 dicarboxylic acid residues and 12.7 basic amino acids per 100 amino acid residues. Amide nitrogen may make up for five to nine equivalents of the dicarboxylic acids. In chloride free acetate buffer of 0.1 ionic strength salivary amylase has an isoelectric *p*H of 5.9[2] which would seem compatible with a slight excess of acidic amino acids.

It would seem that the number of residues of isoleucine, leucine, lysine and phenylalanine are the same. Per 100 amino acid residues the values for these four acids all fall between 4.62 and 4.72.

Caldwell[15] has recently determined the amino acid composition of pancreatic amylase from swine. There are some similarities between the compositions of the two amylases. The values for the following amino acids differ by less than 10%: glutamic acid, glycine, isoleucine + leucine, proline, tryptophan, tyrosine. The differences are over 50% for arginine, alanine, serine and cystine, increasing in that order. The lower arginine content and also the somewhat lower lysine content of the pancreatic

(5) The valuable advice of Drs. S. Moore and W. H. Stein and a tested sample of amino acid mixture on which the method was checked is also gratefully acknowledged.

(6) S. Moore and W. H. Stein, personal communication.

(7) M. W. Rees, *Biochem. J.*, **40**, 632 (1946).

(8) C. H. W. Hirs, *Federation Proc.*, **13**, 230 (1954).

(9) E. L. Smith and A. Stockell, *J. Biol. Chem.*, **207**, 501 (1954).

(10) E. L. Smith, A. Stockell and J. R. Kimmel, *ibid.*, **207**, 551 (1954).

(11) G. H. Beaven and E. R. Holiday, *Advances in Protein Chem.*, **7**, 378 (1952).

(12) P. E. Johnson, Master's Thesis, Mount Holyoke College, 1950.

(13) P. Bernfeld, A. Staub and E. H. Fisher, *Helv. Chim. Acta*, **31**, 2165 (1948).

(14) P. Bernfeld, F. Duckert and E. H. Fisher, *ibid.*, **33**, 1064 (1950).

(15) M. L. Caldwell, E. S. Dickey, V. M. Hamrahan, H. C. Knog, J. T. Knog and M. Misko, THIS JOURNAL, **76**, 143 (1954).

amylase is reflected in the lower total nitrogen, 15.92%.

These two animal amylases are in many respects similar in their enzymatic activity and are both activated by chloride. If the clue to this activation is to be found in the amino acid composition, one might expect to find it related to the amino acids which are found in similar quantities. These are, however, found in amounts that are well within the usual range of protein composition. Work on this problem is being continued.

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The Base-catalyzed Condensation of Aldehydes with Methyl Isopropyl Ketone[1]

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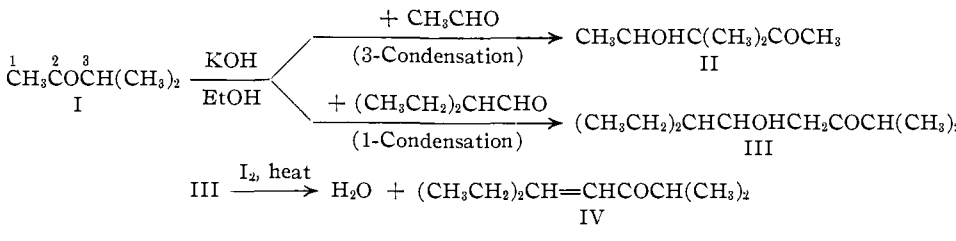
The base-catalyzed condensations of formaldehyde[3,4] and acetaldehyde[3,5] with methyl isopropyl ketone (I) have been reported to occur on the methylidyne group (3-condensation). On the other hand, several aldehydes have been found to condense on the methyl group of this ketone (1-condensation) including isobutyraldehyde,[6a] citral,[6b] benzaldehyde,[6c,d] salicylaldehyde,[6e] *o*-phthalaldehyde,[6f] and furfural.[6g]

In the present work the acetaldehyde condensation has been repeated using ethanolic potassium hydroxide catalyst. The ketol obtained, 4-hydroxy-3,3-dimethyl-2-pentanone (II), produced only traces of water on attempted dehydration by heating with iodine; thus, 3-condensation was indicated to be the main reaction, as previously reported.[5] (Zalkind[5] obtained II in 32% yield using potassium cyanide catalyst and oxidized it to 3,3-dimethyl-2,4-pentanedione.) However, we have found *n*-butyraldehyde and α-ethylbutyraldehyde to undergo mainly 1-condensation with I.



With α-ethylbutyraldehyde, the unsaturated ketone, 6-ethyl-2-methyl-4-octen-3-one (IV), was obtained as the main product in 47% yield; the structure of IV was confirmed by ozonolysis of it to α-ethylbutyric and isobutyric acids. The ketol, 6-ethyl-5-hydroxy-2-methyl-3-octanone (III), obtained in 6.5% yield, gave a 75% yield of IV when heated with iodine (86% water of dehydration). Thus, in this case, 98% or more of the ketone condensation occurred on the methyl group. In the *n*-butyraldehyde condensation the yields of unsaturated ketone, 2-methyl-4-octen-3-one (V) and ketol (VI) were 12 and 28%, respectively; a 6% yield of 2-ethyl-2-hexenal was also formed by self-condensation of the aldehyde. The structure of V was demonstrated by hydrogenation of it to 2-methyl-3-octanone. The present data do not permit determination of the exact amount of 1-condensation; from the dehydration data it is known to be at least 65%. Since VI gave a negative iodoform test it is likely that the actual value is somewhat higher. Under conditions identical with those found to be favorable for the acetaldehyde and *n*-butyraldehyde condensations, propionaldehyde failed to react with I, most of it being consumed by self-condensation to form propionaldol and 2-methyl-2-pentenal. It would appear from this last observation that the course of these reactions is sensitive to the relative reactivities and concentrations of the various carbanions present as well as to steric factors which are evidently operating.

In the presence of acid catalysts methyl isopropyl ketone reacts with bromine[7] and acetic anhydride[8] to give 76 and 68% 3-condensation, respectively; we have found, incidental to this work, that *n*-butyric anhydride gives 74% 3-condensation using Hauser's procedure.[8] In the acid-catalyzed reactions of I, unlike the base catalyzed, the amount of 3-condensation appears to be reasonably independent of the attacking molecule and to be determined by the concentrations of the reacting enols, at least for the limited number of cases so far studied.

If a general procedure were at hand for oxidizing 1,3-ketols to 1,3-diketones in high yields, a simple analytical method would then be provided for the analysis of ketol mixtures formed by condensing aldehydes with unsymmetrical methyl ketones.[9] Our initial experiments to find such a procedure have been only partly successful. We have found N-bromosuccinimide to oxidize 5-hydroxy-2,4,4-trimethyl-3-octanone to 2,4,4-trimethyl-3,5-octanedione in 81% yield (acid potassium permanganate gave 41%[10]). However, at-

(1) Based upon a thesis submitted by Elwin B. W. Ovist in partial fulfilment of the requirements for the degree of Master of Science, University of Idaho, 1951.

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(3) J. E. Dubois, *Ann. chim.* (Paris), **5**, 406 (1951).

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(8) C. R. Hauser and J. T. Adams, THIS JOURNAL, **66**, 345 (1944); *ibid.*, **67**, 284 (1945).

(9) Diketones of the type $RCOCH_2COR$ and $RCOC(R)(R')COR$ (R' = alkyl or H) may readily be separated quantitatively by the procedure of Hauser and Adams (ref. 8).

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