to d^{27}_{27} 1.10769. Correction for a slight abnormality in oxygen isotope ratios and for the difference in thermal expansions of H_2O and D_2O yields d^{25}_{25} 1.10763.

Deuterium concentrations of recombined fractions taken in the electrolysis are in good agreement with values calculated from measured separation coefficients for hydrogen isotopes determined with the same cells, if the above figure is taken to be the specific gravity of pure D_2O, but are inconsistent with Taylor and Selwood's d^{25}_{25} 1.10790.

The experimental observations indicate that Tronstad and Brun's published value d^{25}_{25} 1.10750 is definitely low but yield very close agreement with the revised value d^{25}_{25} 1.10764, attributed to them.

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Heavy Oxygen Exchange Reactions of Proteins and Amino Acids

BY WHITNEY H. MEARS AND HARRY SOBOTKA[1]

Exchange reactions with the heavy oxygen isotope O^{18} which recently has become available[2] promise to become valuable and helpful in the elucidation of a number of structural problems. The tendency of simple organic compounds to exchange, or not to exchange, the oxygen in various functional groups may be recognized in exploratory experiments. Hence, one may deduce the types of oxygen linkage in molecules, whose structure is still questionable, from their exchange behavior against water with different O^{18} abundance. Other applications of heavy isotopes are the study of reaction mechanisms[3] and the earmarking of organic substances in metabolism studies. The use of O^{18} along the latter line of research presupposes the possibility of isolating oxygen from its compounds, a task much more difficult in the case of oxygen than with other elements, as the disintegration of organic materials must be carried out, in the case of O^{18}, in the absence of extraneous oxygen to avoid dilution. The development of a convenient hydrogenation method must precede investigations of this nature (*cf.* ref. 9). Studies on compounds earmarked with O^{18} must be based upon findings on oxygen exchange behavior.

Studies on O^{18} exchange with proteins and their derivatives are reported in this paper. There are at least half a dozen functional groups containing oxygen in proteins, namely, (1) the peptide bond —CO—NH— (or $>\overset{\overset{OH}{|}}{C}—N<$ under the assumption of cyclization), (2) alcoholic hydroxyl groups as in serine, hydroxyglutamic acid, hydroxyproline, (3) phenolic hydroxyl groups as in tyrosine, (4) free carboxyl groups with two oxygen atoms in aspartic and glutamic acid, (5) amide groups in asparagine and glutamine, (6) $—OPO(OH)_2$ in phosphoproteins, and finally water of hydration, as in crystals, or possibly bound otherwise in the hollow of globular proteins. If only one or certain types of such oxygen atoms were subject to exchange, one would obtain information regarding protein structure, since it would be possible to accomplish such exchanges without denaturing the protein.

Exploratory experiments were carried out with a number of derivatives and potential fragments of protein. Model substances for the various oxygenous groups in protein, in which we observed no oxygen exchange, comprise glycylglycine, the simplest peptide (1), tyrosine as a model for a phenol (3), and benzamide for the $—CONH_2$ group (5). The stability of oxygen in peptide and amide linkages toward exchange can only be observed at moderate acidities where these bonds themselves are stable. The stability of aliphatic —OH (2) toward oxygen exchange over a wide range of hydrogen ion concentration was taken for granted on the basis of numerous precedents with alcohols, etc.

In the first runs with individual amino acids O^{18} exchanges up to 21% of the amount theoreti-

(1) Acknowledgments are due to The Rockefeller Foundation through whose aid this work has been carried out, to Professor H. C. Urey for the supply of heavy oxygen water of various concentrations and for helpful suggestions throughout the course of the work, and to Drs. M. Fox and J. E. Gorham for analyses by the mass spectrograph.

(2) J. R. Huffman and H. C. Urey, *Ind. Eng. Chem.*, **29**, 531 (1937).

(3) I. Roberts and H. C. Urey, THIS JOURNAL, **60**, 880, 2391 (1938).

cally possible were observed. Two of these were repeated after careful drying of the sample in the Abderhalden dryer and then no exchange whatsoever took place. As the nearest model for possible cyclic structures in the protein molecule, diketopiperazine and cyanuric acid were investigated with negative results. Only in the case of free carboxyl groups (4) in acid solution did we observe exchange with O^{18}, in fact of both oxygen atoms.[4] This is in agreement with findings of Urey and Cohn on trichloroacetic acid and of Roberts on acetic acid at high acidities.[5]

As a typical protein, egg albumin was tried first. The trifling amount of oxygen exchange observed was well within the limits of experimental error and corresponds to less than six oxygen atoms for a mol. wt. of 36,000. This result leads us to assume that egg albumin, dried under the given standard conditions, which exclude denaturation, retains no water nor does it contain oxygen exchangeable at a hydrogen concentration where the molecule is not subject to denaturation and hydrolytic disruption. Hydrolysis and subsequent reclosure of peptide linkages in the presence of water with high O^{18} abundance obviously would lead to statistical distribution of O^{18}.

Because of our observations[4] with glycine at *p*H 2, we wished to attempt oxygen exchange with a protein at very low *p*H. As a simple acid-stable protein pepsin was chosen, because it may be purified by crystallization, has a relatively well-known amino acid distribution, and because the maintenance of enzymatic activity may serve as an indication for the integrity of the molecule.

Preparation and Purification of Substances.—Glycine (Eastman Kodak Co.) was purified by recrystallization from 20% alcohol. **Glycine hydrochloride** was prepared by dissolving glycine in water and adding an equivalent amount of hydrochloric acid. On evaporation the hydrochloride crystallized and was dried to constant weight in the Abderhalden dryer at 110° (Cl found, 31.6%; calcd., 31.8%). **Tyrosine** (Pfanstiehl); amino N found (Van Slyke), 7.74%; calcd., 7.73%. **Diketopiperazine** (Eastman Kodak Co.) was recrystallized from water and washed with alcohol. **Leucine, cyanuric acid** and **benzamide** were likewise obtained from Eastman Kodak Co. **Albumin.**—Soluble flake egg albumin was dissolved in water and the insoluble material centrifuged off. The water was then removed by the Mudd–Flosdorf method and the substance dried to constant weight on the high vacuum line at room temperature.[6]

Pepsin.—Commercial pepsin (Parke, Davis and Co.) was crystallized according to the method of Philpot.[7] The crystals were washed with distilled water and then dialyzed against distilled water until the sulfate content had dropped below 0.2%. They were then suspended in water, frozen, and the water was pumped off using the Mudd–Flosdorf technique. Before exchange each sample was dried to constant weight on the high vacuum line. The pepsin contained 1.3% ash, some of which was sodium sulfate, as the samples showed 0.2% sulfur and 0.02% sodium. However, such small quantities of impurities could not interfere with the results of the exchange experiments.

The activities of the pepsin samples were measured before and after the oxygen exchange as was also that of a sample of commercial pepsin for comparison. The method was a modification of the one used by Langmuir and Schaefer.[8] A solution of 16 g. of "Breadlac" (for a sample of which we are indebted to the Borden Co.) was dissolved in 100 g. of water. As a buffer 0.82 g. of sodium acetate was added and the solution adjusted with glacial acetic acid to *p*H 5.5, which was checked potentiometrically. The pepsin test solution contained a known amount of pepsin dissolved in water. Both solutions were warmed to 37° and 1 cc. of the test solution was pipetted rapidly into 5 cc. of the milk solution, shaken once by inverting the tube and observed at 37°. When the surface of the liquid would not flow past a 45° angle with the tube held horizontally the clotting point was reached and the time recorded. The solution contained somewhat more water than Langmuir's, since it was more convenient to add a known volume of liquid rather than a weighed amount of dry pepsin. The results were calculated by the equation $U = V/6t$ where t = time in minutes and V = volume of solution coagulated.

The initial sample contained 66,000 units in one gram; after the exchange operations of the second experiment the peptic activity was 45,000 units/g. Thus the pepsin lost less than one-third of its activity during the drying operations and O^{18} exchange to equilibrium. It was not denatured, as it retained its solubility.

Oxygen Exchange Experiments.—The following technique was used in the exchange reactions. The Abderhalden dryer was used for drying amino acids and simpler organic compounds. Where the compound foamed or would have been denatured through heat, the bulk of the water was removed by the method of dehydration through the solid or ice phase, developed by Flosdorf and Mudd.[6] For the final drying to constant weight, desiccation at room temperature in the vacuum of a mercury vapor pump proved satisfactory. The Mudd–Flosdorf apparatus consisted of a manifold to which containers might be attached and sealed off by a metal ring clamp. This manifold was connected with a large trap cooled with dry-ice and methylcellosolve, and finally to a Cenco Hy-vac pump. An adaptation of this method served to remove the water from the protein solution after O^{18} exchange. Here, the large trap was replaced by two small ones in series while the pumping system consisted of a one-stage mercury vapor pump and a Cenco Hy-vac fore-pump.

(4) W. H. Mears, *J. Chem. Phys.*, **6**, 295 (1938).

(5) H. C. Urey and M. Cohn, THIS JOURNAL, **60**, 679 (1938); I. Roberts, *J. Chem. Phys.*, **6**, 294 (1938).

(6) Flosdorf and Mudd, *J. Immunology*, **29**, 387 (1934).

(7) Philpot, *Biochem. J.*, **29**, 2458 (1935).

(8) Langmuir and Schaefer, THIS JOURNAL, **60**, 1351 (1938).

In this case the sample to be dehydrated could be weighed during the process as the reaction flask was equipped with a break-off seal and could be attached to the apparatus through a ground glass joint and stopcock.

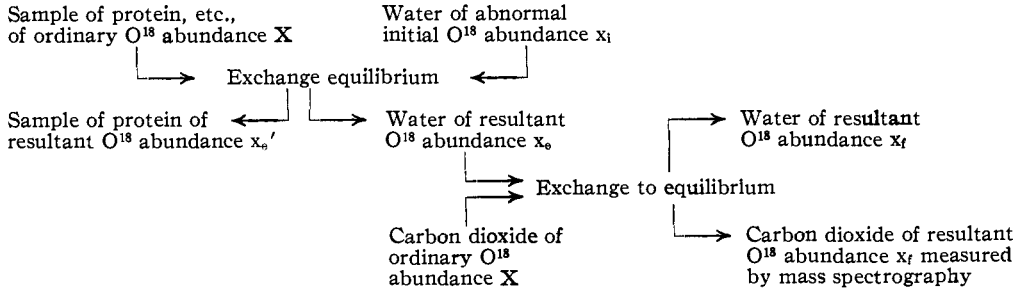
In preparing the samples for exchange, strict vacuum technique served to prevent contamination from the water in the atmosphere. The reaction flask consisted of a 50-cc. flask which was equipped with a side arm used for sealing it to the vacuum line and with a capillary break-off seal for removal of the water after the run. The vacuum system consisted of a trap to protect the pumps, and the mercury and Cenco pumps described above. On the opposite side of the protection trap from the pumps was a long 12-mm. wide tube for connection of the reaction flask with a closed end manometer, and finally with the purification line for the water samples after reaction. This line consisted of several traps equipped with ground glass joints in which a weighed amount of materials for purifying the water could be placed. The same line, slightly modified by the removal of the purification train, was used for the preparation of the mass spectrograph samples. This apparatus and technique are described by Cohn and Urey[5]; the same mass spectrograph was used for the samples in this research.

To prepare for a run, the compound was dried to constant weight in the Abderhalden dryer at an elevated temperature. A sufficient weight of compound to give a 1:2 or 1:3 molar ratio with 1 cc. of water was placed in the reaction flask. The flask was sealed to the vacuum line and evacuated for thirty minutes by the pumping system while the water sample was prepared. One cc. of purified water of known isotopic concentration was placed in a trap connected to the rest of the line through a stopcock and closed by a ground glass joint which served as a cap. After being frozen by a dry ice–ether mixture and completely evacuated, the water sample was thawed and distilled into the reaction flask. This was sealed off and the mixture was kept in contact for a definite time either at room temperature or in a water-bath maintained at 100°. On completion of the run the flask was resealed to the apparatus and evacuated up to the break-off seal. This was then broken and the water distilled to the purification train which contained known amounts of sodium hydroxide, chromic oxide, and sometimes potassium permanganate. To analyze the water of the train, a known amount was equilibrated with carbon dioxide after the method of Cohn and Urey, and analyzed on the mass spectrograph.

At the commencement of this work, an investigation of the possibilities of the falling drop or other density methods was undertaken. However, a number of difficulties arose with the task of adequate purification of the water after exchange without introduction of extraneous oxygen, and also because of the necessity for electrolyzing all initial water samples to avoid the effect of deuterium concentration. Therefore, this was abandoned for the more rapid and convenient mass spectrograph method described above. It is thought, however, that a density method might become serviceable if it were used in conjunction with some hydrogenation train.[9]

While the above technique served satisfactorily when compounds of low molecular weight were investigated, it had to be modified in the study of proteins where a high "oxygen exchange equivalent weight" was expected. In this case the same water sample was exchanged with successive portions of protein of about equal weight, the whole process being similar to a fractional extraction. In this process, protein was carefully weighed into a reaction flask of about 20-cc. capacity equipped with a neck and two break-off seals. The neck was then sealed to a stopcock and the inner member of a ground glass joint, pumped on the high vacuum line to constant weight and sealed off. In this manner dried protein samples could be stored *in vacuo* until needed. To carry out the exchange the sample was resealed at one of the break-off seals to the stopcock and joint, weighed and the water sample to be exchanged distilled onto the protein. Some shaking, usually for a few hours at about 20 strokes a minute, accomplished the solution or suspension of the reactant. To stop the run the solution was frozen and the water pumped off *in vacuo* to be caught in one of a series of traps kept in a dry-ice bath. To exchange the next sample the process was repeated. The reaction flask was weighed before and after each operation to determine the amount of water gained or lost and thus to correct for any water which diluted the reaction sample in isolated instances. The results of two pepsin runs are shown in Tables II and III.

In evaluating the results the following steps must be considered



With regard to the exchange reaction between carbon dioxide and water, the equilibrium between CO^{16}_2 and CO^{18}_2 must be corrected for, and the equilibrium between $CO^{16}O^{18}$ and CO^{16}_2 must be calculated. The following are the equilibria involved[10]

(9) *Cf., e. g.*, H. Ter Meulen, *Rec. trav. chim.*, **53**, 118 (1934); Lindner and Wirth, *Ber.*, **70**, 1025 (1937), and Unterzaucher and Bürger, *ibid.*, **70**, 1392 (1937).

(10) Urey and Greiff, THIS JOURNAL, **57**, 321 (1935).

$CO^{16}_2 + 2H_2O^{18} = CO^{18}_2 + 2H_2O^{16}$ $K = 1.080$ at 298°K.
$CO^{16}_2 + CO^{18}_2 = 2CO^{16}O^{18}$ $K = 3.999$ at 298°K.

The equilibrium constant for the reaction

$$CO^{16}_2 + H_2O^{18} = CO^{16}O^{18} + H_2O^{16}$$

is therefore 2.076 at 398°K. Using the above results, the following expression may be developed for the change in isotopic abundance when water of other than normal abundance is brought into contact with normal carbon dioxide. The isotopic abundance of carbon dioxide after exchange with the water sample to be analyzed is $[CO^{16}O^{18}]_f/[CO^{16}_2]_f$ where the subscript indicates concentrations after exchange has taken place. The species CO^{18}_2 can be neglected for our purposes. By comparison of the peaks on the mass spectrograph, the abundance of $CO^{16}O^{18}$ is found r times higher than the normal abundance $[CO^{16}O^{18}]_o/[CO^{16}_2]_o$, which equals 0.004030, namely, the product of general abundance of O^{18} in oxygen, 0.001947, times the equilibrium constant $K = 2.076$ derived above. The abundances of $CO^{16}O^{18}$ before and after exchange bear the following relation to those of H_2O^{18} in the water sample analyzed

$$w[(H_2O^{18})_e - (H_2O^{18})_f] = c[(CO^{16}O^{18})_f - (CO^{16}O^{18})_o] \quad (1)$$

where w and c are the number of moles of water and carbon dioxide present, and the terms in parentheses represent the abundances of the species of molecules indicated, expressed in mole fractions, *e. g.*

$$(H_2O^{18}) = [H_2O^{18}]/\{[H_2O^{18}] + [H_2O^{16}]\}$$

The subscript "e" stands for the isotopic abundance of the water sample equilibrated with the protein under investigation, "o" for the original abundance of $CO^{16}O^{18}$, and "f" for the resulting abundance after exchange between H_2O and CO_2 has been accomplished. In turn, $c(CO^{16}O^{18})_f - (CO^{16}O^{18})_o = c(CO^{16}O^{18})_o(r - 1)$. Hence, the abundance of heavy oxygen water after exchange with the protein sample, expressed as molecular ratio between the species H_2O^{16} and H_2O^{18}, is

$$x_e = \frac{w(H_2O^{18})_f + c(CO^{16}O^{18})_o(r - 1)}{w(H_2O^{16})_f - c(CO^{16}O^{18})_o(r - 1)} \quad (2)$$

Replacing the mole ratio x_e by the mole fraction N_e of (H_2O^{18}), the denominator of the right side of the preceding equation becomes simply w; hence

$$N_e = (H_2O^{18})_f + \frac{c}{w}(CO^{16}O^{18})_o(r - 1) \quad (3)$$

Since $(CO^{16}O^{18}) = K(H_2O^{18})$, we obtain

$$N_e = (H_2O^{18})_o[r(1 + Kc/w) - Kc/w] \quad (4)$$

In this equation $(H_2O^{18})_o$ is the ordinary abundance of the most abundant heavy isotope of oxygen taken from the work of Manion, Urey, and Bleakney[11] on the oxygen abundance in meteorites and equals 1:514 or 0.001947. The numbers of moles c and w, the equilibrium constant K, and the result of the mass spectrograph measurement r have been defined above: since the concentration of O^{18} is small, it is permissible to calculate c and w on the basis of the molecular weights 44.01 for carbon dioxide and 18.02 for water.

In the exchange reactions summarized in Table I, we had also to consider the diluting effect of the oxygen contained in the salts used for the purification of the water, according to the equation

$$N_e = \frac{x_e w + \Sigma s_n z_n (x_e - X)}{w} \quad (5)$$

where N_e, x_e, and w are used as before. X is the ordinary abundance of O^{18} assumed to obtain in these salts, $s_1, s_2, s_3, \ldots s_n$ is the number of moles and $z_1, z_2, \ldots z_n$ the number of oxygen atoms per molecule, assuming that the salts used in the purification exchange completely under the conditions of the experiments.

To decide whether exchange of O^{18} has taken place or not between a given compound and water, the H_2O^{18} abundance in the equilibrated state, N_e, computed from the mass spectrograph analysis according to equation (4) must be compared with the calculated value for N_e. This is derived from the number of moles w of heavy water of O^{18} abundance x_i and the number of moles p of the substance of O^{18} abundance X and containing z atoms expected to be exchangeable under the assumption that complete statistical distribution of the O^{18} isotope occurs

$$N_e \text{ calcd.} = \frac{x_i w + X z p}{w + z p} \quad (6)$$

The significance of experimental errors on the evaluation of the final isotopic abundance may be illustrated by the following figures. An error of 1% or 10 mg. due to incomplete drying of a 1-g. sample of a substance will cause an error in abundance of O^{18} of *ca.* 0.00002 under the conditions of the experiments. A similar deviation would be produced by a 10% error in the estimation of the volume of carbon dioxide. Finally, an error of 1% in the mass spectrographic determination of the peak ratio would contribute a variation smaller than, or at most equal to, the

(11) Urey and Bleakney, THIS JOURNAL, **56**, 2601 (1934).

TABLE I

OXYGEN EXCHANGE EXPERIMENTS WITH AMINO ACIDS AND RELATED COMPOUNDS AT 100°

Compound	Water sample	Change in isotopic abundance Calcd.	Obsd.	Exchange, % of theory	Time, hrs.	Remarks
Glycine	Light	0.000614	0.000050	8.2	72	Samples insufficiently dried
Leucine	Light	.000371	.000082	21	41	
Tyrosine	Heavy	.000253	.000029	11	46	
Diketopiperazine	Heavy	.001967	.000130	6.8	42	
Tyrosine	Light	.000682	− .000060	..	40	
Diketopiperazine	Heavy	.001700	− .000015	..	48	
Glycylglycine	Light	.000632	− .000020	..	24	
Benzamide	Light	.000300	− .000020	..	23	
Glycine	Light	.000610	.000654	106.5	24	*p*H 2. Two atoms oxygen per mol. exchange
Glycine	Light	.000607	.000644	106.3	70	
Cyanuric acid	Light	.001585	.000026	1.6	40	
Albumin	Heavy	.001935	.000020	1.0	40	Room temperature
Pepsin						See Tables II and III

errors of the chemical operations. Since the usual expected change in O^{18} abundance is about 0.00030 or more (see Table I) and the average errors are of the magnitude 0.00002, the uncertainty of the results is about 6% and proportionally smaller, the larger the expected change in abundance from exchange experiments.

Multiple Exchange Experiments.—To calculate the results of a multiple exchange experiment, it is first necessary to evaluate the equivalent weight of protein for an exchangeable oxygen atom. In the solution of this problem, a mathematical treatment of the process of multiple exchange is in order. Two assumptions are made: first, that the mole fraction of water present in each exchange is constant; and second, that the equilibrium is reached. The first may be shown valid by inspection of the sixth column, molar fraction b, in Table II, whereas the second seems correct since two different runs of widely varying conditions of exchange give concordant results. Identical amounts of oxygen were exchanged (and, hence, identical oxygen exchange equivalents were obtained) in one run with four exchange periods of forty-eight hours each and in a second one with two exchange periods of one hundred and sixty-eight hours each. Moreover, in the second experiment, the water sample used had approximately twice as high an O^{18} concentration.

Multiple exchange follows a course resembling that of a fractional extraction. Let w be again the number of moles of water, and p the number of "equivalents" of pepsin, and b the mole fraction of water $w/(w + p)$. W_p, the average weight of pepsin in grams used in each exchange operation, divided by p yields E_p, the oxygen exchange equivalent of the pepsin. The isotopic abundance of O^{18}, expressed as mole ratio, is x with the subscript i for the initial ratio, n after n exchanges, and e for the final equilibrium value, obtained by mass spectrographic measurement. X represents again the ordinary abundance of O^{18}. Hence,

$$x_n = \frac{wx_i + pX}{w + p} \tag{7}$$

and

$$x_1 = bx_1 - X(b - 1) \tag{8}$$

For the "n"th exchange the equation

$$x_n = b^n x_1 - b^n X + X \tag{9}$$

may be derived, which, solved for b, results in

$$b = \left(\frac{x_n - X}{x_i - X}\right)^{1/n} \tag{10}$$

Using the definition of b and solving for p, one obtains the number of oxygen exchange equivalents

$$p = w\left[\left(\frac{x_i - X}{x_n - X}\right)^{1/n} - 1\right] \tag{11}$$

The mole ratios x. . . may be replaced by the mole fractions N . . . .

The theoretically expected change in abundance is the sum of the partial changes from the four or two pepsin exchanges computed in Table II. The experimentally found change, divided by the change calculated on the basis $E_p = 500$, gives the percentage exchange, which was 99 and 94% for experiments I and II, respectively. Or, using equation (11), one may calculate the number of equivalents p, hence the magnitude of the equivalent E_p which, as given in the last column in the lower half of the table, agrees with the assumption of $E_p = 500$ within the limits of experimental accuracy.

TABLE II

MULTIPLE HEAVY OXYGEN EXCHANGE BETWEEN PEPSIN AND WATER

Exchange no., n	Pepsin = W_p, g.	Water distilled on, g.	Equivalents of pepsin, p	Moles of water, w	Molar fraction, $b = w/(w + p)$	O^{18} abundance, x
			Expt. I			
1	0.71	1.00	0.00142	0.0556	0.974	x_i = 0.004685
2	.91	0.92	.00182	.0508	.969	x_1 = .004614
3	.85	.85	.00170	.0472	.966	x_2 = .004522
4	.78	.84	.00156	.0467	.967	x_3 = .004428[a]
						x_4 = .004349[a]
Measured	..	..	....	...	..	x_e = .004353[a]
			Expt. II			
1	0.693	0.999	0.00139	0.0552	0.978	x_i = 0.008075
2	.634	.982	.00127	.0545	.97	x_1 = .007905
						x_2 = .007770
Measured	..	..	....	...	..	x_e = .007788

	Initial abundance of O^{18}		Final abundance of O^{18}		Av. no. of moles of water, w	Weight, number of equivalents and exchange eq. of pepsin		
	Mole ratio x_i	Mole fraction N_i	Mole ratio x_e	Mole fract. N_e		W_p	p	E_p
Expt. I	0.004685	0.004663	0.004353	0.004334	0.0501	0.81	0.00162	493
Expt. II	.008075	.008010	.007788	.007728	.0548	.663	.00133	502

[a] In the abundances x_3, x_4, and x_e of Expt. I, it was necessary to correct for two slight dilutions which occurred after the third and last exchanges.

Discussion of Results

Considering the various functional groups containing oxygen in proteins, it can be seen from Table I that under the conditions of the experiments exchange occurs only in the case of the carboxylic group at about *p*H 2. The figures in Table I demonstrate that in glycine, glycylglycine, leucine, and diketopiperazine, the carboxyl groups and peptide links do not exchange oxygen at neutral reaction. In leucine the observed change in isotopic abundance must be ascribed to accidental dilution, since in these earlier runs the compound was not dried in the Abderhalden, but only for a short time on the high vacuum line. The other changes are within the maximum experimental error of the measurements, and in view of the rigorous conditions under which these exchanges were carried out, they indicate the stability of these oxygenous groups against exchange. The other oxygen containing groups tested with negative results were: the amide group, the phenolic oxygen of tyrosine, and the CO groups in cyanuric acid. Serine was not studied in view of the above results and the work of Roberts in which the alcoholic hydroxyl is shown not to exchange at neutral or moderately acid reaction.[12]

Since no exchange is found at the neutral point with the oxygen groups of the protein molecule, it is not surprising that no exchange was found in the case of dry egg albumin. Moreover, it is probable that no water of crystallization or adsorption is held by albumin, when dried under our conditions. Exchange would be expected in acid solution in the case of the acid-stable pepsin which contains a number of free carboxyl groups, and this is actually the case as may be seen from Table II. It is worth while noting that the *p*H at which pepsin exchanged was about 4.0, whereas exchange of oxygen from carboxyl groups in acetic acid and glycine becomes significant at about *p*H 2.[4,12] It seems possible that some structural property of the pepsin, or of proteins in general, weakens the C–O link, enabling a faster approach to equilibrium at a relatively higher *p*H.

The close agreement between the two pepsin runs carried under widely differing conditions to equilibrium, encourages one to consider the bearing of the pepsin results on its molecular structure from the analytical data available. These, although incomplete, account for the most important amino acids with regard to oxygen exchange (Table III). 24.07% oxygen in one molecule of mol. wt. 36,000 corresponds to 540 atoms of oxygen. According to our results, 36,000/500 = 72 oxygen atoms per molecule, or 13.4% of the total oxygen, exchange, which is equivalent to 36 free carboxyl groups. The data in Table III are composed of the best information at present available, principally from the work of Northrup,

(12) Roberts, *J. Chem. Phys.*, **6**, 294 (1938).

Calvary, and Herriot.[13,14] The figures indicate that there still remain about 188 amino acids unaccounted for, including glycine, alanine, and their higher homologs, and hydroxyamino acids. There are about 64 dicarboxylic acids of which about 28 are tied down as amides, leaving 36 free carboxylic groups to react, in good agreement with our observations. There are furthermore 104 + 28 = 132 oxygen atoms which must be assigned to aliphatic hydroxyl groups except perhaps 3 oxygen atoms for a single phosphoric acid residue. The figure of about 130 oxygen atoms for 188 amino acid residues (average residue weight, 120) unaccounted for seems rather high; it may be lowered by assuming that the elementary analysis given was performed on a specimen containing, *e. g.*, 1% moisture. This would account for 20 oxygen atoms per molecule, whereas the percentage of hydrogen would be affected in the second decimal only. However, such an additional amount of 20 atoms of oxygen in exchangeable form in our pepsin specimen would not have gone unnoticed. The inclusion of bound water in order to reconcile the number of 288 amino acids with a molecular weight slightly higher than 36,000 is excluded by our findings.

TABLE III

AMINO ACID DISTRIBUTION AND OXYGEN PARTITION OF PEPSIN

Amino acid	Weight per cent. Found[a]	Calcd.	Number of equivalents	Number of oxygen atoms In peptide bond	In side chain
Histidine	0.28	0.42	1	1	...
Arginine	1.29	1.45	3	3	...
Cystine	2.2	2.67	4	4	...
Lysine	1.69	1.62	4	4	...
Tryptophan	2.2	2.27	4	4	...
Tyrosine	10.3	10.03	20	20	20
Aspartic acid	6.8	6.66	18	..	...
Glutamic acid	18.6	18.4 (19.6)	45 (48)	*ca.* 64	128 − *a*[b]
Ammonia	1.35	1.32	28	..	...
Phosphorus	0.078	0.086	1	..	3
Total accounted for			*ca.* 100	100	151 − *a*
Unaccounted for			*ca.* 188	188	101 + *a*
Total			288	288	252
Total number of oxygen atoms				540	

[a] Elementary analysis of pepsin according to Northrup [*J. Gen. Physiol.*, **13**, 775, 778 (1930)], in per cent.: 52.4 C, 6.67 H, 15.3 N, 0.86 S, 0.078 P, 0.22 Cl, 0.40 ash, and 24.07 O; amino acid distribution according to Calvary, Herriot and Northrup [*J. Biol. Chem.*, **113**, 11 (1936)]. [b] a = number of $-NH_2$ groups substituted for $-OH$ in the free carboxyl group of aspartic and glutamic acids. a must be ≥ 28 the number of NH_3 molecules obtained on hydrolysis. If one assumes $a = 28$, then the number of exchangeable oxygen atoms $128 - 2a$ becomes 72 in agreement with the findings by O^{18} exchange.

(13) We are indebted to Professor Edwin J. Cohn of Harvard Medical School for a critical survey of these figures.

(14) Northrup, Calvary and Herriot, *J. Biol. Chem.*, **113**, 11 (1936); *J. Gen. Physiol.*, **18**, 53 (1935).

Summary

The oxygen exchange behavior of proteins and protein derivatives was studied by means of water with high or low O^{18} abundance. The only oxygenous group in amino acids which exchanges its oxygen against that of water was found to be the carboxyl group at acid reaction. Albumin at neutral reaction did not exchange any oxygen, but crystalline pepsin, as representative of a protein soluble at strongly acid reaction, exchanged 13% of its oxygen. This figure corresponds with the amount of oxygen present in the free carboxyl groups of the dicarboxylic amino acids contained in pepsin. The data, in addition to their bearing on the use of heavy oxygen isotopes in metabolic studies, corroborate the present knowledge of amino acid distribution in pepsin.

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