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The Assay of Tritium in the Form of Ammonia and the Measurement of Exchangeable Hydrogen[1]

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Because of the potential utility of tritium for the investigation of hydrogen bonding in biological macromolecules, a new gas-phase method has been developed for the assay of this isotope. The method has been designed to be particularly suitable for the measurement of tritium acquired by exchange of the non-carbon-bonded hydrogen of proteins and nucleic acids with, *e.g.*, tritiated water. Ammonia has been selected as the counting gas primarily because in the presence of methane it gives rise to a plateau in the proportional region and because it undergoes equilibrium exchange with the exchangeable hydrogen of protein. The equilibration is carried out with carefully dried protein at 80° overnight and practically complete recovery of ammonia is also achieved at this temperature. For the development of the method exchange experiments have been carried out with tritiated ammonia of measured specific activity and lyophilized samples of isoionic human serum albumin. It has been found that there is an isotope effect in this exchange which favors the retention of the tritium in the solid phase. The exchange of ammonia with glass and vacuum grease requires that blank corrections be made. It is evident that the tritium content of proteins acquired by exchange can be measured with an accuracy of 1 or 2% with samples of 10 mg. or possibly less.

In recent years the essential participation of the hydrogen bond in the structure of biological macromolecules has received considerable emphasis and clarification. The main developments which have provided this focus are the emergence of the α-helix as a basic feature of the organization of protein molecules and synthetic polypeptides, originally proposed by Pauling and Corey,[2] and the proposal by Watson and Crick[3] that the deoxyribonucleic acid molecule exists as a two-stranded helical structure. In both structures intramolecular hydrogen bonds are assumed to play a decisive role in rendering them stable in aqueous solution. The experimental investigation of this aspect of the structure of biological macromolecules was greatly facilitated by the development, a few years ago, of a new method for studying hydrogen bonding within macromolecules in aqueous solution. This method, as described by Krause and Linderstrøm-Lang,[4] is based on the idea that hydrogen atoms engaged in intramolecular hydrogen bonding, in particular, the imide hydrogen of the peptide group, might exchange sufficiently slowly with deuterated water so as to be detectable by measurement of the exchange rate and distinguishable from other non-carbon-bonded hydrogen atoms not so involved. Such an experimental distinction has indeed been found, and it has been placed on a quantitative basis by the kinetic studies of Hvidt and Linderstrøm-Lang.[5] Prior to this work Lenormant and Blout[6] inferred from infrared spectra that there are two kinds of peptide groups in bovine serum albumin and egg albumin which differ in the ease with which they are deuterated by D_2O. More recently Haggis[7] has made a more extensive study of proton-deuteron exchange between D_2O and proteins by the infrared absorption method.

It has appeared to us that considerable experimental advantage might be gained in such exchange studies by the use of the radioactive isotope tritium, particularly because of its ready availability at low cost and with high specific activity. The main problem which was encountered in such a proposed application was the development of a suitable assay method for tritium. The methods which have been described[8] are generally unsatisfactory for our purpose because of limitations such as insensitivity, inaccuracy and loss of radioactivity during handling of the specimen. We have, therefore, devoted our attention to the development of a new method which would be especially suitable for exchange studies.

In considering the selection of a counting gas, it appeared that ammonia would provide several important advantages. It possesses a relatively low ionization potential and strongly absorbs photons formed during a discharge.[9] Experimentally it was found that tritium-containing ammonia mixed with methane could be counted under appropriate conditions. In addition ammonia is not irreversibly bound to proteins[10] and, as we shall see, can be completely desorbed at elevated temperatures. Of decisive importance for our purpose is the fact that the hydrogen of ammonia can exchange with the non-carbon-bonded hydrogen of dry protein. This exchange, which is quantitatively favored by the presence of three hydrogen atoms per molecule of ammonia, permits the measurement of the tritium content of protein acquired by the exchange of the protein with tritiated water. Under these conditions the carbon-bonded hydrogen does not dilute the radioactivity as it would in methods involving the combustion of the protein.

The method to be described can also be applied to tritium-labeled compounds involving a carbon–tritium linkage. In this case the compound is oxidized to yield tritiated water which is then allowed to exchange with ammonia. After careful removal of water from the recovered ammonia,

(1) These studies were aided by a research grant (H-869) from the National Heart Institute of the National Institutes of Health, Public Health Service.

(2) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Nat. Acad. Sci.*, **37**, 205 (1951).

(3) J. D. Watson and F. H. C. Crick, *Nature*, **171**, 737 (1953).

(4) I. M. Krause and K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **29**, 367 (1955).

(5) A. Hvidt and K. Linderstrøm-Lang, *ibid.*, **29**, 385 (1955).

(6) H. Lenormant and E. R. Blout, *Nature*, **172**, 770 (1953).

(7) G. H. Haggis, *Biochim. Biophys. Acta*, **23**, 494 (1957).

(8) R. F. Glascock, "Isotopic Gas Analysis for Biochemists," Academic Press, Inc., New York, N. Y., 1954.

(9) J. Sharpe, "Nuclear Radiation Detectors," John Wiley and Sons, Inc., New York, N. Y., 1955.

(10) J. Seehof and S. W. Benson, THIS JOURNAL, **77**, 2579 (1955).

the measured activity of the latter permits the calculation of the activity of the original sample.

Experimental

Counting of Tritiated Ammonia.—The counting is carried out in Bernstein–Ballentine counter tubes,[11] purchased from Process and Instruments, Brooklyn, N. Y. The total volume of the tube is about 100 ml. of which about 90% constitutes the sensitive volume. The scaler was obtained from Nuclear Instrument and Chemical Corp., Chicago, Ill. (Model 182X), and was provided with a high voltage supply of 5000 volts. The counter tube is contained in an enclosure formed from iron bricks. Electrical shielding is provided by a rectangular aluminum box surrounding the bricks.

Satisfactory plateaus can be obtained with an ammonia pressure up to 10 cm. and a total pressure of approximately one atmosphere. As shown by oscilloscopic observation of the variation of the pulse height these plateaus occur in the proportional region. The location and extent of the plateau depends on the ratio of the ammonia pressure to the total pressure and on the threshold voltage. As shown in Fig. 1 increase of the methane pressure shifts the plateau

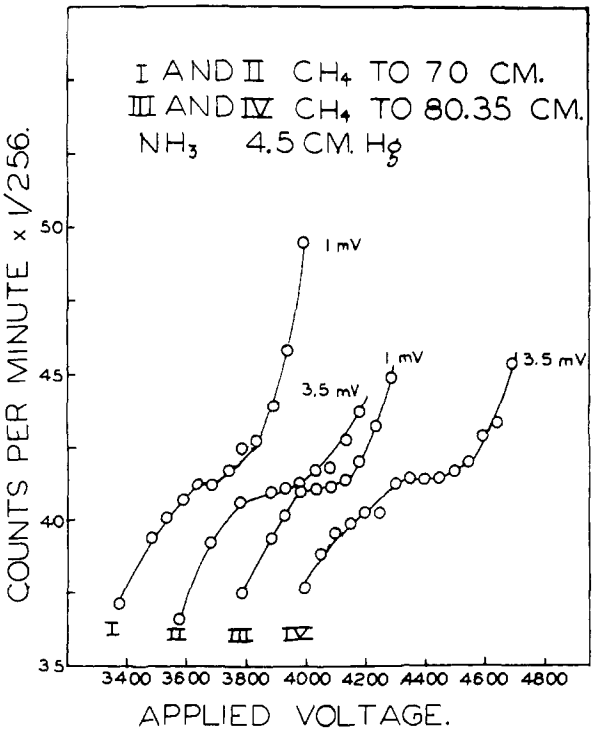

Fig. 1.—The dependence of the counting rate on the threshold voltage and the pressure of methane.

to higher voltage and extends it. On the other hand an increase in the proportion of ammonia decreases the plateau voltage as is expected from the ionization potentials of the individual gases.[9] The values of the counts per minute per unit of tritium determined in the plateau region, however, are practically independent of these variables. It is possible, therefore, to establish counting conditions which will give a plateau of 200 volts with a slope of not more than 0.5%/100 volts and which yield a reliable measure of the activity of the sample. A threshold voltage of 3.5 mv. is routinely used and a new plateau established for every filling of the counter tube. The analytical count is made at a voltage corresponding to a point about 40% beyond the beginning of the plateau. The presence of water vapor in the counter tube leads to spurious counts and precautions must be taken to free the ammonia used in the exchange from water vapor initially associated with it. The background count under the above conditions is approximately 100 per minute.

(11) N. Bernstein and R. Ballentine, *Rev. Sci. Instr.*, **21**, 158 (1950).

Vacuum System.—Vacuum for the system is provided by a mercury diffusion pump backed by a mechanical pump and the pressure is measured by means of a thermocouple vacuum gauge. Before the vacuum system is fabricated the glass components are treated with silicone (General Electric SC-87). The glass is baked at 100–120° for several hours and washed with copious quantities of hot water. It is essential to remove all traces of the unreacted material in order to avoid a large memory effect subsequently. The joints and stopcocks of the system are greased with Apiezon L as this has been found to exchange least with ammonia of several vacuum greases tested. The reaction tubes are the inner members of 14/35 joints sealed and blown out at the end with an over-all length of about 12 cm. They are connected to the vacuum line through the outer members which are permanently connected to the line by way of vacuum stopcocks. The reaction tube manifold is directly connected to the manometric space and is separated from the ammonia supply section of the vacuum line by a stopcock. This arrangement serves to minimize the memory effect associated with the movement of ammonia between the reaction tubes and the manometric space. At each reaction tube site is a cold finger sealed to the outer member of the joint below the stopcock. The cold finger allows the transfer of ammonia to the reaction tube while the latter is immersed in an oil-bath. The reaction tubes can be immersed in oil-baths in groups of four or eight. The oil containers consist of stainless steel rectangular troughs around the outside of which are wrapped heating tapes.

The amounts of ammonia used for the reaction tubes and for counting are obtained from pressure readings in the manometric space. The volume of this space and its variation with the height of the mercury column in the manometer were calibrated according to the procedure described by Glascock.[8]

Preparation of Radioactive Ammonia.—For most of the work described in this report it was necessary to prepare radioactive ammonia of known specific activity. Anhydrous ammonia, which was used for reaction with radioactive protein as well as for this purpose, was obtained from the Matheson Co., East Rutherford, N. J., with a purity of not less than 99.9% according to the manufacturer. The first step in the procedure is to dissolve between 0.1 and 0.2 g. of ammonia in 1 ml. of tritiated water (5–50 microcuries) contained in a tube which serves as a reservoir of labeled ammonia. For any particular experiment an estimated amount in small excess of that required is transferred to a dehydrating tube immersed in a bath of liquid nitrogen. During this transfer the ammonia passes through a Dry Ice–Cellosolve bath which serves to remove the bulk of the water vapor which accompanies the ammonia. The nitrogen bath is then replaced by a Dry Ice–Cellosolve mixture and the ammonia is transferred with liquid nitrogen to a cold finger which is a part of the manometric space. At the same time the last traces of water are retained in the dehydrating tube. After vaporization of the ammonia its pressure in the manometric space is measured and any excess is returned to the reservoir tube. Measured portions of the ammonia are then transferred to the appropriate reaction tubes by condensation of the ammonia into the associated cold fingers. The last portion of ammonia is transferred directly into the Bernstein–Ballentine counter tube for determination of the specific activity.

Protein–Ammonia Exchange.—To date our experiments have been limited almost exclusively to proteins and the quantity of protein used per reaction tube has ranged between 20 and 40 mg. The exhaustive drying of the protein is essential both because of the high affinity of proteins for small quantities of water and because the error in exchangeable hydrogen due to water contamination is magnified about 7-fold relative to its weight fraction. A measured volume of protein solution is shell-frozen in a reaction tube using a Dry Ice–cellosolve bath. The tube is attached to the vacuum system and the frozen protein solution lyophilized with a trap immersed in a CO_2–cellosolve bath. This process is continued until the pressure in the system is less than 0.007 mm. From 3 to 6 hours are usually required to reach this stage. The tubes then are heated with an oil-bath at 80° and the pumping continued overnight.

For the exchange reaction a measured quantity of ammonia is transferred to the reaction tube as described above. For equilibrium exchange the reaction is allowed to proceed overnight at 80°. The ammonia is then removed to the

manometric space with the aid of liquid nitrogen and, after temperature equilibration, its pressure is measured. This information allows calculation of the recovery of the ammonia, which is usually about 98%. The gas then is transferred to the counter tube by immersion of the tube in liquid nitrogen and methane added to a final pressure of about one atmosphere.

The results presented in this paper have been obtained with isoionic human serum albumin. The lyophilized protein was kindly supplied by Cutter Laboratories and was 99% pure by electrophoretic analysis. A 9% stock solution was deionized and rendered isoionic by passage through an ion-exchange column (MB-1, Fisher Scientific Co.). The *p*H of the effluent solution was 4.75. The protein concentration was determined by drying at 105° to constant weight and confirmed by micro-Kjeldahl analysis and measurement of optical rotation.

Results

Sources of Exchangeable Hydrogen.—There are several points in the procedure at which errors can be introduced into the determination of the exchangeable hydrogen of a sample. Reference already has been made to the treatment of the glass (Pyrex) components of the vacuum system with silicone. This treatment serves to reduce the exchange which would otherwise occur between the OH groups of glass[12] and ammonia. With siliconized glass our system contains about 15 microgram-atoms of rapidly exchangeable hydrogen excluding the reaction tubes. This is the quantity of hydrogen which can exchange with ammonia during the transit of the latter between the reaction tubes and the manometric chamber.

The reaction tubes, on the other hand, give rise to a more significant blank correction due, undoubtedly, to their prolonged exposure to ammonia at elevated temperature. The blanks are highly variable, ranging from 0 to 80 microgram-atoms of exchangeable hydrogen per tube, and are greatly dependent on the history of the tube. They are greater with increased temperature and time of reaction, indicating that they are due in part to slowly-exchangeable hydrogen.

Another source of slowly-exchangeable hydrogen is the grease used in the lubrication of stopcocks and joints, particularly the grease at the joints connecting the reaction tubes to the vacuum system. Several vacuum greases were tested for their capacity to exchange with radioactive ammonia. Approximately 200 mg. of each of the greases listed in Table I was spread over an area of about 14 cm.^{2} in reaction tubes and evacuated several hours to remove absorbed gases. The samples were then exposed to radioactive ammonia for 22 hr. at room temperature. The results of this experiment are given in Table I from which it is evident that silicone grease and Apiezon L are to be preferred. When the same experiment was done without prior evacuation all the samples exhibited greater exchange than before. The silicone grease was found to contain the greatest quantity of removable gas containing exchangeable hydrogen. These results were confirmed by back-exchange experiments in which the radioactive samples of grease were exposed to non-radioactive ammonia and the activity acquired by the latter was measured.

(12) G. S. Annis, H. Clough and D. D. Eley, *Trans. Faraday Soc.*, **54**, 394 (1958).

TABLE I

EXCHANGE OF HYDROGEN BETWEEN AMMONIA AND VACUUM GREASES[a]

Type of grease	Amount of exchangeable hydrogen, μgram-atoms
Silicone (Dow-Corning)	43
Apiezon L[b]	42
Apiezon M[b]	62
Apiezon N[b]	70

[a] About 200 mg. of grease was spread out over an area of approximately 14 cm.^{2} and reacted with ammonia at room temperature for 22 hr. [b] Purchased from James G. Biddle Co., 1316 Arch St., Phila. 7, Pa.

Finally, we may note that there is only a slight memory effect in the counter tube which is negligible under ordinary circumstances. The magnitude of this effect is indicated by the observation that only 0.4% of the original radioactivity in the tube was retained after it was flushed with air several times and evacuated for 24 minutes on the vacuum line.

When the exchange of hydrogen between dissolved protein and tritiated water is under study, the origin of the blank correction is somewhat different than that associated with the reaction between radioactive ammonia and dry protein. In the latter case one measures a decrease in the specific activity of the ammonia and any non-protein exchange contributes to this decrease. The relative significance of the blank is not affected by changes in the amount of ammonia but can be reduced by increasing the quantity of protein employed. In the former case non-protein radioactivity is acquired by the reaction tube during the removal of tritiated water by lyophilization. Following the addition of ammonia and its acquisition of radioactivity, any loss of tritium from it can be minimized by an increase in the quantity of ammonia used for reaction with the protein sample. From recent experiments with tritiated protein it has become apparent that with 20-mg. samples of protein the blank correction would comprise less than 1% of the activity of the sample. This indicates the feasibility of using substantially smaller quantities of protein.

Drying Conditions.—In order to establish adequate drying conditions, protein samples (44 mg.) were dried overnight under vacuum at 60, 105 and 125° in reaction tubes and the quantity of exchangeable hydrogen determined with radioactive ammonia. The conditions of reaction are indicated in Table II as well as the effective amounts of exchangeable hydrogen. From the constancy of the results it appears very unlikely that any significant quantity of water was retained by the protein. Our choice of 80° for drying provides assurance that adsorbed water does not contribute to the exchange with ammonia.

Conditions of Exchange.—The primary requirement which must be met by the conditions of exchange is, of course, that all of the non-carbon-bonded hydrogen atoms of the protein molecule establish an exchange equilibrium with the hydrogen atoms of the ammonia. A further important feature is that the recovery of ammonia from the reaction tube should be nearly quantitative. Only in this way is it possible to avoid with

TABLE II

THE EFFECT OF TEMPERATURE OF DRYING ON THE AMOUNT OF EXCHANGEABLE HYDROGEN IN HUMAN SERUM ALBUMIN[a]

Temp., °C.	Ammonia hydrogen used, μgram-atoms	Condition of reaction	Blank μgram-atoms	Net effective amount μgram-atoms
60	1039	1 hr. at −77°	24	760
		1 hr. at room t.		
105	1010	1 hr. at −77°	18	750
		1 hr. at room t.		
125	1095	1 hr. at −77°	12	760
		1 hr. at room t.		
60	2297	2 hr. at −77°[b]		754

[a] The quantity of isoionic protein used was 43.8 mg. per sample. [b] The recovery of ammonia was carried out at room temperature.

certainty an isotope fractionation effect leading to an erroneous specific activity of the recovered ammonia. It is also desirable that the recovery period should be brief, preferably not more than a few minutes.

The reaction conditions which we have selected, overnight at 80°, appear to satisfy the requirements noted above. Thus, as is shown by the results of Table III, a reaction period of 19 hours at 80° yields the same value for exchangeable hydrogen as a seven-day reaction at the same temperature. Furthermore the desorption of ammonia is sufficiently rapid at this temperature to allow recovery of 98% of the ammonia in five minutes. The results of Table III also serve to demonstrate the reproducibility of the analyses which can be regarded as reliable to within about 1%.

TABLE III

THE EYCHANGE OF HYDROGEN BETWEEN AMMONIA AND HUMAN SERUM ALBUMIN AT 80°[a]

Ammonia hydrogen used, μgram-atoms	Reaction time	Blank μgram-atoms	Net effective amount μgram-atoms
1418	7 days	38	770
1477	7 days	38	779
1530	21.5 hr.	34	762
1501	19 hr.	31	778
1493	24 hr.	6	770
1480	20 hr.	26	780

[a] Each reaction tube contained 43.8 mg. of isoionic protein which was dried overnight at 80°.

It is of interest to note that extensive exchange can take place at lower temperatures such as room temperature or even, with sufficient amount of ammonia, at −77°. The results shown in Table II illustrate these points although there is some question whether complete exchange occurred. It has been observed that the rates of exchange at all temperatures are very sensitive to the quantity of ammonia used for the exchange.

The Isotope Effect.—It has been found that there is a substantial isotope effect in the distribution of tritium between gaseous ammonia and dry protein favoring its retention in the solid phase. A quantitative estimate of this effect may be made with the results of Table III. The average value for the effective amount of exchangeable hydrogen contained in 43.8 mg. of protein is 770 microgram-atoms. On the basis of the composition given by Hughes[13] for human serum albumin there are 900 gram-atoms of non-carbon-bonded hydrogen per 65,000 grams of isoionic protein. In this calculation the number of carboxyl groups was taken as 100 based on Tanford's titration results[14] and the number of amide groups was therefore reduced to 24. These figures give a value for the isotope factor of 1.27 (770/606). This value, however, is clearly too large because the composition data account for only 91% of the protein. If a proportional correction is made for the exchangeable hydrogen of the remaining 9% of protein, then the isotope factor is reduced to 1.16. It is noteworthy that such a significant effect exists in spite of the fact that 92% of the non-carbon-bonded hydrogen of the protein is linked to nitrogen. Fortunately the present uncertainty in the isotope factor does not affect the accuracy of the determination of the specific activity of a tritiated protein sample. This is because the amount of ammonia used for exchange contains several-fold as much hydrogen as the amount of exchangeable hydrogen of the protein. Finally we may note that recent experiments with isoionic ribonuclease have yielded an isotope factor of 1.13. This value is in good agreement with that found above for human serum albumin in view of the incomplete composition data for albumin.

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(13) W. L. Hughes, "The Proteins," ed. by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, IIB.

(14) C. Tanford, THIS JOURNAL, **72**, 441 (1950).