The kinetics of the deuterium exchange of insulin with D_2O. An amendment

In continuation of previously reported experiments[1] it has been found that P_2O_5 is unfit for drying deuterium-loaded insulin. However fast P_2O_5 is transferred from bottle to desiccator, it will have time to absorb sufficient water from the air to enable it to replace a considerable quantity of deuterium atoms in the frozen-dried protein by ordinary hydrogen atoms during the subsequent drying process (5 days). The amorphous character of the solid insulin in combination with its extremely light and spongy structure is favorable for the exchange process. The problem of the deuterium exchange of insulin in aqueous solution has therefore been reinvestigated using the improved drying technique indicated below. It was consistently found (16 estimations) that almost all exchangeable hydrogen atoms, *viz.*, 89 ± 1 out of 91 per mole pork insulin (Mw 5777), exchanged in the course of 12–20 hours at 38°. Special control experiments combining determinations of the nitrogen content and weight of the dry protein have secured that the higher value obtained does not originate in insufficient drying of the samples.

In view of these findings our previous exchange-curve must be re-interpreted, *i.e.*, it does not represent the number of hydrogen atoms which dissolved insulin will exchange with deuterium atoms from D_2O, but rather the number of deuterium atoms in the *dry* protein which will *not* exchange back with H_2O from P_2O_5. Since the figure 46 atoms per mole is quite reproducible (± 2 atoms) if the drying is performed with P_2O_5 as described, our previous experiments do reveal something about the stability of the 48 hydrogen atoms in the backbone of solid, amorphous insulin, and show the extreme reactivity of the hydrogen atoms in the side chains even when water is virtually absent.

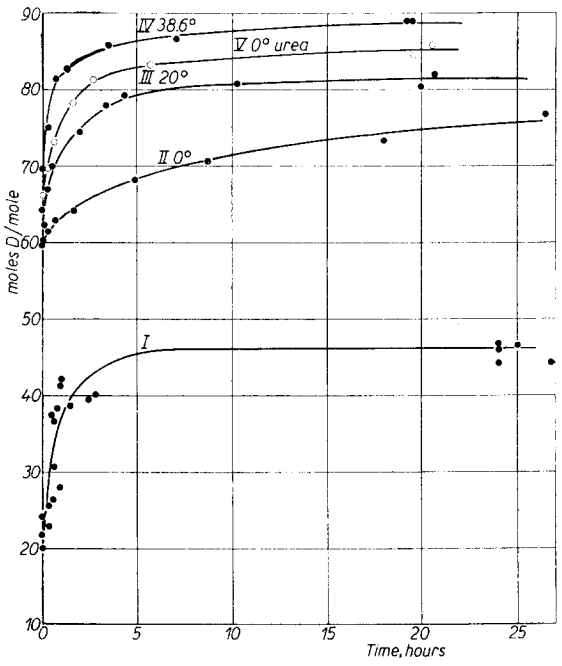

Fig. 1

The question of the true rate of exchange of dissolved insulin was approached in the following way: 200 µl of a 2 % insulin solution, pH 3, were lyophilized and dried over P_2O_5. Next 200 µl 99.7 % D_2O were added to the dry protein and allowed to react with it for 20 hours at 38°, after which time D_2O was distilled off *in vacuo* at low temperature (cryodistilled) using a cold trap (—60° to —80°). When the protein was almost dry it was heated to 60° for 2 to 4 hours to drive the residual water over into the trap. Finally the protein was dissolved as fast as possible in 200 µl H_2O (or urea solution), and samples of 15 µl were taken out at suitable times, frozen to —60°, and cryodistilled. The density of the water in the trap was determined in the gradient tube[2]. Fig. 1, curves II–IV, shows the rate of this back-exchange at different temperatures. It is apparent that about 59 hydrogen atoms exchange at a rate which is too high to be measured. The remaining 32 however are characterized by much a smaller reactivity, especially at 0°, and fall in different groups with greatly differing rate constants. It is too early to discuss the significance if this observation in detail, but we venture to maintain that these slowly exchanging hydrogen atoms are situated in the peptide groups and are hydrogen-bonded in the secondary structure of the protein. Curve V which represents experiments at 0°, pH 3, 5.2 molar urea, strengthens this view in that it shows the activating influence of a denaturing agent upon the hydrogen exchange. It will be observed that curve I[1], will roughly coincide with curve IV if it is displaced upwards a distance corresponding to the 45 odd hydrogen atoms lost in the drying over P_2O_5. Therefore the rate measured in the first, indeterminate part of curve I is probably that of the slow exchange of hydrogen atoms in the backbone of dissolved insulin. The possibility that the curves in Fig. 1 represent rates of dissolution of insulin and of some "ageing" processes in the solution (dissociation of higher complexes in which hydrogen atoms are "masked") may be excluded for several reasons. The following small experiment is interesting in this respect: 100 µl aged insulin solution were cooled to 0° and mixed with 100 µl D_2O at 0°. After 1 min and 60 min 50 µl samples of the mixture were frozen and

lyophilized, and the protein dried at 60° for 2.5 hours as described above. Then 25 μl H_2O were added and after 2 hours back-reaction at 38° the solutions were cryodistilled and the density of the water determined. Exchange values of 60 and 62 respectively were found, in good agreement with curve II.

Our wish to correct our previous errors as quickly as possible had led us to omit a discussion of the behavior of SANGER's A-chain on which experiments are in progress. It should, however, be noted that renewed experiments with the tetrapeptide leucyltriglycine, using the improved technique, have shown that all 6 labile hydrogen atoms exchange instantaneously if the peptide is lyophilized prior to dissolution, so that it is brought into rapid contact with the solvent. The exchange with H_2O during the drying over P_2O_5 is considerably slower than in the case of insulin, which may be due to the fact that the lyophilized peptide is crystalline. A similar assumption may explain why the A-chain does not seem to exchange with P_2O_5[1].

The method and results will be described more fully elsewhere.

Carlsberg Laboratorium, Copenhagen (Denmark) AASE HVIDT
K. LINDERSTRØM-LANG

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Influence of methionine on protein and nucleic acid synthesis in *Pseudomonas hydrophila* *

The recent findings of SCHMIDT *et al.* showed an increase in the biosynthesis of acid-insoluble and acid-soluble adenine and guanine fractions in yeast cells grown in a synthetic medium supplemented with methionine. The present investigation deals with the influence of DL-methionine on nucleic acid and protein synthesis in *Pseudomonas hydrophila*.

Pseudomonas hydrophila NRC 492 was grown at three concentrations of DL-methionine in a synthetic medium[2] for 24 hours with constant shaking. The cells, harvested in a centrifuge, were washed four times with ice cold water, frozen and dried.

The following estimations were made on the freeze-dried material.

a. *Protein:* 20 mg of the freeze-dried material was incubated with 2 ml of 5% trichloroacetic acid (TCA) at 0° C for 2 hours and centrifuged at 4° C. The residue, washed twice at 4° C with 5% TCA, was taken up in 3% NaOH and the protein in a 4 ml aliquot was estimated by the colorimetric biuret method of HILLER *et al.*[3] Intensity of color was measured in a Beckman spectrophotometer at 550 mμ.

b. *Total nucleic acid phosphorus* (TNA-P): 30 mg of the freeze-dried material was incubated with 4 ml of 5% TCA at 0° C for 2 hours and centrifuged at 4° C. The residue was washed four times at 4° C with 5% TCA, once with ethanol, three times with a mixture of ethanol: ether (3:1) at room temperature. The washed residue was extracted three times with 2 ml aliquots of 5% TCA at 90° C for 10 min. The TCA extracts were made up to 10 ml and phosphorus was estimated in an aliquot according to the method of KING[4].

c. *Desoxyribonucleic acid* (DNA-P): 80 mg of the freeze-dried material was incubated with 5 ml of 5% TCA at 0° C for 2 hours and centrifuged at 4° C. The residue was washed with TCA and lipid solvents as described above. The washed residue was taken up in 2 ml *N* KOH and the mixture was kept for 18 hours at 37° C. It was then chilled in an ice bath and DNA and protein were precipitated with 0.4 ml 6*N* HCl and 2 ml 5% TCA. The mixture was held at 0° C for 15 min and centrifuged at 4° C. The residue was washed at 4° C with 5% TCA. The washed residue was extracted three times with 1 ml aliquots of 5% TCA at 90° C for 10 min and the phosphorus in the extracts estimated according to the procedure of KING[4].

d. *Ribonucleic acid phosphorus* (RNA-P): RNA-P was obtained by substracting DNA-P from TNA-P.

The turnover of ribonucleic acid phosphorus as effected by DL-methionine was studied by using ^{32}P. *P. hydrophila* was grown in a synthetic medium[2] with or without added 0.02 *M* DL-methionine. Labeled phosphorus (1.5 μc/ml of medium) was supplied as $H_3{}^{32}PO_4$. Twenty-four hour cultures were centrifuged and the cells were washed six times with ice cold water. The washed cells were suspended in 7% TCA (2.5 ml TCA per g of wet cells) and held at 0° C for two hours. The suspension was then centrifuged at 4° C and the residue was washed six times with 5% TCA at 4° C and with

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