tocin. The differences in the amount of activity regenerated from the inactivation product by the various —SH compounds may reflect differences in the reducing power of the —SH compounds and/or differences in the tendencies of the latter to form mixed disulfides with inactivated oxytocin (or oxytocin), as well as possible differences in the activity of the mixed disulfide species formed thereby. The unexpectedly low potencies obtained in the sodium-ammonia experiments indicate perhaps a greater lability, of undetermined type, toward the alkaline reagent than is the case with oxytocin or its S,S′-dibenzyl derivatives, which yield appreciable activity with this reagent (*14*).

A preliminary determination of molecular weight gave a calculated minimal value of 2250 (uncorrected for moisture) (*15*). The inactivated material is therefore a dimer or other higher molecular weight form of the hormone and may be represented at present as $(\text{oxytocin})_n$; $n \geqq 2$. The inactivation process would appear to involve cleavage of the intramolecular disulfide bond of oxytocin and the formation of intermolecular disulfide bonds between two or more molecules of the hormone. This inactivation of oxytocin, which may also be represented as an interchange between two or more molecules of an unsymmetrical disulfide, may be a prototype of some of the dimerization or aggregation reactions that occur with certain proteins, especially in concentrated solution. Whether it is related in mechanism to the interchange studied by several investigators between two different symmetrical disulfides (*6, 16*), which under neutral or alkaline conditions is thought to proceed through a reaction of the disulfide bond with a mercaptide ion, remains to be determined.

The occurrence of varying degrees of disulfide interchange during the handling of the posterior pituitary hormones may account for some of the losses in biological activity that take place, and may well explain some of the difficulties encountered in obtaining preparations of vasopressin of a uniform high potency. It is possible that the inactivation of these hormones in the body might also proceed through an intermolecular disulfide exchange, where it could be catalyzed enzymically. The inactivated forms of oxytocin and vasopressin could serve perhaps as physiological storage products for these hormones; when needed, activity could be generated through the action of body —SH compounds.

Because the conditions under which these hormones undergo molecular rearrangement with resultant increase in molecular weight are those which are useful for forming -DNP derivatives, it is apparent with cystine-containing peptides and proteins that structural interpretations based on data obtained through the dinitrophenylation procedure, and perhaps through other procedures as well, must be made with caution.

A second inactivation of oxytocin was observed when the polypeptide was allowed to stand in 7*M* urea solution. Changes in optical rotation were also followed (Fig. 1). The potency after 7 days at *p*H 8 was not increased by addition of cysteine to the solution, either before or after dialysis. The findings would suggest that secondary forces such as hydrogen bonding are of importance for biological activity in oxytocin. However, the possibility of chemical change should be ruled out before this inactivation in urea is ascribed to change in the spatial configuration of the oxytocin molecule.

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1. For a review of the subject, see F. W. Putman in *The Proteins*, H. Neurath and K. Bailey, Eds. (Academic Press, New York, 1953), vol. 1B, p. 808. Transformation in aqueous solution suggestive of denaturation has also been observed with a synthetic polypeptide of high molecular weight, poly-DL-alanine ($n \sim 500$) [A. Elliot, *Nature* 170, 1066 (1952)].
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3. Part of this work was done during the tenure of an established investigatorship of the American Heart Association. This work was also supported in part by a grant (H-1675) from the National Heart Institute, U.S. Public Health Service.
4. I express appreciation to Dr. V. du Vigneaud for the samples of arginine-vasopressin and some of the natural oxytocin, as well as for encouragement and stimulating discussion. Thanks are given to Mr. R. L. Tostevin and Miss D. W. Tull for the bioassays and to Mrs. L. S. Abrash for the chromatography on starch.
5. Prepared here from N-acetyl-L-cystine; its preparation from cystine has since been decribed [F. R. Bettelheim, *J. Biol. Chem.* 212, 235 (1955)].
6. The reverse reaction—that is, the formation of mono-DNP-cystine from di-DNP-cystine and cystine—was observed under acidic conditions by A. P. Ryle and F. Sanger [*Biochem. J.* 60, 535 (1955)]. Interchange reactions between symmetrical disulfides, namely, di-DNP-cystine and oxidized glutathione or cystylbisglycine, were studied also at neutral or slightly alkaline *p*H, and were found to be almost completely inhibited by —SH binding reagents.
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11. For assay the solid (1 mg) was dissolved in 50 μl of 1*N* acetic acid and the solution was diluted with water. Whether or not the low activity is due to the presence of traces of residual oxytocin has not been determined.
12. Qualitatively similar results were obtained with arginine-vasopressin. After 3 days its potency in the $NaHCO_3$ reaction mixture fell from 309 pressor units to 17 units per milligram. Treatment with cysteine raised the potency to 225 unit/mg.
13. The decrease in potency observed on treatment of oxytocin with H_2S is in substantial agreement with earlier observations using the guinea pig uterus [J. M. Gulland and S. S. Randall, *Biochem. J.* 29, 378 (1935)]. The results obtained with cysteine or thioglycollic acid are similar to those obtained by H. B. van Dyke, B. F. Chow, R. O. Greep, and A. Rothen [*J. Pharmacol. Exptl. Therap.* 74, 190 (1942)] with their protein-hormone complex. Under other conditions no change in potency was found on treatment of oxytocin with cysteine [R. R. Sealock and V. du Vigneaud, *ibid.* 54, 433 (1935)].
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15. A numbers-average molecular weight was determined on material that had been lyophilized from dilute acetic acid. A thermoelectric osmometer was used [E. J. Baldes, *Biodynamica*, No. 46, 1 (1939)]. I am indebted to Dr. J. R. Rachele for this determination. The theoretical value for a dimer diacetate is 2135.
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Airborne Immunization against Tuberculosis

Abstract. Inhalation of very small numbers of living attenuated (BCG) organisms and their multiplication in guinea pigs results in the development of acquired resistance against subsequent airborne infection with virulent tubercle bacilli. Different strains of BCG have differing capacities to immunize by this means.

It has long been known that certain attenuated mutant strains of tubercle bacilli are capable of limited, though definite, multiplication in animals susceptible to tuberculosis. The strain selected by Calmette and Guérin, BCG, for purposes of immunization, and some of its descendants, manifest this behavior. A wide variety of routes has been used to introduce such organisms into experimental animals or man (*1*), but introduction by the airborne route has not previously been investigated. During the course of such studies (*2*) we have observed that the viable units in certain cultures of BCG, even when inhaled in very small numbers, are able to "infect" and produce tuberculin allergy and immunity against subsequent airborne challenge with virulent tubercle bacilli in guinea pigs.

The apparatus and techniques employed were the same as those previously described (*3, 4*). Three different BCG strains have been used: a commercial BCG vaccine (*5*) prepared for purposes of immunization of human beings; and two different stock laboratory cultures of BCG, "D" and "T," derived from BCG 317, originally obtained from the Henry Phipps Institute (*6*). The "D" strain was maintained for long periods in the Deep-freeze and infrequently transferred in artificial media. The "T" strain had been frequently passed through Tween-albumin liquid medium for several years.

Suspensions of the commercially available BCG vaccine were prepared from glass vials of freeze-dried material: the

content of a vial (40 to 50 mg) was dispersed, as directed on the label, in 1 ml of sterile distilled water, and was shown by plate count to contain 2.5 ($\pm$ 1) $\times 10^8$ viable units per milliliter. Such suspensions of BCG organisms, which contain many clumps of widely varying size, were further diluted in distilled water containing 0.1 percent serum albumin and 0.01 percent Tween 80 before introduction into the nebulizer for airborne infection.

Under the standardized conditions used, which involve a ½-hour exposure of guinea pigs in the airborne infection chamber, 10^3 viable units per milliliter of nebulizer fluid containing a *well-dispersed* suspension of fully pathogenic tubercle bacilli yield approximately one infecting unit per guinea pig (*7*).

In Table 1 are presented the results of exposing guinea pigs by the airborne route to various dilutions and, therefore, to various numbers of viable units of the untreated commercial BCG vaccine, and then challenging them with fully pathogenic tubercle bacilli by the same route. Statistical analysis has shown that the differences between the plate counts of virulent tubercle bacilli in the lungs and spleens of the unvaccinated tuberculin-negative animals and the groups of tuberculin-positive animals are highly significant ($p < .001$). Other typical manifestations of resistance were also seen in the tuberculin-positive animals: they had smaller numbers of grossly visible primary pulmonary lesions, smaller spleens, and smaller mediastinal lymph nodes than the controls. These gross observations were confirmed by microscopic examination of sections from the lungs and tracheobronchial nodes. Only a small proportion of the tubercles in the vaccinated animals had necrotic centers or contained acid-fast rods, whereas nearly all of the tubercles in the controls had necrotic centers and large numbers of stainable organisms. It is evident that four out of six guinea pigs estimated to have inhaled no more than 25 potentially infecting units (2.5×10^4 viable units per milliliter of nebulizer fluid) became hypersensitive to tuberculin and manifested a significant degree of acquired resistance to the subsequent airborne challenge.

In view of the poor degree of dispersion of the bacterial cells in the vaccine suspension used, it seems likely that a large proportion of the viable units and, therefore, of the droplet nuclei formed during nebulization, were too large to infect by inhalation (*8*). With better dispersion it can be anticipated that inhalation of fewer than 25 infective units per animal would be effective in conferring a significant degree of immunity on a majority of guinea pigs exposed under the same conditions.

It seems noteworthy that an increase of 100-fold in the number of viable units inhaled did not result in a further increase in immunity, although it did result in immunization of all of the animals. It is also important to state that the degree of acquired resistance manifested by airborne-BCG-immunized guinea pigs against subsequent virulent challenge is at least as great as guinea pigs show after immunization with many thousandfold larger numbers of viable BCG organisms by the subcutaneous route (*4*).

Table 1. Airborne BCG immunization of guinea pigs against experimental tuberculosis.

No. of animals	Airborne BCG exposure* (0 day)	Tuberculin reactions† (41st day)	Mean bacterial counts‡ after virulent challenge (70th day)	
			Lung (r.l. lobe/50)	Spleen (whole organ/50)
6	2.5×10^4	(2) neg. (4) pos.	2.5×10^4 1.0×10^2	1.1×10^4 2.5×10
6	2.5×10^5	pos.	1.7×10^2	6.0×10
6	2.5×10^6	pos.	0.5×10^2	3.0×10
6	0 (controls)	neg.	1.2×10^4	1.7×10^4

* No. of viable units per milliliter of nebulizer fluid.
† 300 tuberculin units intracutaneously, read after 24 hrs.
‡ Plate counts at 28 days after airborne challenge with about 100 infecting units of virulent tubercle bacilli (H37Rv) per animal.

Gross and microscopic examination of the lungs, mediastinal nodes, spleens, and livers of guinea pigs at 35 days after inhalation of these small numbers of living BCG organisms have revealed no abnormalities except for occasional and slight enlargement of the mediastinal nodes containing a few microscopic collections of epithelioid cells which harbor small numbers of acid-fast rods.

Evidence has been adduced that the development of tuberculin allergy and immunity after airborne exposure to BCG organisms under these conditions is dependent upon actual multiplication of the attenuated organisms in the guinea pig. Thus treatment of the animals with a sterilizing antibacterial agent, isoniazid, for 10 days after airborne exposure to large numbers of BCG organisms completely prevents the development of tuberculin hypersensitivity or immunity.

These results with the commercially available vaccine have been confirmed in other experiments under similar conditions with the stock laboratory strain BCG "D." On the other hand, in several experiments organisms of the BCG "T" strain have been ineffective in conferring tuberculin hypersensitivity or immunity under conditions calculated to provide for inhalation of as many as 300 to 500 potentially infecting units per guinea pig. Especially noteworthy is the fact that this strain is, nevertheless, quite effective in conferring immunity when it is introduced in customary dosage by the subcutaneous route. It is common experience in our laboratory that strains of tubercle bacilli lose infectivity or pathogenicity (or both) on repeated passage through Tween-albumin liquid medium. Thus the striking difference between the BCG "D" and "T" strains on inhalation indicates that the airborne infection route can provide a precisely quantitative means of defining the infectivity of different strains and cultures of attenuated tubercle bacilli.

The observations described in this report have important implications. (i) The consequences of inhalation of small numbers of living BCG organisms by human beings should be investigated. Mass airborne BCG immunization, under appropriate epidemiologic conditions, could have certain advantages over conventional methods. (ii) Possibilities are suggested of airborne immunization of experimental animals against some other types of respiratory tract infection with living attenuated parasites.

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7. When calculations are made from the known aerodynamic characteristics of this airborne infection apparatus (*3*), and on the assumption that each guinea pig inspires 3 liter/30 min, this infection rate corresponds well with the experience described by Lurie [*Am. Rev. Tuberc.* 61, 765 (1950)]—that is, one infective unit out of every three inhaled actually infects to produce a primary tuberculous lesion.
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