hypothesis on which to base further efforts toward the elucidation of a possible specific constituent of insulin.

The action of acids and alkali on insulin to which I have already referred has been studied in more detail by E. A. Evans, Jr., and myself. The results thus far obtained seem to support the postulation of the above grouping in the insulin molecule. Insulin, when heated with N/10 hydrochloric acid in a boiling water bath forms a coagulum with the simultaneous appearance of ammonia. Upon redissolving this precipitate in very dilute alkali and again subjecting the material to the original acidity and temperature, a second coagulum is obtained with the separation of much less ammonia than before. The formation of this acid insoluble coagulum indicates a decrease in the basicity of the protein molecule, such as would result, in the case of the postulated grouping (formula I), from the condensation of the amide group and the amino group with the elimination of ammonia. Solution of this condensation product in alkali would open the lactam ring with the regeneration of the carboxyl group. In case of formula II the ammonia is derived from the amide group, and the condensation of the amino group with the free carboxyl group, giving a diketopiperazine, accounts for the formation of a coagulum. Alkaline treatment of the insulin acid coagulum results in regaining practically the complete physiological activity of crystalline insulin. In terms of the postulated structures, however, this material should differ from the original insulin in the number of free carboxyl groups present, due to the removal of the amide group. This may explain the failure to crystallize this alkali-treated material by the usual method. If these assumptions are correct, the removal of the free amino group should prevent the formation of an acid insoluble coagulum. The treatment of insulin with very dilute alkali splits off ammonia and gives a product which does not coagulate when heated with N/10 hydrochloric acid at 100° C. This seems to point to the removal of the amino group under alkaline treatment. One may infer from these data that the amide group is not necessary for the physiological action of insulin.

Interpretation of the results of alkaline treatment of insulin is somewhat uncertain in reference to the postulated insulin constituents. One can only conclude from the study of alkali action on insulin that the sulfur, or at least a part of it, as the disulfide linkage, and also a free amino group are necessary for physiological activity. The inactive product of alkali treatment gives only a very weak ninhydrin reaction in contrast to the strongly positive test of crystalline insulin. This suggests, though the question remains unsettled, that the ammonia given off by alkaline treatment originates from the physiologically important amino group. The details of the experimental work involved in these conclusions will be published elsewhere.[34]

Reference must also be made to the following experiment. Treatment of insulin with acid alcohol produces a physiologically inactive product giving a negative ninhydrin reaction. Subjecting this material to the action of dilute alkali restores most of the activity with the simultaneous reappearance of the positive ninhydrin reaction of the original insulin. One may explain this by assuming that the carboxyl group of the cystine (formula I), or of the glutamic acid (formula II), after esterification, reacts with the free amino group to form a diketopiperazine. The resultant blocking of the free amino group accounts for the negative ninhydrin reaction. Alkaline treatment opens the diketopiperazine ring with the regeneration of the original molecule, and also of the free amino group which reacts with ninhydrin. These results are also treated in more detail elsewhere.[34]

Although these findings may be explained in terms of the postulated active constituents of insulin, further proof is necessary to establish the validity of these assumptions. It hardly needs to be said that the results can also be explained by assuming a similar combination of another amino acid, such as tyrosine or leucine, with cystine. I believe, however, that cystine is of importance and is a part of the possible active constituent. The similarity in the physiological action of insulin and glutathione suggests glutamic acid as a possible constituent of the active group. The determination of the amino acid, to which the free amino group is attached, is important. The research is being continued in this direction and the synthesis of cystine peptides having the postulated structures is also being attempted. I wish to propose this hypothesis in only the most cautious and tentative manner until further data, either supporting or opposing the postulated structures, are available.

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[34] Jensen and Evans, *Zeit. physiol. Chem.* (in press).