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Arginine and Its Possible Relation to the Physiological Activity of Insulin.

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Recently[1] we stated that insulin (21 units per mg.) gives positive color reactions with picric acid, with *m*-dinitrobenzene, and with *m*-dinitrobenzoic acid (1.3.5). It seemed that the presence of labile sulfur in insulin sufficed to account for the color reactions obtained. But among the aminoacids that yield positive reactions with the above mentioned reagents, arginine was conspicuous for the strength of its picric acid reaction.

Color reactions specific for arginine have been devised by Harden and Norris,[2] and more recently by Sakaguchi.[3] On testing our insulin we found both reactions strongly positive. Insulin, similar in purity to our preparation, yielded with the van Slyke method 35 per cent of diamino nitrogen in one instance[4] and 10 per cent of arginine nitrogen in another.[5] Since only small amounts of insulin were at our disposal, we could not attempt to isolate arginine by acid hydrolysis.[6] We, therefore, tried an enzymatic hydrolysis, which may be carried out with relatively small amounts of insulin (21 mg.). In addition, we expected this enzymatic method would throw some light upon the structure of the insulin molecule, as well as yield information concerning certain enzymatic problems.[7] The method has the advantage of being highly specific, thereby eliminating many sources of error.[8]

We determined the arginine from the amount of NH_3 liberated from 440 units of insulin (21 mg. containing a total of 3.4 mg. of nitrogen) by the combined action of trypsin + trypsin-kinase,[9] erepsin, arginase and urease (18 hours, 37° C., pH = 7.4, 0.2 *M* phosphate buffer). As a control we used the amount of NH_3 which was liberated under identical conditions from the same amount of insulin by the combined action of trypsin + trypsin-kinase, erepsin and urease, but omitting the arginase. Other necessary checks were also run. In this manner we found our insulin[10] to contain approximately 12 per cent of arginine, which figure, however, probably represents only the minimum value of the actual arginine content.

If the digestion of insulin is carried out with erepsin,[11] arginase and urease, but omitting trypsin, practically no arginine can be detected. This experiment shows that the arginine we find in our

insulin preparation is not derived from an accompanying polypeptide. If the digestion of insulin is carried out with trypsin + trypsin-kinase, arginase and urease, but omitting erepsin, no arginine can be detected. This shows that trypsin splits off from the insulin molecule neither free arginine nor an arginine peptide containing a free COOH group in the arginine moiety.[12] It is interesting to note that under similar conditions (*i. e.*, digestion by trypsin + trypsin-kinase, erepsin, arginase and urease) no arginine can be detected in gelatin.[13] From this finding we conclude that insulin is a protein[14] of a different and probably less complicated constitution than gelatin.

In a former publication[1] we stated that by treatment with *N* alkali at room temperature only relatively small amounts of sulfur are split off from insulin. It seemed possible that other factors besides the removal of the sulfur play a part in the alkaline destruction of the physiological activity of insulin. The high arginine content of insulin suggested the possibility that by mild alkaline treatment urea might be split off from insulin.[15] When insulin is boiled for 30 minutes with 0.1 *N* sodium carbonate, the resulting physiological inactivation is associated not only with an alteration in the linkage of the sulfur, but also with a splitting off of definite amounts of urea (about 17 per cent of the arginine-urea in our preparation). Treatment with *N* alkali (4 hours, 37° C.) splits off from insulin about 5 per cent of the total nitrogen as NH_3.

Our recent observations on the lability of the sulfur in cystine derivatives,[1] and their lack of action on the blood sugar level led us to doubt that the (labile) sulfur group is the carrier of the physiological activity within the insulin molecule. Successful investigations by Frank, Nothmann and Wagner[16] seem to offer new possibilities in the search for the physiologically active group within the insulin molecule. These authors, taking advantage of the fact that guanidine lowers blood sugar, investigated a series of guanidine derivatives for their insulin-like action. They found that agmatine, the amine of arginine, lowers blood sugar, while being less toxic than guanidine. Their work culminated in the synthesis of an arginine or guanidine derivative (synthalin), a few mgs. of which, given orally, cause hypoglycemic convulsions. Frank[17] had suggested in 1924 that the "characteristic group of guanidine" might possibly play a part in the activity of the insulin molecule. In 1923 Collip[18] had called attention to a possible chemical relation between insulin, guanidine and certain constituents of plant extracts.

The results obtained by Frank, Nothmann and Wagner,[7] the high arginine content of insulin (probably more than 12 per cent in im-

pure preparations), and the finding that alkaline inactivation of insulin is associated with splitting off of urea, all suggest that the guanidine group of arginine is the carrier of the physiological activity within the insulin molecule. However, the physiological activity of the insulin molecule cannot be due to the presence *per se* of the guanidine group of arginine, but must be dependent on the special way in which the arginine is inserted into the insulin molecule. Concerning the chemical nature of this second factor we know only that this linkage or grouping is attacked and destroyed by trypsin and pepsin.

In regard to the action of insulin[1] it is still undecided whether insulin is directly involved in the enzymatic processes of carbohydrate metabolism or whether it is concerned only in their nervous control. Granted the first possibility to be true, the highly specific structure of enzymes, coenzymes, kinases and similar substances renders it improbable that a compound, even if closely related to insulin, would show an insulin-like action. Substances, however, which differ considerably in their constitution, but which contain the same physiologically active grouping (*e. g.,* pyrocatechol, epinephrine), may have similar effects on the visceral nervous system. If it can be established that the physiologically active group of insulin is the guanidine group, this would further support the view that insulin acts through some nervous mechanism. If this is correct, it seems unlikely that the action of insulin can be demonstrated by *in vitro* experiments at the present stage of our knowledge of nervous mechanisms.

While little is known of the intermediary arginine metabolism, it is certain that it follows several entirely different courses. In one kind of arginine metabolism undoubtedly the enzyme arginase is involved. For that part of arginine metabolism which results from the participation of arginase, Edlbacher and Röthler[19] have established definite relations to sex. These authors found that an increased arginine metabolism begins at puberty. They adduced evidence that in animals the higher arginine metabolism is a specific characteristic of the male. Whether these age and sex differences in arginine metabolism may have important underlying relationship to the pathology of diabetes future investigations must determine.

In view of the previous discussion on the importance of the guanidine group for the activity of the insulin molecule, an interesting enzymatic problem suggests itself. Recently[7] we pointed out that the enzyme-substrate compound between trypsin-kinase and insulin is formed involving the physiologically active group of insulin. We

are now carrying out experiments to establish whether trypsin-kinase combines with the guanidine group of the proteins to form the enzyme-substrate compound and whether entero-kinase* is a necessary link in this combination.

* Felix and Waldschmidt-Leitz have shown that insulin is neither attacked nor inactivated by trypsin free from enterokinase.

[1] Brand, E., and Sandberg, M., *J. Biol. Chem.*, 1926, lxx, 381.

[2] Harden, A., and Norris, D., *J. Physiol.*, 1911, xlii, 332.

[3] Sakaguchi, S., *J. of Biochem.* (Japan), 1925, v, 25. This reaction is far more satisfactory than the diacetyl reaction.

[4] Shonle, H. A., and Waldo, J. H., *J. Biol. Chem.*, 1924, lviii, 731.

[5] Scott, D. A., *J. Biol. Chem.*, 1925, lxv, 601.

[6] The difficulties encountered by Fischer, E., and Suzuki, U., *Ber. Chem. Ges.*, 1905, xxxviii, 4173, when working with arginyl-arginine, did not encourage hydrolysis experiments.

[7] Brand, E., and Sandberg, M., PROC. SOC. EXP. BIOL. AND MED., 1925, xxii, 428.

[8] The method, however, does not exclude the possible presence of the hypothetical isomeric arginine (methyl-arginine). *Cf.*, discussion by Thomas, K., Kapfhammer, J., and Flaschenträger, B., *Z. physiol. Chem.*, 1923, cxxiv, 75.

[9] The trypsin used was a 10 per cent solution, prepared from Fairchild's trypsin (*Cf.*, Northrop, J. H., *J. Gen. Physiol.*, 1924, vi, 337, see p. 340). It was practically free from erepsin and free from arginase. We designate this trypsin solution as "trypsin + trypsin-kinase" in accord with Waldschmidt-Leitz, E., Schäffner, A., and Grassmann, W., *Z. physiol. Chem.*, 1926, clvi, 68.

[10] Another somewhat less pure insulin preparation gave similar results.

[11] This erepsin preparation was practically free from trypsin.

[12] Edlbacher, S., and Bonem, P., *Z. physiol. Chem.*, 1925, cxlv, 69, have shown that a free COOH group is indispensable for the action of arginase.

[13] Previous digestion with pepsin has not yet been tried.

[14] Felix, K., and Waldschmidt-Leitz, E., *Ber. Chem. Ges.*, 1926, lix, 2367, have demonstrated with trypsin-free erepsin that insulin is not a polypeptide.

[15] Bergmann, M., and Köster, H., *Z. physiol. Chem.*, 1926, clix, 179, recently synthesized an arginine derivative which already splits off urea when treated with water.

[16] Frank, E., Nothmann, M., and Wagner, A., *Klin. Woch.*, 1926, v, 2100.

[17] Frank, E., *Klin. Woch.*, 1924, iii, 955.

[18] Collip, T. B., *J. Biol. Chem.*, 1923, lviii, 163.

[19] Edlbacher, S., and Röthler, H., *Z. physiol. Chem.*, 1925, ciii, 273.