rapid and accurate procedure for handling moisture calculations.

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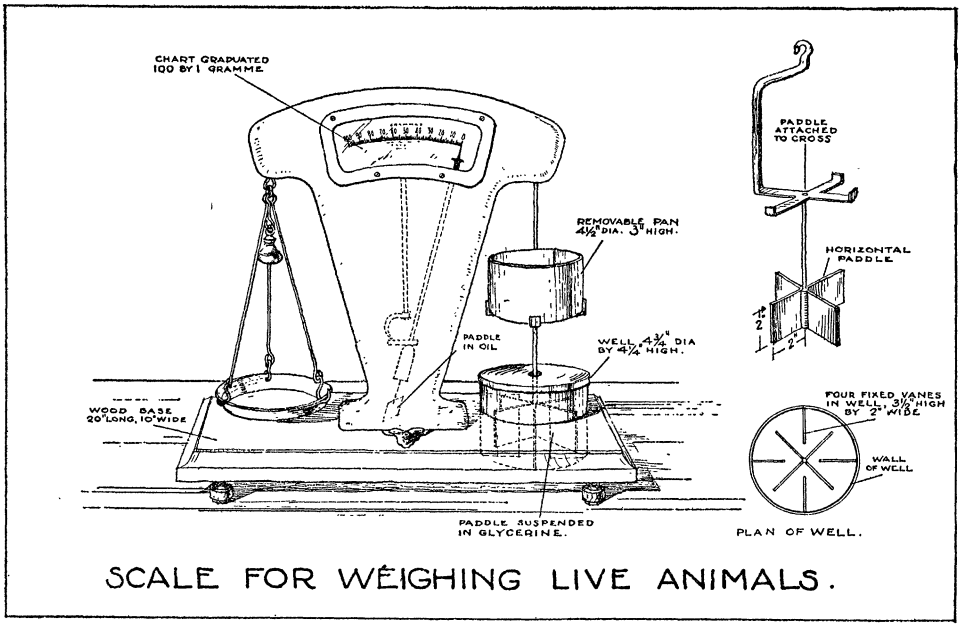
A BALANCE FOR LIVE ANIMALS

CERTAIN experiments in this laboratory have necessitated recording every week the weight of large numbers of rats. A spring balance was found to be unsatisfactory for two reasons, it does not read correctly over a range of 0 to 400 grams, and the movements of a rat on the pan cause such wide oscillations of the pointer that accurate reading is impossible. An ordinary beam balance has the additional disadvantages of being slow in operation because of the time required to manipulate weights, and of being even more affected than the spring balance by the movements of the rat.

The addition of a simple attachment to a commercial direct-reading beam balance gave such satisfactory results that we believe other workers may be glad to have a description of the device. The balance is one of those commercial balances of 1 kilo capacity, reading directly from 0 to 100 grams in one gram intervals, in which the oscillations of the beam are damped by a paddle attachment immersed in oil. Such a balance is most useful for general laboratory purposes, and even for weighing animals. Its performance may be considerably improved, however, by additional damping to overcome the oscillations caused by movements of the animal on the scale pan. We have achieved this by attaching to the underside of the scale-pan a paddle immersed in oil. This successfully damps the sideways or rotatory oscillations. A cup-shaped pan was adopted because rats move about in it very much less than on the ordinary scale-pan. The oscillations of the pointer due to movements of a rat are in this way practically eliminated.

As many as three hundred weighings of rats, accurate to within 1 gram, may, with this apparatus, be made easily in one hour.



SCALE FOR WEIGHING LIVE ANIMALS.

Messrs. W. and T. Avery, of Toronto, who are the makers of the commercial balance which we have adapted, were good enough to construct the additional attachments for us.

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SPECIAL ARTICLES

THE CHEMICAL STUDY OF INSULIN[1]

CRYSTALLINE insulin was first prepared by Abel in 1926.[2] Since then it has been of the greatest interest to ascertain the chemical structure of this substance, which plays so important a rôle in carbohydrate

[1] No. 13 of the series "Studies on Crystalline Insulin." Investigations carried out under grants from the Carnegie Corporation of New York and the Eli Lilly Company, Indianapolis, Indiana.

[2] J. J. Abel, *Proc. Nat. Acad. Sci.*, 12, No. 2, 132, 1926; Abel, Geiling, Rouiller, Bell and Wintersteiner, *J. Pharmacol. and Exp. Therap.*, 31, 65, 1926.

metabolism. It was found that crystalline insulin was of characteristic protein nature, possessing a high sulfur content of 3.2 per cent.[3] Naturally, the question arises whether this crystalline protein is identical with the hormone itself, possessing therefore its specific hypoglycemic action, or whether the protein crystal acts simply as a convenient carrier, the true hormone being adsorbed or loosely bound to the protein surface. The assumption that the crystalline protein is the hormone itself is based upon the following evidence.

Crystalline insulin possesses the maximal amount of physiological activity thus far obtained from insulin preparations, namely, 24 international units per milligram.[4] Attempts to obtain more active products have failed.[5] The preparation, as claimed by Dingemanse,[6] of an amorphous substance four times as potent as crystalline insulin, but extremely unstable, could not be repeated by workers in other laboratories[7] so that further proof is necessary before this can be generally accepted. The physiological activity of the crystals is independent of the method of their preparation[8] and also of the source of the pancreatic tissue from which they are obtained. Crystalline insulin has been prepared from the islet tissue of certain fishes[9] and from pig and sheep pancreas.[10] Insulin crystals obtained from these various sources all possess the same activity, approximately 24 units per milligram, and all have the same sulfur content, 3.2 per cent.[11] All the evidence thus far obtained favors the assumption that this crystalline protein represents the hormone insulin in its purest and most active form.

It may be added here that the ease with which one can obtain crystalline insulin varies, as does the yield, with the commercial product used for its preparation.[12] Using the proper preparation (in this laboratory Squibb's commercial product has given the best yield) satisfactory amounts of crystalline insulin can be obtained. In contradiction to a statement in *Nature*[13] the yield of crystalline insulin in terms of activity is about 50 per cent., using the Squibb's commercial material. It has been shown in this laboratory that the initial hyperglycemia produced by commercial insulin preparations is a non-hormonic action due to the impure nature of these preparations.[14]

It is of fundamental importance to decide whether the activity of insulin is a function of the whole molecule or of a prosthetic group, non-proteid in composition, which is attached to the protein complex. The latter situation exists in the case of hemoglobin, in which globin is the protein component and hematin (an iron-pyrrole compound) constitutes the prosthetic group. However, hematin, which is easily separable from the original molecule, is incapable of assuming the function of hemoglobin in the blood. In the case of crystalline insulin efforts to show the presence of such a prosthetic group, chemically different in its composition from ordinary amino acids, have thus far failed. As yet there is no evidence that such a constituent is present in the crystalline protein.

If one does not assume that the insulin molecule as a whole is responsible for the physiological action another possibility demands consideration. A grouping of certain component amino acids embedded in the molecule may be responsible for the hypoglycemic effect of insulin. In the case of the thyroid protein, iodothyreoglobulin, the physiological activity is a property, not of the protein as a whole, but of a specific constituent, thyroxine, which, moreover, is readily separable from the rest of the molecule.[15] Various attempts to separate such an active component from the insulin molecule have resulted in inactivation. The possibility exists, of course, that an active intramolecular complex, if present, is so unstable that it decomposes as rapidly as it is liberated. Investigation of the intact insulin molecule, therefore, would be necessary to establish the presence of a similar component. Could one definitely assign specific physiological activity to such a group, it is still possible that this constituent, when isolated from the molecule as a whole, would not show all the specific physiological actions of insulin.

The investigation of the possible existence of such groupings in the insulin molecule may be approached in two ways, first, one may separate and characterize the different amino acids obtained by hydrolysis. This method in addition will allow the identification of any constituents in the molecule other than amino acids. Secondly, one might also establish the presence

[3] Abel, Geiling, Rouiller, Bell and Wintersteiner, *J. Pharmacol. and Exp. Therap.*, 31, 65, 1926.

[4] Jensen, Wintersteiner and Geiling, *J. Pharmacol. and Exp. Therap.*, 36, 115, 1929.; Freudenberg and Dirscherl, *Zeit. physiol. Chem.* 180, 212, 1929; Harington and Scott, *Biochem. J.*, 23, 384, 1929.

[5] duVigneaud, Geiling and Eddy, *J. Pharmacol. and Exp. Therap.*, 36, 125, 1929; Jensen and DeLawder, *J. Biol. Chem.*, 87, 701, 1930.

[6] Dingemanse: *Arch. néerl. physiol.*, 12, 259, 1927; van Bronkhurst, *Pharmaceutisch weekblad*, 26, 641, 1930.

[7] See reference 5 and Dirscherl, *Zeit. physiol. Chem.*, 202, 116, 1931.

[8] Harington and Scott, *Biochem. J.*, 23, 384, 1929.

[9] Jensen, Wintersteiner and Geiling, *J. Pharmacol. and Exp. Therap.*, 36, 115, 1929.

[10] Scott, *J. Biol. Chem.*, 92, 281, 1931.

[11] See references 9 and 10.

[12] Jensen, Wintersteiner and Geiling, *J. Pharmacol. and Exp. Therap.*, 36, 115, 1929; Jensen and DeLawder, *Zeit. physiol. Chem.*, 190, 262, 1930.

[13] *Nature*, 126, 810, 1930.

[14] Geiling and DeLawder, *J. Pharmacol. and Exp. Therap.*, 39, 369, 1930.

[15] See reference 8.

of physiologically active groups by treating insulin with chemical reagents having a precise action on certain specific arrangements in the molecule. Investigation of the insulin molecule by these two methods has been in progress in this laboratory since the crystallization of insulin. The expensive nature of the material makes it imperative to use only small quantities for chemical manipulation. In conjunction with the time-consuming assay of the products of chemical treatment this has made progress somewhat slow.

Earlier publications from this laboratory have shown the presence of the following amino acids in the insulin molecule: cystine, about 12 per cent.; tyrosine, about 12 per cent.[16]; and arginine, histidine, lysine and leucine.[17] These amino acids are not sufficient to account for the total nitrogen of insulin. Determination of the distribution of nitrogen in the crystalline protein also indicates the presence of amino acids other than those listed above. In cooperation with Dr. Wintersteiner at Columbia University, New York, I have therefore undertaken anew the hydrolysis of insulin with somewhat larger amounts (4 grams). Only workers in this field will realize the difficulties of isolating various amino acids from such a small amount of original material. One can only hope for a renewed study of the chemical properties and quantitative separation of the various amino acids. Such a research would prove invaluable, not only in the study of insulin, but also for the chemical investigation of other biologically important compounds, protein in nature, and available only in comparatively small amounts.

The new hydrolysis of insulin reveals that the protein is composed of relatively few amino acids. Besides those already mentioned glutamic acid has been isolated and identified.[18] According to the amount of ammonia nitrogen (9.58 per cent. of total nitrogen) found in the determination of the nitrogen distribution in insulin,[19] a comparatively large amount of this amino acid should be present. Evidence of the presence of hydroxyvaline has also been obtained and, furthermore, leucine was found to be present in relatively large amounts. The presence of proline seems doubtful. No evidence for the presence of any other amino acids, or of any other constituent differing in its composition from an amino acid, could be obtained. We, therefore, find the following amino acids in the insulin molecule: cystine, tyrosine, glutamic acid and leucine, each present in quantities of more than 10 per cent.; and histidine, arginine, lysine, hydroxyvaline and proline (the last two are doubtful) in smaller amounts. The details of our work on the hydrolysis of insulin will be published elsewhere.[20]

The problem of linking the physiological activity of insulin to certain amino acid groups has been approached in two ways. First, various derivatives of insulin were prepared and their activity studied. The fundamental idea underlying these experiments was to block the active group or groups and thus to obtain a compound of no or greatly diminished activity. Then, by splitting off the radical which had been introduced into the active group, one might obtain a product, either equally as active as the original crystalline insulin, or at least of higher activity than the unhydrolyzed "blocked" substance. In undertaking these experiments one must bear in mind the marked instability of insulin. By treating insulin with acetic anhydride, acetyl insulin is obtained either with greatly diminished activity[21] or in a completely inactive form.[22] Hydrolysis of the acetyl insulin gives a product of an activity higher than that of the acetylated compound but lower than that of the original insulin. Under the conditions employed acetylation is possible at $-OH$, $-NH_2$ and $=NH$ groups. It is logical to conclude, therefore, that the acetylated group or groups are of importance in the physiological action of insulin.

The treatment of insulin in weakly alkaline solution with benzaldehyde and other aldehydes yields completely inactive products. Reactivation of these compounds has thus far been unsuccessful.[23] Here again one may conclude that the active group or groups have been blocked.

Insulin, when allowed to stand in acid alcohol for several hours, gives a completely inactive product. On treatment of this compound with very dilute alkali, about 60 per cent. of the activity is regained.[24] The hydrolyzed product may be partly obtained again in crystalline form identical with the original crystalline insulin.[25] Various investigators ascribe the inactivation to the blocking of the active group or groups

[16] duVigneaud, Jensen and Wintersteiner, *J. Pharmacol. and Exp. Therap.*, 32, 367, 1928.

[17] Jensen, Wintersteiner and duVigneaud, *J. Pharmacol. and Exp. Therap.*, 32, 387, 1928.

[18] Jensen, *J. Biol. Chem.* (in press).

[19] Wintersteiner, duVigneaud and Jensen, *J. Pharmacol. and Exp. Therap.*, 32, 397, 1928.

[20] Jensen and Wintersteiner, *J. Biol. Chem.* (in press).

[21] Jensen and Geiling, *J. Pharmacol. and Exp. Therap.*, 33, 511, 1928; Freudenberg and Dirscherl, *Zeit. physiol. Chem.*, 175, 1, 1928; Freudenberg, Dirscherl and Eyer, *Zeit. physiol. Chem.*, 187, 95, 1930; Jensen and DeLawder, *Zeit. physiol. Chem.*, 190, 262, 1930.

[22] Charles and Scott, *Trans.* Royal Soc. Canada, Section v, 187, 1931.

[23] Jensen and DeLawder, *Zeit. physiol. Chem.*, 190, 262, 1930; Freudenberg, Dirscherl and Eyer, *Zeit. physiol. Chem.*, 202, 144, 1931.

[24] Carr, Culhane, Fuller and Underhill, *Biochem. J.*, 23, 1010, 1929; Charles and Scott, *J. Biol. Chem.*, 92, 289, 1931; Freudenberg, Dirscherl, Eyer, *Zeit. physiol. Chem.*, 202, 133, 1931.

[25] Charles and Scott, *J. Biol. Chem.*, 92, 289, 1931.

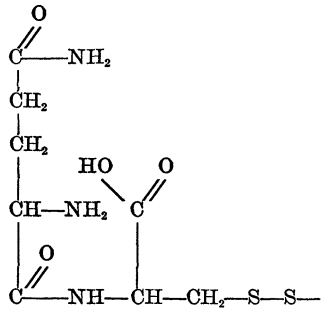
by methylation (using methyl alcohol) of the –OH, $-NH_2$ and =NH group or to the esterification of the –COOH group. Treatment with dilute alkali removes the methyl radical with the subsequent regeneration of the active groups. Later in this paper I shall propose a further explanation for this reaction.

The reduction of insulin, even under the mildest conditions, gives an inactive product which thus far has been found incapable of reactivation. Under the conditions employed probably only the disulfide linkage is affected. Since insulin gives no positive test for the sulfhydryl group it is generally assumed that all the sulfur is present as the disulfide linkage. In my opinion the total sulfur of insulin exists as cystine in the molecule. Even such mild reducing agents as cysteine and glutathione will apparently reduce the disulfide linkage, giving a physiologically inactive product which can not be reactivated.[26] It has been found that under mild conditions initial inactivation coincides with the first appearance of a positive sodium nitroprusside test for the sulfhydryl group.[27] In the above experiments the inactivation of insulin resulted from the alteration of certain physiologically important groups, without, however, removing these groups from the original molecule.

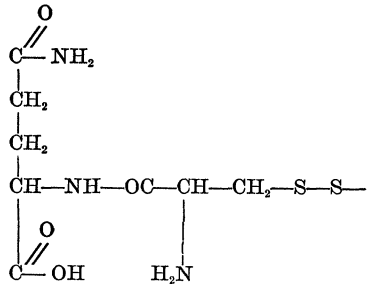
A second line of attack consists in splitting off such active groups and then determining the particular amino acid affected by this treatment. If insulin is heated with N/10 hydrochloric acid in a boiling water bath, an inactive coagulum is obtained. Treatment of this coagulum with dilute alkali gives a product, the activity of which is practically equal to that of crystalline insulin.[28] In spite of its high activity, efforts to recrystallize this material by the usual method have failed.[29] duVigneaud and coworkers[26] express the view that the formation of a coagulum under these conditions is specific for insulin and that the physiologically important groups are involved in this reaction. I shall return to this reaction later. It has also been pointed out in earlier communications from this laboratory that insulin, under the influence of alkali, not only splits off sulfur,[30] but also ammonia.[31] A later and more detailed study of this reaction[32] indicates that treatment of insulin even with very dilute alkali liberates ammonia and leads to the formation of an inactive product. There seems to exist a correlation between the degree of inactivation and the amount of ammonia given off. Under these same conditions the sulfur of insulin is also affected. Thus far it has not been possible to inactivate insulin irreversibly without removing at least a part of its sulfur, and it may be doubted whether one can remove a part of the sulfur without affecting the activity. In opposition to this point of view Freudenberg and his coworkers[33] assume that the sulfur of the insulin molecule plays no rôle in the action of its active component. If one affects only a very small part of the sulfur, as in Freudenberg's experiment, the unfortunate variations of the physiological assay may prevent the clear indication of the resultant slight inactivation. This point is under a more thorough investigation in this laboratory at the present time.

These experimental findings, in conjunction with others omitted because of their detailed nature, have led me to postulate the characteristic constituent of the insulin molecule as composed, wholly or in part, of glutamic acid and cystine and having the following chemical structures:

Formula I



Formula II



I am quite aware that I am unable at the present time to furnish exact proof that the glutamic acid is linked to cystine as indicated in the above formulae. These structures are proposed only as a working

[26] duVigneaud, Fitch, Pekarek and Lockwood, *J. Biol. Chem.*, 94, 233, 1931.

[27] Unpublished results.

[28] duVigneaud, Geiling and Eddy, *J. Pharmacol. and Exp. Therap.*, 33, 497, 1928; Freudenberg and Coworkers, *Zeit. physiol. Chem.*, 180, 223, 1929; 187, 91, 1930; 202, 131, 1931.

[29] Jensen and DeLawder, *Zeit. physiol. Chem.*, 190, 262, 1930.

[30] Abel and Geiling, *J. Pharmacol. and Exp. Therap.*, 25, 423, 1925.

[31] Jensen, Wintersteiner and duVigneaud, *J. Pharmacol. and Exp. Therap.*, 32, 388, 1928; Wintersteiner, duVigneaud and Jensen, *J. Pharmacol. and Exp. Therap.*, 32, 410, 1928.

[32] Jensen and DeLawder, *Zeit. physiol. Chem.*, 190, 270, 1930; Freudenberg, Dirscherl and Eyer, *Zeit. physiol. Chem.*, 187, 192, 1930; 202, 129, 1931.

[33] Freudenberg, Dirscherl and Eyer, *Zeit. physiol. Chem.*, 187, 100, 1930.

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).