THE REDUCTION OF INSULIN

by

J. LENS AND J. NEUTELINGS
Organon Laboratories, Oss (Holland)

The reduction of insulin has already been the subject of several investigations. In particular the reaction with thiol compounds has been studied extensively. FREUDENBERG *et al.*[1] observed the inactivating effect of such reducing agents and claimed to have observed a certain reversibility of this effect[2]. DU VIGNEAUD *et al.*[3] studied the reaction between insulin and cysteine or glutathione at neutral reaction. After a sufficiently long reaction time, an almost complete inactivation resulted. By oxidation, the activity could not be recovered. WINTERSTEINER[4] quantitatively measured the rate of reaction between insulin and cysteine in buffered solutions of p_H 7.2. The reduction was estimated by iodometric determinations of the remaining cysteine. He compared the degree of reduction thus observed with the biological activity. All authors agree that only a fraction of the S–S–bridges present in the insulin need be reduced in order to give a complete or almost complete loss of activity.

STERN AND WHITE[5] investigated the reduction under the influence of thioglycolic acid at a reaction of p_H 1.8–1.9. The thiol groups in the reduced insulin were determined colorimetrically with the FOLIN-MARENZI reagent. The temperature coefficient of the reaction was found to be considerable. According to DU VIGNEAUD *et al.*[3] this reduction is not catalysed by heavy metal ions, because KCN was found to have no influence on the reaction rate. This conclusion is not entirely warranted, as the tendency of the metal ions to form complexes with compounds containing S–S– or –SH might be stronger than the affinity of these ions to KCN. Hence the system, though sensitive to metal ions might be insensitive to poisoning by KCN.

Our investigation showed this to be true to some extent, albeit that KCN was not completely without effect.

Secondly we wanted to study the influence of other solvents on the rate of reduction. It is well known that the ionization constants in aqueous-alcohol solutions vary widely from those in water, and this might influence the reaction rate, as in fact it does. The interpretation of these data will have to await further exploration of the electrochemistry of both insulin and cysteine in these media.

EXPERIMENTAL

The insulin available was crystalline material prepared in our own laboratories according to ROMANS, SCOTT, AND FISHER[6]. In aqueous solution this product is very slightly soluble at neutral reaction, *i.e.*, just in the range in which we wanted to work, whereas the zinc-free amorphous material has a moderate solubility under these circum-

References p. 508.

stances. The crystalline insulin was therefore dissolved in 0.1 N acetic acid and dialysed in cellophane tubes first against 0.1 N acetic acid and subsequently against water.

The insulin concentration in the reaction mixtures was kept at 1%. Reductions were studied with cysteine as reducing agent in a concentration of 0.25% (0.021 N). Some runs have been made with glutathione and with ascorbic acid. The solutions employed were: water, ethanol 60% and 30%, and methanol and acetone, both 60%. All runs were made in a thermostat of 30°, under nitrogen, purified by forcing it through alkaline pyrogallol and through a tube with a red hot copper gauze.

The reductions in aqueous solutions were studied at p_H 7.2 in the presence of 1/30 mol phosphate buffer. The cysteine, available as the hydrochloride, was dissolved in water and neutralized with NaOH shortly before adding it to the buffered insulin solution.

For the fixation of the p_H in the alcohol and acetone solutions we did not succeed in finding a suitable buffer. A phosphate buffer in 60% alcohol shows a decrease of the p_H of several units, when measured with the glass electrode and with appropriate indicators. Though realizing that in these instances the interpretation of the determined p_H values is quite doubtful, we wanted to measure in a range comparable to neutral reaction in water. The apparent p_H of all solutions was therefore adjusted to 7 with ammonia and acetic acid solutions. The "p_H" measured, before and after the reduction, gave identical values. As in alcoholic solutions the reaction is very rapid, the insulin and cysteine solutions were adjusted separately before mixing the two. The cysteine solution, when neutral, is quite liable to oxidation by air and the adjustment of the p_H must be made as quickly as possible, not permitting a precision in the adjustment of 0.1 of a unit. Consequently the values in the final reaction mixtures varied between 7.0 and 7.5. Control experiments indicated that this difference did not perceptably alter the rate of reaction and any change observed cannot be described to these small differences in p_H. For the reduction experiments the method proposed by STERN AND WHITE[5] was adopted in its main lines. Their apparatus was slightly modified after some trial runs, as we did not consider the samples drawn quite representative of the bulk of the reaction mixture, unless the capillaries were first flushed with some freshly withdrawn solution, resulting in a considerable loss of expensive material. We therefore replaced the little burette, suggested by these authors, by a siphon with a capillary bore and provided with a glass stop cock. The liquid which had been in the siphon and accordingly not at the proper temperature was discarded immediately before drawing a sample. A volume, slightly in excess of the desired one, was rapidly withdrawn in a test tube and from the contents the samples of 2 ml were pipetted into centrifuge tubes.

The insulin was precipitated with 3 ml of 6% trichloro-acetic acid from aqueous solutions or with 8 ml of slightly acidified acetone if the sample contained organic solvents. In this way non-sticky, flocculent precipitates were obtained. They were washed four times with fresh solvent to remove the reducing agents completely. The precipitate was dissolved in 8 ml 40% urea solution and 1 ml citric acid-NaOH buffer according to LUGG[7] giving a p_H of 5.8. Subsequently 1 ml of the FOLIN-MARENZI reagent[8] was added. The colour reaches a maximum after 10–15 minutes if the reduction has not proceeded very far. With increasing reduction the reduced insulin gradually becomes less soluble in the reagent solution, resulting in a slower development of the colour. Such solutions must be centrifuged before they can be measured in the photometer.

The instrument used was an electric photometer with a colour filter transmitting

References p. 508.

light of 650–700 mμ. A calibration curve with pure cystine, reduced with Na_2SO_3 had been made for this instrument. The rate of reduction is expressed as "percent cystine reduced", assuming a cystine content of 12% for insulin[9].

RESULTS

1. *Cysteine in aqueous solutions*

The influence of heavy metals on the rate of reduction has been investigated. The results are represented graphically in Figs 1 and 2. The metals were added in the form

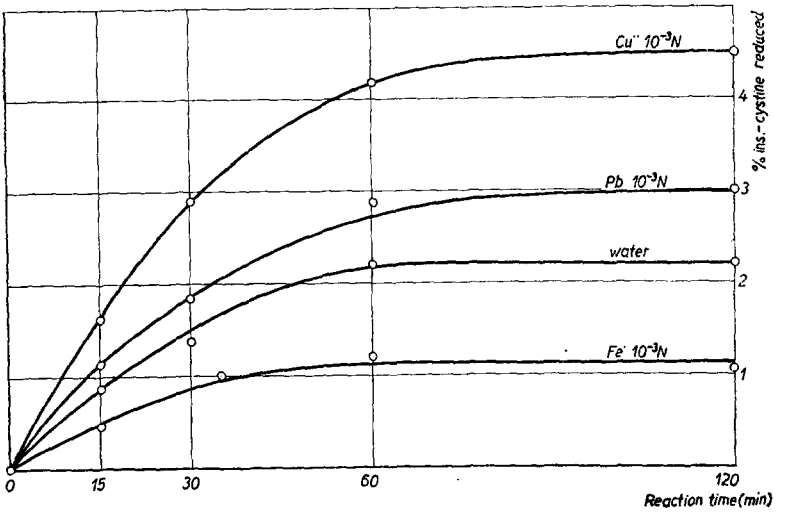

Fig. 1. Reduction of insulin by cysteine in aqueous solutions in the presence of various metal ions. The reduction curves for Mn^{++} and for an aqueous solution without metals almost coincide. Abcissa: reaction time in minutes; ordinate: Percentage insulin-cystine reduced. Insulin concentration: 1%; cysteine-HCl concentration: 0.25%; pH 7.2; temp. 30° C. Normality of metal ions: 10^{-3}.

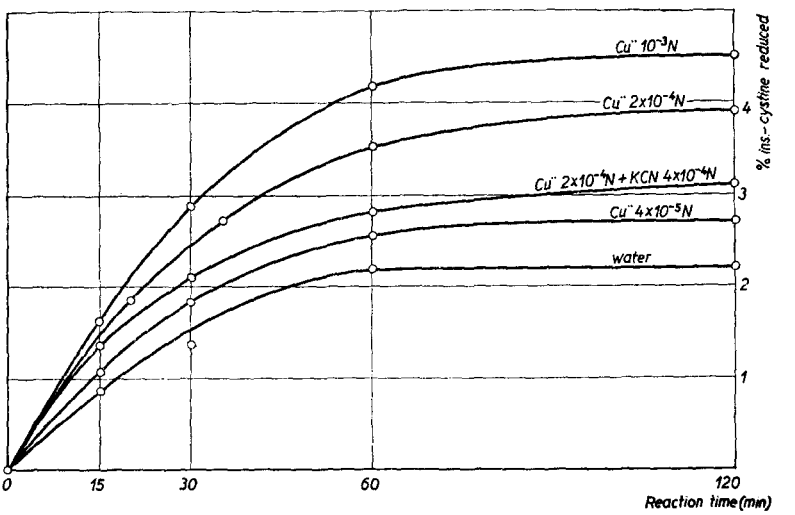

Fig. 2. Reduction of insulin by cysteine in aqueous solutions at various Cu concentrations. Abcissa: reaction time in minutes; ordinate: Percentage insulin-cystine reduced. Insulin concentration: 1%; cysteine-HCl concentration: 0.25%; pH 7.2; temp. 30° C

References p. 508.

of their bivalent chlorides. Cu and Pb catalyse the reaction, Mn has no influence and Fe apparently decreases the reaction rate. The catalytic influence of Pb could only be demonstrated in a qualitative way because of the precipitation of the phosphate. The influence of Cu, as being the most pronounced one and apparently without the complications encountered with Pb, lead us to study its influence in some more detail. It has a marked effect in a concentration as low as $2 \cdot 10^{-4}$ N. Only in dilutions of $4 \cdot 10^{-5}$ N its influence becomes negligible. By the addition of KCN in a molar concentration 4 times as large as that of the Cu the effect is somewhat quenched but still perceptable.

As KCN itself may reduce –S–S–groups the addition of unlimited quantities is not without objections, and has not been investigated for this reason.

Potentiometric titrations as described by EISENBRAND AND WEGEL[10] for cysteine and Zn were carried out to study the complex formation between cysteine and the

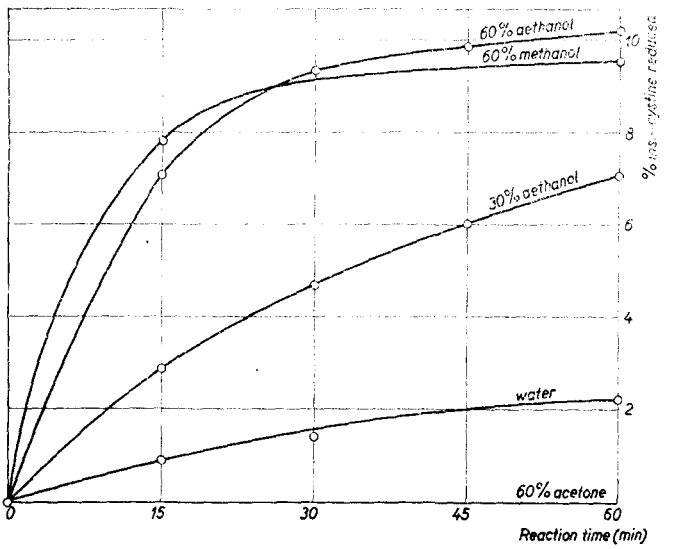

Fig. 3. The reduction of insulin by cysteine in some organic solvents. Abcissa: reaction time in minutes; ordinate: Percentages insulin-cystin reduced. Insulin concentration: 1%; cysteine-HCl concentration: 0.25%; pH 7.0–7.5; temp. 30° C.

above-mentioned cations. Conditions and concentrations of the reagents were exactly those used by these authors. Cu and Pb gave curves comparable to the one they obtained for cysteine and Zn; Mn did not show any sign of complex formation under these conditions. The ferrous ion also gives rise to complex formation.

In all titrations interference by redox potentials was circumvented by the use of a "Cambridge" special glass electrode, which may be used in alkaline solutions as well.

2. Cysteine in alcohol solutions

As demonstrated in Fig. 3 the rate of the reaction is increased considerably by adding of ethanol the solution. At any time in the range studied this increase is proportional to the alcohol concentration. At the higher alcohol concentrations the effect cannot be increased by the addition of Cu though it has been found, again with the potentiometric titration, that Cu complexes of cysteine are formed in 60% ethanol just as easily as in water.

References p. 508.

The curves for 60% methanol and ethanol can be said to coincide.

Amorphous insulin and crystalline Zn insulin (which could be used in these experiments, as it dissolves well in 60% alcohol), gave identical curves.

3. *Cysteine in 60% acetone*

No reduction of the insulin occurs in 60% acetone. Even when the concentration of the cysteine was increased from 0.25 to 1% no reduction was detectable after two hours. With the usual cysteine concentration of 0.25%, a reduction of only 1.2% was found after 18 hours. The difference in behaviour between alcohol and acetone has frequently been checked and confirmed.

4. *Cysteine in acid solution*

Some experiments were performed at p_H 3–4 both in aqueous and alcoholic solutions. Only a very slow rate of reduction was observed under these circumstances.

5. *Glutathione and ascorbic acid*

In aqueous solutions at p_H 7.2, the reduction by glutathione was investigated in a concentration of 0.487%, aequimolecular to that of the cysteine. The curve obtained proved that glutathione behaves as cysteine within the experimental error.

No reduction could be obtained with ascorbic acid under widely varying conditions. In a p_H range from 3–7, both in water and in 60% alcohol, with an insulin and an ascorbic acid concentration both as high as 3.3%, no reaction could be demonstrated.

6. *Reduction and physiological activity*

This subject has been extensively studied by other authors, using aqueous solutions of the insulin. It is only necessary to extend these data to the case of the solutions containing alcohol. The insulin was precipitated from these solutions with acetone as described above and dissolved in dilute hydrochloric acid. The assays were performed on rabbits with the technique of DE JONGH *et al.*[11].

In Table I the results are summarized.

TABLE I

RELATION BETWEEN REDUCTION AND ACTIVITY
1% INSULIN, 0.25% CYSTEINE, p_H 7, 60% ALCOHOL, t = 30°

Time in minutes	Insulin-cystine reduced (%)	Activity left (%)	Activity* calculated
0	0	100	100
5	2.7	60	84
15	7.3	20–35	56
60	10.2	13–14	40

* The calculated activities are based on the assumption of an active sub-unit with 6 S-S-bridges.

The stability of the solutions of reduced insulin is very low, which gives the results more or less the character of the maximum values which can be obtained. If an assay is repeated after some weeks a much lower result is found.

References p. 508.

DISCUSSION

It has been demonstrated that some heavy metal ions catalyse the reduction of insulin by cysteine in very low concentrations. The cations which were found to be active were also those which formed a complex with cysteine, whereas Mn, which has no catalytic influence, does not form a complex.

An exact evaluation of the relation between cation concentration and rate of reaction is not always possible, because many cations give a heavy precipitate with insulin or with the phosphate buffer at p_H 7. In the presence of cysteine, copper remains in solution, probably by the formation of the afore-mentioned complex and the influence of Cu has therefore been chosen for further investigation. It could be followed down to concentrations of $4 \cdot 10^{-5}$ N. This means that the effect still exists if roughly one copper ion per insulin molecule of m.w. 48000 is present. The ferrous ion shows an abnormal behaviour: though this ion also forms a complex with cysteine, it apparently suppresses the reduction of insulin but biological standardizations would be necessary to check this result. We did not go into this problem any further.

The increased rate of reduction in solutions containing alcohol is very pronounced and directly proportional to the alcohol concentration. The effect of Cu in 30% and 60% alcohol is no longer perceptible though complex formation between cysteine and Cu occurs just as well in these solutions. Apparently there is some other influence which entirely dominates the course of the reaction in these cases. The question what this influence might be must be left unanswered, the more so because in solutions containing acetone no reduction was found at all. Ignoring the latter observation, the hypothesis that either the insulin or the cysteine or both must be present as anions would deserve consideration, but this suggestion appears to be incompatible with the behaviour of the reactants in acetone, where qualitatively the same effect occurs, namely increased ionization of the carboxyl groups and suppression of the ionization of the amino groups.

In principle, reduction in acid solutions under the conditions chosen in these experiments is possible. STERN AND WHITE[5] reduced insulin with thioglycolic acid at p_H 1.8–1.9 but the concentration of their reducing agent was 24 times larger, on a molecular basis, than ours and this explains why under our conditions only a very low reaction rate was found.

Ascorbic acid is not able to reduce the dithio-bridges of insulin. This is in accordance with the observation of SCHOCK *et al.*[12] that insulin is not inactivated by ascorbic acid.

REDUCTION AND INACTIVATION

The rapid inactivation of insulin, already described by FREUDENBERG *et al.*[1] and confirmed by STERN AND WHITE[5], by WINTERSTEINER[2] and again found by us, offers the possibility of throwing some light on the problem of the sub-unit of insulin. According to SJÖGREN AND SVEDBERG[13] and confirmed by GUTFREUND[14], the insulin molecule dissociates into 4 sub-units of molecular weight 12000 in aqueous acid solution. SANGER[15] showed that this sub-unit consists of 4 polypeptide chains, linked by 6 dithio bridges.

The residual activities mentioned in Table I can only find a satisfactory explanation by assuming that *not* the sub-unit, but a larger complex is physiologically active. In making this deduction it has been assumed that the reduction of one S–S–bridge is sufficient to give a completely inactive molecule.

References p. 508.

If the sub-unit were the physiologically active group, a reduction of one of the 6 S–S–bridges or of 16.7% would mean a total loss of activity. A proportionally smaller loss of activity would correspond with a smaller reduction.

Going over the figures in Table I, it is at once obvious that this is far from the facts. For instance, 10.2% reduction would mean an average of 0.6 S–S bridges per sub-unit reduced, or 40% activity left, whereas only some 13–14% are found. On the other hand, in assuming that the molecule as a whole with its 24 S-S bridges is the active substance, a 10.2% reduction corresponds to an average of 21.6 S-S bridges intact. This average may be made up by a certain percentage of molecules with 24 bridges (in this case apparently 13–14%), a certain percentage with 23 bridges and so on. The same deductions can be made for the other residual activities measured.

From this argument it follows that not the sub-unit is the physiologically active group but that this group must be composed of several sub-units together. It is impossible to say how many sub-units must associate to give an active complex, but it seems reasonable to assume that this active complex is identical with a whole molecule, consisting of 4 sub-units. This assumption has in fact been made in the example given. It should be pointed out that the figures obtained for the residual activities can be equally well explained by the assumption of an association of two or more sub-units as forming the physiologically active unit.

The poor stability of the reduced insulin might be explained by a possible levelling off of the reduction to the same degree over all molecules present.

Considering the ease with which the reduction of insulin takes place and the presence of thiol groups in the form of cysteine and glutathione in all cells, there seems to be no reason to assume enzymatic inactivation of insulin in the living organism. A number of enzymes, hydrolysing and hence inactivating insulin are known to exist and they may certainly play a rôle in its destruction, but thiol groups will have to be taken into consideration just as well.

SUMMARY

The reduction of insulin by cysteine in neutral aqueous solutions is catalysed by Cu and Pb. Mn has no influence and Fe apparently suppresses the reduction. Cu, Pb and Fe form complexes with cysteine, but Mn does not.

The rate of reduction is increased in solutions containing alcohol and is directly proportional to the alcohol concentration. In 60% acetone no reduction occurs.

In acid solutions the rate of reaction is considerably less than at neutral reaction.

Glutathione behaves as cysteine. Ascorbic acid does not reduce insulin. The loss of activity occurring by reduction has been confirmed.

RÉSUMÉ

La réduction de l'insuline par la cystéine dans les solutions aqueuses neutres est catalysée par le Cu et le Pb.

Le Mn n'a pas d'influence, tandis que le Fe semble s'opposer à la réduction. Le Cu, le Pb et le Fe forment des complexes avec la cystéine, tandis que le Mn n'en forme pas.

La vitesse de la réduction est augmentée dans des solutions alcooliques; elle est directement proportionnelle à la concentration de l'alcool. Dans l'acétone (60%) il n'y a pas de réduction.

Dans les solutions acides la réduction est retardée considérablement.

Le glutathione se conduit tout comme la cystéine.

L'acide ascorbique ne réduit pas l'insuline.

La perte d'activité accompagnant la réduction a été confirmée.

References p. 508.

ZUSAMMENFASSUNG

Die Reduktion des Insulins durch Cystein in neutralen wässrigen Lösungen wird katalytisch durch Cu und Pb beschleunigt.

Mn hat keinen Einfluss, Fe scheint die Reduktion zu verzögern.

Cu, Pb und Fe bilden Komplexe mit Cystein, im Gegensatz zu Mn.

Die Reduktionsgeschwindigkeit wird in alkoholischen Lösungen erhöht und ist der Alkoholkonzentration direkt proportional.

In 60% Azeton findet keine Reduktion statt.

In sauren Lösungen ist die Reaktionsgeschwindigkeit erheblich kleiner als bei neutraler Reaktion.

Glutathion verhält sich wie Cystein.

Ascorbinsäure reduziert das Insulin nicht.

Der die Reduktion begleitende Aktivitätsverlust wurde bestätigt.

REFERENCES

1 K. FREUDENBERG AND H. EYER, *Z. physiol. Chem.*, 213 (1932) 226.
2 K. FREUDENBERG AND A. MÜNCH, *Z. physiol. Chem.*, 263 (1940) 1.
3 V. DU VIGNEAUD, A. FITCH, E. PEKAREK, AND W. WAYNE LOCKWOOD, *J. Biol. Chem.*, 94 (1931–32) 233.
4 O. WINTERSTEINER, *J. Biol. Chem.*, 102 (1933) 473.
5 K. G. STERN AND A. WHITE, *J. Biol. Chem.*, 117 (1937) 95.
6 R. G. ROMANS, D. A. SCOTT, AND A. M. FISHER, *Ind. Eng. Chem.*, 32 (1940) 508.
7 J. H. W. LUGG, *Biochem. J.*, 26 (1932) 2144.
8 O. FOLIN, *J. Biol. Chem.*, 106 (1934) 311.
9 F. SANGER, *Nature*, 162 (1948) 491.
10 J. EISENBRAND AND F. WEGEL, *Z. physiol. Chem.*, 268 (1941) 26.
11 S. E. DE JONGH, J. LENS, AND R. W. SPANHOFF, *Arch. intern. pharmacodynamie*, 74 (1947) 63.
12 E. D. SCHOCK, H. JENSEN, AND L. HELLERMAN, *J. Biol. Chem.*, 111 (1935–36) 553.
13 B. SJÖGREN AND T. SVEDBERG, *J. Am. Chem. Soc.*, 53 (1931) 2657.
14 H. GUTFREUND, *Biochem. J.*, 42 (1948) 156.
15 F. SANGER, *Biochem. J.*, 39 (1945) 507.

Received March 12th, 1949