THE ESSENTIAL GROUPS OF INSULIN

by

JANE FRAENKEL-CONRAT AND HEINZ FRAENKEL-CONRAT*

College of Agriculture, Agricultural Experiment Station, University of California, Berkeley, Calif. (U.S.A.)

Since the crystallization of insulin many attempts have been made to allocate its hormonal activity first to a prosthetic group and later to any one particular part of the molecule. The finding, however, that reduction of the disulfide bonds led to progressive inactivation[1-3] suggested that the hormonal activity depended more on the integrity, possibly the shape, than on any one part, of the protein molecule. Yet, further chemical studies showed that certain protein groups could be modified without loss of activity, while others could not. Thus treatment with ketene caused inactivation only if sufficiently intensive to cause acetylation of some phenolic groups; acetylation of the amino groups did not affect the activity[4]. Iodination of all phenolic groups caused marked inactivation, which was in part reversed when part of the iodine was removed by reduction[5]. Coupling, presumably of phenol and imidazole groups, with as many as 14 equivalents (per mole of protein) of various anionic diazobenzene derivatives caused no appreciable inactivation, but the introduction of cationic residues by the same mechanism led to loss of activity[6]. Sulfation of all aliphatic hydroxyl groups caused no loss in activity[7], but esterification of the carboxyl groups did lead to inactivation[8, 9, 7]. The effects of aromatic isocyanates caused some controversy. The claim that these reagents combined selectively with the amino groups[10] is not supported by more recent studies[11, 12]. A reinvestigation of the reaction of insulin with phenyl isocyanate by HALLAS-MØLLER[13] has led him to conclude that inactivation resulted not from the most rapid reactions-those of the amino and imidazole groups—but from the subsequent acylation of the guanidyl groups.

Evidently, there is much more information available about the essential groups of insulin, than about those of most other biologically active proteins. In connection with the use of insulin as a model protein of singular composition[14-17, 19] a few new derivatives have been prepared, or new methods employed, and it is the purpose of this paper to report the results of assays of these preparations, and the conclusions that may be drawn from them concerning the essential groups of insulin. The possibility that some of the active derivatives might find therapeutic use because of their altered solubilities, possibly in the from of complexes with protamine, is also discussed.

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* On the staff of the Western Regional Research Laboratory, United States Department of Agriculture, Albany, California.

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METHODS AND MATERIALS

Most of the preparative and analytical methods have been described in detail in preceding papers; many of the derivatives have already been analytically characterized[14–19].

Iodination

a. 50 mg samples of insulin were dissolved in 1.8 ml of water by means of a trace of *N* sodium hydroxide. To these solutions was added 0.2 ml of either 3.4 *M* p_H 7.6 phosphate, or 3 *M* p_H 5 acetate buffer, followed by 0.1 *N* iodine (KI_3) solution, 0.1 ml at a time. At p_H 7.6 the iodine was rapidly decolorized so that 0.4 ml could be added within 30 minutes at room temperature. At p_H 5 only 0.2 ml was added over a 24 hour period, and was even then not completely used up. The protein was precipitated from the reaction mixtures by the addition of a few drops of 3 *M* p_H 5 acetate buffer, the precipitate washed, redissolved with a drop of *N* NaOH, reprecipitated at p_H 5, washed, frozen, and dried.

b. To 100 mg insulin, dissolved in 6 ml water with 0.2 ml 0.1 *N* sodium hydroxide and 0.3 ml p_H 7.6 or 6.2 phosphate buffer, was added at room temperature 3.5 ml 0.1 *N* iodine solution. After 10, 20, 30, or 120 min., the dark brown suspension was decolorized with 0.5% sodium sulfite. From half of the reaction mixture the insulin was isolated by isoelectric precipitation, then washed; to the other half was added half again as much of the sodium sulfite solution to reduce the labily bound iodine prior to isolation. Dialysis was also used at times for the isolation of iodoinsulin.

Aromatic isocyanates

Samples of 100 mg protein, dissolved at p_H 7.6 as above, were cooled to 0°, and then treated, with continuous stirring, with amounts varying from 0.02 to 0.2 ml of the isocyanate. The reaction mixtures were dialysed after the isocyanate odour had disappeared (3–12 hours). One experiment was performed with 1.0 ml phenyl isocyanate at 8° (decomposition time 2 hours); in another experiment with 1.0 ml phenyl isocyanate the reaction was terminated after 1 hour at 0°. The soluble fractions were dialysed and analysed. In some instances, the diphenylurea was extracted with ether from the insoluble protein fraction (in a SOXHLET apparatus), and the latter also analysed and assayed.

Assay methods

All preparations were assayed by the convulsion method in mice. Usually rather small groups of mice were used (3–6), but the assay at the proper level (50% convulsions) was repeated several times. A total of about 1600 mouse assays were performed. The accuracy of such tests would not suffice to detect differences smaller than 20%, but served the purpose of the present study, which was to determine which reactions did, and which did not, cause appreciable loss of activity.

Some of the preparations were also assayed by the cross-over technique in a limited number of rabbits. Rabbits were also used for the assay of protamine complexes of standard insulin and of some of the derivatives. Blood samples were in these cases taken over periods of 8–12 hours. In preparing the insoluble complexes it was found necessary to add more protamine to the more acidic derivatives than to standard insulin, to obtain maximum insolubility. For purposes of convenience of injection, the suspensions were prepared more dilute than commercial protamine insulin (only 2, 6, and finally 18 Units per ml, as compared to 40 units per ml). The solubilities of many of the complexes were approximately determined by mouse assay of the supernatants after centrifugation.

The insulin used in this study was zinc insulin crystals, kindly placed at our disposal by ELI LILLY and Co.

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DISCUSSION OF RESULTS

All assay results are listed in Table I, while the detailed analytical characterization of the derivatives is summarized in Table II. The amino groups of insulin may be largely acetylated without appreciable loss of activity. This conclusion of STERN AND WHITE's[4] studies with ketene has now been confirmed, particularly by the use of the specific method of acetylation with acetic anhydride*. Furthermore, substitution of

* In contrast to these authors, we were not able to achieve acetylation of all the amino groups with either reagent.

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the amino with $-CH_2-NH-CO-CH_3$ groups (formaldehyde treatment in the presence of acetamide[14]) affected the activity but slightly. Loss of activity was obtained upon treatment with phenyl and *m*-chlorophenyl isocyanates, but the specificity of this class of reagents has often been questioned[11, 12]. The present experiments show that amounts of the latter reagent in excess of the reduction in amino groups can be introduced into insulin under gentle reaction conditions, thus substantiating the claims of the non-specificity of the isocyanates.

The retention of activity by insulin samples that had been briefly treated with formaldehyde at p_H 11.5 indicates that the most reactive amide and/or guanidyl groups are not essential for the activity. This is in contrast to all other biologically active proteins that have been tested. An alternate method to modify amide and guanidyl groups is available, *i.e.*, by treatment with formaldehyde in the presence of an amine at or below neutrality[14]. This method, however, lacks specificity since the methylol-amine condenses with phenolic groups only a little more slowly than with amide or guanidyl groups*. When alanine was used as the amine in such experiments, insoluble and inactive products were obtained after 3 days of reaction at p_H 3–4 although partial retention of activity was observed after a seven hour period at p_H 6–7**.

To summarize, the more or less marked inactivating effect of formaldehyde in all but quite alkaline solutions, or in the presence of acetamide, is attributed not to reactions with any one group, but to cross-linking between amino groups on the one side and amide, guanidyl, phenolic, and possibly imidazole groups on the other. The participation of the amino groups is definitely shown by the resistance of the amino-acetyl insulin to inactivation by formaldehyde. The protecting action of high concentrations of acetamide against inactivation by formaldehyde is similarly understandable, since cross-linking of all available amino groups with this amide would prevent cross-linking between pairs of protein groups. When both factors were combined, *i.e.*, when acetyl-insulin was treated with formaldehyde in the presence of acetamide (to condense with the few residual amino groups) the activity was fully retained.

The essentiality of the phenolic groups for the activity of insulin has previously been deduced from ketene and iodine experiments[4, 5]. The present study has indicated that iodination of up to half of the phenolic groups did not cause very extensive inactivation. It would thus appear that the iodination of the most reactive phenolic groups may cause less inactivation than their acetylation. The possibility must be recognized, however, that ketene, like phenyl isocyanate, may act on some imidazole and guanidyl groups of insulin, as well as on the phenolic groups, after acylation of most of the amino groups.

Treatment for a short time with a great excess of iodine at p_H 7.6 (method b) was recently shown to introduce iodine also into the imidazole rings of some proteins, particularly lysozyme[19]. The iodine fixed by the histidine residue of lysozyme under such conditions was found to be in part released by immediate treatment with sodium sulfite at neutrality and room temperature. The histidine residues of globin had previously been shown to be remarkably reactive toward iodine[20]. Since no appreciable amounts of iodine were bound in such a labile manner by insulin, and since the total amount of iodine rapidly bound did not exceed the amount apparently bound by the

* Imidazole groups react similarly though at an appreciable rate only at elevated temperatures[15].

** It must be remembered that primary amines, such as alanine may, in conjunction with two moles of formaldehyde, act as crosslinking agents between two protein amide groups[14].

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phenolic groups, it was concluded that the histidine residues of this protein were not similarly reactive as those of lysozyme*. On the other hand, the phenolic groups of insulin appear more reactive than those of any other protein studied. Over half of them reacted within less than one minute with excess iodine, or within 2 hours with gradual addition of iodine. This is in line with other findings concerning the reactivity of the phenolic groups of insulin[12, 21, 22].

TABLE I
BIOLOGICAL ACTIVITY OF INSULIN DERIVATIVES

No.	Reaction and Conditions[a]	Protein Group Involved[b]	Activity % of Original
1	Acetylation (acetic anhydr. (85), p_H 7.5–6.0, o° C., 1 hour)	Amino (90)	>75
2	Acetylation (ketene, 5 min., o° C., p_H 6)	Amino (62)	75
3	Acetylation (ketene, 10 min., o° C., p_H 6)	Amino (>90), phenolic, etc.	29
4	Acetylation (ketene, 20 min., o° C., p_H 6)	Amino (>90), phenolic, etc.	12
5	Formaldehyde (600) (p_H 3.2, 3 days)		<10
6	Formaldehyde (600) (p_H 3.2, 3 days) plus acetamide (850)	Amino (84)	75
7	Formaldehyde (600) (p_H 3.2, 3 days) plus alanine (560)	Amide, guanidyl (70), etc.	4[c]
8	Formaldehyde (600) (p_H 5.0, 3 days)		<3
9	Formaldehyde (600) (p_H 5.0, 3 days) *after acetylation*		50
10	Formaldehyde (600) (p_H 5.0, 3 days) plus acetamide (850) *after acetylation*	Residual amino (12)	75
11	Formaldehyde (600) (p_H 5.0, 3 days) plus alanine (560) *after acetylation*	Amide, guanidyl (89), etc.	19
12	Formaldehyde (600) (p_H 8.8, 3 days)		<3
13	Formaldehyde (600) (p_H 7.8, 3 days) *after acetylation*	Residual amino, some amide, guanidyl (22)	75
14	Formaldehyde (900) (p_H 7.2, 7 hrs.)		19
15	Formaldehyde (900) (p_H 6.5, 7 hrs.) plus alanine (560)	Amide, guanidyl (28), etc.	50
16	Formaldehyde (900) (p_H 11.5, 10 min.	Amide, guanidyl (23)	88
17	Control to No. 16, 43		71
18	Phenylisocyanate (19) (o° C.)	Amino (70), imidazole, phenolic, etc.	25
19	Phenylisocyanate (93) (o° C.)	Amino (65), imidazole, phenolic, etc.	43
20	Phenylisocyanate (190) (1 hr., o° C.)	Amino, imidazole, phenolic, etc.	22
21	*m*-Chlorophenylisocyanate (14) (o° C.)	Amino (65), imidazole, phenolic, etc.	22
22	*m*-Chlorophenylisocyanate (35) (o° C.)	Amino, imidazole, phenolic, etc.	20
23	*m*-Chlorophenylisocyanate (70) (o° C.)	Amino, imidazole, phenolic, etc.	10
24	*m*-Chlorophenylisocyanate (140) (o° C.)	Amino, imidazole, phenolic, etc.	13
25	Iodine[d] (4 × 2) (p_H 7.6, 0.5 hr.)	Phenolic (50) (32% MIT[e])	67
26	Iodine (9 × 2) (p_H 7.6, 5.5 hrs.)	Phenolic (73) (8% MIT)	8

* After more prolonged reaction periods more iodine is bound by insulin than calculated from tyrosine and monoidotyrosine analyses (Table II). This may be in part due to the fact that very low colorimetric values for tyrosine and monoiodotyrosine represent the basis for calculation of high diiodotyrosine values, which would greatly magnify positive errors such as contributed by the (slight) colour of the hydrolysate or the (slight) chromogenic activity of diiodotyrosine. On the other hand, iodination of histidine seems to occur under such conditions in the other proteins studied and may also easily account for the observed discrepancies.

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TABLE I (continued)

BIOLOGICAL ACTIVITY OF INSULIN DERIVATIVES

No.	Reaction and Conditions[a]	Protein Group Involved[b]	Activity % of Original
27	Iodine (2 × 2) (p_H 5, 24 hrs.)	Phenolic (11)	69
28	Iodine (35) (p_H 6.0, 0.5 hr.)	Phenolic (56) (23% MIT)	27
29	Iodine (35) (p_H 7.6, <1 min.)	Phenolic (64) (13% MIT)	16
30	Iodine (35) (p_H 7.6, 10 min.)	Phenolic (74) (24% MIT)	10
31	Iodine (35) (p_H 7.6, 30 min.)	Phenolic (81) (14% MIT)	7
32	Iodine (35) (p_H 7.6, 120 min.)	Phenolic (87) (11% MIT)	<6
33	Diazobenzene sulfonic acid (4.3) (p_H 7.6, 30 min.)	Phenolic, imidazole (40)	67
34	Diazobenzene sulfonic acid, then iodine (35)	Phenolic, imidazole	2
35	Phosphorylation (78% P_2O_5) (3 days)	Aliphatic –OH (100)	38
36	Reduction (20% thioglycol, p_H 5, 24 hrs.)[f]	Disulfide (40)	10
37	Reduction, then partial reoxidation (air)		7
38	Reduction, then complete reoxidation		<5
39	Reduction, (as No. 36, 1 hr.) then iodoacetamide (p_H 8, 10 min.[g])	Disulfide (42)	<6
40	Deamination (*M* nitrite, p_H 4.1, 0° C., 30 min.)	Amino (46)	>75
41	Deamination (*M* nitrite, p_H 4.1, 0° C., 20 hrs.)	Amino (54), phenolic, etc.	25
42	Iodoacetamide (50) (p_H 7.6, 40° C., 3 d.)	Amino, phenolic, etc.	21
43	Iodoacetamide (10) (p_H 11.5, 23° C., 15 min., for control rf. No. 17)		100

[a] Room temperature unless otherwise specified. Amounts of reagents, unless used in great excess, are expressed as equivalents per 10^4 g protein, indicated in parentheses after the reagent.

[b] Approximate extent of reaction indicated in parentheses, in terms of percent of residues affected.

[c] Product insoluble at all but extreme p_H's, therefore assayed in suspension.

[d] Immediately after the iodination of preparations 25–32 treatment with excess sulfite did not effect any regeneration of activity, nor any alteration in the analytical data.

[e] MIT stands for monoiodotyrosine; the percentage is that of the original total tyrosine.

[f] Crystalline zinc insulin was treated with thioglycol in suspension in water.

[g] The protein was dissolved with a trace of alkali, then precipitated in amorphous form by the addition of p_H 5 acetate buffer followed immediately by the reducing agent.

TABLE II

ANALYTICAL DATA OF INSULIN DERIVATIVES[a]

No.	Reaction	Analyses
1	Acetylation (acetic anhydride)	NH_2–N (V)[b]: 0.5, Folin p_H 8/p_H 11[c]: 0.95
2	Acetylation (ketene)	NH_2–N (V): 1.9, Folin p_H 8/p_H 11: 1.02
3	Acetylation (ketene)	NH_2–N (H)[b]: >0.4, Folin p_H 8/p_H 11: 0.67
4	Acetylation (ketene)	NH_2–N (H): >0.4, Folin p_H 8/p_H 11: 0.62
6	Formaldehyde plus acetamide (p_H 3.2, 3 days)	Amide–N: 12.5
7	Formaldehyde plus alanine (p_H 3.6, 3 days)	Acid groups: 21.6, guanidyl groups: 1.7
7a	Formaldehyde plus methylguanidine sulfate (p_H 3.2, 3 days)	Amide–N: 8.0, guanidyl groups: 2.7
8	Formaldehyde (p_H 5.1, 3 days)	Formaldehyde: 4.3
9	Acetylinsulin: formaldehyde (p_H 5.1, 3 days)	Amide–N: 8.5
10	Acetylinsulin: formaldehyde (p_H 5.1, 3 days) plus acetamide	Amide–N: 8.9
11	Acetylinsulin: formaldehyde (p_H 5.0, 3 days) plus alanine	Acid groups: 23.2, formaldehyde: 8.7, tyrosine (T)[d]: 4.1, (F)[d]: 6.5
13	Acetylinsulin: formaldehyde (p_H 7.8, 3 days)	Acid groups: 14.4, formaldehyde: 2.4, tyrosine (T): 4.1, (F): 5.4
14	Formaldehyde (p_H 7.2, 7 hrs.)	NH_2–N (V): 4.9, acid groups: 12.5 tyrosine (F, p_H 8[d]): 3.8

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TABLE II (continued)

ANALYTICAL DATA OF INSULIN DERIVATIVES[a]

No.	Reaction	Analyses
15	Formaldehyde plus alanine (p_H 6.5, 7 hrs.)	NH_2–N (V): 7.8, acid groups: 17.4, tyrosine (F, p_H 8): 3.8
16	Formaldehyde (p_H 11.5, 15 min.)	Formaldehyde: 2.3
18	Phenylisocyanate (0.02 ml)	NH_2–N (V): 1.5
19	Phenylisocyanate (0.1 ml)	NH_2–N (H): ca. 1.5, tyrosine (F, p_H 8): 4.6
20	Phenylisocyanate (1 ml, 0° C., 1 hr.)	Tyrosine (F, p_H 8): 3.4
21	*m*-Chlorophenylisocyanate (0.02 ml)	NH_2–N (H): ca. 1.5, chlorine: 4.0
22	*m*-Chlorophenylisocyanate (0.05 ml)	Chlorine: 5.4, tyrosine (T): 6.6, (F): 7.2
23	*m*-Chlorophenylisocyanate (0.1 ml)	Chlorine: 8.5
24	*m*-Chlorophenylisocyanate (0.2 ml)	NH_2–N (H): 1.4, chlorine: 9.3
25	Iodine (4 × 2) (p_H 7.6, 30 min.)	Iodine: 4.0 (calc.: 4.2)[e], tyrosine (R): 3.1 MIT (R): 2.0
26	Iodine (9 × 2) (p_H 7.6, 5.5 hrs.)	Iodine: 7.5 (calc.: 8.5), tyrosine (R): 1.7 MIT (R): 0.5
27	Iodine (2 × 2) (p_H 5.0, 24 hrs.)	Iodine: 1.4
28	Iodine (35) (p_H 6.0, 30 min.)	Iodine: 5.3 (calc.: 5.7), tyrosine (R): 2.7, MIT (R): 1.3
29	Iodine (35) (p_H 7.6, <1 min.)	Iodine: 7.2 (calc.: 7.2), tyrosine (R): 2.2, MIT (R): 0.8
30	Iodine (35) (p_H 7.6, 10 min.)	Iodine: 8.2 (calc.: 7.8), tyrosine (R): 1.6, MIT (R): 1.5
31	Iodine (35) (p_H 7.6, 30 min.)	Iodine: 9.5 (calc.: 9.1), tyrosine (R): 1.2, MIT (R): 0.9
32	Iodine (35) (p_H 7.6, 120 min.)	Iodine: 10.6 (calc.: 10.1), tyrosine (R): 0.8, MIT (R): 0.7
33	Diazobenzene sulfonic acid (4.3) (p_H 7.6)	Acid groups: 17.6, tyrosine (T): 4.1
34	Diazobenzene sulfonic acid, then iodine (35) (p_H 7.6, 10 min.)	Iodine: 7.3, acid groups: 17.3
35	Phosphorus pentoxide in H_3PO_4	Phosphorus: 7.6
36	Reduction (thioglycol, p_H 5)	–SH groups: 4.0
38	Reduction (thioglycol, p_H 5) then partial reoxidation (O_2)	–SH groups: 1.3
39	Reduction (thioglycol, p_H 5) then iodoacetamide (p_H 8, 10 min.)	–SR groups: 4.2
40	Nitrite (p_H 4.1, 0° C., 30 min.)	NH_2–N (H): 2.7, tyrosine (T): 5.6
41	Nitrite (p_H 4.1, 0° C., 20 hrs.)	NH_2–N (H): 2.3, tyrosine (T): 4.3
42	Iodoacetamide (p_H 7.6, 40° C., 3 days)	NH_2–N (H): 2.3, tyrosine (F, p_H 8): 4.9, (T): 5.2, (F): 6.1
42a	Control to F42 (p_H 7.6, 40° C., 3 days, no iodoacetamide)	NH_2–N (H): 5.6, tyrosine (F, p_H 8): 4.5, (T): 4.4, (F): 7.8
43	Iodoacetamide (p_H 11.5, 15 min.)	Amide–N: 8.6
	Untreated insulin	NH_2–N (V): 5.0, (H): 4.3, amide–N: 8.3, tyrosine (F, p_H 8): 4.8, (T): 6.4, (F): 6.6, (R): 6.2, acid groups: 14.6; guanidyl groups: 1.8; disulfide bonds: 5.0

[a] Numbers in column 1 correspond to those of Table I. All analytical data, unless otherwise specified, represent equivalents per 10^4 g protein.

[b] V stands for analyses by the VAN SLYKE method[28], H for those obtained by the ninhydrin method of HARDING AND MACLEAN[29].

[c] Ratio of Folin tyrosine values given by the unhydrolysed protein, when treated according to HERRIOTT[30].

[d] Tyrosine was determined after alkaline hydrolysis by either the method of THOMAS[31] (indicated as T) or with the Folin reagent (F)[30], or together with monoiodotyrosine (MIT) according to ROCHE's *et al.*[26] (R) with the MILLON reagent. (F p_H 8) indicates analysis without hydrolysis[30].

[e] The values in parentheses represent iodine equivalents, calculated from the observed monoiodotyrosine and the diiodotyrosine, which was calculated by difference[19]. They are to be compared with the found values as listed.

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In connection with other proteins, particularly pepsin, the question has been amply discussed whether partial iodination leads to the formation of appreciable amounts of monoiodotyrosine, or only to that of diiodotyrosine[23-26]. Microbiological assays in the case of iodolysozyme[19] and spectrophotometric analyses[26] of iodoinsulins[19] have indicated the occurrence of monoidotyrosine whenever the reaction had been performed under conditions in which either the time or the amount of iodine used were insufficient for diiodination of all the reactive phenolic groups. Particularly with limiting amounts of iodine (method a), preparations were obtained in which only about 18% of the tyrosine was present as diiodotyrosine but 32% as monoiodotyrosine. Such preparations retained, surprisingly, 67% of the original activity. In contrast, iodination with a great excess of iodine (method b) led to preparations containing always more diiodotyrosine than monoiodotyrosine, and even when the total number of tyrosine groups involved was only 56%, the activity was reduced to 27% of the original, much lower than in the previously mentioned preparation when 50% of the tyrosine was two thirds monoiodinated (Table I–II). The occurrence of monoiodotyrosine and diiodotyrosine was also demonstrated by paper chromotography[19].

The idea has been expressed[12] that coupling with limited amounts of diazobenzenesulfonic acid might represent a specific means of modifying the imidazole groups. It has since been found, however, that the phenolic groups participate at all stages of the reaction[19]. Thus the retention of most of the activity by coupling insulin is no longer regarded as evidence for the nonessentiality of the imidazole groups. The integration of all evidence now available indicates that a certain proportion (one third to one half) of the phenolic and imidazole groups of insulin is not essential, but that more extensive substitution, coupling, *etc.* of the groups causes inactivation.

Reduction of the disulfide bonds of insulin is known to cause inactivation[1-3]. It was hoped that this reaction might be rendered reversible if it were performed on intact crystals suspended in the reaction medium. To this end the protein was treated for 24 hours with a 20% solution of thioglycol buffered at p_H 5. The reduced product was then isolated and thoroughly and repeatedly washed by means of centrifugation. One half was redissolved for –SH analysis (with *p*-chloromercury benzoate) and assay, the rest reoxidized, either by aeration in the presence of a trace of copper sulfate, or with hydrogen peroxide. Tetrathionate was also used for the simultaneous analysis (by potentiometric titration) and reoxidation of the reduced insulin suspension[27]. In all experiments, the activity of the protein was found to be about 10% of the original value after reduction of about one third of its disulfide bonds, and no reactivation, but further loss in activity resulted from all types of reoxidation. The retention of some activity in this experiment may be due to the grossly heterogeneous state of the reaction mixture, part of the hormone being protected from the reducing agent by occlusion within the crystals. When insulin was dissolved and reprecipitated at p_H 5, reduction for only one hour, and subsequent alkylation of the –SH groups with iodoacetamide caused more complete inactivation.

Phosphorylation with phosphoric anhydride in phosphoric acid has recently been shown to transform the aliphatic hydroxyl groups of proteins into phosphate esters, though not without damage to the peptide chain[17]. The reaction proceeds at room temperature and is therefore more harmful to the integrity of the protein molecule than the previously described method of sulfating the aliphatic hydroxyl groups by means of concentrated sulfuric acid at very low temperatures[16, 17]. It is thus not surprising that phosphorylated insulin shows lowered activity.

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A few experiments were performed with sulfated and coupled insulin combined with protamine, to ascertain whether the insulin action could be further prolonged when the complex was formed with acidic insulin derivatives than with regular insulin. Some indications of prolonged action were obtained, particularly with the coupled insulin, but the variability for the same preparation, including commercial protamine insulin or laboratory-preparated complex of untreated insulin and protamine was so great in our hands that a final conclusion must await substantiation in a greater number of test animals.

Tests, by mouse assay, for the solubilities of the various complexes at p_H 7.3 showed that the diazobenzenesulfonic acid coupled insulin yielded a more insoluble complex than did standard insulin. This finding is in agreement with the indications obtained in rabbit assays. A more detailed study of the coupled and possibly other insulin protamine complexes would appear indicated.

Comments to the esterification of insulin. It has been suggested that treatment of insulin with 0.1 *N* methylalcoholic hydrochloric acid at room temperature causes not only esterification of the carboxyl groups, but also unknown reactions involving loss of nitrogen, possibly from amide groups. A reinvestigation has indicated that amide-N is *not* released. If the insulin ester hydrochloride is dialysed, 50 to 60% of the protein passes through the membrane during 3–4 days; if the preparation is first treated with alkali under conditions which partly reverse the esterification reaction[7, 8], only 25% is subsequently dialyzable. Untreated insulin is dialysable to a similar extent when 1% acetic acid is used as solvent. The explanation for these findings appears to be, that esterification, like dilute acid, favours dissociation into the subunits (M.W. 12 000) which are slowly dialysable. The fraction remaining in the bag appears to differ somewhat from the original insulin in containing a higher amide-N, and lower total N, on the weight basis.

SUMMARY

Insulin has been treated under carefully controlled conditions with acetic anhydride, ketene, aromatic isocyanates, iodine, diazobenzene sulfonic acid, phosphorous pentoxide, thioglycol, formaldehyde and other reagents, either singly or in sequence. From assays of the reaction products, and from data in the literature, the following conclusions have been derived:

1. Neither acetylation, nor other reactions involving most of the amino groups, nor sulfation of most of the aliphatic hydroxyl groups causes inactivation of the hormone.

2. Addition of methylol to part of the amide or guanidyl groups, or iodination of half of the phenolic groups, or coupling of part of the latter as well as a few imidazole groups (with diazobenzene sulfonic acid), causes little if any loss of activity.

3. Reduction of most of the disulfide bonds, or esterification of most of the carboxyl groups, or extensive iodination or coupling of phenolic and imidazole groups causes marked inactivation.

4. The partially coupled insulin, combined with protamine, appears to be less soluble and possibly more protracted in its action than standard protamine insulin.

RÉSUMÉ

Nous avons traité l'insuline, dans des conditions strictement contrôlées, par l'anhydride acétique, le cétène, les isocyanates aromatiques, l'iode, l'acide diazobenzène sulfonique, le pentoxyde de phosphore, le thioglycol, la formaldéhyde et d'autres réactifs, soit indépendamment, soit à la suite.

De l'étude des produits de réactions et des données de la littérature nous déduisons les conclusions suivantes:

1. L'hormone ne perd son activité ni par acétylation ou toute autre réaction portant sur a plupart des groupes amino, ni par sulfatation de la plupart des groupes hydroxyl aliphatiques.

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2. L'activité est peu ou n'est pas diminuée par addition de méthylol à une partie des groupes amido ou guanidyl, par ioduration de la moitié des groupements phénoliques ou par copulation d'une partie de ces derniers et de quelques groupes imidazoliques avec l'acide diazobenzène sulfonique.

3. L'activité diminue fortement par réduction de la plupart des liaisons disulfide, par estérification de la plupart des groupes carboxyl, par ioduration énergique ou par copulation des groupements phénoliques et imidazoliques.

4. L'insuline partiellement copulée, combinée à la protamine, semble être moins soluble et avoir une action de plus longue durée que la protamine-insuline qui sert d'étalon.

ZUSAMMENFASSUNG

Insulin wurde unter genau kontrollierten Bedingungen mit Acetanhydrid, Keten, aromatischen Isocyanaten, Jod, Diazobenzolsulfonsäure, Phosphorpentoxyd, Thioglykol, Formaldehyd und anderen Reagenzien einzeln oder nacheinander behandelt.

Aus der Untersuchung der Reaktionsprodukte und den Angaben der Literatur wurde gefolgert:

1. Weder durch Acetylierung oder durch andere, die meisten Aminogruppen angreifende Reaktionen, noch durch Veresterung der meisten aliphatischen Hydroxylgruppen mit Schwefelsäure wird das Hormon desaktiviert.

2. Addition von Methylol an einen Teil der Amid- oder Guanidylgruppen, oder Jodierung der Hälfte der Phenolgruppen oder Kuppeln eines Teiles dieser Gruppen und einiger Imidazolgruppen (mit Diazobenzolsulfonsäure) verursacht geringen oder gar keinen Aktivitätsverlust.

3. Ein bedeutender Aktivitätsverlust tritt dagegen ein, durch Reduktion der meisten Disulfidbindungen, durch Veresterung der meisten Carboxylgruppen oder durch weitgehende Jodierung oder Kuppeln der Phenol- und Imidazolgruppen.

4. Das teilweise gekuppelte und an Protamin gebundene Insulin ist schwerer löslich als das Standardpräparat Protamin-Insulin und möglicherweise hält auch seine Wirkung länger an.

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