CIX. A REVERSIBLE INACTIVATION OF INSULIN.

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EARLY in 1927 a new method of purification for application to large-scale manufacture was devised in the laboratories of "A-B" insulin by which the activity of insulin hydrochloride could be increased from 14 to 20 international units per mg. When, however, later in that year the same process was applied to 1781 g. (25 million units), the resulting material showed a progressive loss of activity, such that, when tested a few months later, only 3 million units remained. The investigation of the cause of this loss resulted in the discovery of the precise conditions under which insulin can be inactivated and reactivated and also led to the ultimate recovery of much valuable material. Since the observations made in the course of the work are of general interest, they are reported in this paper.

Although it has previously been stated that insulin can be inactivated by treatment with various reagents, with subsequent recovery of at any rate some of the potency, it has been supposed that the inactive products are either adsorption compounds, or derivatives in which only part of the activity has been destroyed. While the present work was proceeding, however, Freudenberg and Dirscherl [1928] showed that insulin may be inactivated by treatment with acetic anhydride and pyridine and that 20-30 % of the activity is recovered by the action of weak sodium hydroxide. Jensen and Geiling [1928] repeated these experiments using crystalline insulin. Their inactive product contained only 18 % of the original potency, whilst on treatment with sodium hydroxide 46 % was recovered.

The work now presented shows how insulin can be inactivated by ethyl alcohol and hydrochloric acid, and the activity completely recovered by treatment with aqueous sodium hydroxide. The process may be repeated with only a small amount of degradation. Coincident with the change in activity, the reaction at which the insulin is completely precipitated shifts towards the alkaline side of its original value, reverting to the latter with recovery of the activity.

EXPERIMENTAL.

Methods of assay. All the biological tests were performed on rabbits, the hypoglycaemic response being taken as criterion of activity. The blood-sugar estimations were carried out by the Folin and Wu [1920] technique, as modified by Haden [1923]. For strictly quantitative work, Marks's [1925] "cross test" method was used as modified by Culhane [1929], 8-20 animals being employed for each test. In a few cases, to demonstrate only a small difference between two samples, one was directly tested against the other; but in all other assays the standard used was a specimen of insulin hydrochloride, of which the potency had been previously determined in terms of the International Standard Insulin Powder by a series of cross tests. The standard dose was invariably 0.5 unit per kg. body-weight.

As a preliminary to all "cross tests," a rough test employing two or three rabbits has been used, in order to fix a suitable dose for the more accurate assay. The test that has been found most useful is one in which the blood-sugar is determined hourly until a minimum is reached. The position of the minimum, together with a knowledge of the history of the animal, gives some indication of the activity of the material injected. Thus, the average blood-sugar values (mg. per 100 cc.) for six rabbits injected on different occasions with doses of 0.25, 0.5 and 0.75 units per kg. were:

	Initial	1st hour	2nd hour	3rd hour
0.25 unit	109	91	97	—
0.5 "	105	74	65	75
0.75 "	114	78	65	66

On the smallest dose four reached the minimum at the first hour, two at the second; on the dose of 0.5 unit, five reached the minimum at the second hour, one at the third, and on the largest the values for the second and third in no case differed by more than 3 mg. per 100 cc., the minimum occurring four times at the second hour and twice at the third.

The method has also been used to demonstrate inactivity. Even on a dose of 0.1 unit per kg. an appreciable fall in blood-sugar is obtained in the first hour. Hence, if no fall occurs in 1 hour, the unit can be regarded as not smaller than ten times the dose given.

The isoelectric point. Commercial insulin is insoluble between about p_H 5 and 6, the exact figures depending on the concentration and on the amount of salts present. If ammonia is added to a solution of the hydrochloride, the insulin flocculates out at a reaction (p_H 5.0) which is on the acid side of this "precipitation range." If acid is added to an alkaline solution of insulin, flocculation takes place at a point on the alkaline side. The isoelectric point presumably lies between these reactions.

The figures given in this paper are the precipitation points on the acid side of the range. They will be referred to as the "final precipitation points" throughout this paper. In a few cases the corresponding values on the alkaline

side have been obtained; they are approximately 1 p_H unit more alkaline than the quoted figures.

The method for the determination of the final precipitation point is as follows.

2–5 mg. insulin hydrochloride are dissolved in 1–2 cc. distilled water, and $N/50$ ammonia is added drop by drop until a definite cloudiness is obtained. Then one or more drops of $N/200$ ammonia are added to increase the cloudiness, followed by drops of $N/500$ and $N/2000$ until the precipitate shows signs of flocculation. The solution just prior to flocculation presents a characteristic turbidity. It is left for a few minutes, and then centrifuged for 2–3 minutes. If a clear supernatant fluid results, flocculation is judged to be complete, but if the fluid is opalescent, a drop of $N/2000$ alkali is added and it is centrifuged again after 5 minutes. This process is repeated as often as is necessary to give a clear supernatant fluid. The reaction of the clear solution is then determined, using methyl red and bromothymol blue as indicators, with the appropriate buffer solutions. The determinations are usually duplicated, and repeated if the two results do not agree. The inactivated insulins (especially those obtained by treatment with higher alcohols and glycerol) give gelatinous precipitates and a rather indefinite final precipitation point.

INACTIVATION.

The action of ethyl alcohol containing hydrochloric acid.

When insulin is allowed to stand in solution at room temperature in a mixture of three parts of anhydrous ethyl alcohol and one part of 3 N hydrochloric acid, giving 0.75 N 75 % alcoholic acid, it slowly loses its activity. In one experiment in which such a solution was kept at 20°, portions being withdrawn at given intervals of time, the insulin precipitated with acetone and assayed, it was found that complete inactivation had occurred at the end of about 2 days (see Fig. 1).

Fig. 1. Rate of loss of activity.

Reagent: 0.75 N HCl 75% ethyl alcohol.
Temperature: 20°.

The rate of inactivation is increased by raising the temperature; thus, at 37°, a loss of 51 % of the activity was obtained in 2 hours, the material containing somewhat less than 9 units per mg. instead of the 14 remaining after 2 hours' treatment at the lower temperature.

The rate of inactivation is also increased by raising the concentration of either the acid or the alcohol (see Table I).

For these experiments, the reagents were prepared either by mixing aqueous hydrochloric acid with anhydrous ethyl alcohol or, in the case of those containing acid more than 2.5 *N*, by passing dry hydrochloric acid gas into the anhydrous alcohol to make a 10 *N* solution and diluting the latter with water and alcohol as required.

Table I.

Duration of exposure to reagents: 7 hours.
Temperature: 20°.
Original potency: 17.9 units per mg.

Concentration of alcohol		Concentration of acid	
Reagent	Final potency: units per mg.	Reagent	Final potency: units per mg.
50 % ethyl alcohol with 0.75 <i>N</i> HCl	12.5	0.25 <i>N</i> HCl 75 % ethyl alcohol	13.5
75 % ethyl alcohol with 0.75 <i>N</i> HCl	10.9	0.5 <i>N</i> HCl 75 % ethyl alcohol	8.9
40 % ethyl alcohol with 4.0 <i>N</i> HCl	6.2	0.75 <i>N</i> HCl 75 % ethyl alcohol	10.9
60 % ethyl alcohol with 4.0 <i>N</i> HCl	3.8	1.25 <i>N</i> HCl 75 % ethyl alcohol	6.3
80 % ethyl alcohol with 4.0 <i>N</i> HCl	0.5-1.0	1.75 <i>N</i> HCl 75 % ethyl alcohol	1.7-1.0

Coincident with the loss of activity there occurs a gradual change in the final precipitation point, such that, after 86 hours in 0.75 *N* 75 % alcoholic acid at 20°, it has changed from p_H 5.0 to p_H 6.5. This change was found to be characteristic and was used as a preliminary indication of inactivation.

The rates of change obtained with varying concentrations of acid and alcohol are given in Table II and Figs. 2 and 3.

Table II.

Final precipitation point of insulin: p_H 5.0.
Potency: 12.5 units per mg.
75 % ethyl alcohol. Temperature: 20°.

0.25 <i>N</i>		0.5 <i>N</i>		0.75 <i>N</i>		1.25 <i>N</i>		1.75 <i>N</i>	
Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H
7	5.15								
16	5.2	16	5.5	1	5.0	4	5.3	4	5.4
24	5.3	24	5.6	2.5	5.1	6.5	5.9	7	6.5
50	5.4	40	5.6	4	5.2	7	6.0		
72	5.7	49	6.0	7	5.4	24	6.5		
90	5.8			16	5.75	48	6.9		
120	5.9			30	6.0				

The results demonstrate clearly that the change in final precipitation point is accelerated by raising the concentration of the acid and the alcohol. The shift is also increased by raising the temperature (see Table III).

Table III.

Final precipitation point of insulin before treatment: p_H 5.0.
 Potency: 12.5 units per mg.
 Reagent: 0.75 *N* HCl 75 % ethyl alcohol.

18°		37°	
Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H
2.5	5.1	2.5	5.7
4	5.2	3	6.0
7	5.4	4.5	6.3

Fig. 2. Change of p_H of point of final precipitation: effect of acid concentration.
 75 % ethyl alcohol. Temp. 20°.

Fig. 3. Rate of change of p_H of point of final precipitation: effect of acid concentration.
 Ethyl alcohol 80 %.

Thus a general parallelism exists between the loss of activity and the shift in the final precipitation point towards the alkaline side.

Aqueous hydrochloric acid in concentrations similar to those used above effects no change in the final precipitation point, nor does treatment of insulin with dry hydrochloric acid gas. On the other hand, if insulin is moistened with alcohol and exposed to a current of dry hydrochloric acid gas, the final precipitation point changes to p_H 6.6 in 20 minutes.

REACTIVATION.

Experiments were carried out to discover the mildest conditions under which a totally inactive product capable of complete reactivation could be obtained.

The criterion for complete inactivity was that the material should cause no lowering of blood-sugar when injected into two or three rabbits in a dose of 1 mg. per kg., indicating that the unitage of the "inactive" product was at least 10 mg. It is impracticable to give the high doses required to prove complete freedom from insulin activity, and unnecessary when it is remembered that a unitage of 10 mg. represents a loss of 99.5 % of the insulin employed in these experiments. By treatment with 8 *N* 80 % alcoholic hydrochloric acid at 20° the potency was reduced to 0.25–0.5 unit per mg. in 2 hours, representing a loss of about 98 %; with 0.75 *N* 75 % alcoholic hydrochloric acid, more prolonged treatment was required, complete loss occurring within 63 hours. In both cases a change in the final precipitation point was observed.

Preliminary experiments having shown that treatment with dilute sodium hydroxide effected some recovery of activity, a series of tests was carried out to determine the optimum conditions of reactivation. The time and temperature were arbitrarily fixed at 17 hours and 0°.

From a series of experiments in which sodium hydroxide solutions of various strengths were mixed with equal volumes of the solution of inactive insulin it was found that maximum recovery of activity was attained with *N*/11.875 alkali (see Fig. 4).

Fig. 4. Reactivation. Effect of concentration of sodium hydroxide.

Time 17 hours.

Temperature 0°.

× Single determinations.

⊙ Average of several results.

The reactivation may be complete in less than 17 hours at 0°. After 5 minutes at this temperature, 60 % of the activity was restored by 0.0842 *N* alkali. With the recovery of activity the precipitation point reverted to p_H 5.

The details of the method finally adopted for all reactivations are as follows.

To a 1 % solution of inactive insulin is added with stirring an equal volume of 0.0842 *N* NaOH; the solution is kept at 0° for 17 hours and then acidified to p_H 3.5. Either it is tested directly or the insulin is precipitated as picrate and the hydrochloride regenerated and assayed. The normality of the mixture is somewhat less than half that of the added alkali since some of it is required for neutralisation of the hydrochloric acid and proteins present.

The method was applied directly to material completely inactivated and it was found that recovery of activity is usually quantitative and that the final precipitation point shifts back towards p_H 5.0 (see Table IV).

Table IV.

Exp. No.	Conditions of inactivation	Inactivated material		Reactivated material		Original material	
		Potency units per mg.	Final ppt. p_H	Potency units per mg.	Final ppt. p_H	Potency units per mg.	Final ppt. p_H
1	8 <i>N</i> 80 % alcoholic acid for 1 hr.	0.5	6.0	18.5	4.9	17.9	5.0
2	8 <i>N</i> 80 % alcoholic acid for 2 hrs.	0.25-0.5	6.2	20.0	5.0	17.9	5.0
3	0.75 <i>N</i> 75 % alcoholic acid for 63 hrs.	<0.1	6.25	16.9	5.2	17.9	5.0
4	0.75 <i>N</i> 75 % alcoholic acid for 63 hrs.	<0.1	6.2	14.5	5.0	14.3	5.0
5	0.75 <i>N</i> 75 % alcoholic acid for 86 hrs.	<0.1	6.5	14.3	5.1	14.3	5.0
6	0.75 <i>N</i> 75 % alcoholic acid for 63 hrs.	<0.1	6.4	17.2	5.05	17.9	5.0

The question arises whether the recovered material has the same properties as the original insulin. The final precipitation point reverts to its original value of p_H 5, but, upon repetition of the process, the second recovery of activity is not always quantitative. Thus, following exposure to 0.75 *N* 75 % alcoholic hydrochloric acid for 63 hours at 20°, the second recovery was only 65 %, or, following a relatively mild second inactivation, 71 % (Table V).

Table V.

Exp. No.	Conditions of inactivation	Unitage of			
		First inactive product: units per mg.	First reactivated product: units per mg.	Second inactive product: units per mg.	Second reactivated product: units per mg.
1	0.75 <i>N</i> 75 % alcoholic HCl for 30 hrs.	0.93	15.6	0.5	16.9
2	0.75 <i>N</i> 75 % alcoholic HCl for 63 hrs.	0.1	17.2	0.1	11.1
3	0.75 <i>N</i> 75 % alcoholic HCl for 16 hrs.	—	—	3.2	12.2

When, however, less severe conditions were used, which gave approximately 96 % inactivation at the first treatment, 95 % recovery was obtained after the second reactivation.

Experiments comparing the rate of loss of activity of insulin with that of reactivated insulin have given somewhat conflicting results, but, using

material which had been about 80 % inactivated and recovered, the results were very similar to those obtained with untreated insulin, and appear to fall on the same curve (Fig. 5, Curve I). On the other hand, material obtained by action of alkali on insulin inactivated twice to the extent of over 94 % appears to be more rapidly inactivated (Fig. 5, Curve II).

Fig. 5. Rate of inactivation of treated and untreated insulin.

Reagent: 0.75 *N* HCl 75 % ethyl alcohol.

- Curve I { $\odot$ Untreated insulin.
 $\otimes$ Insulin inactivated once to a potency of 3 units per mg. and reactivated.
- Curve II { $\times$ Insulin inactivated twice (to 1.0 and 0.5 unit per mg.) and twice re-activated.

THE NATURE OF THE CHANGE.

When insulin is treated with a mixture of hydrochloric acid and ethyl alcohol the activity slowly disappears and a coincident change of the final precipitation point occurs towards the alkaline side. The rate of the reaction is increased by raising the temperature and by increasing the concentration of alcohol or acid. It occurs only when alcohol is present; it takes place with a mixture of alcohol, rendered specially free from traces of aldehyde, and chlorine-free hydrochloric acid, and further, the action is reversed by treatment under conditions of a mild hydrolysis.

If the change is an esterification, it should be possible to bring it about by the use of other conditions for ester formation, whilst it should not occur under conditions in which ester formation does not take place. Experiments have been carried out on the action of a variety of alcohols and acids and of certain other compounds. In these, a shift in the final precipitation point

towards the alkaline side has been taken to indicate a loss of activity, and in a number of cases biological tests have confirmed it.

The reagents, in the case of the higher primary alcohols, were made by adding one part 10 *N* acid to three parts of alcohol, or of an alcohol-acetone mixture, since with *n*-hexyl alcohol and higher homologues the reactants are not miscible without the addition of acetone. The experiments were carried out at 20°.

With methyl alcohol the rate of change of both activity and final precipitation point is greater than with ethyl alcohol. Thus, 0.5 *N* 75 % methyl alcoholic hydrochloric acid reduced the potency from 17.9 to 3.7 units per mg. in 7 hours, as compared to 8.9 units per mg. with the corresponding ethyl alcoholic reagent. The changes in the final precipitation point are shown in Table VI.

Table VI.

Final precipitation point of insulin used: p_H 5.0.							
75 % methyl alcohol				75 % ethyl alcohol			
HCl 0.25 <i>N</i>		HCl 0.5 <i>N</i>		HCl 0.25 <i>N</i>		HCl 0.5 <i>N</i>	
Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H
16	5.6	3.5	5.5	16	5.2	16	5.5
24	6.1	19	6.5	24	5.3	24	5.6
41	6.2	26	6.7	50	5.4	40	5.6

Fig. 6. Rate of change of activity and final precipitation point.

Curve I. —□— —○— With 0.75 *N* HCl 75 % glycerol.

Curve II. —×— —•— With *N* HCl 75 % butyl alcohol.

The results obtained with glycerol and butyl alcohol are shown in Fig. 6, and it is of interest to note that whilst the change in the final precipitation point is certainly somewhat more rapid with butyl alcohol than with glycerol, the rate of loss of activity is much greater. Thus, products may be obtained by the two reagents having the same final precipitation point but very

different activities, suggesting that different chemical compounds have been formed in the two cases, rather than that the same change in the configuration of the molecule has been induced by two different mixtures of acid and alcohol. 0.75 *N* 75 % acid propyl alcohol reduced the potency to 11.0 units per mg., whilst a similar concentration of acid ethyl alcohol gave an almost identical figure of 10.9 units per mg. The shift in the final precipitation point was observed with all the other primary aliphatic alcohols up to *n*-octyl, with the aromatic primary alcohols—benzyl and phenylethyl alcohols—and with ethylene glycol. Some of the results obtained are given in Table VII.

Preliminary potency tests have indicated some loss of activity in the case of amyl alcohol and ethylene glycol but have not been carried out after treatment with the other alcohols.

Table VII.

<i>n</i> -Propyl alcohol		2.5 <i>N</i> HCl 75 % alcohol.		<i>iso</i> Amyl alcohol	
Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H
0.5	5.25	2.5	5.6	2.7	6.0
1.5	5.5	5.0	6.6	5.0	6.5
2.5	6.1	7.0	6.8	7.5	6.6
5.0	6.6	24.0	7.1	24.0	6.9
7.0	6.8				
19.0	7.0				

Mixtures of the secondary alcohols, *isopropyl* alcohol and cyclohexanol with 10 *N* hydrochloric acid (3 : 1) gave similar results (Table VIII), but the rate of change was much less than with the corresponding primary alcohol. In the case of *isopropyl* alcohol loss of activity was also observed.

Table VIII.

2.5 <i>N</i> HCl.			
75 % <i>iso</i> Propyl alcohol		75 % Cyclohexanol	
Time hrs.	Final ppt. point p_H	Time hrs.	Final ppt. point p_H
2.7	5.1	3.5	5.1
5.0	5.2	6.0	5.2
24.0	5.7	29.0	5.8

Mixtures of tertiary amyl alcohol and of terpineol (containing 50 % acetone) with 10 *N* HCl (3 : 1) were without effect on the final precipitation point.

It may be concluded that secondary alcohols produce a slower change than primary, and that with tertiary alcohols the change only occurs extremely slowly, if at all. This different behaviour of the various classes of alcohols is well known in the formation of esters with fatty acids.

Hydrochloric acid can be replaced by phosphoric, nitric or sulphuric acids; the last two render part of the insulin insoluble, but the final precipitation point of the soluble fraction is slowly changed towards the alkaline side.

Table IX shows the inactivation produced by different acids and ethyl alcohol. Whilst the activity of the original material is hardly changed by acetic or phosphoric acid, it is reduced to 10.9 units per mg. by hydrochloric acid under the same conditions. Thus, strong acids cause a more rapid change than weak ones.

Table IX.

Original potency: 17.9 units per mg. Temperature: 20°.			
Hydrochloric acid	0.75 <i>N</i> 75 %	Time (hrs.)	Units per mg.
Phosphoric acid	0.75 <i>N</i> 75 %	7	10.9
	(calculated as tri-basic acid)	7	17
	4.0 <i>N</i> 75 %	19	<2
Acetic acid	0.75 <i>N</i> 75 %	7	17.2

3.8 *N* 75 % alcoholic phosphoric acid changed the p_H of the final precipitation point to 5.7 in 24 hours and 6.5 in 46 hours; lower concentrations of acid had only a slight effect. 2.5 *N* 75 % alcoholic sulphuric acid changed the p_H to 5.8 in 3 hours and 6.9 in 26 hours; lower concentrations were much less effective. 0.75 *N* 75 % alcoholic nitric acid changed the p_H to 6.0 in 23 hours. On the other hand, neither 0.8 *N* nor 3.2 *N* acetic acid with 75 % alcohol had any effect on the final precipitation point in 3½–4 days at 20°.

Ethyl alcohol, however, can be replaced by methyl alcohol; thus, 3.8 *N* 75 % methyl alcoholic phosphoric acid changed the p_H to 6.5 in 20 hours, the reaction proceeding more rapidly than when ethyl alcohol was used.

Preliminary experiments on the reactivation of some of these inactive compounds have indicated that alkali causes a recovery of potency and shifts the final precipitation point back towards p_H 5.0.

The effects of a number of other compounds and hydrochloric acid upon the final precipitation point of insulin have also been investigated without the discovery of any which produced the shift towards the alkaline side. Thus no change in 4 days was caused by the following in 2.5 *N* hydrochloric acid: ketones (diethyl, methylethyl, methylisopropyl), mesityl oxide, cresol, phenol, glycollic acid and acetophenone (the last four required the addition of acetone to bring the insulin into solution). It appears therefore that the change only occurs in the presence of an alcohol.

DISCUSSION.

It has been shown that insulin can be inactivated by treatment with ethyl alcohol and hydrochloric acid, and that the original potency can be restored by action of dilute sodium hydroxide. It may be pointed out that the conditions used for reactivation are very similar to those determined independently by Freudenberg and Dirscherl, and Jensen and Geiling for insulin inactivated by acetic anhydride. The former used 0.03 *N* alkali at 1–3° for 24 hours, and the latter 0.01 *N* at 0° for 15 hours, since they found that 0.03 *N* sodium hydroxide destroys 30 % of the potency of crystalline insulin under these conditions. We worked with non-crystalline insulin, and found

that 0.042 *N* sodium hydroxide gave maximal reactivation. A preliminary experiment with crystalline insulin, kindly given to us by Dr D. A. Scott, gave evidence of inactivation and subsequent reactivation, but neither, using our standard conditions, appeared to be complete.

From the fact that inactivation is produced by treatment with hydrochloric acid, and primary or secondary alcohols, but not by ethyl alcohol and acetic acid, whilst the accompanying change in the final precipitation point has been observed also in the case of other strong acids in the presence of ethyl alcohol, but not after treatment with hydrochloric acid and a ketone, phenol, glycollic acid, etc., we are of the opinion that the loss of potency is accompanied by esterification. The conditions used for this and for the reactivation, however, are more gentle than those generally regarded as suitable for the synthesis and hydrolysis of an ester; for this reason their observation may be of particular chemical interest. The fact that after partial inactivation the compounds may show different potencies for similar final precipitation points as in the case of the glycerol and butyl compounds, is also in favour of the change being an esterification. The recovery of the alcohol after hydrolysis of the compound by alkali would be evidence in favour of esterification. Inactivated insulin which had been kept *in vacuo* over sulphuric acid for 5 months showed the presence of a volatile substance oxidisable by potassium dichromate and sulphuric acid, to the extent of 4.1 %, calculated as alcohol. Untreated insulin contained 0.8 %. These figures are suggestive, but there is the possibility of adsorbed alcohol in the insulin.

The inference appears justified that the activity of insulin is dependent on one or more free carboxyl groups.

SUMMARY.

1. Insulin can be completely inactivated by treatment with ethyl alcohol and hydrochloric acid.
2. At the same time the "final precipitation point" shifts towards the alkaline side. Such a change is characteristic of insulin inactivated by this method.
3. Complete recovery of potency and of the original final precipitation point is obtained by treatment with dilute sodium hydroxide.
4. Inactivation and change in final precipitation point can be effected by treatment with mixtures of other strong acids with primary or secondary alcohols.
5. It is suggested that the change undergone by insulin on inactivation is an esterification.

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