CXIX. ELECTROMETRIC TITRATION OF INSULIN. PREPARATION AND PROPERTIES OF IODINATED INSULIN.

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THE work to be described in the present paper represents an attempt to obtain accurate data concerning the acid- and base-binding powers of pure insulin by the method of electrometric titration. The experiments have been extended to a study of the physico-chemical and physiological properties of an iodinated product obtained from crystalline insulin by a method which has been used successfully with other proteins [cf. Neuberger, 1934, 2] and which appears to be limited in its effect to substitution of the tyrosine groups.

The material used in the investigation was a sample of crystalline insulin kindly given to us by Dr D. A. Scott to whom we wish to express our best thanks.

Existing data bearing on acid- and base-binding powers of insulin.

It is unfortunate that, for the obvious reason of the expense of the material, no complete analysis of the constituent amino-acids of insulin has been made which is sufficiently accurate to allow a close estimation of its acid- and base-binding groups.

Recent determinations of the basic amino-acids of insulin by the Van Slyke distribution method [Jensen and Wintersteiner, 1932, 2; Fisher and Scott, 1934] have led to widely divergent results for the individual bases, although the sums of the total basic amino-acids found by the two groups of workers are not greatly different. Assuming a molecular weight of 35,100 for insulin [Sjögren and Svedberg, 1931] the number of basic groups per molecule (including one terminal α -amino-group) is 30 according to the results of Jensen and Wintersteiner [1932, 2] and 22.5 according to those of Fisher and Scott [1934].

No satisfactory information is available concerning the dicarboxylic amino-acid content of insulin, the only experiments bearing on this point being the qualitative isolation of glutamic acid and failure to obtain aspartic acid from the products of hydrolysis of insulin recorded by Jensen and Wintersteiner [1932, 1]. The value of the isoelectric point of insulin however suggests that the number of free carboxyl groups in the molecule must be at least equal to that of the basic groups.

The figures for the tyrosine content of insulin, viz. 12-12.5% [Du Vigneaud *et al.*, 1928; Gerlough and Bates, 1932], rest on a more secure foundation since accurate and specific colorimetric methods are available for the determination of this amino-acid. The value of 12.5% tyrosine, which has been used in the present work, corresponds to 24 tyrosine groups per molecule of insulin; combination of this figure with the approximations deduced above for the basic and carboxyl groups leads to the conclusion that the total base-binding capacity of insulin (tyrosine + carboxyl groups) should be >46 groups per molecule.

Isoelectric point of insulin.

The experiments of Howitt and Prideaux [1932] and those of Wintersteiner and Abramson [1933] suggest that insulin in the amorphous condition or adsorbed on quartz is isoelectric at p_H 5.30–5.35. The optimum p_H for crystallisation of insulin however varies in different buffer solutions from 5.8 to 6.3 as shown by Scott and Fisher [1935]. The latter authors indeed incline to the opinion that the zinc which they find to be an essential constituent of crystalline insulin is bound in salt-like combination with free carboxyl groups.

This view receives some support from the X-ray measurements of Crowfoot [1935] but is rendered of doubtful validity by the following considerations.

(1) Electrophoresis experiments indicate that insulin crystals are isoelectric about p_H 5.0 [Wintersteiner and Abramson, 1933].

(2) The acid-binding capacity of crystalline insulin determined in the present work does not differ greatly from that found for ash-free amorphous insulin by Harvey *et al.* [1934].

(3) If insulin crystals are suspended in CO_2 -free water the solution acquires p_H about 5.3. (The evidential value of this observation is diminished by the very low solubility of insulin at its isoelectric point.)

In the present paper calculations have been made on the assumption that crystalline insulin does not differ in its acid- and base-binding capacities from the pure ash-free protein; the results are therefore subject to future re-calculation if evidence should appear that crystalline insulin is in fact a zinc salt.

Iodination of insulin.

Blatherwick *et al.* [1927] and Jensen *et al.* [1932] have shown that iodine in dilute aqueous solution in the p_H range 6.8–8.0 causes irreversible inactivation of insulin; this inactivation was ascribed by the latter authors to oxidation of disulphide groups. It is possible however to select conditions for the action of iodine on a protein under which the tendency for oxidation is greatly diminished and that for substitution increased.

It was found in the case of zein [Neuberger, 1934, 2] that iodination under such conditions led, so far as could be judged from analytical data, exclusively to the iodination of the tyrosine groups. The same appears to hold good for insulin which takes up an amount of iodine which is almost exactly in agreement with the figure calculated from its tyrosine content.

The titration and the physiological properties of this product, which present points of some interest, are described below.

EXPERIMENTAL.

Titration vessel.

In order to conserve the valuable material it was necessary to devise a method for continuous titration suitable for use with protein solutions having a marked tendency to froth. For this purpose a cell was constructed as shown in Fig. 1 which is self-explanatory.

During the titration purified hydrogen, saturated at the thermostat temperature with the appropriate solvent, was passed through the cell which was rocked in the thermostat with a frequency of about 30 complete rocks per min.; condensation in the connecting tubes was prevented by juxtaposition of a lamp. The rocking device was constructed from Meccano parts and was electrically driven.

The reference electrode was a saturated calomel cell which dipped into a vessel containing saturated KCl (also immersed in the thermostat); junction with the titration vessel was effected by a saturated KCl-agar bridge, the end of which dipped into and moved freely in the saturated KCl vessel.

Fig. 1. Titration vessel; $\frac{1}{3}$ actual size. *A*, inlet for hydrogen; *B*, opening for hydrogen electrode, the height of which is adjusted so that the electrode is alternately covered by the solution and exposed; *C*, opening for agar bridge, the end of which is continuously below the surface of the solution; *D*, opening for burette; *E*, outlet for hydrogen.

This size of vessel is suitable for 10–15 ml. of solution.

Titration were carried out at $25^{\circ} \pm 0.05^{\circ}$. The protein was introduced into the cell in the dry state, the solvent being added afterwards. Titrant was added from a microburette in such a manner that the drops fell directly into the solution. Gold-plated platinum electrodes were used and were only introduced into the cell after all the protein had dissolved.

Steady and reproducible potentials were readily obtained with this technique, and no difficulty was experienced from frothing of the solution. Crystalline ovalbumin, when titrated in this apparatus, gave a titration curve in excellent agreement with that determined by Kekwick and Cannan [1936].

Titration of insulin and iodinated insulin.

Over the range p_H 4.3–7.8 insulin is so insoluble in water that it cannot be titrated accurately, whilst in 80 % alcohol the zone of low solubility is even more extended. No attempt was made to determine potentials except when the solution was perfectly clear.

Iodinated insulin was titrated on the alkaline side only, since in this case the object was to determine the shift in the curve produced by the alteration in the dissociation constant of the phenolic groups.

Iodination of insulin.

Two preparations of iodinated insulin were made by the following slightly different methods.

(a) Crystalline insulin (200 mg.) was dissolved in a mixture of purified methyl alcohol (10 ml.) and aqueous ammonia (2 ml.; sp. gr. 0.880); the solution was cooled in an ice-salt mixture and treated gradually with iodine in potassium

iodide (2.8*N*) in slight excess over the amount calculated for iodination of the tyrosine contained in the insulin; 0.2 ml. of the iodine solution was used in all. About 45 min. after the last addition of iodine the solution, which was still pale yellow, was centrifuged and the clear supernatant liquid treated in the cold with glacial acetic acid (2 ml.). The clear neutralised solution was diluted with water (about 3 vols.), kept in the cooling mixture for an hour and centrifuged; the p_H of the mother-liquor was 4.5.

The precipitate was warmed to 55° with 15 ml. of 5% acetic acid and the mixture centrifuged; separation was bad but a good flocculation was obtained by adding 5 ml. of 0.1 *N* NaOH to the mother-liquor; the mixture was again centrifuged. (The supernatant fluid, which had p_H 3.2, was adjusted to p_H 5.6; this produced a slight precipitate which was probably a trace of unchanged insulin and was rejected.)

The precipitate was suspended in water and again centrifuged, this process being repeated twice, and was finally dried in a vacuum desiccator; the aqueous washings (p_H 3.9–4.4) were slightly cloudy and a further small flocculation was obtained by adding to them sufficient acetic acid-sodium acetate buffer at p_H 4.5 to make a final concentration of *M*/50; this second precipitate was collected and dried as above.

The total yield was 180 mg. (76% of the theoretical) of a cream-coloured powder. The product gave no Millon reaction but gave on the other hand a strong reaction for the *o*-diiodophenolic grouping with nitrous acid and ammonia. It contained 1.42% H_2O (loss of weight on drying at 120°) and 1.98% of ash; on an ash- and water-free basis the iodine content was 15.4% (calculated on the assumption that the original insulin contained 12.5% of tyrosine: I, 15.4%).

The product differed from insulin in being readily soluble in buffer solutions of p_H 6.8 or over. On the acid side its solubility was profoundly influenced by the presence of salt. Solution was readily obtained below p_H 3.0 in the almost complete absence of salt, but even at p_H 1.5 addition of quite small amounts of sodium chloride caused quantitative precipitation.

Attempts to crystallise the material by methods which have been successfully employed with insulin have failed, but it must be remembered that the amount at our disposal has been so small as to preclude any extended exploration of suitable conditions.

(*b*) Crystalline insulin (200 mg.) was dissolved in aqueous ammonia (2 ml.; sp. gr. 0.880) and the solution cooled in an ice-salt mixture. Iodination was effected as above with 0.2 ml. of 2.8*N* iodine in potassium iodide. In this case the addition of each drop of iodine solution caused precipitation of nitrogen iodide which rapidly disappeared on stirring. After about half the iodine had been added a white precipitate began to separate and at the end of the addition the mixture had become a stiff jelly. There was no apparent residual excess of iodine.

Addition of water gave a clear solution which was neutralised with glacial acetic acid (2 ml.). The resulting precipitate was collected at the centrifuge (p_H of mother-liquor 5.4) and re-suspended in water; *N* HCl was added to p_H 3.0 when, on warming to 45°, an almost clear solution was obtained; readjustment of the reaction to p_H 4.5 caused sharp flocculation. The precipitate was again collected, and dissolved in water by the addition of *N* HCl to p_H 2.0; the clear solution was treated with a few drops of saturated aqueous NaCl and centrifuged. The supernatant fluid gave no reaction for protein with salicylsulphonic acid; the precipitate was washed at the centrifuge with 0.35% NaCl and dried in a vacuum desiccator.

The yield was 210 mg. (90 % of the theoretical) of a product identical in appearance and behaviour with that described under (a) above. As might be expected from its mode of separation this preparation (b) contained higher proportions of water (4.0 %) and ash (5.8 %); on a water- and ash-free basis however it again contained 15.4 % I and had 11.7 % N, the latter figure being in good agreement with that calculated from the nitrogen content of the original insulin.

DISCUSSION OF RESULTS.

Acid- and base-binding powers of insulin.

It is not easy to arrive at accurate values for the "maximum" acid- and base-binding capacities of proteins, since most of the latter contain groups which dissociate in very acid or alkaline ranges, under which conditions liquid junction potentials cannot be entirely eliminated. A more serious source of error is the impossibility of making valid blank corrections owing to the fact that the theory of strong electrolytes cannot as yet be applied to multivalent colloidal electrolytes and hence that the activity of hydrogen ions in solutions containing such compounds cannot be evaluated. Furthermore the protein itself may dissociate into smaller molecules at high acidity or alkalinity with resultant liberation of free acidic or basic groups.

Titration in aqueous solution recorded in the present paper have been confined to the range p_H 2.5–11.5 over which the blank correction remains reasonably small and liquid junction potentials can be neglected. The blank correction has been calculated on the assumption that the activities of H^+ and OH^- are equal to their concentrations; this assumption is certainly incorrect and probably leads to values for acid and base bound which are too high; there exists however no sound theoretical basis for any other mode of calculation. For p_{KW} the value of 13.98 has been adopted [Bjerrum and Unmack, 1929].

The results given in Table I and Fig. 2 show that there is no evidence of maximum acid-binding at p_K 2.5. The possibility of including the extreme limits of acid- and base-binding in the titration is greater if the latter be performed in 80 % alcohol owing to the effect of the lower dielectric constant in depressing the dissociation of the groups concerned [Neuberger, 1934, 1]; p_H 3.0¹ in 80 % alcohol, at which the error introduced by the blank correction is still quite small, corresponds to p_H about 1.5 in water. Examination of this part of the titration curve of insulin in 80 % alcoholic solution (Fig. 2) indicates a value of 43 ± 2 groups per molecule as an approximate estimate of the maximum acid-binding capacity of the protein.

Turning to the question of the base-binding power of insulin we find a less satisfactory situation. Owing probably to the fact that for reasons already given no activity correction could be made, the base-binding at p_H 11.5 seems unreasonably high in comparison with the base-binding calculated from analytical data (*v. supra*). Titration in alcoholic solution does not help greatly in the alkaline range. Whereas in the acid range the titration curve in alcoholic solution lies about 1.3 p_H units above that in aqueous solution in accordance with expectation [Neuberger, 1934, 1, 2] in the alkaline range the shift of the alcoholic titration curve varies from a small amount at moderate alkalinity to a large amount at high alkalinity; this deviation is due to the fact that in the range under discussion two sets of groups are dissociating, namely basic groups (*e.g.*

¹ The term p_H in so far as it applies to alcoholic solutions is used in the conventional sense indicated by Neuberger [1934, 1].

Table I. *Titration of insulin.*

A. With acid. 75 mg. insulin; 11 ml. water. Temp. 25°.

ml. 0.1 N HCl	p_H	ml. 0.1 N HCl corrected	Mol. $\times 10^5$ H ⁺ bound per g. protein	Mol. $\times 10^5$ H ⁺ bound per g. mol. protein
0.56	3.368	0.510	71.0	25.0
0.60	3.286	0.540	75.0	26.5
0.66	3.159	0.580	81.0	28.4
0.74	3.000	0.623	87.0	30.5
0.82	2.858	0.654	91.5	32.0
0.88	2.750	0.670	93.5	32.9
0.95	2.668	0.694	97.0	34.0
1.00	2.607	0.710	99.3	34.8
1.10	2.506	0.723	101.0	35.5

Reversed titration.

ml. 0.1124 N NaOH	p_H	ml. 0.1 N HCl corrected	Mol. $\times 10^5$ H ⁺ bound per g. protein	Mol. $\times 10^5$ H ⁺ bound per g. mol. protein
0.21	2.806	0.622	87.0	30.5
0.39	3.091	0.560	78.3	27.5
0.50	3.362	0.483	67.5	23.7
0.60	3.689	0.400	56.0	19.6
0.65	3.870	0.353	49.3	17.3
0.70	4.059	0.304	42.5	14.9
0.75	4.241	0.250	35.0	12.3

B. With alkali. 75 mg. insulin; 12 ml. water. Temp. 25°.

ml. 0.1124 N NaOH	p_H	ml. 0.1 N NaOH corrected	Mol. $\times 10^5$ H ⁺ bound per g. protein	Mol. $\times 10^5$ H ⁺ bound per g. mol. protein
1.5	11.342	1.370	191.5	67.2
1.6	11.392	1.423	199.0	69.8
1.8	11.487	1.570	219.5	77.0

Reversed titration.

ml. 0.1 N HCl	p_H	ml. 0.1 N NaOH corrected	Mol. $\times 10^5$ H ⁺ bound per g. protein	Mol. $\times 10^5$ H ⁺ bound per g. mol. protein
0.2	11.419	1.435	200.6	70.4
0.3	11.351	1.390	194.0	68.2
0.4	11.291	1.330	186.0	65.3
0.5	11.232	1.264	177.0	62.0
0.6	11.167	1.200	168.0	58.9
0.7	11.044	1.152	161.0	56.5
0.8	10.963	1.082	151.0	53.1
0.9	10.826	1.020	143.0	50.0
1.0	10.709	0.940	131.0	46.1
1.1	10.506	0.870	122.0	42.7
1.2	10.267	0.790	111.0	38.8
1.3	9.952	0.710	99.0	34.8
1.4	9.692	0.620	88.0	30.4
1.5	9.173	0.520	73.0	25.5
1.6	8.337	0.420	59.0	20.2
1.7	7.967	0.320	45.0	15.9

guanidino-groups) which are little affected and phenolic groups which are considerably affected by change of solvent.

Between the isoelectric point and p_H 8.5 the base-binding amounts to 21–22 groups per mol. of protein; from p_H 8.5 to 9.5 there is little buffering, as is indeed the case with most proteins. As to the total base-binding between the isoelectric point and p_H 11.5 it is only possible to say that this approximates to 60–70 groups per mol. of protein; from this and from the knowledge of the number of tyrosine groups present it may be calculated that the insulin molecule contains at least

30–35 free carboxyl groups. The protein is indeed remarkable as a whole for the very large number of ionisable groups which it possesses.

It is apparent from Table I and Fig. 2 that reversed titrations gave results in satisfactory agreement with those of the direct titrations in spite of the small change which must have occurred in the ionic strength of the solution; this indicates that no irreversible change was produced in the protein by exposure to high degrees of acidity and alkalinity, a supposition which was confirmed in so far as the aqueous titrations were concerned by the recovery of 75% of the starting material in crystalline form; the simple recrystallisation of insulin gives

Fig. 2. Insulin.

a yield of the same order [Scott, 1934]. The possibility of reversible dissociation at high alkalinity cannot however be excluded; such dissociation may indeed contribute to the abnormally high figure for the total base-binding.

An electrometric titration of insulin has been reported by Harvey *et al.* [1934]. It is difficult to compare the results of these authors with those obtained in the present communication since an amorphous preparation of insulin was used and no details of the technique of titration are given. Nevertheless the value obtained by them for the acid-binding power of insulin in aqueous solution is in agreement with that determined by us.

Iodinated insulin.

Analyses by earlier workers indicate the presence of 23–24 tyrosine groups in the molecule of insulin; the iodine content of our fully iodinated product corresponds with 24 tyrosine groups per mol. on the assumption that the iodine

has entered exclusively the 3:5-positions of the tyrosine residues. The titration curve of iodinated insulin over the range p_H 6.6–11.5 is shown in Fig. 3 in which is included, for purposes of comparison, the titration curve of insulin over part of the same range. The shift in the titration curve owing to the diminution of the p_K of the phenolic groups in the iodinated product can be clearly seen, as can also the fact that the total base-binding of the protein is unaffected by iodination.

Fig. 3. Iodinated insulin.

The curve on the left of Fig. 3 has been obtained by plotting the difference of the number of groups titrated in insulin and iodinated insulin against p_H . The maximum value of this difference is 20 and is therefore somewhat smaller than that calculated from analytical data (23–24; *v. supra*); this slight discrepancy is partly to be explained by the extended range over which the phenolic groups of insulin dissociate and the consequent overlap of the titration curves of insulin itself and of the iodinated product. Such a discrepancy would also be caused by error in the isoelectric point (p_H 5.5) assumed for iodinated insulin; that no great error of this kind was made is shown by the identity of the total base-binding capacities of iodinated insulin and insulin itself.

The general conclusion that the phenolic groups of insulin are free and that these groups alone are affected by iodination under the conditions employed in this work is supported by the results of the titration; it is further reinforced by the extended range of solubility of iodinated insulin in the neighbourhood of p_H 7.0 which has already been mentioned in the description of its properties and which is shown graphically in Fig. 3.

Effect of iodination on physiological properties of insulin.

Since chemical evidence favours the view that iodination under the conditions which we have employed induces no change in the molecule of insulin other than substitution of the tyrosine residues it became of interest to examine the physiological properties of the iodinated product.

For co-operation in this part of the work we are greatly indebted to Mr H. P. Marks, whose results may be summarised as follows.

Mouse tests. The iodinated product caused no convulsions in mice in doses as high as the equivalent of 1/5 unit of the original crystalline insulin, whether given subcutaneously or intravenously; a dose equivalent to 1/4 unit of the original material injected intravenously caused convulsions in 12/20 mice. This would correspond to an activity about 15% of that of the original insulin.

Rabbit tests. The iodinated insulin had very little hypoglycaemic action in rabbits when given by subcutaneous injection; thus in one case iodinated insulin equivalent to 10 units of original insulin gave less effect than 1/2 unit of the latter. After intravenous injection on the other hand the same dose (10 units) of iodinated insulin lowered the blood sugar to an extent of the same order as the lowering produced by 1 unit of insulin.

The general conclusion to be drawn from these results is that the iodinated product retains 5–10% of the physiological activity of the original insulin.

Removal of iodine from iodinated insulin.

It is evident that whilst the results recorded above leave no doubt that the physiological activity of insulin has been greatly diminished by iodination they leave much to be desired from the point of view of quantitative accuracy, since there is a discrepancy between the effects of the iodinated product when given by the subcutaneous and oral routes, which makes its comparison with insulin difficult. Thus no convulsions were produced in mice with any subcutaneous dose of iodinated insulin although an intravenous dose was effective; this is in direct contrast with the greater efficacy of subcutaneous than intravenous injections of insulin itself. Again in rabbits the activity of iodinated insulin appeared to be about 2.5% of that of insulin by the subcutaneous test and 10% by the intravenous test.

The relatively lower effect of subcutaneously injected iodinated insulin suggests that its absorption may be delayed in comparison with that of insulin. In this connection the possibility was considered that the residual activity of the iodinated product might be due to the persistence of a small amount of unchanged insulin, the absorption of which was delayed by its association with a preponderance of totally inactivated material; such a possibility was however practically excluded by the observation that insulin could exercise its normal effect in mice when administered subcutaneously in admixture with the iodinated material.

A doubt nevertheless remained whether the loss of activity on iodination represented total inactivation of 90% of the insulin, the remainder being unchanged or 90% inactivation of all the insulin. In order to gain further information on this point attempts were made to remove the iodine from the iodinated product in the hope that restoration of physiological activity might be obtained. The experiments about to be described will show that this hope has been partly realised.

It will be apparent that the major difficulty to be faced in these experiments was the search for a method of removing iodine which would not at the same

time cause physiological inactivation through the induction of other changes in the molecule. Insulin is well known to be irreversibly inactivated by treatment with reducing agents even under the mildest condition, such inactivation being accompanied by exposure of sulphhydryl groups. The only method which offered any prospect of success therefore appeared to be that of catalytic reduction which was unlikely to attack the disulphide linkages in the protein.

Catalytic dehalogenation of organic compounds is usually effected in strongly alkaline solution with palladium deposited on calcium carbonate or barium sulphate; in this case the use of strong alkali was excluded for obvious reasons and it was therefore decided to use 50% aqueous pyridine as solvent, a preliminary experiment having shown that diiodotyrosine could be satisfactorily deiodinated in this medium.

Iodinated insulin therefore (100 mg.) was dissolved in a mixture of equal parts of water and pure redistilled pyridine (10 ml.) and the mixture shaken in an atmosphere of hydrogen in presence of palladium-barium sulphate catalyst (1 g.). Uptake of hydrogen ceased after about 4 hours; the amount absorbed by the catalyst itself was so much greater than that taken up by the protein that the latter quantity could not be determined with any accuracy. The catalyst was filtered off and the solution evaporated under diminished pressure to remove pyridine; adjustment of the reaction to p_H 5.5 caused flocculation of the protein, which was separated on the centrifuge, redissolved in aqueous pyridine and subjected to further hydrogenation with a fresh lot of catalyst. The whole process was then repeated a third time.

The combined mother-liquors (which showed positive reactions for iodide of decreasing intensity) were freed from pyridine by distillation under diminished pressure after addition of potassium hydroxide; the iodide was then determined by oxidation with bromine followed by titration with thiosulphate and was found to correspond to 10.4 mg. of iodine or approximately 70% of that in the starting material.

The protein was dissolved in water acidulated with hydrochloric acid and reprecipitated at p_H 6.2 from a phosphate buffer (total volume 50 ml.) under the conditions described by Scott [1934] for the crystallisation of insulin. No crystallisation occurred but a flocculent precipitate slowly separated which, after keeping at 0° overnight, was collected and dried. The yield was 35 mg. of a product which gave a strong Millon reaction and only a faint reaction for the *o*-diiodophenolic grouping. It contained 4.2% of iodine, from which it may be concluded that approximately 2/3 of the phenolic groups had been restored to their original condition. The nitroprusside reaction for sulphhydryl groups was entirely negative.

Since the amount of material available was so small it was decided to subject the partly de-iodinated product to physiological test rather than to attempt more complete removal of iodine.

Fig. 4, which is based on tests kindly carried out for us by Dr H. P. Himsworth, shows in a striking manner the very considerable degree of re-activation which accompanies the removal of iodine. The upper curve represents the effect on the blood-sugar of a rabbit of the intravenous injection of 1/50 mg. of fully iodinated insulin and the lower curve the effect of the same dose of the partly deiodinated product. From the lower curve it may be deduced that the latter product possessed at least 50% of the activity of crystalline insulin. A test on mice carried out by Mr Marks showed that the deiodinated product produced convulsions in mice when given subcutaneously in the ordinary way and indicated an activity in the neighbourhood of 30% of that of insulin.

In view of the residual iodine content of the final product an activity of about 60 % of that of crystalline insulin was the maximum to be expected. The results of the rabbit test (Fig. 4) are in accordance with this expectation; those of the mouse test are somewhat lower. It is however clearly established that deiodination has effected a re-activation which is roughly proportional to the amount of iodine removed and therefore to the restoration of the original phenolic groups.

Fig. 4. The curves represent the effects of intravenous injection of 1/50 mg. of the respective products into a rabbit.

It seems reasonable then to conclude that the phenolic groups of insulin are of importance in relation to its physiological activity, since this activity can be almost completely abolished by loading the phenolic groups with iodine and can be restored in approximate proportion as the iodine is removed. We are inclined to believe that the residual activity of the fully iodinated product is intrinsic to this product and is not to be ascribed to the persistence of unchanged insulin although we are unable to offer incontrovertible proof of this point.

It may be pointed out that our conclusion is in accordance with the observations of Jensen and Geiling [1928] and of Freudenberg and Eyer [1932-33] on the partial re-activation of acetylated insulin; the treatment of the acetylated product with dilute alkali adopted by these workers is such as to hydrolyse *O*-acetyl but not *N*-acetyl groups, and the partial reactivation obtained is almost certainly associated with regeneration of free phenolic groups.

SUMMARY.

1. Crystalline insulin has been titrated electrometrically, in both aqueous and 80 % alcoholic solutions, in a specially designed apparatus.
2. From the results it is deduced that insulin has an acid-binding capacity of 43 ± 2 groups per molecule and a base-binding capacity in the neighbourhood of 60-70 groups per molecule.
3. The interpretation of these results in relation to existing analytical data concerning insulin is discussed.
4. Insulin has been iodinated; evidence is presented that the iodinated product differs from insulin only in that the tyrosine groups are substituted with iodine in the 3:5-positions.

5. It is shown that iodinated insulin retains only 5–10 % of the physiological activity of the parent substance, but that partial removal of iodine by catalytic reduction is accompanied by approximately proportional restoration of activity.

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