LXXIX. THE PURIFICATION AND PROPERTIES OF INSULIN.

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It is a matter of common experience that, whilst the purification of crude insulin to a degree of activity represented by a unit of approximately 0.1 mg. presents few difficulties, attempts to exceed this to any great extent usually result in a considerable loss. The methods employed for this purpose may be grouped into two main classes: (a) precipitation at the isoelectric point, and (b) the use of protein precipitants.

With the former method it is usual to find that after repeated precipitation the activity is not further increased, moreover, considerable losses accompany each precipitation. This fact is frequently referred to by various workers, *e.g.* Shonle and Waldo [1924], and although in some cases material has been prepared which greatly exceeds the degree of activity represented by the unit mentioned above, such methods of purification are not ideal for the preparation of insulin in quantities sufficient either for clinical use or for experimental purposes, owing to the small fraction of the original number of units which is recovered in the final product after several precipitations.

The use of the second group of methods, whilst it does not generally involve the loss of active material which accompanies isoelectric precipitation, results in little purification beyond the limits referred to above. Moreover, it is frequently impossible to purify by this means certain specimens of insulin, notably those prepared from pancreas in which, owing to insufficient freezing, tryptic action has occurred, so as to obtain material the unit of which is below 1 mg. or even more. The following investigations were undertaken with a view to determining the reasons underlying the failure by these methods.

In order to characterise the group of protein fission products to which insulin belongs it was thought that the application of the recently described method of Wasteney and Borsook [1924] would prove to be of value. These workers showed that if trichloroacetic acid were added to a solution containing a mixture of proteins and their disintegration products in sufficient quantity to make up the concentration in the solution to 2 %, then proteins and meta-proteins alone were precipitated. On applying this method to insulin solutions, it was found that the whole of the potency was precipitated under these con-

ditions. The supernatant fluid from this precipitation was always found to give a further precipitate on the addition of picric acid, even when insulin of considerable purity was used. In order to test the effect of this 2 % trichloroacetic acid precipitation quantitatively it was necessary to devise some method whereby the insulin could be recovered from the trichloroacetic acid precipitate, and it was found that if this were dissolved in dilute acid, the activity was completely precipitated by the addition of sodium chloride. This precipitate was free from trichloroacetic acid, and could easily be converted into the hydrochloride.

In this way, a quantitative recovery of the activity results, together with considerable purification. If a specimen of insulin which has been through this process is subjected to treatment with 2 % trichloroacetic acid again, it is found that the whole is precipitated and that the supernatant fluid yields at most a trace of picrate, and that therefore nothing is to be gained by repeating this treatment. This experiment would show, according to Wasteneys and Borsook, that the active fraction of insulin must be associated with the groups of protein or metaprotein, but the application of these views to the question of the constitution of insulin is discussed later. It is clear, however, that the greater part of the material precipitated by 2 % trichloroacetic acid must consist of proteins or metaproteins, and this suggested that physico-chemical means might be applied to the problem of further purification.

Hardy [1905] showed that at or near their isoelectric point globulins can be dissolved by neutral salt solutions. More recently Cohn [1924] has shown that the degree of such solubility is a characteristic property of each individual protein, and it was thought that the application of this principle to insulin might yield valuable results. Accordingly an isoelectric precipitate of insulin was washed with solutions of potassium chloride, both the material dissolved and the insoluble residue being converted to hydrochloride. Tests upon rabbits demonstrated that the active fraction is relatively insoluble in dilute salt solution, whereas some of the inactive portion dissolves more readily; thus purification has been effected.

Hardy also showed that bivalent and trivalent anions are capable of holding in solution more globulin than are univalent anions. After some preliminary trials it was decided to investigate the action of oxalates and citrates upon insulin, but it was clear that an experiment similar to the one quoted above with sodium chloride could not be performed, because these salts are in themselves weak buffers, the solutions having a p_H of 7 or over, and if the isoelectric precipitate of insulin is washed with a solution of potassium oxalate, then the precipitate dissolves, owing to the fact that the p_H rises above that of the isoelectric point.

The effect of adding a $N/8$ solution of potassium oxalate to a solution of insulin hydrochloride (which is strongly acid) was then observed. On adding such a solution drop by drop it was found that a precipitate began to appear when the p_H had reached about 3.7, and reached a maximum at about p_H 4,

and then the solution began to clear as the p_H rose. The formation of a maximum precipitate between p_H 3.7 and 4.2, instead of at the normal isoelectric point (p_H 4.5–5.0 with this particular material), appeared to be capable of explanation by the work of Michaelis and Szent-Györgyi [1920], who showed that the isoelectric point of certain proteins can be definitely lowered by salts. The experiments described confirm this, in the case of insulin, and the lowering of the isoelectric point is shown to depend upon the concentration of oxalate present. Great purification was obtained in this manner with little or no loss of activity.

In the light of the foregoing experiments it appears that the following explanation may be advanced to account for this phenomenon. The addition of a weak buffer of potassium oxalate slowly raises the p_H of the solution and at the same time lowers the isoelectric point of the insulin until a precipitate is produced. The potassium oxalate holds in solution inactive fractions which will be precipitated during the course of an ordinary isoelectric precipitation.

This type of method has certain objections both from a practical and a theoretical aspect. In the first place, it is difficult to determine the end-point of the titration except by centrifugation after each small addition of oxalate, and, secondly, since the amount of inactive material held in solution must vary with the quantity of oxalate present, it is often impossible to obtain a sufficiently high concentration of oxalate without raising the p_H to a level above the isoelectric point, thus dissolving some of the active portion. A method was therefore devised in which the insulin solution could be added to a buffer solution of suitable p_H value which contained sufficient oxalate to hold the relatively greater quantities of inactive material in solution. It was found that to produce a p_H of 4, the point at which maximum precipitation occurs in the above experiment, 100 cc. of potassium oxalate solution must be added to 20 cc. of oxalic acid solution. The p_H of the solution of insulin hydrochloride was adjusted to 4, and it was then run into two volumes of the above buffer solution with vigorous stirring. A precipitate was produced which was found to contain all the original potency in a much higher concentration, showing that a large amount of inactive material had been removed.

By combining the trichloroacetic acid precipitation, the acid and salt precipitation and the oxalate precipitation, a very efficient purification method was obtained. Similar results are obtained when sodium citrate is used in place of potassium oxalate. The details of these investigations are given in the experimental section.

In the above examples we have employed solutions of approximately p_H 4, since, for the particular specimens of insulin used (isoelectric point at or about p_H 5), this represents the optimum condition. We have observed, however, that many specimens of insulin hydrochloride possess an isoelectric point much higher than that recorded hitherto. This applies more especially to some specimens of material prepared by the acetone-picric acid process, the isoelectric point of which is frequently at p_H 5.9, or even higher. If such solutions

be brought to p_H 5.7 there is often no sign of precipitation. In this case, the oxalate mixture at p_H 4 is clearly too acidic and only a small fraction of the activity is precipitated on mixing the solutions at this hydrogen ion concentration. By first performing an isoelectric precipitation on a portion of the solution and determining the hydrogen ion concentration at maximum precipitation this difficulty can be avoided. In such cases the reactions of the oxalate solution and the insulin solution should be brought to a higher p_H value (approximately 5.0) before mixing, or the original method of running the solution of potassium oxalate into the solution of insulin hydrochloride to maximum precipitation may be employed.

EXPERIMENTAL.

The precipitation of insulin by trichloroacetic acid.

(a) *Effect of concentration.* Crude insulin hydrochloride (19.7 g.) was dissolved in water to give a 1.5 % solution. A 10 % solution of freshly distilled trichloroacetic acid was added drop by drop with constant stirring until the concentration of the acid was 1 %. There was no precipitation. A further measured quantity of trichloroacetic acid was added to bring the concentration up to 1.5 %. The precipitate which was obtained was allowed to stand for 2 hours and was then centrifuged as completely as possible. Although this operation was performed at a high speed, the supernatant fluid was still faintly turbid. It was decanted from the precipitate and trichloroacetic acid was added to it to bring the concentration up to 2 %. A further precipitation resulted, and after centrifugation the supernatant fluid was perfectly clear. The precipitates were dissolved in $N/10$ hydrochloric acid and, on the addition of half the volume of saturated sodium chloride solution to each, a precipitate formed. This was collected in the centrifuge, dissolved in water, and the material was obtained from the solution in the usual way by precipitation as picrate and conversion to the hydrochloride [Dudley and Starling, 1924]. The two fractions thus obtained weighed 1.86 and 2.84 g., and standardisation showed that they contained 1230 and 2840 units respectively. The initial material had 4500 units. This experiment shows that by means of the precipitation 15.0 g. of almost completely inactive material had been removed. It is also clear that, whereas a 1.5 % concentration of trichloroacetic acid is insufficient for complete precipitation of the activity, 2 % trichloroacetic acid causes almost the whole of the activity (90 % in this example) to be precipitated. The addition of further quantities of 10 % trichloroacetic acid brought about the precipitation of inactive material.

In the above example very crude insulin hydrochloride was used. Similar experiments with highly purified material have also shown that the activity is completely precipitated by 2 % trichloroacetic acid.

(b) *Fractional precipitation with trichloroacetic acid.* An experiment performed in a manner similar to that described above was carried out, using

concentrations of trichloroacetic acid of 2, 3 and 5 %. The precipitates in each case were converted to hydrochloride. The results are shown in the following table. The initial weight of material (34.1 g.) contained 34,000 units. Precipitate A below, the unit of which proved to be 0.44 mg., contained 31,000 units.

Of these fractions A is the only one which behaves as a typical protein or metaprotein, *e.g.* it is precipitated at the isoelectric point, completely precipitated by half saturation with ammonium sulphate and by copper sulphate. B gives only a very faint turbidity at the isoelectric point, whilst solutions of C and D remain perfectly clear, and all three yield a further precipitate on full saturation with ammonium sulphate and are not precipitated by copper sulphate. The precipitates of B, C and D, obtained on adding potassium ferrocyanide and acetic acid, clear on warming, indicating the presence of primary proteose in these fractions.

The distribution of amino- to total nitrogen in these fractions is shown in Table I. It will be seen that there is a gradual rise in the ratio of amino- to total nitrogen with each fraction.

Table I.

Trichloroacetic acid	Amino-N	Total N	Amino-N Total N
	%	%	%
(A) Up to 2 %	0.82	14.06	5.8
(B) 2 to 3 %	0.94	14.65	6.4
(C) 3 to 5 %	0.98	14.80	6.6
(D) Not precipitated by 5 % trichloroacetic acid	1.41	15.00	9.4

Solubility of the isoelectric precipitate in salt solutions.

(1) *Potassium chloride.* Qualitative experiments having shown that the inactive fractions, separated from insulin during the course of purification by various means, tend to dissolve much more readily when potassium and sodium chloride solutions are added to their isoelectric precipitates than does the active portion, the following roughly quantitative experiment was performed in order to estimate the partition of active and inactive material in solution and precipitate. Crude insulin hydrochloride (110 mg.) was isoelectrically precipitated by the addition of *N*/100 potassium hydroxide solution (6.5 cc.); the precipitate was collected in the centrifuge, the supernatant fluid

discarded, and the precipitate well stirred with $N/2$ potassium chloride solution. A portion dissolved and was recovered in the usual way from the supernatant fluid obtained after centrifugation. The precipitate was also converted into the picrate and hydrochloride. The undissolved portion weighed 16 mg. and contained 100 units. The solution yielded 21 mg. hydrochloride containing 30 units. The solubility of the isoelectric precipitate in water alone was shown to be negligible under the same conditions by a control experiment.

(2) *Potassium oxalate*. To a solution of insulin hydrochloride (4.00 g. = 34,000 units) in water (50 cc.) a $N/2$ solution of potassium oxalate was run in with stirring. The addition of 10.0 cc. produced the first permanent turbidity. The initial reaction of the solution of hydrochloride, as determined by the hydrogen electrode, was p_H 2.35, and after addition of 24.8 cc. of oxalate this had risen to p_H 3.46. Precipitation was now complete, and precipitate and supernatant fluid were separately converted to picrate and then to hydrochloride. The precipitate after washing and drying weighed 1.14 g. and contained 28,400 units. In this experiment considerable activity was contained also in the supernatant fluid which yielded 1.79 g. hydrochloride containing 3670 units. (In this case the portions insoluble in acid alcohol, during the preparation of the hydrochloride from the picrate, were rejected.)

This experiment shows that oxalate may be used in the same way as potassium chloride for dissolving a considerable portion of material of low activity. Repetition of the process gave a final product which could not be further precipitated by this means without considerable loss. In these later experiments better results were obtained by using more dilute solutions of hydrochloride and of oxalate. Finally, for reasons to be discussed later, $N/8$ potassium oxalate was added to a 1.5 % solution of the hydrochloride. In this way, after four more precipitations, 0.483 g. of hydrochloride was obtained, the unit of which, obtained according to the method laid down in the League of Nations Report [1926], proved to be 0.016 mg., a total recovery of 30,000 units after five precipitations in all. A loss of 10 % on each stage would thus have resulted in a final yield of only 60 %, whereas actually the recovery was almost quantitative. Isoelectric precipitation, carried out by adding $N/10$ sodium hydroxide to the hydrochloride in the usual way, resulted in a loss of approximately 25 % of the activity in each precipitate.

The above example shows that when used for purifying insulin which is already of the order of 0.1 mg. per unit this type of process gives satisfactory purification and yields. Even for this purpose, however, it is troublesome to determine the end-point when complete precipitation is reached, this necessitating repeated centrifugation and addition of further small quantities of oxalate. Moreover, when cruder specimens of insulin are used losses occur. In order to obtain precipitation under uniform conditions the solution was first adjusted by the addition of dilute alkali to p_H 4 ($\pm$ 0.05), experiments having shown that this is an average figure with this material for precipitation in the presence of oxalate. In the following experiment this solution contained 1.0 g.

of crude insulin (= 1250 units) in 25 cc. It was run into 50 cc. of a solution of potassium oxalate and oxalic acid containing five volumes of $N/8$ oxalate to one volume of $N/8$ oxalic acid. The p_H of this solution was 4.05. Although the hydrogen ion concentration remained constant, on running in the insulin solution a precipitate at once began to form. This was collected and converted to hydrochloride, when it weighed 0.264 g. and contained 1200 units. The supernatant fluid, converted to hydrochloride through the picrate, weighed 0.622 g., and the unit was considerably above 2 mg.

(3) *The concentration of oxalate.* These results now made it possible to investigate the optimum concentration of oxalate which should be used, the hydrogen ion concentration remaining constant at p_H 4, in order that a maximum quantity of inactive material might be held in solution, whilst the activity should be found in the precipitate. For this purpose, oxalic acid-potassium oxalate solutions were prepared such that all were at p_H 4 (± 0.05), the concentration of oxalate ions being respectively $N/4$, $N/6$, $N/8$ and $N/10$. A solution of crude insulin (3.75 g. = 4300 units) in 250 cc. water was adjusted to p_H 4.02. Equal portions of 60 cc. each were then run into 120 cc. of the above oxalate solutions. After standing for 2 hours, the mixtures were centrifuged, and the precipitates converted into hydrochloride. The results are shown in the following table:

Final dilution of oxalate	p_H after addition of insulin	Wt. of hydrochloride g.	Unit in mg.	Total units recovered %
$N/6$	3.99	0.174	0.17	100
$N/9$	4.00	0.148	0.14	100
$N/12$	3.99	0.113	0.11	100
$N/15$	3.98	0.062	0.15	40

From this it is clear that between $N/12$ and $N/15$ there is an abrupt transition, and that at the lower concentration more than half of the activity remains unprecipitated. The use of higher final concentrations than $N/12$ is to be avoided, however, since a greater proportion of inactive substance accompanies the precipitate, whilst by the use of a concentration of $N/12$ all the activity is carried down with a minimum of other material. The necessity of allowing the mixture to stand for 2 hours in order to ensure complete precipitation should be emphasised.

The effect of concentration of oxalate upon the hydrogen ion concentration at which precipitation of insulin from solution occurred was investigated. The specimen of insulin used had a normal isoelectric point of p_H 5.1.

A solution of insulin hydrochloride (1.00 g.) in water (40 cc.) was treated with $N/10$ sodium hydroxide solution until maximum precipitation occurred (10.2 cc.). The suspension of isoelectric insulin was well shaken and divided among a series of tubes in equal portions. To each of these was added an equal volume of a solution of oxalic acid of double the strength of that finally required. The precipitates dissolved immediately and potassium oxalate of the required normality was run into the clear solutions until precipitation occurred. The

hydrogen ion concentrations were then determined, and the results are shown in Fig. 1.

Fig. 1.

Complete purification process.

The three operations already described were now combined. The following is a typical example. 10.0 g. of insulin containing 85,600 units were dissolved in water and the solution centrifuged to remove a small quantity of insoluble material. The clear solution was diluted to 667 cc. and to this was added with stirring a 10 % solution of trichloroacetic acid (167 cc.). The precipitate settled fairly rapidly, leaving the supernatant fluid only faintly opalescent. After 2 hours the main portion of this was removed by means of a syphon, and the precipitate was collected in the centrifuge. The supernatant fluid gave further precipitates on bringing the concentration of trichloroacetic acid successively up to 3 % and 5 %. These precipitates were collected separately, and these and also the material remaining unprecipitated by 5 % trichloroacetic acid were converted to hydrochloride through the picrate.

The precipitate obtained by 2 % trichloroacetic acid was not converted directly to hydrochloride, but, while still moist, it was dissolved in $N/10$ hydrochloric acid (260 cc.), and to this 130 cc. of saturated sodium chloride solution was run in with good stirring. The precipitate was centrifuged, and the supernatant fluid was neutralised, and, after dilution to avoid precipitation of sodium picrate, the picrate was precipitated. This was converted to hydrochloride also. Meanwhile the salt precipitate was dissolved in water (200 cc.), its reaction was adjusted to p_H 4.0, and the solution was run with good stirring into 400 cc. of the $N/8$ oxalate mixture (p_H 4.05). The precipitate which formed was collected in the centrifuge after 2 hours, the precipitate dissolved in the minimum quantity of dilute hydrochloric acid, the solution diluted to about 200 cc., and, after neutralisation of the excess acid, half its volume of saturated picric acid solution was added. This picrate was well washed in the centrifuge with dilute picric acid solution, and was then converted into the hydrochloride.

Similarly, the material in the supernatant oxalate solution was also converted to picrate and hydrochloride. The weights of hydrochloride obtained at each stage are given in Table II.

Table II.

It sometimes happens that, owing to much salt remaining adherent to the precipitate, on adjusting the hydrogen ion concentration to p_H 4 a precipitate occurs. This may contain a considerable proportion of the activity. It is collected in the centrifuge and redissolved in dilute acid, when the solution may usually be adjusted to p_H 4 without any precipitation occurring. Both the solutions are then run into the oxalate solution separately in the usual way, and the final product is obtained by combining the two precipitates. If the purification by means of oxalate buffer be repeated there will be no precipitate at p_H 4 on adjusting the reaction of the solution prior to oxalate precipitation.

Table III shows the results of a series of experiments where the full procedure was followed.

Table III.

Initial weight g.	Initial unit mg.	Total initial units	No. of oxalate precipitations	Weight recovered g.	Final unit mg.	Total units recovered	Purification ratio
130	6.0	21,600	1	9.0	0.5	18,000	12
136	1.05	129,500	1	15.0	0.125	120,000	8.4
30.5	1.0	30,500	2	2.70	0.09	30,000	11.1
10.0	0.116	85,600	1	3.30	0.045	73,000	2.3
			2	2.53	0.035	72,400	3.3
			5	1.82	0.030	60,600	3.9

Use of sodium citrate. To a solution of insulin hydrochloride (1.0 g. = 1000 units) in 67 cc. water, $N/10$ solution of sodium citrate was added drop by drop. The initial p_H values of the hydrochloride and citrate solutions were 2.4 and 7.3 respectively. After 16 cc. had been added a permanent turbidity appeared, the reaction being p_H 4.5. Complete precipitation was obtained after adding 28 cc. of citrate, when the hydrogen ion concentration had fallen to p_H 5.05.

The precipitate was collected in the centrifuge, and both the precipitate and the material contained in the supernatant fluid were converted into hydrochloride in the usual way. The precipitate yielded 0.254 g. of substance containing 960 units. From the supernatant fluid the weight obtained was 0.52 g., the unit of which was approximately 8 mg. From this experiment it is clear that citrate acts similarly to oxalate in holding in solution a large proportion of inactive material.

Qualitative tests.

The following tests were performed on specimens of insulin the rabbit units of which lay between 0.016 and 0.03 mg. Solutions containing 2 mg. or more per cc. were used. The Pauli reaction was strongly positive with all the specimens tested, whether in the usual way or after destruction of the tyrosine by nitration by Brunswick's method [cf. Shonle and Waldo, 1924]. The biuret, Millon and xanthoproteic reactions were also always positive. The reaction for tryptophan and the Molisch reaction were negative. If carefully neutralised before performing the test, all specimens gave a positive ninhydrin reaction. Jaffé's reaction with alkaline picric acid was positive, which may indicate, according to Abderhalden, the presence of diketo-piperazine rings. Although these experiments were performed quantitatively whenever possible, in no case was the intensity of the reaction proportional to the activity of the preparations used.

Heat coagulation. A solution of purified insulin hydrochloride in 1.5 % aqueous solution is not coagulated if the temperature is slowly raised to 100°. When a suspension of the isoelectric precipitate is heated in the same way it coagulates, the activity being contained almost completely in the precipitate. The coagulated precipitate so obtained may be dissolved in dilute acid, showing that this is not an irreversible coagulation. The precipitate obtained on adding sodium chloride solution to a solution of insulin hydrochloride, when treated similarly, is irreversibly coagulated.

Dialysis. A solution of insulin hydrochloride (0.500 g. containing 2000 units) previously purified by precipitation with 2 % trichloroacetic acid, as described above, was dialysed in a collodion thimble against *N*/10 hydrochloric acid for 18 hours. At the end of this time the level of liquid inside the thimble was higher than that outside, and the outer liquid had taken on a yellowish tinge. Both the inside and outside fluids were neutralised, and the dissolved material was precipitated as picrate. It was found that 0.147 g., almost a third of the total weight of substance, had passed through the membrane, and when tested upon rabbits this was found to contain approximately 15 % of the total initial activity. A similar result is reported by Shonle and Waldo [1924]. The dialysate is completely precipitated by 2 % trichloroacetic acid and there was only a slight difference in the ratio of amino- to total nitrogen, determined first on the original material and secondly on the dialysate, as shown in Table IV. No difference was observed in the unit of the dialysate and dialysed solution, when material which had been previously precipitated

Table IV. *Dialysis.*

	Weight in g.	Amino-N %	Total N %	$\frac{\text{Amino-N}}{\text{Total N}}$ %
Inside	0.259	0.845	14.4	5.85
Outside	0.147	0.98	14.2	6.9
Original material	0.500	0.817	14.1	5.8

with 2 % trichloroacetic acid was employed. After prolonged dialysis (5 days) the whole of the material was found to pass through a similar membrane, the outer liquid being replaced by fresh distilled water at 24-hourly intervals.

None of the material passed through a parchment membrane (Schleicher and Schüll) when treated in an exactly similar way.

Precipitation by tungstic acid. A solution of insulin hydrochloride was precipitated by tungstic acid, as in the Folin method of blood analysis, the whole of the activity being carried down.

Effect of heat on the trichloroacetic acid precipitate. It was observed that on warming a suspension in 2 % trichloroacetic acid of the trichloroacetic acid precipitate, which had previously been washed in the centrifuge with 2 % trichloroacetic acid, the suspension cleared slightly. The solution was warmed to 60° and was filtered through analytical filter paper in a jacketed funnel maintained at 60°. The clear filtrate, on cooling, became turbid. This was centrifuged, and the precipitate was converted to hydrochloride in the usual way. This precipitate contained 6 % of the weight of the original specimen and approximately 6 % of the initial activity. Experiments on this point are being continued. Similar results were obtained on heating a suspension of the precipitate, caused by the addition of sulphosalicylic acid to insulin solutions.

An attempt was made to prepare from our highly purified specimens insulin in a crystalline state, following the method of Abel [1926], but without success.

Quantitative determinations.

Inorganic constituents. By the process described it is possible to obtain material which leaves a very slight ash on incineration. 4.663 mg. (unit = 0.03 mg.) yielded 0.004 mg. ash.

The *sulphur* content of insulin preparations of varying degrees of activity was determined by Carius' method. The results are shown in Table V.

Table V.

Unit (mg.)	1.5	1.5	1.0	0.3	0.25	0.2	0.15	0.06	0.03
Sulphur %	1.7	2.0	1.9	1.6	1.5	1.9	2.0	2.0	1.55

The sulphur content shows but slight variation even in specimens of insulin differing in activity in the ratio of 50 to 1.

Nitrogen. The amino-nitrogen and total nitrogen content of insulin hydrochloride has been determined in a number of cases. The values obtained vary only slightly for specimens of very different degrees of purity, the units of specimens investigated lying between 0.15 mg. and 0.016 mg. The total nitrogen

varied from 13.7 to 15.0 %, the ratio of amino-nitrogen to total nitrogen from 5.33 to 6.9 %. All these preparations had previously been precipitated by 2 % trichloroacetic acid. These results are similar to those obtained by Scott [1925]. We have also analysed by van Slyke's method a specimen of insulin the unit of which was 0.03 mg. We have added for comparison the figures obtained by us on a specimen of very crude insulin hydrochloride containing 1.5 mg. of solids per unit. The results are given in the following table:

Unit	1.5 mg.	0.03 mg.
Ammonia-N	9.43	9.59
Melanin-N	1.29	0.52
Cystine-N	6.06	5.93
Arginine-N	15.28	9.37
Histidine-N	2.93	4.29
Lysine-N	5.01	0.21
Amino-N (non-basic)	52.64	67.61
Non-amino-N (non-basic)	10.48	5.14
Total N (sum)	103.1	102.6

Apart from a marked difference in the cystine nitrogen and a reduction in the lysine content in our purified material, there is no very striking difference between the figures obtained by Scott and by us, although the material analysed by us was approximately four times purer than his. These results show the danger of speculating on the "purity" of insulin preparations on account of similarity between such analyses; see, for example, the conclusions of Somogyi, Doisy and Shaffer [1924].

SUMMARY.

1. Insulin is completely precipitated with the protein and metaprotein class, following the analytical method of Wasteneys and Borsook.
2. On this basis a method of purification has been developed, depending on the action of salt solutions at the isoelectric point, which can be used to produce insulin of a very low physiological unit (about 0.02 mg.).
3. The results of various qualitative tests on the purified product are described.
4. Analytical results on specimens of insulin of varying degrees of purity are given.
5. The bearing of these observations on the probable nature and constitution of insulin is discussed.

REFERENCES.

- Abel (1926). *Proc. Nat. Acad. Sci.* **12**, 132.
 Cohn (1924). *J. Biol. Chem.* **59**; *Proc. Amer. Soc. Biol. Chem.* iv.
 Dudley and Starling (1924). *Biochem. J.* **18**, 147.
 Hardy (1905). *J. Physiol.* **33**, 251.
 League of Nations Report (1926). *The Biological Standardisation of Insulin* (Geneva).
 Michaelis and Szent-Györgyi (1920). *Biochem. Z.* **103**, 178.
 Scott (1925). *J. Biol. Chem.* **65**, 601.
 Shonle and Waldo (1924). *J. Biol. Chem.* **58**, 732.
 Somogyi, Doisy and Shaffer (1924). *J. Biol. Chem.* **60**, 31.
 Wasteneys and Borsook (1924). *J. Biol. Chem.* **62**, 1.