XLVI. OBSERVATIONS ON INSULIN.

PART I. CHEMICAL OBSERVATIONS.

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PART I.

Since the first announcement by Abel [1926] of the isolation from commercial insulin of a crystalline substance exhibiting in a high degree the specific activity of the hormone, it has become a matter of the greatest interest to attempt to determine whether this crystalline material is indeed to be regarded as insulin itself, or whether it is in reality an inert constituent of the pancreas which, in the process of preparation, has become associated with the hormone by adsorption or otherwise. The final proof of the identity of the compound with insulin, if such identity exists, can naturally only be afforded by synthesis; since, however, we have to do with a substance which is to be classed among the simpler proteins the prospects of such a synthesis remain infinitely remote, and other, less direct, evidence bearing upon the question must be sought. Later papers from Abel's laboratory [Abel *et al.*, 1927; Jensen and Geiling, 1928; du Vigneaud *et al.*, 1928] have not increased the volume of such evidence to any considerable extent. Slight modifications have been introduced into the original method of preparation of the crystalline material, but these modifications do not successfully meet the criticisms which may be levelled at the claim to designate the substance pure insulin. In particular have the workers in Abel's laboratory failed to meet the criticism based on the varying degree of physiological activity reported for different samples of a supposedly uniform substance. If we turn to the experience of other workers with this material we find that a similar state of affairs seems to exist, for whilst there is no doubt that crystals identical with those

described by Abel have been prepared in other laboratories by his method, no consistency is to be found in the degree of physiological activity assigned to them by different workers, or indeed sometimes by the same worker. It has even been stated [Dingemanse, 1928] that it is possible by adsorption on charcoal followed by elution with phenol and extraction of the material recovered from phenol with phosphate to obtain an amorphous preparation of a higher degree of activity than that ascribed by Abel to the crystals; this statement is, however, contested by the workers in Abel's laboratory [du Vigneaud *et al.*, 1928].

The present uncertain position of the problem seemed to us to warrant a renewed attempt to obtain more decisive evidence upon the point at issue, and we therefore set out on the present investigation with three main objects in view: (*a*) to obtain the material by Abel's method and to subject it to thorough physiological assay, (*b*) to seek for alternative methods of preparation, and (*c*) to carry out certain experiments with the crystalline substance designed to test the question of its identity with insulin.

As starting material in this work we have employed in the main two commercial preparations of insulin. The earlier experiments were made with a powder prepared at the Connaught Laboratories in Toronto and assayed at 13 international units per mg., and the later experiments with a powder of approximately similar unitage kindly supplied to us by the British Drug Houses, to whom, and in particular to Dr F. H. Carr, we wish here to express our best thanks. We have also had the opportunity to make a few experiments with a powder prepared by Messrs Burroughs Wellcome and assayed at 21 international units per mg., for which we are indebted to Dr H. A. D. Jowett. Our first experiments consisted in attempts to prepare crystalline material from the Toronto insulin by the method described by Abel. After several failures, we were eventually able to obtain a small amount of the crystals by this method; our experience with the technique was discouraging, however, since we found it necessary to carry out many more reprecipitations than are indicated by Abel, with the result that the manipulation became excessively laborious and the yields of crystalline material were exceedingly small. As soon, therefore, as we had accumulated sufficient material for the purpose of physiological assay, we passed on to the second object of our investigation, namely the search for an alternative method of preparation.

The first impression which we, and apparently Abel himself, formed (although subsequent work has caused us to modify this view) was that the success of Abel's method depended on the efficient buffering achieved by the complex mixture of weak bases which he employed; it was thought that such a system enabled the reaction of the solution to be adjusted so gently and so exactly to the isoelectric point that it was possible in effect to obtain a supersaturated solution from which the protein might be expected to separate slowly, with consequently increased tendency to crystallise. With this idea in mind we sought to substitute a simple phosphate buffer for the mixture

of brucine acetate and pyridine employed by Abel, the insulin being dissolved at a slightly alkaline reaction, and the p_H of the solution adjusted by the very gradual addition of carbon dioxide; even the very slowest introduction of the latter, however, caused an immediate amorphous precipitate to form and by no fractionation on these lines were we able to obtain crystalline material. In the light of our subsequent experiments we are inclined to think that the function of the brucine acetate-pyridine mixture is not merely, as we first supposed, that of a delicate buffering system, but that other physical properties of these substances are involved, *e.g.* the lowering of the surface tension of the solution, thus bringing into play an effect on the properties of the commercial insulin analogous to that about to be described. Some such explanation of the rôle of brucine is indeed suggested by Abel himself.

The method for the preparation of the crystalline material which has met, in our hands, with a certain amount of success, and which we wish to describe in this paper, rests on the observation that the properties of commercial insulin are profoundly modified by the presence in the solution of an active saponin. As to what exactly constitutes an "active" saponin from this point of view we shall have more to say later; at this point let us assume that we are speaking of one which has been found by experiment to be satisfactory for the purpose in hand. It is well known that the commercial purification of insulin consists, in its final stages, of repeated precipitations at the isoelectric point, the latter lying at about p_H 4·8–5·0; a good commercial product, such as either of those which we have used in this work, precipitates practically quantitatively when the solution is adjusted to this reaction. If, however, a sufficient amount of saponin be introduced into the acid solution, the reaction may be carried, by the gradual addition of alkali, over the isoelectric point without the occurrence of any precipitation whatever. It might be thought at first sight that we have to do here merely with an instance of inhibition of precipitation or supersaturation such as is known to occur in simpler cases in the presence of saponin; that such a straightforward explanation fails to fit the facts is shown, however, by the effect of lower concentrations of saponin. The saponin possesses apparently the additional power of, so to speak, sharpening the isoelectric points of the proteins present. If a solution of insulin in dilute acetic acid containing about 1 % of saponin is treated gradually with ammonia, a definite turbidity is observed when the p_H is about 4·5; in the absence of saponin, turbidity occurs earlier and a gross flocculation is already apparent at this reaction. On keeping the saponin-containing solution, a flocculent precipitate separates in the course of about 30 minutes, and may be removed on the centrifuge, when it is found to represent about 30 % of the weight of the starting material but only 15–20 % of the activity. Whilst continued addition of ammonia to the solution of insulin in dilute acid in the absence of saponin causes steadily increasing precipitation up to about p_H 5·0, further addition of alkali to the saponin-containing solution after removal of the precipitate at p_H 4·5 causes no visible

change until the reaction reaches p_H 5·6, at which point an opalescence develops; if the addition of alkali be interrupted and the solution kept overnight a precipitate forms which, in a satisfactory experiment, is already partly crystalline. A second similar isoelectric precipitation from more concentrated solution in presence of saponin results in a product which is almost entirely free from amorphous material, although not yet of very definite form. The precipitate may now, however, be successfully recrystallised by isoelectric precipitation from a phosphate buffer solution without the aid of saponin, and, by the selection of appropriate conditions of temperature and concentration, may be obtained in remarkably large and well-defined crystals.

Given, therefore, a suitable saponin we possess, in the technique summarised in the last paragraph, a simple and rapid means for the preparation of crystalline material from a commercial insulin powder of the unitage indicated. There are many factors, however, which render the situation less simple than is indicated by the above brief description. The difficulties of standardising any method requiring the delicate adjustment of physicochemical conditions, which involves the use of saponin, are obvious. Commercial saponins are crude substances of varying origin and variable properties; from the commercial point of view the value of a saponin is determined solely by its frothing capacity, and we were soon able to show that this property by itself fails to give an indication of the efficiency of a saponin for our purpose. We are indeed quite unable to offer any rational explanation of the mechanism of the action of saponin in promoting the crystallisation. That it is not, entirely at any rate, a question of the lowering of surface tension is indicated by the failure of substances such as the higher alcohols, which lower surface tension to a still greater extent, to achieve the same object, at least with a similar efficiency. Part of the effect is undoubtedly to be ascribed to the power of saponins to inhibit precipitation in general, and in so far as this is concerned, there is some indication that a high frothing capacity on the part of a saponin implies also a high capacity for promoting supersaturation. A not less important factor, however, is the apparent ability of certain saponins to effect the sharpening of the isoelectric points of the mixture of proteins which constitute commercial insulin, with the result that it becomes possible to precipitate the proteins individually at fairly widely differing and clearly defined points instead of throwing down the mixture *en masse* over a more extended range of p_H. It is evident that from the preparative standpoint these two properties of the saponin are to some extent mutually antagonistic. The more saponin used the greater is the degree of supersaturation induced and hence the lower is the yield of crystals; on the other hand, the crystallisation fails entirely to occur if enough saponin be not used to effect the separation of the isoelectric points (if we may so designate the phenomenon discussed above) in a well-defined manner. By a fortunate coincidence the first sample of saponin which we tried possessed eminently satisfactory properties in both respects. The next sample, although ostensibly

of similar quality, was almost entirely useless for our purpose; various subsequent samples of saponin have exhibited differing degrees of efficiency, as determined by the number of precipitations necessary for the purification and by the yield of crystalline product obtained. The cruder samples contained considerable amounts of calcium sulphate and traces of heavy metals; with such samples, as might be expected, immediate precipitation of part of the insulin occurred even in fairly acid solutions, and an unduly large amount of material was therefore removed with the first acid precipitate at p_H 4·5. Apart from the elimination of such gross impurities, however, it was difficult to detect any distinguishing feature by which a saponin might be recognised as being suitable for the purpose in hand. In the search for such a criterion we investigated the haemolytic power of the various saponins employed, for help in which part of the work we are indebted to Dr H. D. Wright; as will be seen from the results recorded below there does appear to exist at least a rough parallelism between the haemolytic indices of the different saponins and their efficiencies in promoting the crystallisation; as an approximation it may be said that, as far as our experience extends, a saponin with a haemolytic index of less than 1 : 8000 is unlikely to be of much use for the purpose.

Apart from the variability of the different saponins themselves, a further complication is introduced by the effect of surface. The method was first worked out for 20 mg. samples of the crude insulin powder, and we were for a long time troubled by our inability to extend the process to larger amounts. Thinking that the difficulty might be due to the importance of preserving correct time-relationships between the different stages of the manipulation, we performed many experiments on the adjustment of these relationships, all however to no purpose. We were then reminded of the fact that when a solution of saponin is introduced into a glass vessel a gradual concentration of the saponin occurs at the liquid-glass interface, and this was apparently the key of the situation. In the earlier small-scale experiments mentioned above the solution was always transferred, at the crystallising point, to 15 cc. centrifuge cups in which the liquid-glass interface was about 25 $cm.^2$ for a total volume of 9 cc.; in the larger experiments involving greater volumes of liquid, the final solution was left in a beaker, or transferred to larger centrifuge cups of a different shape; in either case the ratio of liquid-glass interfacial area to volume of solution was significantly lower than that in the small experiments; in the case of the beaker the surface was only 45 $cm.^2$ for a volume of 45 cc. Such larger experiments led to no good result, and it was only when, after many variations, we restored this ratio to its original value by dividing the final solution, as soon as it was adjusted to the correct p_H over a large number of the 15 cc. cups, that we were successful in achieving the crystallisation of larger amounts. We were subsequently able to bring about the same result by leaving the solution in the beaker and introducing the requisite number of glass rods to bring the interfacial area to the same proportionate amount as in the small tubes. It may be noted here that it is

in the first separation from the crude insulin powder that the surface relationship is of paramount importance; once the precipitate has been obtained even partly crystalline this factor becomes of less significance. In all probability there exists an optimum relationship between the concentration of saponin employed and the surface-volume ratio which we have just discussed, and the particular suitability of the 15 cc. centrifuge cups for our purpose is decided by the concentrations of saponin which we have arbitrarily selected. Much further experimental work, however, will be required to clear up this point, and, for the present, we have contented ourselves with the preservation of the correct relationship by the simple expedient indicated, since we have not attempted to extend the method to larger quantities than 0·5 g. of the crude powder. It is hoped in the near future to carry out further experiments with the object of defining the optimum conditions more exactly. In order to make the method, at least for small quantities, readily reproducible, we have worked out the details for the crystallisation using pure digitonin instead of a commercial saponin; the conditions for the use of ordinary saponins can at present only be described in somewhat general terms, the exact details having to be worked out for each sample.

Perhaps the least satisfactory feature of the whole method is the poorness of the yield. With the different saponins of which we have had experience this has varied from 5 to 15 % of the crude powder, which, considering the relatively small access in activity on crystallisation, evidently leaves much to be desired; we have, however, done little or nothing towards working up residues, and it is not unlikely that a systematic application to this task would effect a considerable increase in the total yield obtainable.

It will be apparent from what has been said that a large amount of experimental work remains to be done in order to realise to the full the possibilities of the method which we describe. Nevertheless we are publishing our results at the present stage, partly because our immediate association in the work can unfortunately not be continued, but also because we feel that, even so far as our experiments have gone, we have some evidence to offer bearing on the question of the identity or non-identity of the crystalline compound with insulin.

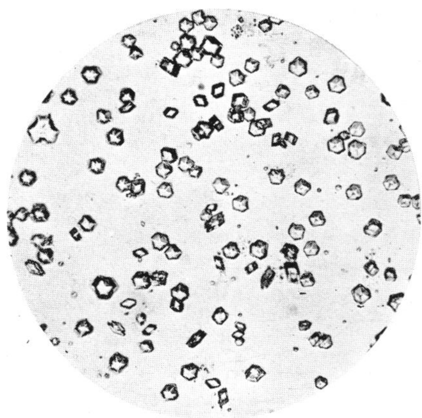
It may be said at once that the strongest evidence on the last-mentioned point is to be found in the results of the physiological assay contained in Part II of this paper. The impressive consistency of the results reported by independent workers for the activity of four different samples of crystals, two obtained by Abel's method, and two by our own, must, we think, be accepted as a powerful argument in favour of the identity of the compound with insulin. In addition to the results recorded in Part II assays were performed by the mouse method on samples of crystals prepared from the B.D.H. insulin by the use of saponin and from the insulin of Messrs Burroughs Wellcome by the aid of digitonin. In both cases the product showed an activity of 25 international units per mg.

To obtain evidence as impressive from the chemical side is a difficult matter, but there is no doubt that the results of our experiments point in the same direction. The crystals themselves exhibit a clearly defined structure, which is well depicted in the photograph (Plate I) (kindly taken for us by Mr J. E. Barnard); all preparations present an appearance of absolute uniformity, not only to our eyes, but to those of a crystallographer[1]; the possibility that they may represent a mixed crystal containing insulin as one component seems therefore remote. There remains the more likely suggestion that they may owe their physiological activity to the adsorption of a trace of a highly active substance, and this criticism is the more difficult to meet, since the crystals can only be obtained under one set of physico-chemical conditions, namely by isoelectric precipitation. We think, however, that the success of the saponin method does afford some evidence against the adsorption theory, since it is difficult to suppose that the conditions of adsorption would not be modified by the presence in the solution of so highly surface-active a substance as saponin, and yet the saponin method and that of Abel lead to products of identical activity. Further, we have been able to show, in confirmation of Abel, that the crystallisation may be effected from 50 % alcohol instead of from water without modification of the activity of the product. Negative evidence on the same point is provided by the failure of our attempts to recover any crystalline material from solutions of the crystals which had been subjected to the mildest chemical treatment consistent with destruction of the physiological activity.

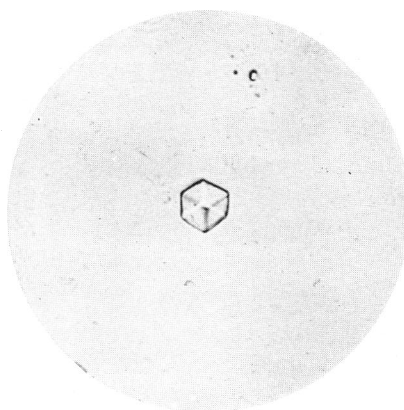
The conception that insulin, with its high degree of specific physiological activity, should be a protein without any obvious distinguishing chemical property is admittedly not readily acceptable; still less acceptable to many minds will be the notion that the best commercial products, prepared by empirical methods, should represent almost pure insulin. The latter objection is, however, based on prejudice rather than on reason, and we suggest that both difficulties may be largely removed by a hypothesis which we wish tentatively to advance.

Let us suppose that we take thyroid gland, extract therefrom the iodothyreoglobulin, and, by some manipulation, succeed in obtaining the latter in the crystalline form. We should then have in our hands a crystalline protein exhibiting (although, owing to its large molecular weight, not in a very high degree) the specific physiological activity of the gland; we might, therefore, not unjustifiably, designate the product the pure active principle of the thyroid. In the light of our present knowledge we recognise that such a designation would be correct only in a limited sense, since the actual physiological activity is due, not to the protein as a whole, but to its specific constituent thyroxine. In a similarly limited sense we are inclined to regard the crystalline protein

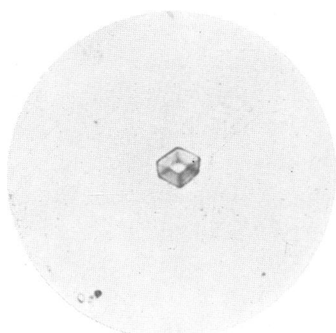
[1] The crystals were kindly examined for us by Dr T. V. Barker of the Department of Mineralogy, University of Oxford, who expressed the opinion that the preparation had the appearance of "a definite substance with well-defined crystallophysical properties." The crystals were too small to permit of complete crystallographic measurements.

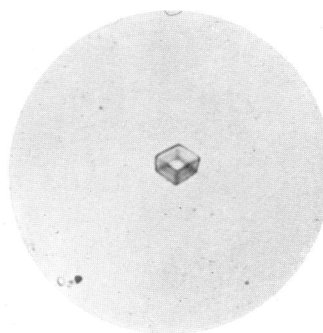


General field of crystals. × 120



Single crystal. × 340





Stereoscopic photograph of single crystal. × 340

PHOTOMICROGRAPHS OF INSULIN CRYSTALS

from the pancreas as being the true active principle of the gland in so far as the function of the latter in relation to carbohydrate metabolism is concerned. We feel that in this case, as in that of iodothyreoglobulin, the physiological activity is a property, not of the protein as a whole, but of some specific constituent or grouping contained within its molecule. The thyroid protein is differentiated from other proteins in an obvious manner by its iodine content, and the specific constituent, being a simple amino-acid, can be readily separated from the rest of the molecule; the insulin protein bears no such chemical earmark, unless it be the high content of sulphur. The analogy which we have drawn between the thyroid protein and the insulin protein must not be taken to indicate that we believe such a separation of the specific group to be necessarily practicable in the latter case. As a theoretical possibility, however, it seems worth bearing in mind.

Experimental.

The method of Abel. The first experiments consisted in attempts to apply the method described by Abel *et al.* [1927] to the preparation of crystalline material from the Toronto insulin powder assayed at 13 international units per mg. Although we followed the directions given by Abel with the greatest care, all our solutions being carefully checked, we met at the outset with a puzzling discrepancy in that we failed to obtain any precipitate on the addition of pyridine. In this respect the crude insulin was apparently behaving in a manner similar to that noted by Abel as characteristic of the crystalline material. We, therefore, proceeded with the addition of ammonia, using a 0·85 % solution as indicated by du Vigneaud *et al.* [1928][1]; after addition of the specified amount of ammonia we obtained a precipitate which was removed on the centrifuge, forming a somewhat gummy mass; nothing separated from the mother liquor on keeping the latter in the ice-chest, and, on testing, the solution was found to be considerably more acid than it should be according to Abel's description; further ammonia was added (3 cc. for every 0·1 g. of original insulin) to bring the reaction[2] of the solution to p_H 5·6. At this point an opalescence developed, and, on keeping the solution in the cold, a precipitate settled out, which was, however, amorphous. By repeatedly putting this precipitate through a similar process in continually diminishing volume (using always more ammonia than indicated by Abel) the material was eventually obtained in the crystalline condition as described by Abel, and was recrystallised in the absence of brucine either by the method given in his paper, or better from a phosphate buffer as described below. No explanation of the differences observed by us can at present be given. We mention our experience for the possible interest of other workers who may

[1] In the paper by Abel *et al.* [1927] the use of 0·65 % ammonia for the crystallisation is described; a subsequent paper by du Vigneaud *et al.* [1928] from Abel's laboratory gives 0·85 % as the concentration of this reagent, no reason for the change being given so far as we can ascertain.

[2] All p_H determinations mentioned in this paper were made colorimetrically.

be attempting to carry out this manipulation. The discrepancy was not due to excess of acid adherent to or combined with the original insulin, since this was neutralised at the outset; attempts at purification of the crude powder by the phenol treatment of Abel or by continuous extraction of an isoelectric suspension with butyl alcohol under diminished pressure failed to bring the behaviour of the insulin into line with that of the sample which Abel employed, although the butyl alcohol extraction, which removed a small amount of physiologically inert material (possibly of a lipoid character) did appear to improve the conditions for crystallisation to a small extent. At the best, however, the yields were so minute and the method so time-consuming that we soon passed on to the attempt to find an alternative procedure, particularly as we were anxious to break away entirely from the use of brucine—a substance which, in a case such as the present, has very obvious disadvantages.

Experiments with carbon dioxide. It was thought that the reaction of an alkaline buffer solution containing insulin might be adjusted so delicately by the gradual introduction of carbon dioxide that we might in this way achieve the necessary supersaturation at the isoelectric point, dispensing with the elaborate mixture of weak bases. With this object 0·1 g. of insulin was dissolved in 4 cc. of water together with 0·3 cc. of 0·1 N hydrochloric acid, and this solution was mixed with 10 cc. of a phosphate buffer at p_H 7·0 to which had been added 1·5 cc. of 0·1 N sodium hydroxide. A slow stream of carbon dioxide was passed into the solution through a capillary tube; after 3 minutes an opalescence was apparent. The addition of carbon dioxide was interrupted and the solution allowed to stand for some hours; the precipitate was separated on the centrifuge, and the mother liquor further treated with carbon dioxide; this caused almost immediate further precipitation, and by the time the reaction had reached about p_H 6·4 the greater part of the insulin had separated in the amorphous condition; no tendency to crystallise was observed at any stage, nor did any fraction obtained by precipitation with carbon dioxide appear to be more suitable for crystallisation by the brucine method than the original crude powder. These experiments were, therefore, not pursued further.

Saponin method. The observations on which this method is based have been discussed in the introductory part of this paper and need not be further referred to here; nor shall we do more than mention the very numerous experiments which have been made on the effect of variations in temperature and in the time-relations of different parts of the process, and on the optimum point at which to remove the first precipitate. In this section we propose to describe (*a*) the method by which we gauge the most suitable amount of saponin to employ, (*b*) the crystallisation with the aid of a good commercial saponin, (*c*) the method with the use of digitonin.

(*a*) *Determination of correct amount of saponin.* A 6 % solution of the saponin in distilled water is made. (This solution should be prepared freshly

each day owing to the rapidity with which it becomes infected.) A stock solution of insulin is then prepared by dissolving 0·1 g. of the commercial powder in 5 cc. of 10 % acetic acid and making the volume up to 25 cc. with distilled water. This stock solution is divided into five equal portions, and these are treated with 0·5, 0·75, 1·0, 1·5 and 2·0 cc. of the saponin solution respectively. The solutions are warmed to 35° and to each portion is added 1·6 cc. of 0·85 % ammonia. After standing for 30 minutes at 35° the precipitates which have formed are removed on the centrifuge, and the mother liquors are adjusted to p_H 5·6 by the addition of a further 1·7 cc. of the 0·85 % ammonia and transferred at once to 15 cc. centrifuge cups. It is important to adjust the reaction to p_H 5·6 as rapidly and as sharply as possible; the exact amount of ammonia necessary will vary slightly with different samples of insulin according to the amount of acid present. The tubes are allowed to stand, with occasional scratching with a glass rod, and are examined next day. With a good saponin the general result of such an experiment will be that in the tubes with the lower concentrations of saponin there is a large amount of amorphous precipitate whilst in those with the high concentrations the precipitate is mostly crystalline but small in amount; it is usually fairly easy to pick out that tube of the series in which conditions are best, *i.e.* where there is the largest amount of precipitation consistent with the absence of grossly amorphous material. It must be understood that, in speaking of crystalline material at this stage we do not mean to indicate that it already has a well-defined form; this is not the case, but with some experience it becomes quite easy, on examination under a high power of the microscope, to distinguish between material which, while not yet of definite form, will recrystallise satisfactorily, and the ragged grossly flocculent impurities which must be avoided. It is really only by experience that these differences can be recognised; a more precise verbal description is difficult to give. However, those tubes in which conditions are good are usually macroscopically distinguished by the separation of material on the sides of the vessel where it has been scratched with the glass rod. In the case of our best commercial saponin optimum conditions obtained in the tube to which 1·0 cc. of the saponin solution had been added; in this case, therefore, a typical experiment was as follows.

(*b*) *Crystallisation with commercial saponin.* To 5 cc. of the stock solution of insulin in 2 % acetic acid (representing 20 mg. of the crude powder) was added 1 cc. of 6 % saponin solution; the mixture was warmed to 35°, treated with 1·6 cc. of 0·85 % ammonia, and kept at 35° for 30 minutes; the precipitate was removed on the centrifuge and the mother liquor adjusted as rapidly and sharply as possible to p_H 5·6 by the addition of 1·7 cc. more of the ammonia, and transferred immediately to a 15 cc. centrifuge cup. At this point the solution was still clear or only very slightly opalescent; on standing and scratching with a glass rod a turbidity soon developed, and overnight a precipitate separated which was largely micro-crystalline in

character. Five such tubes were usually prepared simultaneously, or 100 mg. of the crude powder was worked up as a whole, the final solution being divided over 4 or 5 tubes; after separation was complete the precipitates were collected with the aid of the centrifuge and combined in solution in 2·5 cc. of 10 % acetic acid and 10 cc. of water; 2·5 cc. of the 6 % saponin were then added and the reaction adjusted immediately to p_H 5·6 by the addition of 8·25 cc. of 0·85 % ammonia. This time the precipitate was almost entirely microcrystalline, although not yet of definite form. A further crop of crystalline material could sometimes be obtained by saturating the mother liquor with butyl alcohol. The final crystallisation was effected as follows. The whole of the precipitate after the second separation in presence of saponin (representing 100 mg. of crude insulin) was dissolved in 4 cc. of water and 0·4 cc. of 0·1 N hydrochloric acid; the solution was poured with stirring into a mixture of 16 cc. of a phosphate buffer at p_H 7, prepared according to Clark [1923], to which had been added 0·8 cc. of 0·1 N sodium hydroxide; the resulting solution was warmed to about 55°, treated with 1·0 cc. of 0·1 N hydrochloric acid, which brought the reaction to p_H 5·6, and set aside. Within a few hours there separated a precipitate which was now wholly crystalline and exhibited the characteristic form. The size of the crystals could be increased by one or two further separations from phosphate solution on the same lines. The best results in this last crystallisation were obtained if the solution, after adjustment to the isoelectric point, were left entirely undisturbed so that the material should separate as slowly as possible. The yield varied with the saponin and the crude insulin employed, amounting to 5–15 % of the starting material. A physiological assay in one case indicated that 50–60 % of the activity remained in the combined mother liquors, 15–20 % being lost in the first acid precipitate, which however amounted to 30 % of the weight of the original insulin; this partition probably varies somewhat with different saponins and different preparations of insulin. We have, up to the present, made no attempt to subject the material recovered from the mother liquor to crystallisation; that it would almost certainly be possible to do so with more or less success is indicated, however, by an experiment in which we were able to obtain crystals from a crude powder of as low an activity as 8 international units per mg.

We have had experience in all of nine samples of saponin of which the first was considerably the best; this was a sample of "white saponin" purchased from the British Drug Houses. The second sample, purchased under the same description, turned out to be practically useless; subsequently we tried a number of commercial samples, all of which were more or less useful for our purpose, although some of the cruder ones, containing as they did large amounts of inorganic matter, even including traces of heavy metals, caused an apparent denaturation of a part of the insulin. In the attempt to find some correlation between a measurable physical property of a saponin and its usefulness for our purpose we have compared in Table I the frothing

capacity and haemolytic power of the various saponins with their efficiency in promoting crystallisation. In this table the frothing number is given as that concentration which on shaking for 15 seconds in a tube of 16 mm. diameter produces 1 cm. of froth measured after 15 minutes. The haemolytic power is expressed as the minimum concentration necessary to produce complete haemolysis of a 5 % suspension of rabbit's washed red blood-corpuscles in 0·9 % sodium chloride during 2 hours at 37°. (For these determinations we are indebted to Dr H. D. Wright.) Unfortunately the data are not available for the first and best of our saponins, since we had exhausted the supply of this material before running into the difficulties which persuaded us to make the comparison. The true haemolytic value for digitonin should probably be somewhat higher than that given, since part of the digitonin is precipitated, presumably by the cholesterol from the cells, and is thus removed from the solution. The general result of the comparison is to indicate that, from the point of view of efficiency in promoting the crystallisation, the haemolytic index of a saponin is of more significance than is its frothing power.

Table I.

Saponin	Frothing number	Haemolytic index	Crystallising efficiency
White No. 1	Not determined	Not determined	+ + +
,, ,, 2	1 : 25,000	1 : 4,000	Useless
,, ,, 3	1 : 25,000	1 : 8,000	+ +
A	1 : 20,000	1 : 32,000	+ +
B	1 : 16,000	1 : 32,000	+ +
C	1 : 15,000	1 : 32,000	+ +
D	1 : 15,000	1 : 32,000	+
E	1 : 9,000	1 : 16,000	+
F	1 : 10,000	1 : 32,000	+ +
Digitonin	1 : 30,000	1 : 8,000	+ +

Digitonin method. The insulin powder (0·1 g.) is dissolved in 5 cc. of 10 % acetic acid and the solution is made up to 25 cc. with water; 0·125 g. of digitonin dissolved in 2·5 cc. of water is now added, followed by 8 cc. of 0·85 % ammonia; after standing 30 minutes, the precipitate is removed on the centrifuge and the mother liquor adjusted to p_H 5·6 by the addition of 8·5 cc. of 0·85 % ammonia; the solution is then divided over four or five 15 cc. centrifuge cups and set aside; after standing for some hours (preferably overnight) the precipitate is separated on the centrifuge and subjected to a similar process, using half the quantities of all reagents; after the second separation the precipitate may be successfully crystallised from a phosphate solution exactly as described above. In the case of the digitonin, as in those of some of the commercial saponins, there may be little sign of actual crystallisation as the result of the first two precipitations, the visible improvement consisting rather in the elimination of the grossly flocculent amorphous material; so long as the removal of the latter is complete, however, the final crystallisation from the phosphate solution succeeds. The yield by the digitonin method has not been more than 10 % of the starting material.

Miscellaneous experiments.

(*a*) *Butyl alcohol.* The observation noted above that a further crop of crystalline material may be obtained from saponin-containing mother liquors, combined with the consideration of the part which must be played in the whole process by the lowering of the surface tension of the solution produced by saponin, led us to try some experiments on the effect of butyl alcohol alone. Crude insulin was, therefore, subjected to a process similar to the phosphate crystallisation described above except that 5 % of purified *iso*butyl alcohol was added to the alkaline solution immediately before adjustment to the isoelectric point. In this way we were indeed successful in obtaining some material which appeared partly crystalline, but attempts at recrystallisation did not lead to a satisfactory product. It was, moreover, noticed that part of the material had lost its acid-solubility as the result of the butyl alcohol treatment, *i.e.* was partly denatured, and these experiments were, therefore, not pursued further.

(*b*) *Bile salts.* In the search for a substitute for saponin of a more reproducible character than the latter, an experiment was made to see whether bile salts could be employed; it was found, as was anticipated, that they were useless in solutions of the acidity which we were employing.

(*c*) *Inactivation experiments.* In order to obtain some evidence bearing on the question of the identity of the crystalline material with insulin, particularly in relation to the adsorption theory discussed in the introductory part, attempts were made to recover crystals from solutions of the latter which had been subjected to the minimum treatment with alkali which was necessary to bring about more than 80 % inactivation. The crystals (20 mg.) were dissolved in an alkaline phosphate solution (40 cc.), the p_H being approximately 10·5; in order to produce the required degree of inactivation, this solution had to be heated at 70° for 30 minutes; after such treatment, on readjustment to p_H 5·6, only amorphous material separated, nor could any crystals be obtained by subjecting this material to the saponin treatment. The inactivation by alkali was apparently irreversible, *i.e.* no regeneration of activity or of potentially crystalline material was observed on making the reaction acid and keeping the solution.

Appearance and analysis of the crystals.

The preparations of crystalline material present always an appearance of absolute uniformity. Seen under a low power the most conspicuous feature is the hexagonal outline noted by Abel. Examined more closely under a high power the crystals present the appearance of cubes, or rhombohedra approximating to cubes, which are standing on one corner, thus giving rise to the hexagonal outline already mentioned. This description has naturally not the slightest crystallographic significance, and the crystals are too small for complete angular measurements to be made. They are weakly doubly-refracting and have a refractive index of approximately 1·58. The optical appearance of

the crystals is better depicted in the accompanying photographs (Plate I) than it can be described in words.

For analysis, some of the material was dried in a vacuum desiccator over sulphuric acid at the ordinary temperature and then allowed to come to equilibrium with the air; analysed in this condition it gave the following figures, which agree closely with those given by Abel for his air-dried substance[1].

4·861 mg.	gave	8·79 mg. CO_2, 2·9 mg. H_2O
4·960	,,	8·93 mg. CO_2, 3·08 mg. H_2O
2·945	,,	0·365 cc. N_2 at 21·5° and 767 mm.
3·028	,,	0·364 cc. N_2 at 22° and 767 mm.
11·200	,,	2·535 mg. $BaSO_4$

Whence	C	H	N	S
	49·61	6·81	14·49	3·11
	49·11	6·95	14·03	—

PART II.

In Part I of this paper is described the preparation from insulin of crystals, indistinguishable from those obtained by Abel and his colleagues, by the aid of a simpler method than that which they have used. Preliminary trials showed that these crystals possessed the specific activity of insulin in a high degree. It appeared to be important to have this physiological activity measured as accurately as the available methods allow. Two questions concerning the activity of such insulin crystals seemed to us to require more definite answers than they have hitherto received.

1. Do the crystals, apparently pure and identical, obtained by different procedures, show a uniform activity, or an activity which varies from batch to batch? A precise answer to this question would have an obvious bearing on the claim of the crystals to be regarded as insulin itself in pure form. A wide variation in activity from batch to batch would be inconsistent with such an identification; uniform activity, on the other hand, in batches prepared by different methods from different samples of the crude insulin, while not affording conclusive proof that the crystals are pure insulin, would be difficult to reconcile with the view that they consist of a non-specific, inert material, on which the insulin is merely adsorbed.

2. If the crystals show uniform activity, what is the degree of that activity, expressed in the commonly accepted units?

In spite of the large amount of work on the subject during the past two years, the information on these two points, obtainable from the literature, is curiously incomplete and unsatisfactory. In his first paper on the subject Abel [1926] did not attempt to assign a definite unitage to the crystals, merely indicating that 1/100 mg. in one experiment, and 1/125 in another, had sufficed to reduce the blood-sugar of certain rabbits to the traditional

[1] Analyses performed by Dr Ing. A. Schoeller, Berlin-Schmargendorf, Tölzerstrasse 19.

"convulsant level" of 45 mg. per 100 cc. and even to produce actual convulsions. In a later paper, published with Geiling, Rouiller, Bell and Wintersteiner [1927], he assigned a value of 90 units per mg. to a sample of the crystals. More recently du Vigneaud, Geiling and Eddy [1928] have recorded a value of 54 units per mg., and Jensen and Geiling [1928] one of 45 units per mg., for different samples of the crystalline product[1].

Taken at their face value, these estimates do not give an impression of uniform activity in the different samples; nor, with estimates spread over so wide a range, is it possible to select one as representing the true and characteristic activity of the crystals; though the fact that actual convulsions are recorded in certain rabbits, receiving as little as 0·01 mg., suggests an activity higher than that of the purest insulin obtained by ordinary methods. On none of these points, however, can we regard the evidence of these measurements as satisfactory or convincing, since they were all obtained by the old method of direct calculation from the degrees of hypoglycaemia produced in unstandardised rabbits. It is not possible to attach a precise significance to unit values which have not been measured by comparison with, and expressed in terms of the International Standard, which since 1925 has afforded the basis for the unit by international agreement and general use. Such measurements of the activity of the insulin crystals, in relation to the International Standard, have been published by Freudenberg and Dirscherl [1928], who obtained the values of 23 and 26 units by different comparative methods. The difference between these two figures is not greater than the error of any method yet available; but the potency which they indicate is only about one-half of those more recently published from Abel's laboratory.

Plan of the investigation.

Four lots of the crystals were selected, two prepared by Abel's method, and two by the new method described in Part I. Taking them in the order in which they were examined they were as follows.

A. Crystals obtained by Harington and Scott, using Abel's method, from Toronto insulin having a unit value of 13–15 units per mg.

Test solution A made by dissolving 10·2 mg. in 50 cc., *i.e.* 0·204 mg. per cc.

B. Crystals from the same Toronto insulin, prepared by Harington and Scott's "saponin" method.

Test solution B contained 11·8 mg. in 50 cc., *i.e.* 0·236 mg. per cc.

C. Prepared from the same raw material, and by the same method as B, but on a different occasion.

Test solution C contained 10·2 mg. in 50 cc., *i.e.* 0·204 mg. per cc.

[1] In a recent private communication to Dr Dale, Prof. Abel states that his colleagues are now obtaining values of 3 to 3½ times the activity of the International Standard (*i.e.* 24–28 units) for different samples of the crystals assayed in comparison with that Standard.

D. A mixture of two small lots of crystals kindly furnished by Prof. Abel, and prepared by his methods in his own laboratory.

Test solution D contained 8·2 mg. in 50 cc., *i.e.* 0·164 mg. per cc.

It was decided that each should be assayed independently by the four authors of this part, each of whom has long familiarity with the standardisation of insulin for practical use. Two different types of method were used. Scott and Trevan used the essentially statistical method, based on the relative frequencies of the appearance of hypoglycaemic convulsions in a large number of mice injected with a dose of the preparation under test, and in an equal number of similar mice injected at the same time with a control dose of the standard preparation, and observed under identical conditions. The method was independently elaborated, and with some differences of detail, by Trevan and Boock [1926], and by Krogh [1926]. Both Marks and Culhane used the method based by Marks [1925, 1926] on parallel observations of the degree of hypoglycaemia produced, by a dose of the preparation under test and by a dose of the standard preparation, in the same series of rabbits on different occasions; changes in the sensitiveness of the whole series being eliminated by injecting half the number with each preparation on each occasion, and reversing the groups on the two days of the test. Variations in the details of the application of these tests are mentioned below.

So that no question should arise of differences in the weighing out or solution of the preparations, a sufficient quantity of solution for the tests of all four investigations was prepared by Dr Harington from each of the batches of crystals. Physiological saline, acidulated with HCl to p_H 2·5, was used for the solution of the crystals, and the solutions were not made up to identical strengths of the crystalline material. In each case a quantity in the neighbourhood of 10 mg., but varying in the four cases from 8·2 to 11·8 mg., was dissolved and made up to 50 cc.

A common stock solution of the International Standard Insulin was similarly prepared by Marks, who weighed out and dissolved the dry powder in distilled water, making a solution of acidity about p_H 3·5 which contained 2·5 mg. of the standard preparation, and therefore had an activity of 20 units per cc. For each test each of us received a sufficient quantity of the standard solution, containing 20 units per cc., and of the solution of one of the crystalline preparations, of which the concentration as well as the activity was unknown to him. It was understood, however, that, in order to save time and animals, the solutions of A, B, C and D would not differ widely in concentration.

Each of us completed the determinations on any one of the preparations, A, B, C and D, without consultation with the others. In each case we handed our estimates independently to Dr Harington or Dr Dale, and they were not divulged or submitted to us for comparison until all four had been deposited.

ESTIMATES BY D. A. SCOTT.

The experiments were carried out at the National Institute for Medical Research, with the equipment there available. The mouse method, as advocated by Krogh and by Trevan and Boock, was used. The details followed were mostly those given by Trevan and Boock [1926]. The thermostat bath, in use at the National Institute to maintain the temperature in the mouse-boxes during the experiment, follows the general lines of Trevan's description, but has a motor-driven stirrer, an electrically-controlled thermostat, and a convenient weighting of the boxes.

The method has been for some time in use in the Connaught Laboratories, University of Toronto, with the following departures in detail from Trevan and Boock's description.

1. Mice showing convulsions in the course of a test are restored by injection of glucose, and after a week the whole stock are found to be as suitable for a further test as a fresh stock of mice.

Krogh's experience and practice are here confirmed and followed.

2. The volume of solution injected into each mouse is 0·25 cc. According to our experience a larger injection volume entails danger of leakage. Since the mice used in any experiment are all of about the same weight, not varying more than 1 g. in either direction from the mean, the mean weight can be accepted for all, and the required dose per g. of standard or test solution made up so as to occupy 0·25 cc. per mouse.

3. Observation was completed in 1½ hours, in place of the 2 hours indicated by Trevan. Since about 90 % of the mice which show convulsions do so within the first hour, the time of observation could probably be made even shorter.

The mice used in these tests were all of weights between 19 and 21 g. All but a few were bred at the National Institute, and were from 2½ to 3 months old at the time of their first use for these tests. The diet of all consisted of white English oats and stale brown bread, the latter being moistened with water. The mice to be used in an experiment were transferred to an empty cage and deprived of food at 5 p.m. on the previous afternoon. The injections were begun at 10.30 a.m. Injections were subcutaneous, and were made with a convenient precision-syringe of 0·25 cc. capacity. Each mouse, after injection, was immediately transferred to its appropriate box in the thermostat bath, which was kept throughout the experiment at 37·5–38°. The mice were kept as quiet as possible, and the number showing actual convulsions, or equivalent symptoms of collapse, was recorded. Trevan's test, depending on the inability of a mouse to right itself immediately, when placed on its back, was found useful in the recognition of symptoms "equivalent" to convulsions. Such mice, when the reaction was established, were removed and given a hypodermic injection of 0·2 cc. of 20 % glucose. At the end of 1½ hours the score was regarded as completed, and the mice which had not

shown definite "convulsions" or "symptoms" were removed, and food was given to the whole stock.

The determination of the unknown dose of the solution under test, from the relation between the proportions of convulsions produced by it and by the known dose of the standard, was made from the curve published by Trevan and Boock. After a preliminary orientating test, giving the approximate unit value of the unknown solution, an attempt was made in subsequent tests to give this and the standard in approximately equivalent dosage, and in each case to produce a convulsion-rate in the neighbourhood of 50 %. From the actual rates observed the true dose can then be read off the curve with a relatively small error.

The protocols are given at the end of the next section. From these it is seen that the figures obtained range from 24·2 units per mg. for D to 25·35 units for C.

ESTIMATES BY J. W. TREVAN.

The tests were carried out by the mouse method as originally described by Trevan and Boock [1926], with the modification that the weights of the mice used were matched for the two solutions under test. Mice of 15–18 g. were used for one set of tests, mice from 18 to 25 g. (a very few being over 25) in another. None was used below 15 g. We have taken this precaution for some time.

The protocols are given at the end of this section. The figures in the last column of each table are calculated from the standard curve given in the original description of the method. The values for tests 1 and 2 on A are calculated from convulsion rates which are too low for much reliance to be placed on the calculated potency, although the results do agree with the later ones. However, the deduction can be made that the characteristic for insulin A is not significantly different from that of the International Standard given in the paper mentioned above.

One test (No. 5) on A was made, in which only low dilutions were used, and minute doses both of the standard and of A were injected by means of micrometer syringes. This was done to check whether in high dilutions the purer insulin was destroyed by surface action or shaking. No significant difference was found.

From the protocols, it is seen that the average figures vary between 22·8 units per mg. for C and 25·8 units for B.

Discussion of mouse test results.

The results of the mouse test by both workers are collected and compared in Table I. The degree of uncertainty of the final results is represented by the columns headed "range of possible values." These figures are calculated from the shape of the mouse insulin characteristic already published, and from the number of mice used, by the use of the formula given by Trevan

26—2

Table I. *Combined results and limits of error of mouse tests.*

	Scott		Trevan		
	Mean value (Units per mg.)	Calculated possible range	Mean value (Units per mg.)	Calculated possible range	Combined result
A	24·9	21·75–28·60	24·2	23·4–26·4	24·4
B	25·0	21·85–28·70	25·8	23·6–28·2	25·6
C	25·35	21·15–29·0	22·8	20·9–25·0	23·6
D	24·2	21·65–27·5	24·4	21·8–27·3	24·3
	Mean 24·8		Mean 24·3		Mean 24·5 (23·6–25·4)

[1929], assuming that the average convulsion rate is 50 %. This gives the error corresponding to approximately twice the standard deviation of the convulsion rate, and represents the range inside which the true value may be expected to fall with a probability of approximately 0·95. The discrepancy from the total average value of any single set of tests is not greater than might be expected to occur by errors of random sampling of mice. The most discrepant result is that of Trevan for C. The difference of this from the corresponding result by Scott on the same solution is, however, only 1·26 times its standard deviation. It is interesting that the individual values obtained for C by Trevan were much more concordant than those obtained

Protocols of mouse tests carried out by D. A. Scott.

	International standard 20 units per cc.			Solution of crystalline insulin			
Test No.	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Units per cc.
			Sample A.				
1	1/300	0·25 cc.	11/24	1/75	0·25 cc.	13/24	5·42
2	,,	,,	19/24	1/90	,,	15/24	5·05
3	,,	,,	18/24	1/75	,,	16/24	4·56
4	,,	,,	13/24	1/75	,,	15/24	5·42
						Mean	5·09
			Sample B.				
1	1/300	0·25 cc.	15/24	1/75	0·25 cc.	20/24	6·30
2	,,	,,	17/24	,,	,,	18/24	5·18
3	,,	,,	18/24	,,	,,	21/24	5·88
4	,,	,,	15/24	,,	,,	20/24	6·30
						Mean	5·9
			Sample C.				
1	1/400	0·25 cc.	20/24	1/100	0·25 cc.	19/24	4·72
2	,,	,,	16/24	,,	,,	18/24	5·45
3	,,	,,	17/24	,,	,,	20/24	5·70
4	,,	,,	16/24	,,	,,	15/24	4·80
						Mean	5·17
			Sample D.				
1	1/400	0·25 cc.	11/24	1/80	0·25 cc.	11/24	4·0
2	,,	,,	13/24	,,	,,	10/22	3·53
3	,,	,,	12/24	,,	,,	15/24	4·49
4	,,	,,	13/24	,,	,,	17/26	4·87
5	1/350	,,	15/24	1/70	,,	12/24	3·55
6	,,	,,	12/24	,,	,,	11/24	3·84
7	,,	,,	13/24	,,	,,	11/24	3·69
						Mean	3·97

for the other solutions, and this furnishes a good example of the uncertainty of relying on mere concordance of a limited number of observations as a guarantee of the accuracy of a result. The average result of the potency of all samples by the mouse test is 24·5 with a range of 23·6 to 25·4, the latter being worked out from the total number of mice used, namely, 2652.

Protocols of mouse tests carried out by J. W. Trevan.

	International standard 20 units per cc.			Solution of crystalline insulin			
Test No.	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Units per cc.
				Sample A.			
1	1/1000	0·5 cc.	25/30	1/1000	0·5 cc.	1/30	5·38
	,,	,,	19/30	,,	,,	0/30	
	,,	,,	21/29	,,	,,	0/29	
	,,	,,	19/30	,,	,,	1/30	
2	,,	,,	21/30	1/500	,,	2/30	4·37
	,,	,,	17/30	,,	,,	0/30	
	,,	,,	21/30	,,	,,	4/30	
3	1/1333	,,	19/30	,,	,,	15/30	6·61
4	,,	,,	18/30	1/400	,,	12/30	5·02
5	,,	,,	13/30	,,	,,	7/30	4·69
6	,,	,,	25/30	,,	,,	15/30	4·26
7	1/16	0·008 cc.	21/30	,,	0·008 cc.	24/30	5·75
8	1/100	0·5 cc.	24/30	1/250	0·5 cc.	16/30	3·82
9	,,	,,	18/30	,,	,,	10/30	4·68
10	,,	,	15/30	,,	,,	10/30	4·17
11	,,	,,	16/30	,,	,,	18/30	5·33
					Mean omitting tests 1 and 2		4·92
					Mean including tests 1 and 2		4·92
				Sample B.			
1	1/1000	0·5 cc.	20/30	1/250	0·5 cc.	18/30	4·67
2	,,	,,	17/30	,,	,,	21/30	5·70
3	,,	,,	19/30	,,	,,	24/30	6·00
4	,,	,,	23/30	,,	,,	25/30	5·40
5	,,	,,	12/30	,,	,,	25/30	7·85
6	,,	,,	14/30	,,	,,	17/30	5·51
7	,,	,,	12/30	,,	,,	23/30	7·22
8	,,	,,	21/30	,,	,,	26/30	6·15
						Mean	6·08
				Sample C.			
1	1/1000	0·5 cc.	21/30	1/250	0·5 cc.	15/30	4·11
2	,,	,,	17/30	,,	,,	17/30	5·00
3	,,	,,	22/30	,,	,,	18/30	4·46
4	,,	,,	22/30	,,	,,	20/30	4·65
5	,,	,,	20/30	,,	,,	19/30	4·83
6	,,	,,	18/30	,,	,,	17/30	4·83
7	,,	,,	24/30	,,	,,	22/30	4·61
						Mean	4·64
				Sample D.			
1	1/1000	0·5 cc.	13/30	1/250	0·5 cc.	6/30	3·69
2	,,	,,	10/27	,,	,,	8/27	4·61
3	,,	,,	16/30	,,	,,	8/30	3·74
4	,,	,,	10/30	1/200	,,	11/30	4·12
5	,,	,,	22/30	,,	,,	16/30	3·69
						Mean	3·97

Estimates by H. P. Marks.

For estimating the potency of the various samples of crystalline insulin, the rabbit cross-over method was used, on the lines laid down in the original communications on this test [Marks, 1925, 1926], but with the following modifications, suggested by later experience.

(1) *Weight of rabbits and dosage of insulin.* Culhane [1928] states that the test gives more consistent results when the two groups of rabbits are so chosen that, for each animal in one group, there is a corresponding animal of approximately the same weight in the other group. This procedure was therefore adopted, and it has the added advantage that the same dose of insulin may be given to all the rabbits regardless of body-weight, without disturbing the balance of the two groups.

With one or two exceptions, rabbits weighing between 2 and 3 kg. were used, and the dose of standard insulin throughout was 1 unit per rabbit, injected subcutaneously.

The standard solution was diluted ten times for use, so that the volume injected was in every case 0·5 cc. Either 10 or 12 rabbits were used for a test.

The danger of choosing a dose falling on the upper, flat portion of the dosage-response curve has already been pointed out [Marks, 1926]. In order to avoid obtaining a false match between sample and standard, the dose of sample to be compared with 1 unit of the standard was accordingly varied from one test to another, and, in all cases, this was reflected as a change in relative response.

(2) *Treatment of blood samples.* The simplified method of dealing with the samples of blood already suggested in the communications cited above was adopted. It consists in combining the five hourly blood samples taken from the same rabbit after insulin. The blood-sugar value determined on this composite sample, when subtracted from the initial value, then gives the average fall in blood-sugar during the five hours.

According to Sahyun and Blatherwick [1928], if a rabbit is given a certain dose of insulin after a fasting period of 24 hours, and the fasting continued and the same dose of insulin given again after a further 24 hours, the hypoglycaemic response to each dose is approximately the same. This observation seemed to offer such an attractive possibility of shortening the time required for the cross-test that the matter was immediately investigated, and it was found that to the same dose of insulin, repeated on the second day, the response was slightly greater than on the first day. Although there were occasional exceptions on the part of individual rabbits the results were certainly no less consistent than if the rabbits had been fed after the first test, and prepared again for a second test two or three days later.

In all tests except the first test on A, and the final test on D, the following procedure was therefore adopted.

Food was withdrawn from the rabbits in the afternoon, and on the following day the first part of the cross-test was performed as usual. Instead of feeding the rabbits after the test, fasting was continued and the second part of the test was carried through on the next day, after which the rabbits were fed. Rabbits used in this way regularly once a week (*i.e.* one complete test per week) suffered no ill effects, and increased in weight at the usual rate.

The results of the tests are collected in Table II.

Table II.

Sample	Dose of solution in cc. per rabbit	Estimated units in this dose	Units per mg. of crystals
A	0·167	0·88	26·0
	0·2	1·00	24·4
		Average	25·2
B	0·2	1·07	22·6
	0·187	0·92	20·8
		Average	21·7
C	0·2	0·90	22·0
	0·22	0·96	21·4
		Average	21·7
D	0·2	0·89	27·4
	0·225	0·99	26·9
	0·25	1·01	24·6
		Average	26·3

The estimated activity of the dose was in all cases calculated by reference to the dosage-response curve already published.

ESTIMATES BY K. CULHANE[1].

The determination of the potency of the four solutions was carried out by the cross-over method of Marks [1926] with no modifications except that certain precautions were taken with regard to the selection of suitable animals and the distribution of their weights [Culhane, 1928].

(1) *Choice of rabbits and dosage of insulin.*

The rabbits were selected from young animals which had been used previously for testing a few of our purest samples and found to give consistent results. These were divided into groups of approximately equal weights, the weights of all falling between 1600 and 2400 g. The standard was diluted so as to contain 2 units per cc. and the samples were also diluted to a volume expected to contain the same amount per cc., all doses, both of the sample and of the standard being adjusted to the weight of individual rabbits: the dose of the standard was always 0·5 unit per kg. of body weight.

In almost all of the tests 20 rabbits were used, but on rare occasions one or two were excluded from the result. This was only done when the rabbit

[1] It is a pleasure to acknowledge my indebtedness to Dr S. W. F. Underhill, Director of the Insulin A.B. Physiological Laboratories, for his valuable help and criticism during this investigation.

showed signs of illness during the course of the test or went into convulsions on one of the doses or gave a percentage reduction greater than 50 %, since such an animal is considered too sensitive for quantitative work.

(2) *Preliminary starvation period.*

All animals were starved for 22 hours preceding the test and fed immediately after the fifth hour after the injection. The second half of the test was carried out three or four days later after the same period of starvation.

(3) *Treatment of blood samples.*

To enable us to use so large a number of animals in one test the five hourly blood samples for each rabbit after injection were mixed and a practical average thus determined.

(4) *Calculation of results.*

Experience has shown that results which differ greatly from 100 % are often inaccurate, and, whilst there appears to be a general tendency for the error to cause them to approach nearer to 100 %, we have found it more useful to disregard such tests, except as an indication of a more suitable dose for the next, than to include their results in an average after applying the correction suggested by Marks [1925]. Hence, the result of the final test gives our conclusion for the potency of any solution. In one case only have we departed from this procedure, namely that of the last sample, of which only a small quantity was available; as all the results come within 86 and 89 % there seemed no reason for regarding one test as more accurate than another and the three have therefore been averaged, allowance being made for the number of rabbits used in each.

(5) *Results of the tests.*

The tests are summarised in Table III.

Table III.

Sample	Dose of solution in cc. per kg.	Result as percentage of standard	Units per cc.	Units per mg. of crystals
A	(1) 0·083	79	4·7	
	(2) 0·106	87	4·1	20·1
	(3) 0·125	102	4·1	
B	(1) 0·125	107	4·3	18·2
C	(1) 0·119	104	4·4	21·6
D	(1) 0·119	89	3·7	
	(2) 0·135	86	3·2	20·7
	(3) 0·135	89	3·3	

SUMMARY OF RABBIT TEST RESULTS.

Since not more than three rabbit tests were performed by either worker on any one sample, it is impossible to form any opinion as to the comparative degree of accuracy of the two sets of figures. It is readily apparent, however, that Culhane's figures show less variation in activity between the different samples than do those of Marks. If all the tests carried out by each worker

respectively are considered on the assumption that all the samples have the same real activity, we find that the average of all Culhane's results (excluding test A 1, as being below 80 %) is 20·3 with a standard deviation of 0·5 for the mean, while the corresponding figures for Marks's tests are 24·0 and 0·75 respectively. Put in another way we may say that the probability is 0·95 that the true mean value lies between 19·3 and 21·3, according to Culhane's figures, or between 22·5 and 25·5 according to Marks's figures. Taken as a whole, all the rabbit tests give an average figure of 22·3 with a standard deviation of 0·7 for the mean, *i.e.* the probability is 0·95 that the true value lies between 20·9 and 23·7.

Conclusions and discussion of combined results.

If we review the results obtained by the different investigators, as presented in the preceding sections, it becomes clear at once that there is no wide divergence between the values obtained by any one of us for the four different samples. The largest such difference is that between B or C and D obtained by Marks, but as this is not supported by the other investigators, no significance can be attached to it.

A comparison of Culhane's results with those obtained by Scott and Trevan would suggest that the rabbit test gives a consistently lower value for the crystals than the mouse test, but against this must be set the relatively close agreement between Marks's average of 23·7 for all four samples, and the average of 24·5 for all the mouse tests. It would indeed not be surprising if the rabbit test and mouse test should give somewhat different results when comparing insulin preparations of different degrees of purity, since the physiological reaction forming the basis of measurement is different in the two cases. Even if we assume that the crystals represent pure insulin, we must suppose, from our own data, that the standard preparation contains approximately two-thirds of its weight of other substances, of which some may not be physiologically inert. If the resultant effect of these should be to inhibit, even slightly, the convulsant action on mice, while disturbing less the average hypoglycaemic effect produced in a rabbit over a period of hours, the result would be to show a higher unit value for the crystals in the mouse test than in the rabbit test. Again, differences in the rate of absorption of the injected insulin, if such occurred, might account for the discrepancies observed. We can only say that the course of the hypoglycaemia, which was studied in some of the rabbit tests, gives no clear indication of any difference in this respect between the crystalline insulin and the International Standard. Realising these inevitable uncertainties of the comparative methods of biological measurement, we consider that the fairest procedure is to average the results for the whole series. In Table IV, therefore, we have averaged the results of each investigator for all four samples, and of all four investigators for each sample.

Table IV.

	A	B	C	D	Average
Marks	25·2	21·7	21·7	26·3	23·7
Scott	24·9	25·0	25·3	24·2	24·8
Trevan	24·2	25·8	22·8	24·4	24·3
Culhane	20·1	18·2	21·6	20·7	20·2
Average	23·6	22·7	22·9	23·9	

Looking at the results as a whole, we think that the two questions which we propounded at the outset may fairly be answered as follows.

1. Different batches of the crystals, prepared by different methods, have no differences of activity which the available methods of assay can detect.

2. The activity shown by all the four samples assayed in comparison with the International Standard, and based on the average of the values obtained by the four investigators for each sample, may be assessed at 23·3, with a standard deviation for the mean of $\pm$ 0·6.

This latter result is in close conformity with the values of 23 and 26 units obtained by Freudenberg and Dirscherl [1928] for the particular sample of the crystals which they tested. We can state with some confidence that a sample of such crystals as we have tested, if adopted in the future as a stable standard of reference, might be assumed to possess three times the activity of the present standard, *i.e.* 24 units per mg., without any significant disturbance of the biological unit, as defined in terms of the current International Standard.

The uniform activity of the four batches of crystals seems to us to indicate that their substance has a closer relation to the specific insulin activity than that of an inert adsorbent of an intensely active contaminant. On the other hand, the activity of the crystals is little, if at all, different from that of the most active amorphous insulin, obtainable by pushing to the limit the fractionations used in ordinary manufacture. We are familiar with preparations, commercially available in the ordinary way, in which the unit value per mg. is always in the neighbourhood of 20 and sometimes as high as 22 units per mg. The production of the crystals from such material would accordingly not entail the separation of a highly active principle from a crude mixture, but rather the creation of conditions allowing the crystallisation of a substance already almost pure. It would be analogous rather to the crystallisation of serum-albumin, for example, than to the isolation of such a hormone as adrenaline.

In conclusion we wish to express our indebtedness to Dr H. H. Dale, F.R.S., for his continued interest in this work, and for placing the facilities of his laboratory at the disposal of one of us (D.A.S.) for the animal experiments. We wish especially to thank him for his cooperation in arranging the physiological assay, which could not have been so extensive without his help.

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