THE ASSAY OF INSULIN *IN VITRO* BY FIBRIL FORMATION AND PRECIPITATION

BY G. E. FOSTER, JEAN MACDONALD AND J. V. SMART

From the Wellcome Chemical Works, Dartford

Received June 22, 1951.

IT is well known that when a solution of insulin in dilute acid is heated a series of changes takes place which finally result in the insulin being thrown out of solution as a flocculent precipitate. This phenomenon was first reported by Blatherwick *et al.*[1] in 1927, and a year later was studied in greater detail by du Vigneaud *et al.*[2], who reported that the "heat precipitate" was biologically inactive, but that it could be reactivated by dissolving in dilute sodium hydroxide solution and subsequently acidifying. It was stated that the insulin lost about one fifth of its potency during the cycle.

More recently Waugh[3,4,5,6] has reported a series of investigations which have greatly increased our knowledge of the mechanism of the heat precipitation of insulin. According to the conditions of heating, a clear gel, exhibiting either flow or static double refraction when viewed between polaroid screens, or a precipitate is formed, and Waugh was able to follow the course of the reactions involved by observing the double refraction and viscosity of his solutions. The first change was found to be the formation of fibrils, which the electron microscope showed to possess diameters of about 150 Å and lengths of 15,000 to 40,000 Å. Fibrils elongate when present in solutions of insulin and, under suitable conditions, a precipitate composed of flocs of spherites, whose subunits are radially orientated fibrils, is formed. Moreover, when a solution of insulin, well within the stability range in respect of temperature and *p*H, is seeded with a cooled suspension of fibrils formed at 80° to 100°C. the fibrils elongate at the lower temperature and quantitatively remove the insulin from solution in the fibrous form. It was also observed that insulin could often be separated from impure preparations by seeding solutions with fibrils, which specifically brought about the precipitation of the insulin. Regeneration of the latter by treatment with alkali and acidification afforded a product indistinguishable from insulin.

These observations suggested the possibility of the assay of insulin *in vitro* by fibril precipitation, and Waugh *et al.*[7] have recently published such an assay process. These workers seeded their test solutions, adjusted to *p*H about 2, with a suspension of fibrils, stirred the mixture at 38° to 48° C. until precipitation was complete and subsequently removed, washed, dried and weighed the precipitates. Excellent agreement between biological assays and fibril assays was reported for a series of insulin solutions and crude insulin concentrates.

The biological assay of insulin preparations is such a costly process that any more economical and reliable process would obviously be of

the greatest value. Accordingly, we have made a careful investigation of the process, proposed by Waugh *et al.*[7], and our results are described in the present report.

General Assay Process

During the course of our work we followed closely the methods described by Waugh *et al.*[7] with minor amendments. The process consists of three operations, (i) preparation of insulin gel, (ii) preparation of seeding fibrils and (iii) standard assay. It is convenient to describe the operations under these headings.

Preparation of Insulin Gel. 2·0 per cent. solutions of crystalline insulins from pig, ox and sheep pancreas respectively, were prepared in 0·05 N hydrochloric acid and 2 ml. portions were sealed in neutral glass ampoules. The ampoules were immersed in boiling water for 1 to 2 minutes, withdrawn, cooled and the solutions frozen rapidly by immersion in a mixture of solid carbon dioxide and acetone. They were then thawed and subjected to a second heating in boiling water for 2 minutes and again frozen and thawed. The process was continued until the contents of the ampoules exhibited strong static double refraction when examined with a glass strain viewer.

Preparation of Seeding Fibrils. In order to break up the long fibrils of the gel before use the contents of an ampoule were repeatedly drawn into and expelled from a syringe fitted with a long 16 s.w.g. hypodermic needle. The mixture was then frozen by cooling in a mixture of solid carbon dioxide and acetone and thawed rapidly. The cycle of operations was repeated and 0·1 ml. (equivalent to 2 mg. of insulin) of the mixture, accurately measured, diluted with 1 ml. of 0·05 N hydrochloric acid. The resulting solution was added to the insulin solution to be assayed.

Standard Assay. If necessary the solution to be assayed was filtered until crystal clear when 70 ml., accurately measured, was transferred to a centrifuge tube, 1 ml. of 20 per cent. sulphuric acid added followed by 2 mg. of insulin, as a suspension of seeding fibrils prepared as above described, and the volume made up to 80 ml. with water. The tube was then mounted in a thermostatic bath at 48°C. and the mixture stirred mechanically for 16 to 18 hours with a straight glass rod, in such a manner that no air was stirred into the solution. The resulting precipitate was separated by centrifuging until the supernatant liquid was crystal clear (45 minutes), and washed once with a solution (15 ml.) containing 1 per cent. ammonium chloride in 0·01 N hydrochloric acid containing 65 per cent. alcohol. It was found convenient to transfer the precipitate to a smaller centrifuge tube at this stage by suitably using a 5 ml. hypodermic syringe and the washing solution.

The precipitate was then subjected to two washings, each of 15 ml., with 0·01 N hydrochloric acid in 65 per cent. alcohol and finally washed with acetone (15 ml.). Some difficulty was experienced with the removal of the last traces of precipitate from the original reaction tube. It was found that this was facilitated by dividing the 2nd and 3rd washings, of 0.01 N hydrochloric acid, into three portions, each of 5 ml., consisting of

0·01 N hydrochloric acid in 95 per cent. ethanol, 0·01 N hydrochloric acid in water and 0·01 N hydrochloric acid in 95 per cent. ethanol. When the tube was washed out with the portions in the order indicated any precipitate adhering to the glass was loosened.

Finally the precipitate was spread by means of a small glass rod over the inside of the tube, which was then dried, together with the rod, in a vacuum over phosphorus pentoxide until of constant weight. The precipitate was then removed by washing with dilute sodium hydroxide solution, the tube and rod washed well with water, dried and weighed. The difference in weight gave the weight of the insulin fibrils, which were taken for the purposes of our work, to be equivalent to a potency of 25 I.U./mg. A correction was made for the weight of the seeding fibrils (2 mg.).

Injection of Insulin.

In preliminary experiments in which the fibril assay was applied to solutions, prepared with crystalline insulin and using pig insulin fibrils for seeding, excellent results were obtained and the recoveries were almost quantitative. It was therefore decided to carry out a series of assays on two routine batches of injection of insulin B.P. The results are summarised in Tables I and II.

TABLE 1

Fibril assay on 70 ml. of insulin injection (B.4340)—40 i.u./ml.
[Biological assay :—potency = 37·8 i.u./ml. Limits of error (P = 0·95) = 33·9 and 42·2 i.u./ml.]

Assay No.	Weight of fibril precipitate g.	Mean weight of precipitate g.	Equivalent potency per ml., calculated from mean weight I.U./ml.
1	0·1011	—	—
2	0·1038	—	
3	0·1068	—	—
4	0·1077	—	—
5	0·1082	0·1058	37·8
6	0·1013	—	—
7	0·1114	—	—

The standard deviation for the weights of precipitates recorded for assays Nos. 1 to 7 is 0·0038 g. corresponding to equivalent limits of error (P = 0·95) on the potency of ±1·25 I.U./ml.

The very good results obtained with these freshly prepared injections made it of special interest to ascertain whether the fibril assay will detect loss of potency in an injection on storage. Encouraging evidence on this point has been reported by Waugh *et al.*[8], who found good agreement between biological and fibril assay figures in a heat test on one sample of insulin solution. It was, therefore, fortunate that we had available a sample of insulin injection (40 I.U./ml.) which had been stored in sealed glass ampoules at room temperature for 5½ years. A biological assay on

this preparation gave the mean potency as 31·4 I.U./ml. with limits of error (P = 0·95) of 26·3 to 37·6 I.U./ml. Two fibril assays, however, gave the potency as 40·95 and 39·0 I.U./ml. respectively. The injection

TABLE II

FIBRIL ASSAY ON 70 ML. OF INSULIN INJECTION (B.4360)—40 I.U./ML.
[BIOLOGICAL ASSAY :—POTENCY = 41·8 I.U./ML. LIMITS OF ERROR (P = 0·95) = 38·9 AND 45·0 I.U./ML.]

Assay No.	Weight of fibril precipitate g.	Mean weight of precipitate g.	Equivalent potency per ml., calculated from mean weight I.U./ml.
8	0·1121	—	—
9	0·1134	—	—
10	0·1077	—	—
11	0·1114	—	—
12	0·1126	0·1111	39·7
13	0·1097	—	—
14	0·1106	—	—

The standard deviation for the weights of precipitates recorded for assays Nos. 8 to 14 is 0·0019 g. equivalent to limits of error (P = 0·95) on the potency of ±0·61 I.U./ml.

exhibited flow double refraction suggesting the formation of fibrils during storage, although the preparation did not show any obvious increase in viscosity.

Among observations made during our work on injections, the supernatant liquid separated from the fibril precipitate during an assay was found to be devoid of biological activity. A solution of regenerated insulin, prepared from a sample of dry fibril precipitate by alkali treatment and subsequent acidification, had a biological activity equivalent to a mean potency of 17 I.U./mg. calculated for the dry material used. The fibrils themselves were found to be devoid of activity.

IMPURE INSULIN PREPARATIONS

In order to explore the application of fibril precipitation in the assay of crude insulin preparations a sample of impure insulin, having a mean potency of 11 I.U./mg., was employed. A solution containing a mixture of crystalline and crude insulin was prepared and a series of fibril assays carried out using various volumes of solution, suitably diluted, for the assays, and the results calculated in terms of potency of the original solution. The results were most erratic and no reliance could be placed on them. It was concluded that satisfactory precipitation only occurs when solutions of substantially pure insulin are employed. We therefore carried out a series of assays on some samples of concentrates, representing the three final stages in the manufacture of crystalline insulin. The results are summarised in Tables III, IV and V.

Stages A and B were not assayed in the case of the preparation from sheep pancreas as the amount of insulin present was considered too small before concentration.

TABLE III

FIBRIL ASSAYS ON INSULIN CONCENTRATES FROM OX PANCREAS (BATCH 8355)

Stage of process	Description of solution assayed	Weight of fibril precipitate g.	Fibril assay result as per cent. of yield of insulin from batch
A.	20 ml. from solution before adding second salt.	0·0779	113·0
B.	20 ml. from solution before adding sodium hydroxide for iso-electric precipitation.	0·0718	104·0
C.	5 ml. from solution before pouring into buffer.	0·0739	107·0

TABLE IV

FIBRIL ASSAYS ON INSULIN CONCENTRATES FROM OX PANCREAS (BATCH 8422)

Stage of process	Description of solution assayed	Weight of fibril precipitate g.	Fibril assay result as per cent. of yield of insulin from batch
A.	40 ml. from solution before adding second salt.	0·2764	101·0
B.	40 ml. from solution before adding sodium hydroxide for iso-electric precipitation.	0·2777	101·5
C.	5 ml. from solution before pouring into buffer.	0·1370	100·0

TABLE V

FIBRIL ASSAYS ON INSULIN CONCENTRATES FROM SHEEP PANCREAS (BATCH 8375)

Stage of process	Description of solution assayed	Weight of fibril precipitate g.	Fibril assay result as per cent. of yield of insulin from batch
C.	20 ml. from solution before pouring into buffer.	(1) 0·2300 (2) 0·2256	(1) 107·5 (2) 105·5

INJECTION OF PROTAMINE ZINC INSULIN

In order to investigate the behaviour of injection of protamine zinc insulin when subjected to the fibril assay process, experiments were carried out with a solution of protamine sulphate of the same concentration as is present in the injection (80 I.U./ml.). Unseeded protamine sulphate solution gave no precipitate when stirred under the assay conditions at 48°C. for 16 hours, while seeded solutions afforded precipitates, the dry weight of which was only equal to that of the seeding fibrils. It

was concluded that protamine is not precipitated under the assay conditions and a series of fibril assays was, therefore, carried out on a routine sample of injection of protamine zinc insulin B.P. - 80 I.U./ml. The results are summarised in Table VI.

TABLE VI

FIBRIL ASSAY ON 40 ML. OF INJECTION OF PROTAMINE ZINC INSULIN (BATCH 149) 80 I.U./ML. [BIOLOGICAL ASSAY :—POTENCY=83·0 I.U./ML. LIMITS OF ERROR (P=0·95) =74·6 AND 92·4 I.U./ML.]

Assay No.	Weight of fibril precipitate g.	Mean weight of precipitate g.	Equivalent potency per ml., calculated from mean weight I.U./ml.
15	0·1282	—	—
16	0·1245	—	—
17	0·1223	—	—
18	0·1332	—	—
19	0·1271	—	—
20	0·1305	0·1283	80·2
21	0·1306	—	—
22	0·1285	—	—
23	0·1301	—	—

The standard deviation for the weights of precipitates recorded for assays Nos. 15 to 23 is 0·00335 g., equivalent to limits of error (P=0·95) on the potency of ±1·56 I.U./ml.

During these assays it was found that the 40 ml. of injection of protamine zinc insulin assayed remained opalescent when acidified with the sulphuric acid but became bright and clear when diluted, as directed in the standard assay process. The injection was well shaken to obtain a uniform suspension before sampling.

INJECTION OF PROTAMINE ZINC INSULIN

Some preliminary experiments were performed in order to ascertain whether the fibril assay would succeed in the case of injection of globin zinc insulin. Globin solutions, of the same strength as used in the injection, afford little if any precipitate when stirred at 48°C. for 16 hours, under the assay conditions. When the experiment was repeated using globin solution which had been seeded with insulin fibrils, however, a precipitate equivalent to about 45 per cent. of the globin was formed. Several assays were tried with a batch of injection of globin zinc insulin (80 I.U./ml.) but in all cases the results for the estimated potency were far too high.

DISCUSSION

The results described in this communication leave no doubt as to the merit of the assay process proposed by Waugh *et al.*[7]. In fact, it is the most promising *in vitro* process which we have tried for the assay of insulin. Our work has shown, however, that fibril precipitation of native

insulin is not entirely specific and the assay only succeeds under controlled conditions using solutions of substantially pure insulin. Although success was achieved with freshly prepared injections of insulin, injections of protamine zinc insulin and final concentrates from the manufacturing plant, no reliable results were obtained with a solution of crude insulin (11 I.U./mg.) or with injections of globin zinc insulin. A large discrepancy between the biological and fibril assay results were also observed for a 5½ year old sample of injection of insulin which exhibited flow double refraction, indicating the formation of fibrils in the injection on storage.

Waugh *et al.*[7] used both pig and ox insulin fibrils for seeding purposes and concluded that the former were more reliable. They reported that while ox insulin fibrils would quantitatively precipitate ox insulin they only partially removed pig insulin from solution. On the other hand, pig insulin fibrils were effective with both types of insulin. We have used fibrils prepared from pig, ox and sheep insulin in our work and confirmed that pig insulin fibrils give the best results. In some assays using ox and sheep insulin fibrils very low results were obtained, but in other cases quantitative precipitation occurred. We formed the opinion that for the assay to succeed with ox and sheep insulin fibrils the reaction mixture required more vigorous stirring than with pig insulin fibrils. The insulin solutions used in these experiments were prepared with ox insulin, mixed with small percentages of pig and sheep insulins.

The practical details of the fibril assay are so simple that there is little upon which to comment. Special attention, however, must be paid to the stirring of the reaction mixture. Waugh *et al.*[7] recommended a speed of 2,500 r.p.m. for the straight rod stirrer but we have found lower speeds equally successful if the rod is mounted slightly eccentrically in the chuck of the motor. No air should be stirred into the mixture and it is desirable to have a suitable cover for the reaction tube in order to reduce evaporation from the reaction mixture. There must be no friction between the cover and the stirrer as this will result in the formation of powder which will fall into the mixture and vitiate the assay.

As a result of our experiments the following conclusions have emerged. The fibril precipitation process has a limited but useful application in the assay of insulin preparations. While it cannot be relied upon to indicate the biological activity of a product, of unknown history, it may be applied successfully for routine control of manufacturing operations under conditions which have been experimentally established as giving satisfactory results. In our view, its most valuable application is likely to be in the testing of concentrates during the final stages of the manufacture of crystalline insulin. Fibril formation may also be used to detect loss of potency of insulin injections on storage. Gel formation in insulin injections seldom occurs in this country but in tropical regions it is not uncommon to find a phial of injection containing a gel, having thixotropic properties. When this occurs deterioration is obvious, but if the fibril formation has not reached the gel stage it may be detected by the flow double refraction exhibited by the injection.

SUMMARY

1. The recently-published method for the assay of insulin *in vitro* by fibril precipitation has been investigated.

2. Reliable results were obtained in assays on freshly-prepared injections of insulin, protamine zinc insulin and concentrates, representing the last three stages in the manufacture of crystalline insulin.

3. The fibril precipitation assays failed with a solution prepared with low potency insulin (11 I.U./mg.) and also with injections of globin zinc insulin.

4. A significant discrepancy was found between the biological and fibril assay results for a 5½ year old injection of insulin, which exhibited flow double refraction.

5. It was concluded that the fibril assay is only applicable under controlled conditions with preparations of substantially pure insulin.

We wish to thank Dr. A. C. C. Newman and Mr. R. N. Fox for samples of pig, ox and sheep insulins and for insulin concentrates used in this work. We also wish to thank the Directors of The Wellcome Foundation, Ltd., for permission to publish our results.

REFERENCES

1. Blatherwick, Bischoff, Maxwell, Berger and Sahyun, *J. biol. Chem.*, 1927, **72**, 57.
2. du Vigneaud, Geiling and Eddy, *J. Pharmacol.*, 1928, **33**, 497.
3. Waugh, *J. Amer. chem. Soc.*, 1944, **66**, 663.
4. Waugh, *ibid.*, 1946, **68**, 247.
5. Waugh, *Fed. Proc.*, 1946, **5**, 111.
6. Waugh, *J. Amer. chem. Soc.*, 1948, **70**, 1850.
7. Waugh, Thomson and Weimer, *J. biol. Chem.*, 1950, **185**, 85.
8. Waugh, Thomson and Weimer, *ibid.*, 1950, **185**, 94.

DISCUSSION

The paper was presented by DR. G. FOSTER.

The CHAIRMAN asked whether the phenomenon described was likely to appear during normal storage.

DR. F. HARTLEY (London) said it was unfortunate that the method under discussion was unlikely to fulfil the hopes which were aroused when it was first published in America. In his view it was a little too much to expect that the loss of potency which occurred in insulin injection on storage could be assessed by that method. For instance, one sample after 5½ years showed a very much greater loss of potency when examined by a biological method of assay than by the fibril method.

Dr. D. C. GARRATT (Nottingham) confirmed from his own experience that the method described gave good results on fresh material.

DR. FOSTER, in reply, said that gel formation could occur on storage, but did not often happen in this country. Fibril formation might start without proceeding to the gel stage, and could be detected by the double refraction which resulted. If fibrils were formed during storage it might be possible to filter off and assay the filtrate, but he agreed that the method would not be reliable for testing the biological activity of an insulin injection after storage.