From muck to molecule: insulin discovery over 50 years

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Abstract

A pancreatic extract which was successful in lowering glucose in diabetes was developed and commercialized with leadership from the University of Toronto in 1921-1922. The active principle remained unknown, though it was assumed to be the 'insulin' (or 'isletin' or 'insuline') identified microscopically in the islets of Langerhans from work in the previous 50 years. Within four years the active principle was crystallized by Abel and co-workers, and convincing proof given that it was a peptide. Determining the amino acid sequence of this relatively small protein proved a 30-year task for science, due to the confounding effects of two short chains united by di-sulphide bridges. Even then it was a mystery how the sequence related to insulin activity.

That remained the case when the early X-ray diffraction work in the 1930s by Crowfoot (Hodgkin) matured in 1969 with the determination of the 3-dimensional structure of the insulin hexamer. Meanwhile 25 years of work, much in industry, invented useful extended-acting insulin preparations and, over an even longer time course, insulin preparations of high enough purity to be non-immunogenic in clinical practice. In the 1960s and 1970s work on radioimmunoassay and on glucose clamps provided tools that would prove critical to the further development of insulin as a medication over its second 50 years.

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The pancreatic extract

A legal assignment signed on 19 December 1922 by Frederick G Banting MD, Charles Hubert Best and James Bertram Collip, and effected 01 January 1923, specifically concerns an 'Extract obtainable from the mammalian pancreas or the related glands of fishes', detailed wording indicating that the assigners 'invented an extract'.[1] There is no mention of insulin. Indeed, while the extract prepared from slaughterhouse beef pancreas by Collip produced the first clear lowering of blood and urine reducing sugars on 23 January 1922 (following very unclear results from Banting and Best's extract on 11 January of that year),[2] there was no evidence of what the active principle was, and indeed no prior evidence of what those pancreatic β-cell granules which disappeared in diabetes might be.[3-6]

The concentration of the active principle was also unknown, and is difficult to establish in retrospect. Collip seems to have been producing 2-4 cc injection aliquots soon after the first successful injection,[2] so perhaps purity was as low as 10% of all extracted matter. Thus, insulin had not been isolated, and certainly not 'discovered'. The assignment transferred the rights to the extract to the University of Toronto, and was formally supervised by JJR Macleod, who himself was already a tenured member of the University. The assignment followed heated and close to violent exchanges over the previous months, notably between Banting and Collip, the latter indicating at one stage that he intended to patent his process.[2] Banting believed strongly that preparations containing insulin would need to be made available quickly and at lowest possible cost across the world and, aided by advice on patents and agreements from George Clowes of Ely Lilly, the assignment allowed the University to freely license the process.[2]

Clowes, like Macleod of British extraction, was himself an active researcher with his own laboratories far from the headquarters of the company for which he was research director. Clowes and Macleod knew each other: they had met well before Banting's proposal to Macleod was made, and could well have implanted in Macleod's mind the idea (see article by Alberti and Bailey in this supplement) of a pancreatic extract.[7] The relationship with Clowes became important, as it was with Lilly that Toronto partnered in translating the laboratory process to commercial production, notably harnessing the skills and knowledge of the production chemist George Walden, who set on its way the path from pancreatic extract to isolation and definition of insulin.

Purifying, crystallizing, and identifying insulin

Walden knew how important the quality of reagents was in obtaining consistency in handling biological materials, and quickly established a reproducible process that overcame the erratic performance of the methods of Banting and Best, and of Collip.[2] At one point Lilly were supplying their insulin preparation to Toronto hospitals, when the local researchers' methods unaccountably failed. Walden also set in motion the movement to successful isolation of insulin itself by the introduction of isoelectric precipitation, which produced an amorphous, non-crystalline precipitate of insulin which was perhaps 60-70% insulin. In Toronto the University was closely linked with Connaught Laboratories (early insulin formulations for injection had both institutions named on the label), and Connaught had its own

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Table 1 Steps in the discovery of insulin

Year	New knowledge	Researcher	Note
1877	Pancreatic origin of diabetes	Lancereaux[4]	Cawley speculated on the relationship of the pancreas to diabetes, 1788 Confirmed by pancreas removal, von Mering and Minkowski, 1889
1901	Islets of Langerhans source of anti-diabetic substance	Opie[6]	Islet morphology described by Langerhans, 1869
1914-1921	Diverse demonstrations of a glucose-lowering pancreatic extract in animals	Notably Zeulzer, Paulesco, Banting and Best	See article by Alberti and Bailey in this issue[7]
1922	Clinically useful pancreatic extract and commercialization	Banting, Best, Collip, Macleod, Walden[1,2]	Quickly licensed globally
1926	Insulin crystals	Abel[9]	
1928	Insulin a protein	Abel and colleagues[10]	Atomic composition, amino acids detected
1935	Insulin crystals have structure	Crowfoot (Hodgkin)[13-15]	Purely qualitative in 1935, data-loaded in 1938, 1939
1956	Amino acid sequence; di-peptide chain	Sanger[17]	Defines the insulin monomer, 2-dimensionally
1969	3-D structure of the insulin hexamer	Blundell, Dodson, and Hodgkin[15]	Insulin has other conformations

laboratories investigating biological products (including protamine, Canada being a good source of salmon and trout).

It had been known for some time that whatever the active principle (named 'insuline' or 'isletin') might be it was in the pancreas (Table 1), and it was inactivated by oral ingestion. Though that might be due to chemical instability, destruction by digestion would imply a protein (or fat or carbohydrate or adduct of two of these). Later, a number of very early clinical attempts at oral administration of commercial insulin preparations failed.[8]

John Abel and colleagues in 1926 were able to use redissolved amorphous precipitate to produce 'highly refractive' insulin crystals, which when redissolved proved very potent at lowering blood sugar levels in rabbits.[9] He noted the substance contained sulphur, and given the potency described it as a 'hormone'. A positive ('beautiful') biuret reaction identifies the presence of nitrogen, strongly suggesting a protein; this was supported by a positive Millon's reaction (detecting the phenolic ring of tyrosine) and ninhydrin test (detecting lysine amine side chains). Crystals are only formed with higher purity, likely here over 90%, and so this paper marks the first isolation of the insulin molecule, the true 'discovery' of insulin (Table 1).

Cameron, writing only two years later, summarizes the further work from Abel's laboratory, including insulin's molecular content $C_{90}H_{150}0_{34}N_{22}S_2$ (or multiple thereof), and the presence of disulphide bonds and various amino acids.[10] The complexity compared to 'adrenine' (epinephrine) and thyroxine is noted, and the general properties are said to be those of a 'proteose' (a polypeptide). In discovery terms this is rather like finding a new vertebrate fossil, being aware it has bones (and thus muscle and blood and a nervous system) but not having any idea of what the animal might have looked like.

Understanding insulin

Insulin action is still not fully understood, partly because it is a very flexible molecule when in solution, and indeed changes shape on receptor interaction.[11] But understanding of the importance of zinc in insulin crystallization, notably by David Scott in Toronto in the early 1930s,[12] allowed the production of more substantial crystals that could be subject to novel techniques of investigation. Crystals from the Boots Pure Drug Company, a sub-licensee of the rights to insulin production in the UK, were the source of the self-described most wonderful moment in one lady's academic life, when Dorothy Crowfoot (later Hodgkin) in Oxford in 1934, using early, low-power, X-ray crystallography, determined asymmetries in the diffraction pattern, meaning that insulin had structure.[13] A curious anecdote here is that another hero of insulin development, Hans Christian Hagedorn, a few months later found no diffraction pattern in insulin crystals, seemingly unaware of Crowfoot's publication in Nature that year (1935). Crowfoot's work advanced rapidly so that by 1939 she could describe different types of insulin crystal in some detail, including a molecular weight and the insight that the molecule had a substructure of perhaps three or six parts.[14,15]

Full elucidation of the hexameric structure had to wait until 1969, and members of the Zoology Department in Oxford still remember the celebrations around Hodgkin's team that summer when the latest X-ray diffraction patterns were finally interpreted.[16]

That was of course aided by understanding of the amino acid sequence of insulin. As noted above, many amino acids were identified in Abel's laboratories in the late 1920s, but the two-peptide chain sequence of insulin, united by cystine bonds, and the difficulties of determining sequence rather than content, defied chemists until the work of Frederick Sanger in the early 1950s.[17] Even then, the Nobel Prize-winning work took some five years. As with Hodgkin's work there was a limit to the understanding, rather than knowledge, of insulin gained. Asked at a lecture at Manchester University in 1965 what insight the 2-D structure gave into the mechanism of action of insulin, Sanger replied in one word: 'None'. (I was a schoolboy in the audience – the question was asked by my biology teacher.)

Inventing clinically useful insulin preparations

The first 50 years after the invention of the pancreatic extract is marked in retrospect by two major developments in the nature and quality of insulin preparations, and two scene-setting advances in clinical science that would be applied to great effect subsequently.

Purification

As noted above, rapid advances were made in Toronto and by Walden at Eli Lilly in reducing the mainly protein impurities in the early extracts. Isoelectric precipitation and then recrystallization were key advances, and became the standard methods into

the 1960s. But that decade saw advances in chromatographic techniques applied on a commercial scale, initially gel filtration and then ion-exchange chromatography.[18,19] The so-called monocomponent and rarely immunogenic animal insulins essentially abolished the immune problems of injection-site lipodystrophy, immunological insulin resistance and neonatal hypoglycaemia, at least in those patients/mothers who had never been exposed to earlier insulin preparations.

These advances proved their worth beyond the first 50 years, when the introduction of fermentation-manufactured (bacteria, yeasts) insulins produced a new imperative for purification by chromatography, one that has not failed to date.

Extended-acting insulins

It became clear, in the first year of insulin use, that a single subcutaneous injection of the unmodified extract would not control glucose and ketones for 24 hours. While two injections were widely used, it can be speculated that this was only possible because of the retarding properties of the impure preparations and insulin antibodies. Anecdotal reports are of physicians with diabetes, such as Robin Lawrence, giving themselves up to seven injections a day. Accordingly, the hunt was on for extended-acting preparations, though with limited understanding of insulin physiology this was seen as being aimed at a once-daily preparation.

Clinically useful extended-acting preparations took 25-30 years to develop, though the scene-setting begins with Scott and Hagedorn's work in the 1930s on interactions with zinc and protamine.[20,21] Other approaches which were tried included gum arabic, oils, tannin, lecithin, adrenaline, vasopressin, cholesterol and diverse proteins, and while some could prolong action, their erratic effects prevented standardization. Although DA Scott worked at Connaught Laboratories, and Connaught were already manufacturers of protamine preparations, he attributes the idea of combination with protamine to Hagedorn. It took more than a further decade to establish the right conditions for the preparation of neutral protamine crystals, major challenges being the zinc content, optimal pH, the precise ratio of insulin:protamine, and the critical role of phenol and its derivatives.[22]

Rivalry in north Copenhagen was strong, however, between the Nordisk Insulinlaboratorium and Novo Terapeutisk Laboratorium, both having been licensed insulin manufacturers since 1923. Knud Hallas-Møller, who joined the latter in 1937, built on the experience of zinc concentrations on solubility to devise the insulin-zinc suspension series of insulin preparations, semilente, lente and ultralente.[23] Lente had a very similar profile of effect as NPH insulin,[19] and was a mainstay of twice- daily insulin therapy (together with unmodified insulin) in many clinical services. It only dropped out of use because of its unsuitability for use in fine-needle pen-injectors, and the less effective profile of the necessary preservative, methyl parahydroxybenzoate.

Methodological advances

Radioimmunoassay was developed in the 1960s by Solomon Berson and Rosalyn Yalow, the latter gaining a Nobel Prize (Berson meanwhile had died), and was rapidly applied to insulin pharmacokinetics. Notable here was a paper from Robert Turner, somewhat misleadingly called 'Measurement of the insulin delivery rate in man', misleading because insulin secretion rate was not calculated. The paper did, however, show that insulin half time in plasma was very short,[24] making a nonsense of clinical practice for the management of diabetic ketoacidosis (DKA) at the time, in which as much as 100 U of insulin was given initially intravenously. The paper records a similar unpublished observation from Peter Sonksen. Together with other understandings of insulin-dose response curves this led to the low-dose insulin regimens for DKA introduced by George Alberti and colleagues in 1971.[25]

Like radioimmunoassay, insulin glucose clamps only really became methodologically important in the development of the new insulins and analogues beyond the scope of the present paper. Clamps were developed in the 1960s to study insulin resistance,[26] and were only 'reversed' to study insulin action in the 1980s, becoming an essential part of the measurement of insulin pharmacodynamic profiles.[27,28]

Key messages

- Banting and Best produced a useful pancreatic extract in 1922, but had no knowledge of the active principle it contained
- Abel and colleagues crystallized insulin, and showed it to be a peptide in the late 1920's, but it took science 30 years to provide the amino acid sequence, and >45 years to define the 3-D shape
- Activity in industry improved purification, taking 50 years to provide truly pure insulin preparations, but also developing useful extended-acting insulins after 25 to 30 years

Conclusion

Fifty years is a long period in the development of any medication, and while the isolation and identification of insulin occurred fairly quickly once reliable methods had become available for pancreatic extraction, defining its amino acid sequence took more than 30 years and the 3-dimensional structure 48 years. Nevertheless, in that time insulin has contributed to or generated six Nobel Prizes. The major clinical advances in purification and prolongation of action for subcutaneous administration in those times grew out of lengthy work by the pharmaceutical industry, though founded in the early studies of insulin chemistry. Insulin has also benefitted and stimulated methodological advances in laboratory science, which in turn have been harnessed for development of more recent insulin products.

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