CRITICAL ASSESSMENT **The pathophysiological mechanisms which finally lead to the diabetes phenotype (hyperglycemia, hyperinsulinemia and insulin resistance) exhibited in the various animal disease models for non-insulin dependent diabetes do not necessarily be identical to those in human disease. Therefore, detailed knowledge about the (patho-)physiology of these animal disease models is a prerequisite for interpretation of experimental results and their value for the human disease.**

Hypoglycemic effects

Blood sugar lowering effect in rabbits

PURPOSE AND RATIONALE A biological assay of insulin preparations in comparison with a stable standard using the blood sugar lowering effect in rabbits has been proposed already in 1925.

The biological assay of insulin using the blood sugar lowering effect in rabbits has been until recently the official assay in several pharmacopoeias, such as European Pharmacopoeia, Second Edition 1980; Deutsches Arzneibuch 1986; British Pharmacopoeia 1988; United States Pharmacopoeia 23 and The National Formulary 18, 1995.

The rabbit blood glucose bioassay as well as the mouse convulsion assay and the mouse glucose assay were used for establishing international standards for highly purified human, porcine and bovine insulins.

In several pharmacopoeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia, 3rd Edition 1997; but the rabbit blood sugar method is still valid in the United States Pharmacopeia USP 24, 2000.

PROCEDURE Four groups of at least 6 randomly distributed rabbits weighing at least 1.8 kg are kept in the laboratory and maintained on a uniform diet for not less than one week before use in the assay. About 24 h before the test each rabbit is provided with an amount of food that will be consumed within 6 h. The same feeding schedule is followed before each test day. During the test all food and water is withheld until the final blood sample has been taken. The rabbits are placed into comfortable restraining cages to avoid undue excitement.

Immediately before use two solutions of the standard preparation are made, containing 1 unit and 2 units of insulin per ml, respectively, and two dilutions of the preparation being examined which, if the assumption of potency is correct, contain amounts of insulin equivalent to those in the dilutions of the standard preparation. As diluent, a solution is used of 0.1–0.25% w/v of either m-cresol or phenol and 1.4 to 1.8 w/v of glycerol being acidified with hydrochloric acid to a pH between 2.5 and 3.5.

Each of the prepared solutions is injected subcutaneously to one group of rabbits, using the same volume, which should usually be between 0.3 and 0.5 ml for each rabbit, the injections being carried out according to a randomized block design. Preferably on the following day, but in any case not more than 1 week later, each solution is administered to a second group of rabbits following a twin crossover design. One hour and 2.5 h after each injection a suitable blood sample is taken from the ear vein of each rabbit.

Blood sugar is determined by a suitable method, preferably using glucose oxidase.

EVALUATION The results of the assay are calculated by standard analytical methods (e.g., USP 23, 1995).

CRITICAL ASSESSMENT OF THE METHOD The classical bioassay based on blood-sugar lowering activity in rabbits has been replaced by chemical methods in some pharmacopoeias but is still included in USP 24, 2000; and will be still necessary for evaluation of synthetic insulin derivatives.

MODIFICATIONS OF THE METHOD An assay of insulin activity after intraperitoneal injection in rats has been described.

It is reported as one of the first on an implantable potentiostat-radiotelemetry system for in vivo sensing of glucose, implanted into the paravertebral thoracic subcutaneous tissue of a dog. An enzyme electrode sensor measures the oxidation current of hydrogen peroxide formed by the stoichiometric conversion of the substrate glucose and oxygen as a cofactor in an immobilized glucose oxidase layer.

It is also described the development of a compact, low power, implantable system for in vivo monitoring of oxygen and glucose concentrations.

Hypoglycemic seizures in mice

PURPOSE AND RATIONALE The biological assay of insulin using hypoglycemic seizures in mice has been suggested already. The biological standardization of insulin using the mouse convulsion method has been published in detail by the Health Organisation of the League of Nations in 1926 and has been until recently the official assay in several pharmacopoeias, such as European Pharmacopeia, Second Edition 1980; Deutsches Arzneibuch 1986; British Pharmacopoeia 1988. In most

pharmacopoeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia, 3rd Edition 1997).

PROCEDURE Ninety-six mice of either sex (but not of mixed sexes) weighing 20 ±5 g are randomly distributed into 4 groups. The mice are deprived of food 2–20 h immediately preceding the test. Solutions of the insulin standard and of the test preparation containing 30 and 60 milliUnits/ml are prepared by diluting the original solution with 0.9% NaCl solution, pH 2.5. 0.5 ml/20 g mouse of these solu-tions are injected subcutaneously. The mice are kept at a uniform temperature, between 29 and 35 °C, in transparent containers within an air incubator with a transpar-ent front. The mice are observed for 1.5 h and the num-ber of mice is recorded that are dead, convulse or lie still for more than 2 or 3 s when placed on their backs.

EVALUATION The percentage of mice of each group showing the afore mentioned symptoms is calculated and the relative potency of the test solution calculated using a 2 + 2 point assay.

CRITICAL ASSESSMENT OF THE METHOD Attempts to replace the tests in mice and rabbits by in vitro tests, such as the rat diaphragm test, the rat epididymal fat pad test, or even the radioimmunoassay failed due to several reasons. Nevertheless, for industrial production and for stability studies,the classical bioassays based on hypoglycemic seizures in mice or hypoglycemia in rabbits have been replaced by chemical methods.

Blood sugar determinations in mice

PURPOSE AND RATIONALE Eneroth and Ahlund recommended a twin crossover method for bio-assay of insulin using blood glucose levels in mice instead of hypoglycemic seizures giving more precise results. This test was induced into the British Pharmacopoeia 1980 and continued up to 1988. Moreover, the test is included as alternative in the European Pharmacopoeia, Second Edition 1980; and in Deutsches Arzneibuch, 9. Ausgabe, 1986.

PROCEDURE Non-fasting mice of the same strain and sex are used having body masses such that the difference between the heaviest and lightest mouse is not more than 2 g. The mice are assigned at random to

four equal groups of not less than 10 animals. Two dilutions of a solution of the substance or of the preparation to be examined and 2 dilutions of the reference solution are prepared using as diluent 0.9% NaCl solution adjusted to pH 2.5 with 0.1 N hydrochloric acid and containing a suitable protein carrier. In a preliminary experiment, concentrations of 0.02 IU and 0.10 IU are tested. Each of the prepared solutions (0.1 ml/10 g body weight) is injected subcutaneously to one group of mice according to a randomized block design. Not less than 2.5 h later, each solution is administered to a second group of mice following a twin crossover design. Exactly 30 min after each injection, a sample of 50 µl of blood is taken from the orbital venous sinus of each mouse. Blood glucose concentration is determined by a suitable method, such as described by Hoffman. **EVALUATION** The potency is calculated by the usual statistical methods for the twin-cross-over assay.