E. J. Verspohl

Recommended Testing in Diabetes Research

Abstract

Diabetic disease is increasing rapidly and vast amounts of resources are spent in all countries. Thus, the screening of new compounds including plant extracts for antidiabetic effects is mandatory. In this review both simple assays [e.g., on blood glucose (after or without a glucose load), plasma insulin and extrapancreatic effects] are described as well as specific *in vivo* tests in diabetic animals and *in vitro* tests with respect to the mechanism of compounds. In total, approx. 30 selected tests are evaluated and references are given. Thus, the investigator is guided through the tests and is advised that measuring only one parameter such as glucose will not be sufficient. In the case that the financial resources are poor for the investigator, more than glucose still has to be measured. A balance is made by describing absolutely necessary investigations while concentrating on those at low cost. It has to be started with simple assays; to use one test only, however, means oversimplifying the diabetes disease; additionally antidiabetic effects may be missing. The investigator is guided through the advantages and limitations of diabetic animal models and is advised about specific *in vitro* tests to look at the mechanism of action. All investigators should profit from these details, not only the phytoresearchers.

Key words
Antidiabetic drugs · phytopharmaceuticals · diabetic testing · blood glucose · insulin sensitive tissues

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Introduction

Diabetes increases tremendously; 4 million people are identified in Germany [1] and this number will be doubled within the next 20 years. Diabetes therapy is extremely costly; an efficient therapy, therefore, is needed at low costs although it has to be admitted that only 27% are spent for therapeutics. New drugs/compounds have to be screened. Phytotherapy is not yet included in evidence-based medicine and guidelines with respect to diabetes therapy but can close a gap if screening and profiling a drug is done in a proper way. The aim of this paper is to give a critical overview for all investigators, including those in phytoresearch, with respect to all relevant methods and animal models used knowing well that an investigator will focus only on a few of them. The reader will find a balance between testing at low costs and getting the necessary information without oversimplification/limiting the interpretation of data.

In Table **1** different types of diabetes with their specific characteristics are shown of which mainly but not exclusively type 2 diabetes will be the aim of phytotherapy. For each type of experiment an evaluation and the most representative reference is given. More information can be asked for by contacting the author.

Basic Investigations

It is generally recommended to start with simle tests which should provide highly reproducible results and should not be time-consuming. It has, however, to be warned to oversimplify diabetes by solely measuring a change in glucose levels. Glucose levels are the starting point. Phytochemists are advised to start with crude extracts given orally and/or *i.p.* To reduce the possibility to miss an effect by any other antagonising compounds, gra-

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Received July 6, 2001 · **Accepted** January 20, 2002

Bibliography
Planta Med 2002; 68: 581–590 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943

Table **1** Features of diabetes subtypes

Characteristics	***Type 1 (IDDM)***	***Type 2 (NIDDM)***
Symptoms	Polyuria, polydipsia, weight loss	Often asymptomatic in early years
Age	< 35 (common in youth)	> 35 (frequent in adults)
Onset	Abrupt (days to weeks)	Months to years
Nutritional status	Undemourished	Majority is overweight
Insulin	Mandatory	Required in <30%
Diet	Mandatory	Controls 30 to 50% cases
β-Cells	Complete loss	Varies
Islet cell antibodies	Yes	No
Family history	+ in 10%	+ in 30%

IDDM = insulin dependent diabetes mellitus.
NIDDM = non insulin dependent diabetes mellitus.

dually less lipophilic extracts should be used (e.g., four subsequently obtained extracts).

The researcher is advised to:
1. to use (and start with) simple methods (blood glucose in whole animals);
2. insulin determination is important (to avoid oversimplification of glucose results);
3. look at the possible mechanism (*in vitro* tests, extrapancreatic effects);
4. it may be interesting to include diabetic animal models.

In Table **2** an overview is shown how to start and continue a screening. The table does not explain itself, but is referred to during the whole review. It should be started with basic investigations with respect to glucose and insulin in whole animals. Then pancreatic effects (insulin secretion) either *in vitro* or *in vivo* and extrapancreatic effects (insulin effects) have to be investigated. All these tests can be modified later by using diabetic animals. Additionally, there exist various specific *in vitro* tests in order to look at an isolated mechanism of action. All tests mentioned in Table **2** (overview) are addressed later one after the other in detail.

Glucose

Many investigators correctly start with blood glucose measurement (Table **3**). The glucose lowering activity test is mostly used in rats, mice or guinea pigs. It is not suggested to use rabbits (though used in the Eur. Pharmacop. in order to, e.g., standardise insulin) or even other animals. It has to be decided whether fasted or non-fasted animals are used since both have specific advantages as outlined (comment in Table **3**). The change in blood glucose can be alternatively observed after a glucose load (Table **4**). This test gives an impression on the reactivity of the organism and the handling of elevated glucose when a test compound is present. The lowering of glucose can be better seen in this type of assay of glucose tolerance.

This basic investigation on glucose lowering activity is shown. This test is simple and many authors think this test is sufficient for a publication but, in fact, it is not. Glucose is very well balanced and homeostasis is easily reached by counter-regulatory glucose production. There exist 3 possibilities (glucose oxidase/peroxidase method, hexokinase/glucose-6-phosphate dehydrogenase method and glucose dehydrogenase method); most people expect a decrease in glucose accompanied by an increase in insulin, thus thinking that insulin measurement is not necessary.

For determination of glucose three major enzymatic methods exist and are described in biochemical text books (glucose oxidase/

Table **2** Flow chart of diabetic screening (latin numbers are the same as used in the text and help to be guided through the text explanations; abbreviations are explained in the text)

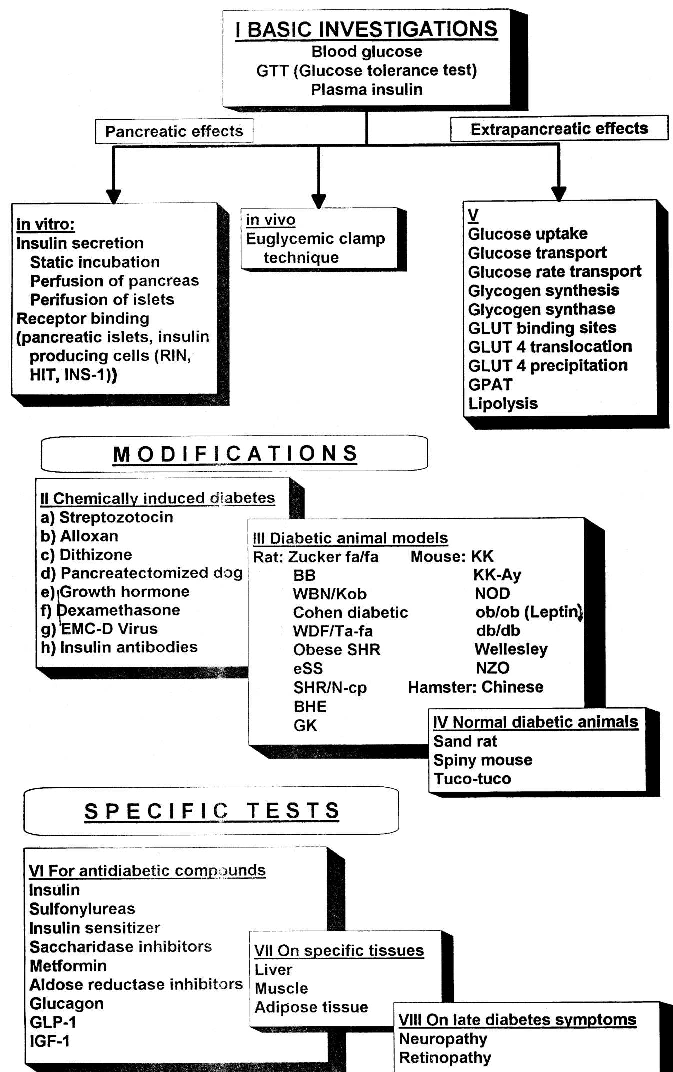

Table **3** Blood glucose lowering activity (healthy animals) [2]

Method:	Non-fasted animals treated orally or *i.p.* with test compound (control group vehicle only)
	Blood is withdrawn from the tip of the tail or retrobulbic system
	Determination of glucose (see below)
Comment:	Fasted animals: controlled situation (small fluctuation)
	Non-fasted animals: more physiological situation
Plotting:	Blood glucose values are plotted versus time using several dosages
	Instead of original glucose values, delta changes or percentage AUC (area under the curve): integrated values over time
Evaluation:	Basic test lacking any information on the mechanism

Verspohl EJ. Recommended Testing in… Planta Med 2002; 68: 581 – 590

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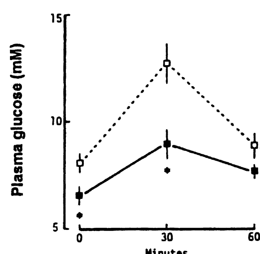
Table **4** GTT (glucose tolerance test) [3]

Aim:	Looking at peripheral glucose utilization
Method:	Glucose load (2 g/kg BW mouse) *i.p.*
	Acute or chronic experiments
Plotting:	Blood/plasma glucose levels vs. time



peroxidase method, hexokinase/glucose-6-phosphate dehydrogenase, glucose dehydrogenase method). Glucose can be determined from capillary blood or serum, plasma or venous blood; urine, however, is not suitable due to the highly variable kidney threshold for glucose. Venous blood is not recommended due to decreased glucose levels induced by quick degradation (low reproducibility of results). Blood can be used as native material, being deprived of protein, hemolyzed or stabilized (inhibition of glycolysis). Glucose concentrations decline progressively in whole blood (by 70% within 24 hours) which is negligible at 4 °C. Values are higher in plasma or serum compared to whole blood since glucose is restricted to the water volume; water volume is different in plasma (92%) and blood cells (70%). Strips as introduced for patient control should not be used for glucose assay in the laboratory because they are not accurate enough.

Insulin

It has to be emphasized that during the same animal protocol/experiment plasma insulin should be determined [4], e.g., by radioimmunoassay (radioactivity lab is necessary, ^{125}I) or enzyme-linked immunoassay (more costly than a radioimmunoassay). For each determination 100 μL plasma are necessary which has to be reminded that this is 1% of the whole plasma of a 300 g rat. Not very often used are RP-HPLC or capillary electrophoresis methods. Other assays like bioassays (epididymal fat pad of rats, using [^{14}C]glucose ($^{14}CO_2$ is trapped in alkaline medium and counted), or lipogenesis assay, ([^{3}H]glucose incorporated into lipids is determined) are not as precise as a radioimunoassay and are only of historical interest.

Blood is drawn by various methods: a short anesthesia is performed, preferentially with halothane but not diethyl ether; halothane is inert with respect to the diabetic situation. A small heparinized, sharpened glass pipette is pushed from nasal into the orbita from the retrobulbaric plexus. Puncture of the tail is not very effective and not recommended.

Pancreatic effect

The information from changes in blood glucose is small because there is no information concerning the underlying mechanism, e.g., a pancreatic or extrapancreatic effect. The next step, therefore, could be to look at pancreatic effects; i.e., insulin secretion (Tables **2** and **5**).

The drawback of static incubations is that the released insulin being accumulated in the medium inhibits further insulin release [5] (see Table **5**). An alternative method could be to perfuse a rat pancreas (Table **6**) or to perifuse isolated rat pancreatic islets. Since both the methods are not a static incubation, they give information on kinetics of insulin secretion and there exist no feedback phenomena with respect to insulin release. Instead of using rat pancreas or isolated pancreatic islets (both are time consuming and tricky methods); cell cultures should be preferred (Table **7**). INS-1 cells are recommended. HIT cells (syrian

Table **5** Insulin secretion *in vitro* (static incubation)

Aim:	Direct pancreatic effects
Method:	Isolated islets from various animals; alternatively an insulin secreting cell culture
	Collagenase digestion of whole rat/mouse pancreas and isolation of islets of Langerhans
	Passage of cultured cells (e.g., INS-1, RIN, HIT) (Table **7**)
Control Experiments:	Lowering incubation temperature; omission of Ca^{++}; blockers of protein synthesis (this is to discriminate between real and toxic effects leading to insulin release)
Plotting:	Insulin release (medium insulin) vs. drug concentration
Advantages:	Rapid information about initiators and modulators of insulin release
Disadvantage:	Complicated isolation technique (holds for pancreatic cells) only static incubations, no kinetic investigations (accumulation of released products with disturbing feedback phenomena on insulin release)

Table **6** Perfusion of rat pancreas [6]

Aim:	Same as with islets, but kinetic investigations without interference with released compounds (feedback mechanisms)
Method:	Circulation through the preparation from arterial to venous effluent (portal vein); perfusate is collected
Evaluation:	Useful tool to study the degree and time course of secretion after adding sulfonylureas or plant extracts

Table **7** Cell lines [7]

RIN (Insulin producing tumor cells) induced by X-ray in rats
RINm (transplantated, e.g., into mice)
Disadvantage: RIN cells are not glucose-dependent (express low amounts of hexokianse IV [= glucokinase])
INS-1 (Rat insulinoma)
Advantage: glucose-dependent insulin release

Table **8** Euglycemic clamp technique [8]

Aim:	Quantifying *in vivo* sensitivity to hypoglycemic agents in humans or animals
	Infusion of test compound with insulin sensitizing (glitazone) – or insulin releasing effect (sulfonylureas)
Method:	A variable glucose infusion is delivered to maintain euglycemia
	Catheters in jugular and femoral vein for blood collections and infusion of glucose and test compound
	Blood glucose concentrations are determined from samples
	Influx and efflux of glucose can be calculated from infusion rate by using [^{3}H]glucose disappearance in a steady state
Modification:	Distinct rat tissues instead of whole rat

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virus 40-transformed hamster β-cell line) are also used but a gene lab is necessary.

An euglycemic clamp technique reflects pancreatic and extra-pancreatic effects (Table **8**). Glucose concentration is clamped by infusing various amounts of glucose; the uptake and metabolism of glucose can thus be calculated.

Concluding remarks

Publication referring only to blood/plasma glucose is not sufficient since antidiabetic effects can be missed. At least plasma insulin should be determined; in case this is positive, an *in vitro* insulin secretion experiment should be performed.

Chemically Induced Diabetes

Many of the above-mentioned types of experiments can be modified by using animals that are diabetic. Diabetes can be investigated either in animals made diabetic by chemical compounds or diabetic strains can be used as is outlined in the overview in Table **2**. There may be a specific damage of insulin producing β-cells, a temporary inhibition of insulin release and/or production or a decreased efficacy of insulin in target tissues. From all compounds mainly streptozotocin and alloxan are used to induce diabetes (Tables **9** and **10**).

Type 2 diabetes after STZ (Table 9)

The glucose moiety of STZ directs this agent to the pancreatic β-cell where it binds to a membrane protein, probably the glucose transporter GLUT2. A process of methylation [production of carbonium ions (CH_3^+ causing DNA breaks by alkylating DNA bases)] may be involved as well as free radical generation (only indirect evidence by protection through superoxide dismutase). Nitric oxide (NO) production may also be important. The cytotoxic action of STZ is initiated by the highly reactive nitrosourea side chain of streptozotocin.

When authors are planning to use streptozotocin (STZ) they are adivsed to use it in a proper manner; in many cases it is forgotten that depending on the conditions (protocol of STZ application) animals with either type 1 (IDDM) or type 2 diabetes (NIDDM) will result (Table **9**). Authors are also advised to use the proper controls to know about the physiological situation of the animals. A single dose of STZ (e.g., 90 mg/kg *i.v.*) is given to 2-day-old neonatal rats. This induces β-cell injury which is followed by limited regeneration (short-term normalization of glycemia), primarily as a result of ductual budding rather than mitosis of pre-existing β-cells. Necrotic and degranulated β-cells cause an initial rise of serum insulin values, resulting in a hypoglycemic phase followed by a persistent hyperglycemia. At 6 to 15 weeks of age an impaired glucose disposal rate and significant β-cell secretory dysfunction (type 2) is observed. The STZ model does not completely resemble human type 2 diabetes since, e.g., insulin resistance is not induced; type 2 diabetes is characterised by many other features, e.g., with respect to insulin relase:

1. missing first phase,
2. less dose-depending from glucose,
3. less potentiating effect of various compounds,

though total insulin output is not diminished. β-Cells are alive but stunned.

Under modified protocols a rat resembling more type 1 diabetes can be obtained; this STZ-model for type 2 diabetes can, however, be modified to create type 1 diabetes: STZ plus Complete Freund's Adjuvant is given in multiple low doses (instead of a single high dose) which induces immune pancreatic insulitis in rats mimicking immune type 1 diabetes in humans.

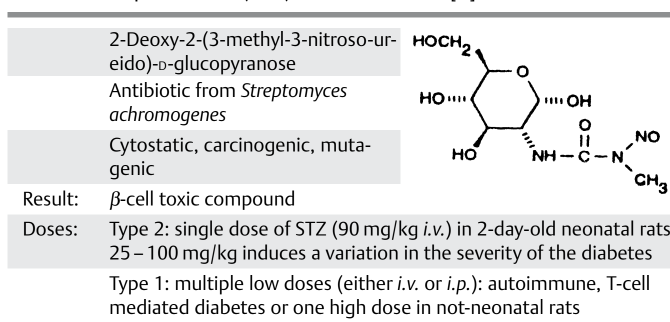
Precautions for the use of STZ have to be kept in mind: STZ is not stable in solution (fresh daily, optimum pH: 4). There exist batch differences in activity. Susceptibility of animals appears to depend on age and animal strain [WKY (Wistar Kyoto) rats are more susceptible than other rats]. Animals must be kept properly because of high urine volume, diarrheas etc. and they must eventually be on insulin on a long run. Continuous blood glucose monitoring is mandatory. It is concluded that it is hard to reproduce identical animal states. Animals, therefore, must be well characterized for publication (weight gain, fasting plasma glucose levels, GTT results, plasma lipid levels). Hard criteria for a diabetic situation have to be created, e.g., 13 mM plasma glucose as cut off.

One advantage of this model is a real diabetes, not a result of diabetes as a consequence of obesity as in many other cases (see below). When the STZ-model has to be evaluated it can be said that STZ is most commonly used, produces a mild and stable form of diabetes, partly resembling type 2 human diabetes (no adequate insulin release). It is a model of hypoinsulinomimetic diabetes rather than a model of tye 1 diabetes. The residual functioning β-cells allow the animal in many cases to survive without exogenous insulin.

The residual insulin-secreting capacity is an advantage in that the animals are easier to maintain than completely insulin-dependent animals.

Alloxan is used very often as a diabetogenic agent (Table **10**). It has to be mentioned that not all animals respond to alloxan with diabetes which has to be checked out. In comparison: The dose of alloxan to produce the desired severity of diabetes without mortality is less predictable than the dose of STZ needed; alloxan, on the other hand, is less expensive than STZ.

Table **9** Streptozotocin (STZ) diabetes model [9]



	2-Deoxy-2-(3-methyl-3-nitroso-ureido)-D-glucopyranose	
	Antibiotic from *Streptomyces achromogenes*	
	Cytostatic, carcinogenic, mutagenic	
Result:	β-cell toxic compound	
Doses:	Type 2: single dose of STZ (90 mg/kg *i.v.*) in 2-day-old neonatal rats 25 – 100 mg/kg induces a variation in the severity of the diabetes	
	Type 1: multiple low doses (either *i.v.* or *i.p.*): autoimmune, T-cell mediated diabetes or one high dose in not-neonatal rats	

Genetically Diabetic Animals

Hitherto chemically induced diabetes was summarized. On the other hand genetically diabetic animals can be used as well (Ta-

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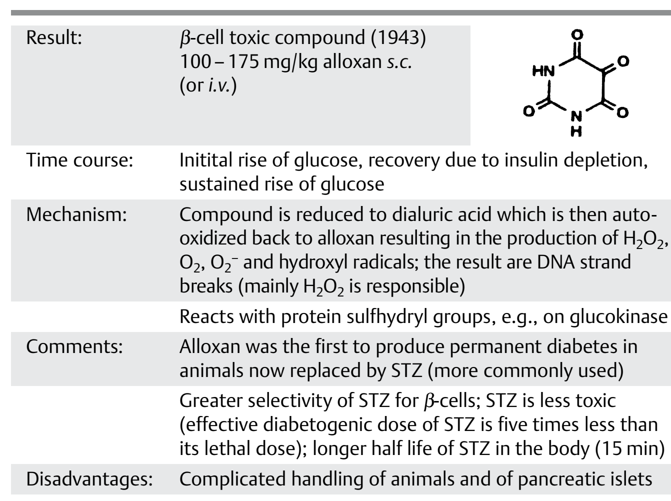
Table **10** Alloxan (diabetes model) [10]



Result:	β-cell toxic compound (1943) 100 – 175 mg/kg alloxan *s.c.* (or *i.v.*)
Time course:	Initital rise of glucose, recovery due to insulin depletion, sustained rise of glucose
Mechanism:	Compound is reduced to dialuric acid which is then autooxidized back to alloxan resulting in the production of H_2O_2, O_2, O_2^- and hydroxyl radicals; the result are DNA strand breaks (mainly H_2O_2 is responsible)
	Reacts with protein sulfhydryl groups, e.g., on glucokinase
Comments:	Alloxan was the first to produce permanent diabetes in animals now replaced by STZ (more commonly used)
	Greater selectivity of STZ for β-cells; STZ is less toxic (effective diabetogenic dose of STZ is five times less than its lethal dose); longer half life of STZ in the body (15 min)
Disadvantages:	Complicated handling of animals and of pancreatic islets

ble **11**). Though genetically obese animals (summarized in Table **11**) are generally of interest, they are not discussed in the review since they are not primarily diabetic. Animals like ob/ob mice [12] or the obese SHR rat [13] are insulin resistant because of extreme obesity.

Type 2

The most prominent animal model is the fa/fa Zucker diabetic fatty rat (ZDF rat) (Table **12**) which is derived (inbred form) from the fa/fa Zucker rat [15]. The ZDF rat [14] is a good model for type 2 diabetes since it is a model for an age-dependent change in glucose and insulin levels. Male littermates show up with obesity, insulin resistance and type 2 diabetes between 7 and 10 weeks of age; their female littermates are similar, but do not exhibit type 2 diabetes (serve as controls).

Sometimes GK rats are used which possess the following characteristics (decreased insulin sensitivity, insulin receptor number, insulin receptor tyrosine kinase activity, increased gluconeogenesis). In addition to rats there exist several mice models as is outlined below (Table **13**). The db/db mouse as well as ob/ob mouse and fa/fa rats (gene Lepr^{ob}) have a leptin problem. Leptin is released from adipocytes depending on fat cell size and serum insulin and inhibits food uptake via a hypothalamic leptin receptor. In db/db mice this feedback is disturbed as well as in ob/ob mice

Table **11** Animal models of diabetes

Most are complex: multiple lesions affecting numerous processes
Type 2:
Produced through selective breeding, spontaneous mutations or genetic engineering
Animals show up with various degrees of hyperglycemia, insulinemia and obesity
Include fa/fa Zucker rat, fa/fa diabetic Zucker rat, GK rat [11], BHE rat, db/db mouse
More typical for obesity than for diabetes: fa/fa Zucker rat, ob/ob, KK, NZO
Strictly non-obese: GK and BHE
Type 1: caused by a reduction (> 90 %) in the number of pancreatic β-cells
Genetic models include BB rat and NOD mouse

Table **12** ZDF rat (Zucker diabetic fatty rat) type 2 diabetes [14]

Characteristics:	Inbred form of fa/fa rat (developed from an animal model for obesity with a (fa) gene on chromosome 5 (Lepr^{fa})
	↑ (6-fold) triglycerides and total cholesterol, dyslipoproteinemia, hypertension male littermates: obesity, insulin resistance and type 2 between 7 and 10 weeks of age
	female littermates: similar, but no type 2 diabetes
Advantage:	mostly used
	Good model for type 2 diabetes since it is a model of an age-dependent change in glucose- and insulin levels

Table **13** DB/db mouse (C57 BKS db/db) type 2 diabetes [16]

Characteristics:	Rather the same as for Zucker rat
	Autosomal recessive mutation having occurred spontaneously in mice of the C57BL/KsJ strain; Lepr^{ob}
	Early onset of hyperinsulinemia, weight loss
	Later on: low insulin levels due to degeneration of β-cells
	Transition from a hyperinsulinemic-normoglycemic state to a severe hypoinsulinemic-hyperglycemic state occurs at about 2 to 4 months of age; early death (type 1) eventually
	Symptoms dependent on animal age: morbide adipositas, metabolic syndrome, type 2 diabetes
	Short and inactive form of leptin
Comment:	Often used (energy metabolism to thermoregulation)

and fa/fa rats (gene Lepr^{ob}). It has to be mentioned that leptin disturbance is no good model for human obesity.

Polygenetic mouse models for adipositas and insulin resistance are NZO and KK mouse. Those models are not discussed here [17], [18]. If a mixed model for both obesity and hypertension is needed, the SHR rat may be useful [13].

Another model of type 2 diabetes is the chinese hamster (*Cricetulus griseus*). These hamsters have an hereditary diabetes mellitus; the remaining islets are histologically abnormal and the animals are not obese.

An overview of models showing up with more or less obesity or diabetes is given in Fig. **1**.

Type 1

There also exist specific animals resembling type 1 diabetes. The most prominent model is the BB rat (Table **14**). It is clear that these animals are not easy to handle since they are on insulin. Rather the same characteristics for BB rats hold for the NOD mouse (Table **15**). Very recently the LEW.1AR1/Ztm-iddm rat was described [21] which has the advantage that diabetes appears exactly at 58 ± 2 days after birth allowing observation of the exact mechanism.

Other models are more complicated and, therefore, not recommended: e.g., transgenic mouse and knock-out mouse (k.o. mouse). Normal or mutated proteins are overexpressed or genes are deleted (knock-outs): Deficiency of receptor kinase, IRS1-substrate, GLUT4, β_3-adrenergic receptors; overexpression of

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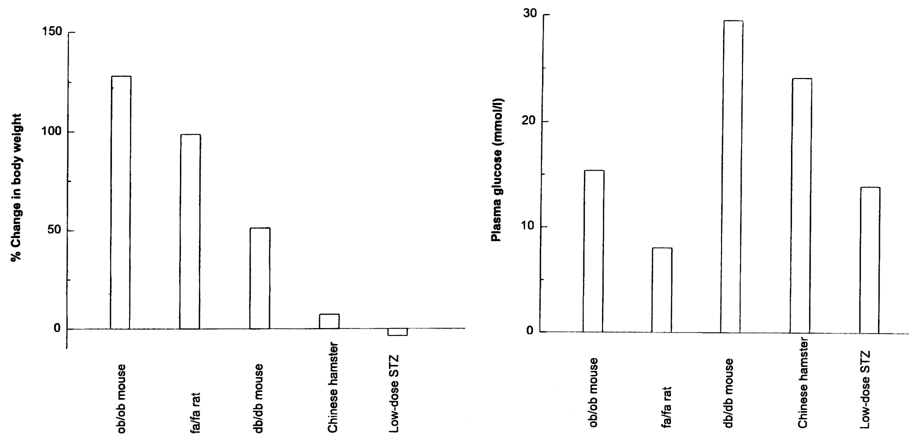

Fig. **1** Models that are representative preferentially for diabetes and/or obesity.

Table **14** BB rat (Bio breeding) [19]

Characteristics:	Autoimmune destruction of pancreatic β-cells (typical for type 1 diabetes), insulitis
	Virtually no β-cells left
	ICA (iselt cell antibodies) present, antibodies against carboxypeptidase, GAD subunits
	Autosomal recessive trait
	Hyperglycemia, glycosuria, weight loss within one day of onset; ketoacidosis within several days
Advantages:	Unique model of human type 1 diabetes
	Testing of immunosuppressive agent

Table **15** NOD mouse [20]

Characteristics:	Similar to BB rat
	Autoimmune destruction of pancreatic β-cells in association with insulitis and autoantibody production
	Insulitis (= heavy leukocytic infiltration of pancreas, primarily $CD4^+$ and $CD8^+$ T cells)
	Onset of diabetes abruptly between 100 and 200 days of age, as well as rapid weight loss, polyuria, polydipsia and severe glucosuria
Comment:	Not all animals become diabetic

glucokinase (= hexokinase II); mutation of the TNF-α gene; all these were performed.

It has to be concluded that no single diabetic animal model accurately represents type 2 in man, but individual syndromes closely resemble aspects of the human disease. On the other hand, the type 1 animal models such as BB rat are much closer to what happens in humans than all type 2 models.

Normal Diabetic Animals (No Models)

There also exist animal models in which diabetes is not induced by chemicals or by a genetic defect. Some animals become diabetic in captivity because the environment in the lab does not fit the area where they come from. The sand rat is very seldom used; it lives in desert regions in the Middle East. It is lean and normoglycemic as long as it lives there but develops diabetic symptoms when fed laboratory chow in captivity [22]. Some are infertile in captivity and there exist even other problems. Other comparable animals are: Spiny mouse (*Acomys cahirinus*, *Acomy russatus*) [23] and Tuco-tuco (*Ctenomys talarum*) [24].

There exist some models which combine diabetes and hypertension: in this case combination of STZ with deoxycorticosterone (both are diabetogenic compounds) along with sodium chloride intake is used.

Extrapancreatic Effects

Next the extrapancreatic effects have to be discussed (see overview in Table **2**). Important are glucose uptake, transport, production, glycogen synthesis and the glucose transporters GLUT. Glucose uptake by adipocytes is shown in Table **16**; Fig. **2** demonstrates an example of 2-deoxyglucose uptake as an experimental result (adapted from [26]). A glucose transport assay was de-

Table **16** Glucose uptake by adipocytes [25]

Aim:	Insulin-stimulated specific transport
Principle:	Utake (= unindirectional transport) of 2-deoxy-D-[1-^3H] glucose or [1,2-^3H]glucose
	Transport, phosphorylation, no metabolization
Methods:	3T3 cells or adipocyte cell suspension, 2 nM insulin, 25 °C, 10 (max. 20) min [^3H]glucose/well
	counting radioactivity of washed and lysed cells
	alternatively centrifugation on top of dinonyl phthalate, tube is cut
	alternatively stop incubation with ice-cold $HgCl_2$, filter
Control:	Non-specific uptake/binding/extracellular adsorption in the presence of 20 μM cytochalasin B (has to be subtracted from all values)
Calculation:	Stimulated specific uptake minus unstimulated uptake (cytochalasin controls are either subtracted)
Plotting:	Glucose uptake is plooted vs. compound concentration
Advantage:	Simple assay with low variability linear uptake within at least 10 or 20 min extrapancreatic effects – even of sulfonylureas – can be shown
Comment:	Short incubation necessary due to intracellular equilibirum changes
	3-*O*-Methylglucose can be used with limitations (5 – 10 sec experiments due to fall in ATP)

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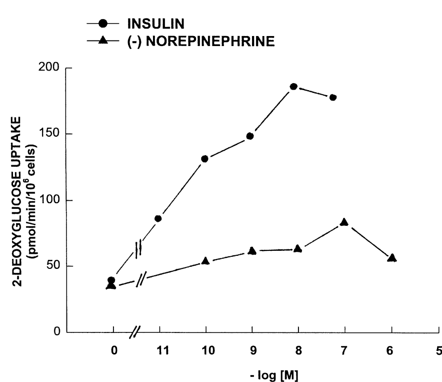

Fig. **2** Experimental result for glucose uptake using 2-deoxyglucose.

scribed [27], [28]. Glucose rate transport is measured within the first 5 – 15 sec of the experiment. Glucose production (gluconeogenesis) as measured from Fao cells [29] is not recommended because it does not work very well.

Glucose production (*in situ* liver perfusion) [30] is a method by which rat liver with arterial and venous flow is prepared. It is not easy since constant pressure, no bubbles in the medium and expensive human albumin are necessary.

A very good working method is the measurement of glycogen synthesis (Table **17**). A biochemical method is the determination of the activity of glycogen synthase by using the supernatant of homogenized smooth muscle incubated with 0.2 mM [U-^{14}C]UDP-glucose [32]. Glucose transporters (GLUT) exist of which mainly GLUT2 (liver) and GLUT4 (adipocytes, skeletal muscle) are insulin-sensitive and can be included in specific experiments (Tables **18** and **19**). Glucose transporters are immunoprecipitated or the transporter translocation is determined in Western blots by specific antisera [35]. There are differences whether a polyclonal or a monoclonal antibody is used for precipitation.

Rarely used is GPAT (glycerol-3-phosphate-acyltransferase) which is affected by insulin. GPAT activity is necessary for synthesis of phosphoglycerides. Determined are radiolabelled products formed from [^3H]glycerol-3-phosphate during incubation with GPAT in adipocyte homogenate [36].

Specific Tests for Antidiabetic Compounds

There exist other specific tests shortly outlined below which are not recommended for general screening. These tests are important if there exists a preliminary idea with respect to the mechanism of action. These tests are:

– Insulin receptor binding (binding characteristics of insulin analogues), testing of insulin-like properties using an insulin target tissue such as adipocytes, liver cells and [^{125}I]Tyr^{A14}-monoiodinated insulin [37].
– Sulfonylureas (binding to, e.g., intact insulin releasing cells (e.g., RIN cells)) of labelled sulfonylurea, e.g., [^3H]glimepiride [38].
– Sulfonylureas ($^{86}Rb^+$ efflux measurements for quantitating the inhibition of ATP-sensitive K^+ channels; $^{86}Rb^+$ is used instead of radioisotope K^+ due to longer $T_{1/2}$) [39].
– Insulin sensitizer (thiazolidinediones; acting via PPARγ receptors): Blood glucose changes in ob/ob mice, increased glucose utilization or GLUT4 expression are only a prerequisite; expression of adiponectin in 3T3-L1 adipocytes and increased adiponectin plasma concentrations; Immunoassay of TNF-α released from mononuclear cells and transcription of genes in target cells [40], [41].

Other specific tests are for glucosidase activity (Table **20**). Another method for evaluating glucosidase activity is the everted sac technique: Rat small intestine segments are turned inside out and glucose liberated within the sac volume in response to inhibitor concentration is determined [42].

Table **17** Glycogen synthesis [31]

Aim:	Quantification of the effect of insulin and substances with insulin-like effects
Principle:	[^3H]- or [^{14}C]glucose incorporation into glycogen
Method:	Smooth muscles (e.g. diaphragms) of rats or HEPG2 cells incubated with labeled glucose, insulin or test compound
	After lysing cells/tissue glycogen but not glucose is precipited in EtOH (– 20 °C)
	Radioactivity of pellet (glycogen) is counted
Plotting:	Radioactivity of glycogen versus insulin or insulinomimetic compounds

Table **18** Glucose transporter (GLUT) binding sites (structure of cytochalasin B) [33]

Aim:	Measurement fo GLUT concentration, e.g., in a membrane fraction (Cytochalasin B method)
Principle:	GLUT concentration by [4(n)-^3H]cytochalasin B-binding
Method:	Stimulation of, e.g., rat adipocytes
	Bound is separated by centrifugation or spotting on filter papers
	Presence or absence of 500 mM glucose
	tracer amounts of [^{14}C]-urea or [U-^{14}C]-sucrose to correct for trapped, unbound [^3H]-cytochalasin B
	2000 mM cytochalasin E to decrease non-specific binding (NSB)
Plotting:	Scatchard plot: B_{max} and K_d were determined from a linear plot derived by subtracting curves generated in the presence of glucose from those generated in the absence of glucose
Comment:	Complex procedure (multiple centrifugation steps)

Table **19** Glucose transporter translocation (immunoblotting) [34]

Aim:	Insulin-stimulated translocation of GLUT (e.g., 4) from intracellular pools to plasma membrane
Principle:	Measurement of GLUT concentration separately in both cytosolic and membrane fraction
	Preparation of cytosolic and plasma membrane probes (see below)
Details of Method:	Immunoblotting/Western: SDS-PAGE (= electrophoresis)
	Transfer to, e.g., nitrocellulose sheets (= blotting)
	Detection by GLUT antibody being labelled
	Identification of first antibody bound by horse-raddish peroxidase (HRP)-labelled or ^{125}I-labelled procedure; secondary antibody; visualization of immunolabelled bands by autoradiography or chemiluminescence
	Laser densitometric scanning of bands

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Table **20** Saccharidase inhibitors (enzyme activity) [42]

Background:	Used for treatment of diabetes and obesity
	Peak blood glucose levels are decreased by inhibiting starch (glucose-polysaccharide) and glycogen degradation to absorbable monosaccharides. Intestinal α-glucosidases include amylase, dextrinase, glucoamylase, isomaltase etc.
Aim:	IC_{50} values of enzyme inhibition are determined
Method:	Glucosidase from rat/porcine intestinal mucosa or pancreas
	Incubation of enzyme solution with inhibitor to be tested
	Liberated glucose is measured by the glucose oxidase method

For metformin or metformin-like compounds no specific test system exists; the decrease in liver gluconeogenesis, the increase in muscle glucose uptake, the decrease in intestine glucose utilization and the increase in lactate production by increased anaerobic glycolysis are involved and can be determined. Mostly the hepatic gluconeogenesis inhibition is used for metformin-like compounds.

There is no aldose reductase inhibitor in the German market in contrast to other countries because there was no clinically significant effect in preventing/abolishing late diabetic secondary failures; testing is outlined in Table **21**.

Some diabetics are hyperglucagonemic or gluconeogenesis has to be suppressed by antiglucagonic compounds. Glucagon may be necessary to be determined by a RIA or by a biological assay [increase in rabbit blood sugar; 1st International Standard for Glucagon (procine, 1973) [45].

In cases where insulin effects are measured, the cross-reactivity with insulin-like growth factor (IGF-1, somatomedins) should be determined [46].

Effects on Specific Tissues

Effects on liver

- Liver perfusion: Several parameters can be determined in the effluate, e.g., glucose production from glycogen or from lactate.

Table **21** Aldose reductase inhibitor (*in vitro*) [44]

Background:	Key enzyme when insulin-stimulated glycolysis is not effective
Aim:	*In vitro* testing of enzyme activity
Method:	Isolation of aldose reductase (lenses of calf eyes)
	Reaction mixture with NADPH, DL-glyceraldehyde and the enzyme plus test compound
Blanks:	Non-specific reduction of NADPH and absorption by compounds
Reaction:	Glyceraldehyde + NADPH $\xrightarrow{\text{Aldose reductase}}$ glycerol + NADP
Plotting:	Percentage of inhibition vs. various concentrations; IC_{50}-value NADPH oxidized per min (enzymatic activity)
Disadvantage:	Aldose reductase has pathophysiological impact; aldose reductase inhibitors have no clinical impact (2 drugs)

- Isolated hepatocytes or Hep G2 cells (a minimal deviation of human hepatoma) [46].

Effects on muscle

Experiments are performed as shown in Table **22**.

Effects on adipose tissue

Experiments are performed as shown in Table **23**.

In this review is not included the testing on late diabetic syndromes such as neuropathy and retinopathy, avoidance of cataracts (see VIII in Table **2**).

Many investigators focus on plant extracts and their diabetic effect. Most of the plants that have been investigated in diabetes research are summarized (Table **24**). Interestingly, in very recent screening a possible "insulin pill" was detected from *Pseudomassaria*; the isolated compound L-783281 does not interact with the insulin receptor but stimulates insulin action by interacting with insulin receptor tyrosine kinase [49]. Thus, also a remedy for type 1 diabetes may be created from the wealth of plants; researchers should take their chance.

Table **22** Perfused hind limb in rats (hindquarter) [47]

Aim:	To study the muscle metabolism *ex vivo*
Method:	Aorta is incised and a catheter is introduced
	Vena cava is cannulated
Evaluation:	Several parameters determined in the effluate such as glucose for net uptake or lactate for metabolic formation, after adding hormones and drugs as well as after electrical stimulation
Advantage:	A useful approach

Table **23** Lipolysis [48]

Aim:	Inhibition of lipolysis (e.g., antilipolytic effect of insulin)
Principle:	Measurement of the release of glycerol or fluorescent fatty acids from prelabelled rat adipocytes
Method:	Fluorescent labelling of adipocyte lipids
	Filtrate contains released glycerol and fatty acids
	TLC plate (total area) is fluorimetrically scanned

Table **24** Plants and compounds with blood glucose lowering activity (overview may not be complete)

Herbs and spices
Aconitum carmichaelii (glycan) (root)
Agrimonia eupatoria (leaves)
Albizia spec. (seed)
Allium spec. (bulb)
Aloe spec. (sap)
Arctium lappa (leaves)
Artemisia dranunculus (leaves)
Atractylodes japonaica (glycan) (rhizome)
Azadirachta indica (= *Antelaea azadirachta*) (leaves)
Bridelia ferruginea (leaves)
Bumelia sartorum (root)
Capsicum frutescens (seed)

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Table **24** cont.

Centaurea corcubionensis (leaves, flower)
Coccipia indica (leaves)
Coix lacryma-jobi (glycan) (seed)
Coprinus comatus (culture)
Coriandrum sativum (seed)
Cuprinum nigrum (seed)
Dioscorea japonica (glycan) (rhizome)
Eleutherococcus senticosus (eleutherane) (root)
Ephedra distachya (herb)
Eriobotrya japonica (leaves)
Euphorbia prostrata (herb)
Fumaria parviflora (herb)
Ganoderma lucidum (glycan) (fruiting body)
Guar gum (from *Cyanopsis tetragonoloha*)
Glycyrrhiza glabra (root)
Gymnema sylvestre (leaves)
Lithospermum erythrorhizon (glycan) (root)
Lythrum salicaria (flower)
Malvaceae (mucilages) (root, leaves)
Momordica charantia (polypeptide-p) (seed, fruit)
Morus alba (root)
Myrtus communis (total plant)
Oryza sativa (glycan) (seed, root)
Panax ginseng (glycan panaxane) (= *P. pseudoginseng*) (root)
Panax quinquefolius (quinquefolane) (root)
Plantago asiatica (mucilage) (root, leaves)
Poterium ancistroides (tomentic acid) (= sanguisorbala) (herb)
Rubus fruticosus (leaves)
Saccharum officinarum [(–)-epicatechin (peduncle)]
Salvia lavandulifolia
Salvia officinale (leaves)
Taraxacum officinale (root, leaves)
Thymus vulgaris (leaves)
Tinospora crispa (peduncle)
Trigonella foenum-graecum (seed, fibre fraction)
Urtica dioca (aerial parts)
Vitex peduncularis
Zingiber officinale (root)
Vegetables
Agaricus bisporus (fruiting body)
Allium cepa (bulb)
Allium porrum (aerial parts)
Allium sativum (bulb)
Apium graveolens (aerial parts)
Brassica oleracea (leaves)
Brassica rapa (root)
Lactuca sativa (leaves)
Phaseolus vulgaris (pod)
Pisum sativum (seed)
Solanum tuberosum (tuber)
Fruits
Citrus limonum (fruit)
Humulus lupulus (leaves)
Juniperus communis (berry)
Rubus fructicosus (leaves)
Rubus idaeus (fruit)
Sambucus nigra (leaves)
Tilia europaea (fruit)

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