EFFECT OF INSULIN ON ULTRASTRUCTURE AND GLYCOGENESIS IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

DENISE BERNAERT, JEAN-CLAUDE WANSON, PIERRE DROCHMANS, and ANYA POPOWSKI

From the Laboratoire de Cytologie et de Cancérologie Expérimentale, Université Libre de Bruxelles, 1000 Bruxelles, Belgium

ABSTRACT

Insulin in the presence of high concentrations of glucose has a beneficial trophic effect on the development of primary cultures of hepatocytes. Compared to the situation observed in hormone-free control cultures, the flattening of the reaggregated hepatocytes is enhanced, and the reconstituted cell trabeculae are enlarged and tend to form a confluent monolayer after 3 days; the survival time is prolonged from 3 to 5 or 6 days. Ultrastructural modifications are also initiated by insulin; numerous glycogen particles appear, after 24 h, in between the cisternae of the proliferated smooth endoplasmic reticulum. After 48 h, large amounts of glycogen are stored, and numerous polysomes are present. A small number of cells show an increased synthesis of lipid droplets in the lumen of the smooth endoplasmic reticulum and form liposomes at the same time. After 72 h, cytolysosomes filled with glycogen develop, simulating glycogenosis type II. Simultaneously, microtubules and microfilaments, closely related to numerous polysomes, appear in cytoplasmic extensions constituting undulating membranes.

The biochemical data demonstrate that, in the absence of insulin, a high concentration of glucose stimulates glycogenesis and hinders glycogenolysis. This effect of glucose on polysaccharide synthesis is progressively lost. The addition of insulin to the cultures induces, after 48 and 72 h, a three- to fivefold increase of the glucose incorporation into glycogen, as compared to the controls. The presence of insulin is required to maintain the hepatocyte's capacity to store glycogen. Glycogen synthetase is converted into its active form under the influence of glucose. Insulin increases the rate of activation.

Primary cultures of hepatocytes isolated from adult rats were used to study the effect of insulin on various metabolic pathways in the liver. As stated by Krahl (48), the primordial function of insulin consists of promoting the formation and storage of macromolecules such as glycogen, lipids, and proteins. Particular efforts were devoted to the study of the effects of insulin on glycogenesis, but contradictory results on the rapid effect of the hormone were obtained during in vivo (6, 10, 22, 24) and in vitro investigations (35, 55, 64, 65). In isolated hepatocytes, the early stimulation of glycogen synthetase and of the synthesis of polysaccharide by insulin has not been demon-
Hepatocyte Culture paper (79). The glucose concentration, as stated in the culture, was either 20 or 40 mM. When insulin was added to the dishes, 20 mU/ml were used. The following contaminating hormonal and substrate concentrations of fetal calf serum were determined in order to check their possible interference with the experimental parameters: insulin (≤3.5 μU/ml), glucagon (119 pg/ml), growth hormone (≤1 ng/ml), cortisol (undetectable concentration, <20 ng/ml), glucose (114 mg/100 ml), and lactate (170 mg/100 ml).

Light and Electron Microscopy of Monolayers

Incubated hepatocytes were examined under the usual and the inverted phase-contrast microscope and under the electron microscope according to the methods described in the preceding paper (79). The diameters of lipid droplets were measured with the Zeiss TGZ 3 particle size analyzer (Carl Zeiss, Inc., New York, N. Y.).

Incorporation of [14C]Glucose into Glycogen

The plated cells were incubated in the presence of 0.80–0.96 μCi[14C]glucose per milliliter for periods of 24 h. At the end of the incubation time, but before collecting the cells, the cultures were washed twice with 2 ml of Hanks' solution kept at 37°C and equilibrated with Carbogen in order to obtain a pH of 7.4. Cells detached by scraping the bottom surface of the petri dishes with a policeman were resuspended in 0.5 ml of Hanks' solution, and two fractions of 0.2 ml were used for radioactivity measurements and colorimetric determinations of glycogen.

Glycogen Extraction and Radioactivity Determinations

Glycogen was extracted from the cells by the method of Good et al. (37). The cells were treated with 30% KOH at 100°C for 30 min. After this chemical digestion, glycogen was precipitated with ethanol at a final concentration of 66%. This precipitated material was stored overnight at 0°C and was then centrifuged at 1,600 g for 30 min. Pellets were used for the glycogen and radioactivity determinations. Glycogen determinations were made by applying the colorimetric technique of Krisman (51). The radioactivity was measured according to the method of Witters et al. (72). Therefore, the glycogen pellet was resuspended in 0.25 ml of distilled water, and two aliquots of 0.1 ml were adsorbed on 31 ET Whatman filters; the filters were dipped into 66% ethanol at 0°C, washed twice in ethanol of the same grade and in acetone, and finally dried. The filters loaded with glycogen were counted in vials containing 10 ml of scintillation medium (4 g 2,5-diphenyloxazole (POPOP), 0.12 g 1,4-bis-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) in 1 liter of toluene).

MATERIALS AND METHODS

Isolation of Hepatocytes

Female adult Sprague-Dawley rats, weighing 200–250 g and fed ad lib. with pellets of standard rat food, were used in our assays. Isolation of hepatocytes was performed by the continuous enzymatic perfusion technique (3) slightly modified as mentioned in previous papers (25, 79). The isolated liver cells were mainly parenchymal cells. The usual criteria of preservation, the Trypan blue exclusion test, and the microscope examinations were applied to each cell preparation. In our experience, the cell preparations yield 85–95% viable cells. Cell counting was carried out with a hemocytometer.

Hepatocyte Culture

The culture conditions were given in the preceding paper (79). The glucose concentration, as stated in the text, was either 20 or 40 mM. When insulin was added to the dishes, 20 mU/ml were used. The following contaminating hormonal and substrate concentrations of fetal calf serum were determined in order to check their possible interference with the experimental parameters: insulin (≤3.5 μU/ml), glucagon (119 pg/ml), growth hormone (≤1 ng/ml), cortisol (undetectable concentration, <20 ng/ml), glucose (114 mg/100 ml), and lactate (170 mg/100 ml).
UDPG-Glycogen Glucosyltransferase
(Glycogen synthetase;
EC 2.4.1.11) Determinations

The method of Thomas et al. (72) which was used consists of incubating the cell preparations in the presence of UDP-[14C]glucose as substrate and measuring the incorporation of the radioactive glucose moiety into glycogen. The assay mixture contains 6.7 mM [14C]-glucose-labeled uridine diphosphoglucose (UDPG), buffered at pH 7.8 with 50 mM Tris-HCl, 5 mM EDTA and 10 mg glycogen/ml. The specific radioactivity of the medium amounts to 0.0744 Ci/mmol. In the case of total enzyme activity measurements, 6.7 mM glucose-6-phosphate is added to the medium. The cell preparations to be tested were manipulated as follows: the petri dishes containing the cultured cells were rinsed twice with a Hanks'-5 mM EDTA solution maintained at 37°C; the cells were scraped off the plastic surface and collected in 5 mM EDTA, pH 7.8 and homogenized by 20 strokes in a 2-ml glass tissue homogenizer with a Teflon pestle (Tri-R Instruments, Inc., Rockville Centre, N. Y.). From the final 1-milliliter vol of homogenate, 0.2-ml samples were added to 0.4 ml of the substrate medium and incubated at 30°C. At different time intervals, 0.05-ml samples were transferred to Whatman filters as described in the preceding section. The loaded filters were dipped into 66% ethanol to precipitate glycogen, and were washed, dried, and counted.

Protein Determinations
The protein content was determined by the method of Lowry et al. (53).

Chemicals
Falcon dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.), Dulbecco’s culture medium, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), fetal calf serum, collagenase, and hyaluronidase were purchased as mentioned in the preceding paper (79). o-[U-14C]glucose (>230 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England, and UDP-[U-14C]glucose (>200 mCi/mmol) from NEN Chemicals, GmbH (New England Nuclear Corp., Boston, Mass.). Porcine insulin was a gift of Novo Research Institute (Copenhagen, Denmark). UDPG was purchased from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp. (Cleveland, Ohio). Glucose-6-phosphate was obtained from BDH Chemicals Ltd., Poole, England. Other products were obtained from Merck AG, Darmstadt, Germany or from Sigma Chemical Co., St. Louis, Mo.

RESULTS
To study the effects of glucose and insulin on cultured hepatocytes, we used Dulbecco’s medium with fetal calf serum as described in the preceding paper (79), but containing either 20 or 40 mM glucose and supplied or not with 20 mU insulin/ml. This considerably high hormone concentration was used in order to maintain adequate hormone concentrations in the culture for 24 h, taking into account its progressive degradation. Concentrations of 100 and 500 mU insulin/ml, which are close to physiological values, were also tested on the cultures, but their effects on cell plating, survival time, and glycogenesis were much less evident than those obtained with higher hormone concentrations.

Cell Plating
Under the phase-contrast microscope, no important modifications in the process of cell adhesion were observed during the first 24 h of incubation, in the glucose- and insulin-enriched Dulbecco’s medium. Hepatocytes adhere, within 4 h of culture, to the plastic surface of the petri dishes, as they do in the original Dulbecco’s medium, but flatten more rapidly on the bottom of the dishes and, after 24 h, form anastomosed trabeculae. Hepatocytes cultured for 48 h in the presence of insulin show an entirely different aspect, when compared to the control situation; cells are remarkably more flattened, become polyhedral in shape, and constitute trabeculae which enlarge to such an extent that cell confluence is reached at some places. Another striking difference from the controls is a significant and reproducible increase of the survival time of the cultures from 3 to about 6 days. Fig. 1 illustrates a 48-h culture of hepatocytes grown in the presence of insulin. Large undulating membranes extend from the periphery of the cells (Fig. 1, arrowheads) and tend to reduce progressively the open spaces. After 72 h of culture, cells constitute a confluent monolayer (Fig. 2), resulting from their increased spreading. No mitoses are observed during the 6 days of culture, either with phase-contrast microscopy or in smear preparations. Notice, in a few places, the round, nonviable cells (Fig. 2, arrowheads) which cover limited areas of the monolayer, as seen also in the control.

Ultrastructure
The morphological characteristics of hepatocytes cultured in the presence of 20 mM glucose were described in the preceding paper (79). Glycogen particles are sparse and dispersed in the cytoplasm of hepatocytes cultured for 24 h or 48
h. No striking ultrastructural features are observed when glucose is added at a final concentration of 40 mM. Glycogen particles, however, are more abundant but present no specific relationships with other cell organelles.

The addition of insulin, with either one or the other glucose concentration, promotes the following ultrastructural modifications. After 24 h, hepatocytes reveal a well-developed network of smooth (ser) and rough (rer) membranes (Fig. 3). The classical continuity between smooth and rough endoplasmic reticulum is particularly well demonstrated in this figure. The parallel, stacked rough cisternae exhibit clearcut limits (Fig. 3, arrows). In between the smooth tubules, numerous glycogen particles of various sizes are distributed; β- and α-glycogen particles appear to be intimately related to the membranes of the reticulum. In some regions, specific contacts are encountered; particles are connected to the membranes by simple contacts or by means of a kind of pedicle (Fig. 4, circles).

The exact nature of this relationship is still unknown. The presence, however, in between the smooth tubules of numerous glycogen particles of various sizes may represent the morphological expression of the initial steps of glycogen synthesis, specifically observed when glucose and insulin are added to the incubation medium. The typical relationships between these two cell organelles are not observed in hepatocytes incubated without insulin.

Numerous peroxisomes are present in the regions rich in glycogen and smooth membranes (Fig. 3, Pe). The classical cell membrane differentiation, described in the preceding paper (79), namely tight junctions and desmosomes, are well developed in the presence of insulin. Newly formed bile canaliculi associated with the development of a biliary polarity have the same morphological characteristics described earlier.

Hepatocytes incubated for 48 h in the presence of insulin exhibit characteristic morphological features, namely accumulations of glycogen particles, synthesis, storage, and extrusion of lipid droplets of varying size, and also an important polysome formation. Fig. 5 illustrates the enrichment of hepatocytes in glycogen (GI) and the presence of numerous, highly osmiophilic lipid droplets (Li), which are detected in the lumen of the smooth endoplasmic reticulum (Fig. 6, ser). A size-distribution histogram was established by measuring the diameter of 1,000 particles; the gaussian distribution is characterized by an average diameter of 120 nm and a mode of 100 nm, a standard deviation of 20 nm and extreme values of 50 and 200 nm. These lipoprotein-like particles differ in size from the classical 30-80-nm VLDL particles, described in liver cells in situ (17, 26) and in the perfused organ (39, 47). Few small particles were detected in the cisternal and vesicular compartment of the Golgi complex, but most of them were found in the cisternae of the smooth endoplasmic reticulum and in vesicles derived from it. These particles show a clear increase in size in the course of their evolution, the smallest particles being located mainly in the tubular portion of the ser and the largest particles in the isolated vesicles (Fig. 6). Finally, these particles are extruded into the intercellular space (Fig. 7).

Liposomes, devoid of a detectable surrounding membrane, are generally larger in size; the smallest droplets may coalesce to form large ones. With the fixation procedure described under Materials and Methods, lipid droplets appear to be intact and not solubilized. Finally, numerous polysomes are detected as free or membrane-related structures, when insulin is present in the medium (Fig. 6, circles). The increased formation of polysomes, induced by insulin, agrees with the results published by numerous authors (31, 57, 58, 59, 62, 74).

Nearly all the hepatocytes incubated for 72 h in the presence of insulin exhibit an important accumulation of glycogen particles in their cytoplasm. Confluent areas of densely stained glycogen particles are observed (Fig. 8, GI). This important storage of polysaccharide which was systematically encountered at this stage of culture corresponds to a three- to fivefold increase in the glycogen content measured in the isolated hepatocytes by biochemical methods. A fine, granular and filamentous material, the nature of which is unknown, is clearly detectable in between the glycogen particles (Fig. 8, inset, arrow). A large number of autophagic vacuoles are frequently observed in the cytoplasm of hepatocytes (Fig. 9). They contain glycogen particles in different degrees of degradation; few intact α-glycogen particles, similar in size and density to the particles of the cytosol, are still present in the vacuoles. On the other hand, polysaccharide chains composed of small β-subunits (Fig. 9, circles) represent polysaccharide degradation. The presence of membrane residues and electron-dense material confirms the autophagic properties of the vacuoles. In between the
vacuoles, single cisternae of the rough and smooth endoplasmic reticulum are closely related to the membranes surrounding the autophagic vacuoles. Finally, at the periphery of the cells, large cytoplasmic areas, which form undulating membranes, appear devoid of organelles such as mitochondria or membranes of the smooth and rough endoplasmic reticulum. They correspond to thin, flattened lamellae of cytoplasm, containing numerous, thin, parallel filaments, 70 Å in diameter (Fig. 10, fi), and microtubules (Fig. 11, tu) 200 Å in diameter, surrounded by a clear cytoplasmic area. At this stage of culture, a large accumulation of polyribosomes occurs, mainly detectable at the periphery of the cells, in areas rich in microfilaments. Synthesis of these filamentous structures by polyribosomes is suggested by the intimate relationships frequently observed between these two cell organelles.

The development of ser, the accumulation of newly synthetized glycogen particles and of lipid droplets, and the presence of numerous polyribosomes constitute the morphological features which characterize the effect of insulin on incubated adult hepatocytes. Administration of glucose alone without insulin, even at a high concentration, does not modify the classical submicroscopic aspects of cultured cells, described in the accompanying paper.

**Glycogen Synthesis**

Isolated hepatocytes are capable of synthesizing glycogen, using glucose from the medium, at a rate which is dependent on the glucose concentration, the age of the culture, and the presence of insulin.

Incorporation of labeled glucose into glycogen, measured for periods of 24 h of culture, is proportional to the glucose concentration in the medium; by raising the glucose concentration from 20 to 40 mM, the rate of glycogen synthesis almost doubles (Fig. 12a). This ratio is not observed when one uses a glucose concentration close to the physiological value; 7 mM glucose added to the medium induces a very low incorporation into glycogen. Therefore, high glucose concentrations were chosen in our assays, in order to amplify the effects of glucose on glycogenesis and also to reduce, to a large extent, glycogenolysis and the resulting isotopic dilution. The glucose incorporation rate, measured under both culture conditions, using 20 and 40 mM glucose in the medium, decreases with time in culture; considering the incorporation rate on the 1st day as being 100%, its value is 60% on the 2nd day and 45% on the 3rd day.

The glycogen content of isolated hepatocytes varies greatly from one preparation to another; a value of the order of 0.143 ± 0.055 mg/mg protein can be obtained by calculating the mean value for the different determinations. The values for 24-h cultures in the presence of 40 mM glucose are definitely higher than those for cultures containing 20 mM glucose (Fig. 12c). For both glucose concentrations, the glycogen content decreases on the 2nd and 3rd days. It follows that cultured hepatocytes do not accumulate glycogen in their cytoplasm. A glycogenolysis is superimposed on the glycogen synthesis, but it is appreciably reduced by raising the glucose concentration of the culture medium to 40 mM. These determinations were not carried out for longer periods of culture because, in the absence of insulin, a sudden involution of the cells occurs after the 3rd day.

**Effect of Insulin on Glycogen Synthesis**

Glycogen synthesis was studied in hepatocytes cultured in Dulbecco's medium containing 20 or 40 mM glucose, supplemented with insulin (Fig. 12b). Insulin added to the culture medium at the time of plating of the hepatocytes does not stimu-
late glycogen synthesis after 4 h of incubation and affects only slightly glucose incorporation into glycogen after 24 h of culture. One observes, however, a more important effect of insulin on the G20 than on the G40 system; an increase of about 30% is measured in the former and of about 10% in the latter. The more pronounced effect of insulin in the presence of lower concentrations of glucose confirms the observation of Akpan et al. (1) on isolated hepatocytes maintained in suspension. On the 2nd day of culture, the glucose incorporated into glycogen is increased three to five times under the influence of insulin, and this stimulation of synthesis is observed equally in both culture media containing 20 or 40 mM glucose.

On the 3rd day of culture, glycogenesis is maintained in both systems and even increased. Now, the difference in behavior between the cultures provided with insulin and the controls becomes more evident. The incorporation rates measured in the presence of insulin are comparable for both concentrations of glucose (G20 and G40). It should be noted, however, that greater discrepancies in the results are found in the 3-day culture of G20 to which insulin was added.

No variations were found, after 24 h of culture, in the amounts of glycogen determined in both systems, G20 and G40 provided with insulin, and the controls. The glycogen content of 48-h cultures containing insulin is about three times higher than that of the insulin-free controls (Fig. 12 d). It was observed that, in the G40 + insulin condition, twice as much glycogen accumulates as in the G20 + insulin condition, a ratio which does not parallel the incorporation rates. On the 3rd day of culture, the glycogen content is still increased and the G20 condition is affected more than the G40, but here also a greater standard of deviation is found.

In cultures containing physiological concentrations of glucose (6–7 mM), the incorporation of glucose into glycogen decreases with time to the same extent as in G20 and G40 cultures, but the absolute values are much lower. This result is due to the intensive glycogenolysis that occurs during the first 24 h of culture. Insulin induces, under such culture conditions, a stimulation of the incorporation of glucose into glycogen comparable to that measured in G20 and G40 conditions. The low glycogen content is doubled 48 h after the addition of insulin and tripled after 72 h. It may thus be concluded that a similar stimulation of glycogenesis is observed with physiological glucose concentrations and with higher concentrations.

For the interpretation of the preceding results, it appeared essential to verify the integrity of the insulin receptor sites and the degree of preservation of the hormone during the incubation.

The low response to the hormone measured during the first 24 h of culture may not be related to a deficient fixation of insulin to the receptor sites. Indeed, specific receptors for this hormone were detected on the isolated hepatocytes.1 These results will be published separately.

The massive degradation of the hormone does not account for the low stimulation of glycogen synthesis. About 3% of the insulin added at the start of the culture was recovered after 24 h and 41% during the following 24 h of culture, the medium including insulin being renewed after the 1st day. Despite this breakdown of hormone, enough insulin subsists to produce an effect, since the residual concentration of insulin, evaluated by the TCA method, exceeds the physiological concentration found in portal blood.

Insulin added repeatedly during the 1st day of

FIGURES 3–4 Hepatocytes cultured for 24 h in Dulbecco's medium containing 40 mM glucose and insulin (20 mU/ml).

FIGURE 3 A well-developed network of smooth tubules meanders in extended regions of the cytoplasm, presenting classical continuities (arrows) with the rough endoplasmic reticulum (rer). In between the smooth membranes, numerous glycogen particles of various sizes are detected. Notice the presence of peroxisomes (Pe) in the vicinity of the rer. × 14,500.

FIGURE 4 Detailed view of a cytoplasmic region rich in glycogen particles and smooth membranes. Numerous particles of various sizes and types, from the unitary β-particles to the composed α-particles, are distributed in between membranes of the rer. They appear free or intimately associated to the membranes of the rer (circles). × 40,000.
FIGURE 5  Hepatocytes cultured for 48 h in the presence of 40 mM glucose and insulin (20 mU/ml). Glycogen particles accumulate in the cytoplasm in areas devoid of smooth membranes (Gl) and form uniform and compact masses. Lipid droplets of various sizes (Li) are detected in the lumen of the ser. Large fat droplets are frequently observed in many cells. × 9,500.
culture, with the object of compensating for the rapid degradation of the hormone, does not modify the rate of glycogen synthesis.

The presence of insulin in the medium during the first 24 h of culture is not a prerequisite for inducing an important stimulation of glycogen synthesis after 48 h. As shown in Fig. 13, the addition of insulin 24 h after plating gives a similar stimulation. When insulin is added after 48 h of culture, the degree of stimulation obtained does not change, as compared to the previous situation. The ability of the cells to respond to insulin is thus totally preserved, but many problems remain to be solved concerning the weak response of the cells to insulin on the 1st day of culture.

Glycogen Synthetase

In addition to the glucose incorporation studies described above, the activity of the glycogen synthetase was measured in hepatocytes cultured for 24 h, 48 h, and 72 h. The total activity of the enzyme and the proportion of its active form (synthetase α) were compared in three conditions: in the presence of 20 mM glucose (considered as the control), of 40 mM glucose, and, finally, of 40 mM glucose and insulin. The results are presented in Table I. In the hepatocytes cultured for 24 h, the total synthetase activity in most cases amounts to 60–67% of that measured in the initial cell population. After 48 h of culture, at the time the stimulation of glycogen synthesis by insulin is fully taking place, the G40I system shows a total synthetase activity similar, or even superior, to that of the previous day. The G20 and G40 systems, on the other hand, significantly reduce their total enzyme activity. After 72 h of culture, the reduction in the total activity is more pronounced and drops to similar values in the three systems. From the results summarized in Table I, we may conclude that high concentrations of glucose, 20 mM and 40 mM, do not stabilize the total synthetase activity of the hepatocytes, but that the G40I system does stabilize it up to the 2nd day of culture. Synthetase determinations carried out after 24 h of incubation of the cells do not show an increase in the amount of active synthetase, but a close study of shorter incubation times demonstrates the existence of a transitory activation phase of the enzyme (Fig. 14). In the initial population of hepatocytes, the glycogen synthetase is essentially present in its inactive form; after 1 h of incubation of the cells in the culture medium, the proportion of the active form of the enzyme, measured in systems G40 and G40I, is increased and reaches 18 and 25%, respectively; after 4 h of incubation, the proportion of active synthetase has regressed in both systems; finally, after 24 h of incubation, synthetase returns almost entirely to its inactive form. On the 2nd day, the enzymic determinations, obtained 1 h after renewal of the culture medium, reveal a new activation phase of synthetase. The activation rate of the enzyme at that time reaches, in the system provided with insulin, a value identical to that found on the 1st day; it is relatively lower in the insulin-free system. After 4 h of incubation, the active form of the enzyme has greatly declined in both systems, and after 24 h the percentage of the active form, measured in both systems, does not present a significant change. These results show that an activation of glycogen synthetase occurs at the time of stimulation of glycogen synthesis.

DISCUSSION

Hepatocytes in culture, being independent of the complex hormonal interactions which normally control their metabolic activities in vivo, offer the possibility of studying separately and for relatively long periods the factors which regulate glycogenesis.

We will discuss successively the effects of the glucose concentration and of insulin on the hepatocyte cultures and their influence on the changes which occur in the enzymes involved in glycogen metabolism. We will examine then the main ultrastructural modifications produced in the hepatocytes by the addition to the cultures of glucose at high concentration and of insulin.

Effect of Glucose

Cultured hepatocytes gave evidence of glycogen synthesis when high concentrations of glucose were added to the culture medium. A direct effect of glucose on the regulation of glycogenesis was shown in experiments carried out in vivo (22, 41, 50) in perfused livers (14, 35, 56) and also in isolated hepatocytes (1, 45, 64, 65). Glucose stimulates the inactivation process of phosphorylase and induces in this way the conversion of glycogen synthetase to its active form. The sequence of enzyme stimulations and inactivations under the influence of glucose has been described by Stalmans et al. (68).

The results presented in this paper show that hepatocytes in culture respond to glucose stimulation by a synthesis of glycogen, which is pro-
portional to the concentration of substrate in the medium. Of course, the amount of labeled glycogen formed after 24 h does not reflect the rate of glycogen synthesis but rather the equilibrium between synthesis and degradation. Despite the high concentrations of glucose used in our assays, the results reveal, on the 2nd and 3rd days of culture, a reduction of the incorporation rate and the absence of a net glycogen synthesis. The highest concentration of glucose, however, was found to inhibit more strongly the breakdown process of glycogen. All these results demonstrate that the effect of glucose decreases with time in culture.

**Effect of Glucose in the Presence of Insulin**

During the first 24 h of culture, insulin affects very slightly hepatocytes cultured in the presence of either one or the other glucose concentration, and the amount of glycogen accumulated is not modified by the hormone. On the 2nd and 3rd days, however, the incorporation of glucose into glycogen is greatly enhanced as compared to that of the 1st day and to the controls lacking insulin. The cells, at that time, store large amounts of glycogen. Some discrepancies were noticed between the values of synthesized glycogen determined colorimetrically and those calculated from the incorporation rates of glucose. A possible explanation would be a progressive diminution of the specific radioactivity of glucose. A notable production of endogenous glucose would be necessary to modify significantly the glucose concentration and, as a consequence, the specific radioactivity. The cells would form glucose either by glycogenolysis of unlabeled preexisting glycogen or by gluconeogenesis using gluconeogenic substrates, such as glucogenic amino acids, pyruvate, or lactate. This latter path appears unlikely under the experimental conditions we used, because the enzymes of the gluconeogenesis have very low activity in the presence of high concentrations of glucose. In favour of a gluconeogenic activity, we may refer to the observations of Seglen (66) which demonstrate a new regulatory function of glucose; it consists of stimulating the conversion of gluconeogenic substrates into glycogen. The simultaneous presence of glucose and fructose or of glucose and lactate in the incubation medium has a synergistic effect on the synthesis of glycogen. Under our experimental conditions, lactate may be formed from glucose present in the incubation medium. On the other hand, a relatively high concentration of lactate was detected in the fetal calf serum, so that the addition of serum to the culture medium amounts to a supply of about 3 mM lactate to the culture.

With respect to the influence of insulin on the gluconeogenesis, it was demonstrated that the hormone represses the key enzymes involved in this process (16, 34, 49, 80, 81).

**Hepatocytes Cultured in the Presence of an Excess of Glucose and in the Absence of Insulin Compared with a Diabetic State**

The behavior of hepatocytes cultured without insulin is similar to that of cells in the diabetic state, because the regulation effect of glucose is progressively lost; these cells are no longer capable of storing glycogen. Miller et al. (56) showed that a 3-h insulin deficiency, induced by injection of anti-insulin, produces a partial loss of control of hepatic glycogenesis by glucose and a 50% reduction of glucose incorporation into glycogen.

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**Figures 6-7** High-magnification electron micrographs of hepatocytes cultured for 48 h in the presence of glucose and insulin.

**Figure 6** Portion of hepatocytic cytoplasm, illustrating the presence of numerous electron-dense osmiophilic lipid droplets in the lumen of the ser. A membrane surrounds the droplet, and the droplets (arrows) are of various sizes, from 50 to 200 nm in diameter. Notice the presence of glycogen particles in between the anastomosing and branching tubules of the ser. Polysomes (circles) are frequently observed, associated with the membranes of the rer. pp: paraplasmic smooth membranes. × 20,000.

**Figure 7** Electron-dense lipid droplets of various sizes are extruded into the intercellular space. They accumulate in large clusters as illustrated in this micrograph, but they also appear as isolated particles. × 16,500.
Hepatocytes incubated for 72 h in the presence of glucose and insulin. Large areas of cytoplasm are filled with electron-dense glycogen particles of various sizes which are present in profusion in nearly all the cultured hepatocytes. In between the aggregated glycogen particles, portions of the cell matrix are characterized by the presence of a dense granular and filamentous material (circles). Microfilaments (fi) are detected at the periphery of the cell. Go: Golgi complex. × 14,500. Inset: detailed view of the cytoplasmic matrix, revealing thin filamentous structures, closely related to the glycogen particles (arrow). × 26,500.
Hepatocytes incubated for 72 h in the presence of glucose and insulin. Numerous autophagic vacuoles are observed in the peripheral cytoplasm. They contain glycogen particles at different steps of degradation and, less frequently, membrane derivatives. Thin polysaccharidic chains, composed of small β-particles, are observed in the cytolyosomes (circles). × 16,500.
**FIGURE 12** Effect of glucose and insulin on glycogen synthesis of cultured hepatocytes. (a) Glycogen synthesis measured in hepatocytes cultured in the presence of 20 mM (G20) and 40 mM (G40) glucose. Each column indicates the rate of glucose incorporation into glycogen after 24-h labeling with [U-14C]glucose. (b) Effect of insulin on glycogen synthesis of cultured hepatocytes. Insulin (20 mU/ml) is added to the G20 and G40 systems, at the time of cell plating. Rates of glucose incorporation into glycogen are measured after 24-h labeling with [U-14C]glucose in the presence of the hormone. The dotted lines represent the values of the corresponding systems (G20 and G40) incubated without insulin, i.e., the values reported in graph a. (c) Amount of glycogen determined in hepatocytes after 1, 2, and 3 days of culture, under conditions similar to those specified in a. (d) Amount of glycogen determined in hepatocytes cultured in the presence of insulin. In dotted lines, values obtained in the absence of insulin (the same as in c). The mean values are calculated from the number of experiments indicated in parentheses. Standard deviations are represented by the vertical lines.

**FIGURES 10 and 11** Peripheral cytoplasmic extensions of 72 h cultured hepatocytes.

**FIGURE 10** Numerous filaments (fi) disposed in bundles run in all directions. Notice the large amount of free and membrane-related polysomes (arrow). × 22,000.

**FIGURE 11** Longitudinally sectioned microtubules (tu), 20 nm in diameter, are frequently detected in the undulating membranes. Po: polysomes. × 22,000.
Friedmann et al. (28) observed that, in the diabetic rat, with severe glycaemia, the capacity to store glycogen is severely altered. These results and those of Hers et al. (41, 42) suggest that the hepatic threshold of glucose is particularly high in the diabetic state.

The mechanism by which the liver cells in the case of diabetes fail to respond to the glucose stimulus may be related to a deficiency of insulin-dependent enzymes: glycogen synthetase, glycogen synthetase phosphatase (7) or glucokinase. Insulin restores the effect of glucose on glycogen synthetase in diabetic rats (56) and induces, according to Steiner and King (70), the neosynthesis of this enzyme within about 40 min. Insulin also re-establishes the activity of synthetase phosphatase (36) and of the glucokinase (19).

The synthesis of this latter enzyme is induced by insulin, but it requires a relatively long time to be

**FIGURE 13** Rate of stimulation of glycogen synthesis by insulin, when added to the cultures at time 0 (GI), after 24 h of culture (G + I24h) and after 48 h of culture (G + I48h). As reference, glycogen synthesis measured in the absence of insulin (G). All cultures contain 40 mM glucose. The rates of glucose incorporation into glycogen are measured for periods of 24-h labeling with [U-14C]glucose. Notice the difference in activities between these assays and those of Fig. 12a and b, although the same type of development of the cultures is observed.

**TABLE 1**

Glycogen Synthetase Activity in Isolated and Cultured Hepatocytes

| Isolated cells: 10.92 ± 0.61 (12) |

| Monolayer cultures | G20 | G40 | G40I |
|---------------------|-----|-----|-----|
| Time h | nmol/min/mg protein ± SD | % | nmol/min/mg protein ± SD | % | nmol/min/mg protein ± SD | % |
| 24     | 7.30 ± 0.77 (7) | 100 | 7.14 ± 0.54 (5) | 100 | 6.55 ± 0.33 (6) | 100 |
| 48     | 4.82 ± 0.41 (5) | 66  | 5.00 ± 0.60 (4) | 70  | 8.00 ± 1.30 (5) | 122 |
| 72     | 3.77 ± 0.88 (4) | 52  | 4.15 ± 0.89 (2) | 58  | 4.10 ± 0.50 (3) | 62  |

G20 = Dulbecco's medium containing 20 mM Glucose; G40 = Dulbecco's medium containing 40 mM Glucose. G40I = Dulbecco's medium containing 40 mM Glucose and 20 mU Insulin/ml. The mean values are calculated for the number of experiments indicated in parentheses.
achieved; the maximum level is obtained only after 16 and even 48 h (28, 61, 70).

**Delay of the Response to Insulin**

We have shown that the addition of insulin to hepatocyte cultures in the presence of high concentrations of glucose strongly stimulates glycogenesis, but with a considerable delay. As a matter of fact, an effect of insulin on the activation of glycogen synthetase is observed from the very 1st hours of culture, but it does not lead to an increased glycogen storage at the 24th h. The reasons for this delay are numerous. It is clear that, at the beginning of the culture, the cells have to recover; the high rate of insulin degradation indicates an increased catabolic activity of the cells during that stage of culture. At the same time, the cells reorganize trabeculae and reconstitute cell membrane differentiations. This initial phase of restoration and formation of cell cords may necessitate glycogenolysis. Another factor that requires a long lag for response to hormones has to be taken into account, the induction of the synthesis of enzymes controlled by hormones. It is known that stimulation of the synthesis of transferrin and albumin by insulin in isolated hepatocytes requires between 24 and 48 h (46). It is probable that the increased glycogen synthesis observed on the 2nd day of culture in the presence of insulin may be the result of a manifold hormonal action; it would consist not only of an effect of insulin on glycogen synthetase, but also of the preservation by insulin of the synthetase phosphatase activity and of an induction of glucokinase.

**Glycogen Synthetase Activity**

One of the earliest effects of insulin that was shown to influence glycogenesis concerns the activation of glycogen synthetase in fed and starved normal animals and also in diabetic animals (1, 6, 8, 10, 30, 55, 56, 82). Our results show that the total synthetase activity is analogous in the cultures, whether provided or not with insulin, after the first 24 h and on the 3rd day of culture. After 48 h, the culture which contains insulin is distinct from the controls by a significantly increased total synthetase activity. If stimulation of glycogen synthesis is related to increased synthetase activity on the 2nd day of culture, this relationship does not exist on the 3rd day. In order to explain the difference in the glycogen synthesis measured in the controls and in the cultures to which insulin was added, an increased activity of glucokinase has to be taken into consideration.

Concerning the pattern of activation of the synthetase observed in our cultured conditions, in the course of the 1st day, it appears that the rate of enzyme activation measured after 1 h of incubation in the presence of insulin is higher than in the controls containing glucose alone; on the 2nd day, the effect of insulin on the synthetase activation is still more evident. Our data show that the activation of synthetase is not maintained for long periods of time, despite the high glucose concentration of the medium and the presence of insulin. The fact that synthetase is reactivated after renewal of the culture medium suggests that inhibiting substances accumulated in the medium could induce its inactivation. The rate of the enzyme activation varies with the residual concentration of phosphorylase $a$, which in turn depends on a complex equilibrium between various regulating factors. Glycogen itself regulates its proper synthesis. At high concentrations, it inhibits synthetase phosphatase (23, 42, 76) and phosphorylase phosphatase (67). This effect would not be exerted directly but via the formation of a complex with their substrate (76).

**Influence of Insulin on the Cell Survival**

In the presence of insulin, the survival of hepatocytes in culture is practically doubled as compared to that observed for cells cultured without the hormone. This observation may be related to the effect of the hormone on cell growth, although insulin does not induce cell divisions in our experimental systems. Several authors established that insulin promotes the growth of various cells in culture (31, 44, 71), and it was even demonstrated that insulin could replace serum (31, 43, 71). Cell growth is in fact due to the anabolic effects of insulin, namely to an enhanced synthesis of DNA, RNA, proteins, lipids, glycogen, to an increased uptake of metabolites, and also to a reduced degradation of macromolecules (38, 83). All reactions involved in these broad insulin effects are known as the "pleiotypic response" (43).

**Smooth Endoplasmic Reticulum and Glycogen**

The morphological analysis of the hepatocyte reveals a frequent close relationship between glycogen particles and smooth-walled endoplasmic
It is a common observation that the ser proliferates during synthesis (18, 21) and degradation (15) of glycogen. All observations made concerning this matter suggest a functional relationship between the polysaccharide and the membranous organelle, although the majority of enzymes involved in the metabolism of glycogen are linked to the glycogen particle (63, 75).

Relationships between β-glycogen particles and sarcoplasmic vesicles have also been detected in striated muscle (77). Furthermore, an association between a polysaccharide-enzyme complex, comprising phosphorylase b and phosphorylase b kinase, and sarcovesicles has been shown (78). Flash activation of these enzymes by calcium suggests the possible physiological role of these complexes in glycogenolysis during contraction (40, 78).

The morphological and biochemical results obtained in our hepatocyte cultures are compatible with a participation of the ser in glycogenesis. Various arguments have been put forward to explain the role of the ser in the metabolism of glycogen. Cardell (15) postulates a correlation between the proliferation of the ser during glycogen degradation and the stimulation of glucose-6-phosphatase activity. Friedmann et al. (28) noticed that glucose-6-phosphatase presents also a phosphotransferase activity which may be the prevailing one in some conditions. More recently, Berthillier and Got (4) discovered the existence of a new biosynthetic pathway for UDPG, localized in the microsomes. Moreover, the same workers (5) showed the presence of several glucosyltransferases in the microsomal fraction, one of which has a glycogen synthetase function.

It seems safe to extrapolate that these various enzymes that are linked to the metabolism of glycogen and integrated in the membranes lining the endoplasmic reticulum (er) are responsible for the changes in the ser which are concomitant with variations in the amount of glycogen.

**Lipid Accumulation**

The ultrastructural events related to lipid metabolism, which were observed in our cultures under the influence of insulin, were particularly evident after 24 and 48 h of incubation. An important accumulation of lipid droplets was detected in the cisternae of the er. These lipid inclusions differ from the lipoprotein particles by their heterogeneity in size, by their large mean diameter, and also by their particular distribution throughout the cisternal system of the cell. It is obvious that in hepatocytes cultured in Dulbecco’s medium supplemented with fetal calf serum, glucose, and insulin, the lipid droplets were detected essentially within the ser; in the classical distribution of the lipoprotein particles described by Jones et al. (47) and confirmed by many workers (33, 39, 54), the relatively homogeneous population of particles was found in the smooth-surfaced terminal ends of the rough endoplasmic reticulum, in the ser, and as clusters in the Golgi saccules (17, 39). This last characteristic site of accumulation of the lipoprotein particles, which is enhanced after alcohol treatment of the animals (26), is completely absent under the culture conditions described here. The export of the lipid droplets, on the other hand, is a feature common to lipoprotein particles and lipid droplets described in our cultures.

Although no proof as to the exact nature of the lipid droplets can be put forward in the present state of our investigations, this lipid material may be considered as accumulations of triglycerides. This assertion is based on similar observations reported in the early phase of triglyceride accumulation leading to the fatty liver, observed after partial hepatectomy (73) and after ethionine (52) or ethanol poisoning (69). Moreover, these lipid droplets are similar to those detected in parenchymal cells after perfusion with free fatty acids for long periods of time, up to 2 h (39).

The stimulation of lipogenesis by insulin may be related to the finding of Gibson et al. (32) who noted that this response was due to induction of lipogenic enzymes. Similar results were obtained with hepatocytes isolated from adult rats and incubated in Ham’s medium, supplemented with fetal calf serum and physiological concentrations of insulin (29). High glucose concentrations enhance the effect of insulin on glucose incorporation into hepatic triglycerides (27, 60). These experimental conditions, which fit perfectly with those used in our cultures, may thus explain the accumulation of lipid droplets.

**Autophagic Vacuoles**

Autophagic vacuoles are detected in the control situation, in relatively large amounts after 48 h of incubation. They contain membranous debris and only a few glycogen particles. Hepatocytes cultured in the presence of glucose and insulin, on the other hand, display a large accumulation of glycogen and only few autophagic vacuoles in their cytoplasm after 48 h of incubation. However,
after 72 h of incubation, numerous glycogen-filled autophagic vacuoles are observed. This situation is similar to that encountered in a specific glycogen storage disease, type II glycogenosis, characterized by a nearly complete deficiency of the lysosomal acid α-glucosidase (2, 20). Although no enzymatic determinations were carried out in our preparations, the morphological observations reveal the dissociation of α-glycogen particles and their breakdown into small subunits inside the autophagic vacuoles. This degradation process was never observed in the cytoplasmic glycogen where intact rosettes are present. Our pictures suggest, therefore, that, in some conditions of intensive glycogen synthesis leading to large accumulations of polysaccharide, the lysosomal pathway of polysaccharide degradation would take over part of the catabolic activity. This autophagosomic activity may correspond to a secondary physiological pathway by which an acute breakdown of storage material occurs, and would be preferential to the phosphorylase degradative pathway, inhibited by the high glucose concentration of the medium.

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