Role of Cortisol on the Glycogenolytic Effect of Glucagon and on the Glycogenic Response to Insulin in Fetal Hepatocyte Culture*

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The effects of insulin and glucagon on glycogen metabolism were studied in cultured fetal hepatocytes transplanted from 15-day-old fetuses. The effects of these hormones were examined just after transplantation, when the cells contained only minute amounts of glycogen, and during the 3 to 4 day culture period, when the hepatocytes were exposed to 10 μM cortisol and actively accumulated glycogen. At all stages of the culture, glucagon addition (10 nM) followed by a rapid depletion of labeled glycogen, previously synthesized during a pulse labeling with [14C]glucose: this effect was mimicked by N4',O2'-dibutyryl adenosine 3':5'-monophosphate (dibutyryl cyclic AMP) (0.3 to 1 mM). Such a glycogenolytic effect of glucagon was observed even 6 hours after transplantation, i.e. at a time when cortisol was not present. In addition, glucagon clearly induced cyclic adenosine 3':5'-monophosphate (cyclic AMP) accumulation in cells grown for 18 hours in the absence of cortisol. With cells grown for 3 days in the presence of cortisol, glucagon-dependent glycogenolysis was also obtained when cortisol was removed from the medium 20 hours before hormone addition. Thus the presence of cortisol is not necessary either to maintain a response to glucagon or for the onset of the glycogenolytic effect of glucagon. Insulin addition (10 nM) stimulated [14C]glucose incorporation into glycogen at all stages of the culture when grown in the presence of cortisol; no glycogenic response to insulin was observed 6 hours after transplantation where cortisol was not previously introduced. In addition, if the hepatocytes were grown in the presence of insulin alone (i.e. in the absence of cortisol) no significant storage of glycogen occurred. Maximal storage (or labeling) of glycogen was observed when hepatocytes were grown in the presence of both cortisol and insulin. The presence of cortisol was therefore necessary for the expression of the glycogenic effect of insulin. These data show that marked differences exist between the onset of developmental responses towards glucagon and insulin. The glucagon-dependent regulatory pathway should be present very early in fetal development and should not depend on cortisol. On the contrary, the onset of the insulin-dependent regulatory pathway seems to be induced during culture, and it is likely that this is caused by cortisol.

It has been demonstrated that the presence of glucocorticoids in the plasma of the rat fetus is necessary for the accumulation of hepatic glycogen in late gestation (1-5). The presence of high levels of insulin in the plasma of the rat fetus during this period is well established (6-11) and the implication of insulin in the glycogen storage process has been suggested (12). Glucagon has been considered as an hormonal factor contributing to the mobilization of glycogen stores at birth (4, 13, 14). In vitro studies performed with perfused rat liver have clearly shown both a glycogenolytic effect of glucagon (15) which is mediated by cyclic AMP1 (16) and a glycogenic effect of insulin (17).

1The abbreviations used are: cyclic AMP, cAMP, adenosine 3':5'-monophosphate; dibutyryl cAMP, Br,cAMP, N4',O2'-dibutyryl adenosine 3':5'-monophosphate.
glycogenolytic response to glucagon was also present before any glycogen storage and in the absence of cortisol. On the contrary, a marked glycogenic effect of insulin was observed only in hepatocytes that have been previously exposed to cortisol.

**EXPERIMENTAL PROCEDURE**

Methods—Primary cultures of hepatocytes were obtained from 15-day-old rat fetuses (Sprague-Dawley), using a culture procedure which already has been described (18, 20). This procedure involves dissociation of liver cells by treatment with trypsin, plating of a mixed cell suspension (on a collagen substratum to which only the hepatocytes adhered), and subsequent removal (after 6 hours) of the nonadhering hematopoietic cells (20). In some experiments, in order to study the effects of glucagon and insulin at earlier stages of the culture, the separation step was performed after 2 hours. The hepatocytes were grown for up to 4 days, the culture medium being replaced every 24 hours. Cortisol (10 μM) was introduced in the culture medium at the time of separation of the cells and after each 24-hour period together with the fresh medium. In other words, cortisol was always absent during the first 6 hours of plating. Several experiments were also performed without the presence of cortisol during the culture period chosen (2 to 6 hours to 4 days). The composition of the culture medium was as follows: NCTC 109, 90 parts/fetal calf serum, 10 parts. Fetal calf serum was carefully selected from several batches. The glucose concentration of the fresh medium was 1.05 mg/ml. The medium was replaced every 24 hours in order to minimize the variations in glucose concentration throughout the 4-day culture period. In short labeling experiments (4 to 6 hours of labeling), [14C]glucose was added 16 hours after replacement of the medium; at this time the glucose in the medium was measured and found to vary from 0.90 mg/ml at Day 1 to 0.78 mg/ml at Day 4. Thus the variation in medium glucose concentration during the culture period, where the effects of insulin and glucagon were measured, varied only by about 10%. In some experiments the specific radioactivity of the glucosyl residues of glycogen was determined as previously described (18).

Measurements of glycogen and protein contents (18), of glycogen labeling in the cultures grown in the presence of [G-14C]glucose, of glucose concentration in the medium (18), and of cyclic AMP levels in the cells (19) were performed as previously reported. The results were expressed per million hepatocytes (18), counting of the cells being performed on parallel cultures. The number of cells relative to wet weight was evaluated. For instance, after 3 days of culture in the presence of cortisol, one million hepatocytes corresponds to 380 μg of protein. By referring to the protein content per mg of fresh 4-day-old rat liver, one million hepatocytes corresponds to about 2.7 mg wet weight.

RESULTS

**Development of Glucagon-dependent Glycogenolytic Response and Effect of Cortisol—**Glucagon clearly produced a breakdown of glycogen stored in fetal hepatocytes taken from 15-day-old fetuses and grown for 4 days in the presence of cortisol (19). This glycogenolytic effect of glucagon was reproduced by cyclic AMP and the addition of glucagon was followed by cyclic AMP accumulation in the cells (19). However, it was not clear from these experiments whether the glycogenolytic response to glucagon was induced by cortisol or whether 15-day-old hepatocytes were already competent to respond to glycogenolytic agonists. To answer these questions the effect of glucagon or dibutyryl cAMP upon [14C]glucose incorporation into glycogen was studied at different times of the culture. Glucagon (10 nM) or dibutyryl cAMP (1 mM) were introduced together with [14C]glucose in 15-day-old hepatocytes grown for 1, 2, 3, or 4 days in the presence of 10 μM cortisol (Table I). Glycogen radioactivity was measured 4 hours after addition of the label. Table I shows that glucagon produced a clear inhibition of net [14C]glucose incorporation into glycogen not only at Day 4 of culture, when the rate of glycogen synthesis was high but also at Days 3 and 2, and even at Day 1, where no effect of cortisol on glycogen storage function was observed (18). To examine further the effects of glucagon in the absence of cortisol glucagon was added to cultures of 15-day-old hepatocytes 2 to 24 hours after plating in a medium lacking cortisol. Glucagon markedly inhibited the accumulation of 14C label in glycogen in cells cultured without cortisol for either 6, 18, or 24 hours. Negative results were obtained only when glucagon was added 2 hours after plating the cells (Table II). Dibutyryl cAMP (1 mM) clearly inhibited net [14C]glucose in-

**Table I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Glycogen radioactivity after glucagon or dibutyryl cAMP addition at:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>Experiment A</td>
<td>None</td>
</tr>
<tr>
<td>10 nm glucagon</td>
<td>1,500</td>
</tr>
<tr>
<td>Experiment B</td>
<td>None</td>
</tr>
<tr>
<td>1 mM BtCAMP</td>
<td>960</td>
</tr>
<tr>
<td>Experiment C</td>
<td>None</td>
</tr>
<tr>
<td>10 nm glucagon</td>
<td>2,020</td>
</tr>
<tr>
<td>1 mM BtCAMP</td>
<td>720</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Glycogen radioactivity after glucagon or dibutyryl cAMP addition at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hr</td>
</tr>
<tr>
<td>None</td>
<td>3,370*</td>
</tr>
<tr>
<td>10 nm glucagon</td>
<td>3,090*</td>
</tr>
<tr>
<td>None</td>
<td>3,900*</td>
</tr>
<tr>
<td>1 mM BtCAMP</td>
<td>1,000*</td>
</tr>
</tbody>
</table>

The cultures were performed in the presence of 10 μM cortisol, cortisol being introduced in the medium at 6 hours, the time when the hepatocytes were freed from the hematopoietic cells. At times indicated in the table, [14C]glucose (1.25 μCi/mg) was introduced together with or without glucagon or dibutyryl cAMP (BtCAMP). The radioactivity (in counts per min per million hepatocytes) present in the newly synthesized glycogen was measured 4 hours later. Each value is the mean of two cultures.
corporation into glycogen 2 hours after plating the cells (Table II). Throughout the culture period, dibutyryl cAMP produced similar (but larger) effects than 10 nM glucagon (Tables I and II). The finding that dibutyryl cAMP was more effective than glucagon when employed at a saturating dose was not reproduced with all the dibutyryl cAMP batches (19); thus, this observation cannot be considered as significant.

A second group of experiments was performed under the same conditions except that glucagon (or dibutyryl cAMP) was added only after labeling of the cells during 4 hours with [14C]glucose. Radioactivity incorporated into glycogen was measured after 4 and 6 hours of labeling, i.e. before, and 2 hours after glucagon or dibutyryl cAMP addition. The glycogenolytic effect of these agonists was estimated by the decrease in glycogen radioactivity. Glucagon (Fig. 1) and dibutyryl cAMP (Table III) stimulated throughout the culture period the degradation of the glycogen, newly synthesized in the presence of cortisol. A clear glycogenolytic effect of glucagon and dibutyryl cAMP was also observed 6 hours after plating, i.e. in the absence of cortisol (Fig. 1 and Table III). Therefore the hepatocytes responded to glucagon after 6 to 24 hours of culture in the absence of cortisol (Fig. 1 and Table III). This response was accompanied by an accumulation of cyclic AMP in these young cells. For instance, after 18 hours of culture in the absence of cortisol (Fig. 2), glucagon increased cyclic AMP levels, the peak occurring within minutes. The cyclic AMP level remained elevated for the 4 hours of glucagon exposure. Similar results have been previously described with hepatocytes grown during 4 days in the presence of cortisol (19). Cyclic AMP accumulation was also observed when glucagon was added 6 hours after plating the cells in the absence of cortisol (results not shown).

Glycogenolytic Effect of Glucagon after Cortisol Removal—
The experiments reported above suggest that the effect of glucagon on glycogen metabolism in fetal hepatocytes does not depend on the presence of cortisol. It has been shown (18) that the presence of cortisol was necessary to maintain high glycogen levels and that glycogen was markedly depleted 24 hours after removal of cortisol from the medium. In order to determine whether the presence of cortisol is necessary to maintain a glycogenolytic response to glucagon the following experiments were performed. Hepatocytes were grown for 3 days in the presence of [14C]glucose and 10 μM cortisol. At Day 3, when the medium was replaced, 10 nM glucagon was added, cortisol being removed from some of the cultures. Fig. 3 shows that glucagon induced, within 4 hours, a clear breakdown of glycogen both in the presence and the absence of cortisol. After 8 hours in both hormonal situations, a partial restoration of the glycogen content was observed. A clear effect of cortisol removal was observed only after 16 to 24 hours. However, since cortisol action is slow (18), it would have been surprising if its removal interfered with the immediate effect of glucagon addition. Another experiment was therefore performed where

| Table III
| Glycogenolytic effect of dibutyryl cAMP at different times of culture |
|-----------------|-----------------|
| Duration of pulse | Glycogen radioactivity after Bt,cAMP addition: |
| Addiition | No cortisol | Cortisol present |
| 6 hr | 24 hr | 48 hr | 72 hr | 96 hr |
| 4 hr | None | 3,260 | 8,510 | 8,510 | 7,300 | 21,020 |
| 6 hr | None | 2,990 | 5,710 | 8,510 | 7,300 | 21,020 |
| 6 hr | 0.3 mM Bt,cAMP | 1,310 | 3,370 | 4,490 | 3,230 | 11,160 |

Fig. 1. Glycogenolytic effect of glucagon at different times of the culture. At times indicated on the abscissa, the effect of 10 nM glucagon on the rate of degradation of newly synthesized glycogen (4 hours labeling in the presence of [14C]glucose) was measured. Time 6 hours, hepatocytes freed from hematopoietic cells 2 hours after plating and then grown in the absence of cortisol. Time 24, 48, 72, and 96 hours, hepatocytes freed from hematopoietic cells 6 hours after plating and then grown in the presence of 10 μM cortisol. Four hours before the time indicated on the abscissa, [14C]glucose (1.25 μCi/mg) was introduced and glycogen radioactivity measured after 4 hours (left bars) and 6 hours (right bars) of labeling. Right hatched bars, cultures exposed to 10 nM glucagon after 4 hours of labeling and grown for a further 2 hours. Left and right empty bars, control experiments with no glucagon addition. Two cultures were used for each point, the individual values being represented by a horizontal line on each bar.

Fig. 2. Cyclic AMP levels of hepatocytes grown in the absence of cortisol after exposure to glucagon. Hepatocytes were grown for 18 hours in the absence of cortisol. At zero time, 10 nM glucagon and 10 nM theophylline were added to the medium and the cultures were analyzed for cyclic AMP content after 2 and 5 min of incubation. Cyclic AMP content was also measured after 4 hours of incubation (II) performed in the presence of glucagon and in the absence of theophylline. Control culture which has received only theophylline (10 mM) at zero time.
glucose. Table IV shows that in the absence of cortisol, no
insulin, 0.93 (Day 2), 0.99 (Day 3), and 1.0 (Day 4) rCi/mg of
presence of cortisol alone, specific activity was 1.0 (Day 2), 0.95
significantly modified by the presence of insulin: in the
specific activity of glucose units in the stored glycogen was not
be uniformly labeled. Moreover it was verified that the
these conditions, since the glycogen pool is very low at the time
replacement of medium, cortisol was either withdrawn (O, △), or not
(●, △), from the medium and 10 nM glucagon was added. Both
glycogen content (B) and radioactivity present in glycogen (A) were
determined on the same cultures.

10 nM glucagon was added only 20 hours after cortisol removal.
Fig. 4 shows that glucagon still induced a clear fall in the level of
labeled glycogen. Thus maintenance of a glycogenolytic
response to glucagon does not seem to depend on cortisol.

Development of Insulin-dependent Glycogenic Response;
Effect of Cortisol—The glycogenic effect of insulin was first
tested by [14C]glucose labeling experiments, hepatocytes being
grown in the presence of cortisol from the beginning of the
culture. [14C]Glucose was introduced at Days 1, 2, 3, and 4 of
culture, with or without 10 nM insulin, and the radioactivity of
glycogen was measured 4 hours after addition of the label. Fig.
5 shows that at Days 2, 3, and 4, when cortisol-dependent
glycogen storage was present, insulin markedly stimulated
glycogen labeling. Insulin produced little glycogen labeling at
earlier times when cortisol was ineffective with regard to
glycogen metabolism. On the other hand very little, if any
increase of glycogen labeling by insulin could be observed 2
hours after plating of the cells in the absence of cortisol (Fig. 5).
Further experiments were performed to test if the insulin
glycogenic effect was cortisol dependent. The glycogen content
of the cells was measured in cultures grown in the presence and
in the absence of cortisol, with or without 10 nM insulin (Fig. 6).
Glycogen content was carefully expressed per million hepato-
cyes, as the presence of these hormones was able to change the
rate of proliferation of the cells.* Fig. 6 shows that in the
absence of cortisol the glycogen content remained low, whether
10 nM insulin was present or not. On the contrary the highest
glycogen levels were obtained in the presence of both cortisol
and insulin. The stimulatory effect of the combination of both
hormones was clearly expressed at Days 3 and 4. Since low
glycogen contents present at Days 1 and 2 were more difficult
to measure, another experiment was performed with
[14C]glucose present throughout the culture period. Under
these conditions, since the glycogen pool is very low at the time
of transplantation in 15-day-old hepatocytes, glycogen should
be uniformly labeled. Moreover it was verified that the
specific activity of glucose units in the stored glycogen was not
significantly modified by the presence of insulin: in the
presence of cortisol alone, specific activity was 1.0 (Day 2), 0.95
(Day 3), and 0.97 (Day 4) μCi/mg of glucose; with cortisol plus
insulin, 0.93 (Day 2), 0.99 (Day 3), and 1.0 (Day 4) μCi/mg of
glucose. Table IV shows that in the absence of cortisol, no

* M. C. Fulchignoni and C. Plas, unpublished results.

stimulation by insulin was detected at any time of the culture.
In the presence of cortisol, stimulation was present at Days 2,
3, and 4 of the culture as indicated by direct measurement of
glycogen content (Fig. 6).

DISCUSSION

In late gestation glycogen synthesis becomes important,
whereas at earlier stages only minute amounts of glycogen are
synthesized. If 16-day-old hepatocytes are grown for 4 days in
the presence of cortisol, both glycogen accumulation (18) and
a clear glycogenolytic response to glucagon (19) have been
previously demonstrated. It has been shown that this effect is
mimicked by cyclic AMP and potentiated by theophylline
(19); glucagon addition (10 nM) was also followed by an
increase of the tissue levels of cyclic AMP which was maximal
after 2 to 5 min (19). Therefore the plasma membranes of
15-day-old hepatocytes grown in the presence of cortisol very
likely contain a specific receptor for glucagon and a hormone-

FIG. 3. Glycogenolytic effect of glucagon, added just after cortisol
removal. Hepatocytes were grown throughout the culture period in the
presence of [14C]glucose, the medium being replaced every 24 hours
(specific radioactivity of glucose in fresh medium: 0.80 μCi/mg). After
3 days of culture in the presence of 10 nM cortisol, at the time of
replacement of medium, cortisol was either withdrawn (O, △), or not
(●, △), from the medium and 10 nM glucagon was added. Both
glycogen content (B) and radioactivity present in glycogen (A) were
determined on the same cultures.

FIG. 4. Glycogenolytic effect of glucagon, added 20 hours after
cortisol removal. The experimental protocol was similar to that
described under Fig. 3 except that the specific radioactivity of glucose
in fresh medium was 0.20 μCi/mg and that the effect of glucagon
addition was tested only 20 hours after replacement of the medium. On
Day 3, at the time of medium replacement, cortisol either was removed
(O, △), or not (●, △) from the medium. The glycogen radioactivity was
measured 20, 21, 22, 23, and 24 hours after medium replacement. △, △,
10 nM glucagon added at time 20 hours. C, ○, control experiment with
no glucagon addition.

FIG. 5. Glycogenic effect of insulin at different times of the culture.
At times indicated on the abscissa, the effect of 10 nM insulin on the
rate of incorporation of [14C]glucose into glycogen was measured. Time
2 hours: hepatocytes freed from hematopoietic cells 2 hours after plat-
ing and then grown in the absence of cortisol. Time 24, 48, 72, and 96
hours: hepatocytes freed from hematopoietic cells 6 hours after plating
and then grown in the presence of 10 μM cortisol. [14C]Glucose (1.25
μCi/mg) was introduced together with or without 10 nM insulin, and
the glycogen radioactivity was measured 4 hours later. Pointed bars, 10
nM insulin was added together with [14C]glucose. Empty bars, control
experiments with no insulin addition. Two cultures were used for each
point, the individual values being represented by a horizontal line on
each bar.

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resulting either from increased synthesis or from decreased
an increase in glycogen labeling might be interpreted as
glycogen labeling with \([Y!]\)glucose. The use of such isotope
incorporation studies requires two comments. First, a decrease
in glycogen labeling could be the result of either decreased
the experiments were performed by determining the changes in
measurement of cold glycogen was difficult to obtain. Therefore,
early stages of development of the hepatocyte, a direct mea-
accumulation in the absence of cortisol is minimal during the
role of cortisol on the effects of glucagon. Since glycogen
However these data did not provide information concerning the
ent protein kinase was found present in these hepatocytes8
sensitive adenylate cyclase as demonstrated in the adult liver
(21). Both activation and inactivation of the cyclic AMP-
dependent degradative and synthetic glycogen pathways were
observed (19). In addition, a fully active cyclic AMP-depend-
ent protein kinase was found present in these hepatocytes.3
However these data did not provide information concerning the
role of cortisol on the effects of glucagon. Since glycogen
accumulation in the absence of cortisol is minimal during the
early stages of development of the hepatocyte, a direct mea-
surement of cold glycogen was difficult to obtain. Therefore,
the experiments were performed by determining the changes in
glycogen labeling with \([^{14}\text{C}]\)glucose. The use of such isotope
incorporation studies requires two comments. First, a decrease
in glycogen labeling could be the result of either decreased synthesis or enhanced breakdown of the glycogen; conversely, an increase in glycogen labeling might be interpreted as resulting either from increased synthesis or from decreased
breakdown of the glycogen. The term "glycogenolytic effect" (or "glycogenic effect") used here does not mean that the
absolute rate of glycogenolysis (or glycogenesis) is stimulated,
but indicates a final effect at the level of glycogen. However in
the prelabeling experiments, glucagon induced the loss of \(^{14}\text{C}\)
from glycogen, indicating that the hormone had triggered the
net breakdown of glycogen. It is likely that glucagon produced
this effect at least by stimulating the absolute rate of glycogen-
olysis. In a previous work (19), it was shown that the glycogen
half-life was 50 min just after glucagon addition whereas, in
the absence of glucagon, the glycogen half-life was 12 to 14
hours; in these experiments the effect of glucagon was mea-
sured after 4 days of culture in the presence of cortisol. Second,
the amount of labeled glycogen depends on the specific activity
of the intracellular glycogen precursor pool which is different from
that of the specific activity of the extracellular labeled
\[^{14}\text{C}\]glucose (18). Changes in extracellular glucose concentration
could alter the specific activity of the glucose precursor pool.
The experimental procedure we have used permits us to
maintain the extracellular glucose concentration throughout
the culture period without large variations; most of the
experiments were performed with a glucose concentration
varying from 0.90 to 0.78 mg/ml. This excludes the possibility of variations of glucose concentration in the effect of the
hormones in glycogen metabolism, particularly that for insulin.
For instance, the specific activity of glucose units in glycogen
stores was not significantly modified by the presence of insulin.
These observations allowed us to study the role of cortisol on
the response to glucagon and insulin at the earliest stages of the
culture.
The experiments reported in this work show that a glycogen-
olytic response to glucagon was maximally expressed before
significant amounts of glycogen were stored (Fig. 1). In
addition, this glycogenolytic response was not found to be
induced by cortisol (Table II and Fig. 1). Removal of cortisol
for 20 hours did not suppress the glycogenolytic effect of
 glucagon (Fig. 4). Consequently, the presence of cortisol in the
medium is not required either for the onset of the glycogeno-
lytic response or for the maintenance of the response to this
hormone for at least a period of 20 hours. The absence of
response to glucagon (and insulin) after 2 hours of plating may
be explained by assuming that the membrane receptor for
 glucagon (or insulin) has been inactivated by trypsinization
(22), the method which is used to isolate the cells. This
assumption is supported by the finding (Table II) that dibu-
tryl cAMP was inhibitory to \(^{14}\text{C}\) labeling in glycogen 2 hours
after plating. On the contrary, 6 hours after plating, a
glycogenolytic response to glucagon was obtained, thus sug-
gesting that the glucagon receptor was either repaired or
synthesized de novo. It is not clear from the literature whether
in 15-day-old liver fetuses there are glucagon receptors in the
active form of adenylate cyclase. Adenylate cyclase activity
has been shown to be present (23, 24) but the ability to respond
to glucagon at this stage is controversial (23, 24). In addition,
the adenylate cyclase thus detected may belong, at least
partly, to the hematopoietic cells, which represent at this stage
60% of the liver population (25, 26). In late gestation (18-day-
old fetuses), glucagon stimulated both adenylate cyclase activ-
ity (23, 27, 28) and glycogenolysis (4, 13, 14). In the system
used in this work, cultured 15-day-old hepatocytes, cyclic AMP
accumulation was induced by glucagon 6 and 18 hours (Fig. 2)
after plating, in the absence of cortisol. Therefore these young
hepatocytes plated in the absence of cortisol are competent, or

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**TABLE IV**

Effects of cortisol and insulin on glycogen labeling in hepatocytes
grown in presence of \[^{14}\text{C}\]glucose

Hepatocytes were grown in the presence of \[^{14}\text{C}\]glucose (1.25
µCi/mg) throughout the culture period, the medium being replaced
every 24 hours. Glycogen labeling was measured at different times of
the culture performed in four hormonal situations as already described
under Fig. 6, cortisol alone, cortisol plus insulin, insulin alone, and no
hormonal addition. A typical experiment is presented, each value
represents one culture.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Glycogen labeling after a culture period of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>10 µM cortisol</td>
<td>12,300</td>
</tr>
<tr>
<td>10 µM cortisol + 10 nm insulin</td>
<td>12,700</td>
</tr>
<tr>
<td>None</td>
<td>7,200</td>
</tr>
<tr>
<td>10 nm insulin</td>
<td>7,000</td>
</tr>
</tbody>
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3 M. Pavlovic-Hournac and D. Delbauffe, personal communication.
rapidly become competent, within a 6-hour period, to respond to glucagon. However in the adult perfused liver, Exton et al. (29) have shown some effects of adrenalectomy on the glycogenolytic response to glucagon.

On the other hand, a glycogenic effect of insulin was clearly shown, when cortisol-dependent glycogen storage was present (Fig. 5). From the results obtained when a combination of cortisol and insulin was used during a 4-day period (Fig. 6 and Table IV), it might be assumed that these two hormones produce additive glycogenic effects. In the absence of cortisol, insulin alone might stimulate glycogen synthesis. Fig. 6 and Table IV show that no glycogen storage was obtained in the presence of insulin alone. A similar result was obtained by Eisen et al. (30) with 16-day-old liver in explant culture. However, these authors found that cortisol alone is also ineffective in stimulating glycogen synthesis, but that previous exposure to the steroid hormone for 40 hours permits the expression of the glycogenic effect of insulin. They assumed that when cortisol by itself stimulates glycogen synthesis in our culture system (18), some insulin could be introduced with the fetal calf serum contained in the culture medium (38). Careful examination of the immunoreactive insulin content of the fetal calf serum (batch 60, Sorga) used in the experiments reported above, revealed that the immunoreactive insulin content was only 5 microunits/ml; this activity was not detectable in the medium NCTC 109 supplemented with 10% fetal calf serum. Moreover, a much higher concentration of insulin (500 to 1,500 microunits/ml) than theoretically present in medium NCTC 109 supplemented with fetal calf serum (0.5 microunits/ml) is needed to obtain a clear glycogenic effect. Therefore, the cortisol-dependent glycogen storage obtained in the absence of insulin cannot be attributed to the presence of this hormone in the serum-containing medium. It could not be excluded also that fetal calf serum contains nonsuppressible insulin-like activity. The nonsuppressible insulin-like activity content of the medium used in these experiments was found to be very low (fat pad assay: lower than 25 microunits/ml, i.e. a value below the sensitivity of the assay; binding assay: 7.9 microunits/ml). However, even if the cortisol effect upon glycogen accumulation should depend on the presence of some unknown factor present in the serum, it remains clear that insulin (10 nm) is still able to induce a stimulation (Figs. 5 and 6, Table IV). In addition, stimulatory effects of cortisol were obtained in cultured liver explants (31) in the absence of both fetal serum (i.e. in absence of nonsuppressible insulin-like activity) and insulin. The present data strongly suggest, therefore, that cortisol is responsible for the onset of glycogen storage and that insulin only potentiates this effect. No true additivity could be shown but, rather, permissive effect of cortisol towards insulin for the development of the glycogen synthetic pathway. Cortisol might induce some step necessary for insulin action. If this step were to occur later in gestation, it would explain why insulin alone is effective in stimulating glycogen synthesis in liver explants from term fetuses (32) and in perfused adult liver (17). This step might also be the rate-limiting enzyme, glycogen synthetase, as it has been suggested that cortisol might be responsible for the synthesis of this enzyme during development (5, 18, 30, 33). It may be also concluded from in vivo (34, 35) and in vitro (18) studies that in the presence of cortisol, glycogen synthetase is mostly in the active form. Insulin might produce a further increase of the activity of the glycogen synthetase, as was suggested by the results obtained by studies performed with adult perfused liver (17) and fetal liver explants (30, 36).

In conclusion, a parallel study between glucagon and insulin response in cultured young fetal hepatocytes has been performed in this work. Marked differences have been found between them. Glycogen storage potential depends on cortisol and develops during the culture. The glucagon-dependent regulatory pathway is present very early in development, before any net storage of glycogen occurs and does not require cortisol. The onset of an insulin-dependent, regulatory pathway parallels that of the glycogen synthetic pathway and seems to depend on a permissive effect of cortisol. In the fetal hepatocyte in culture, cortisol appears as a hormone required both for the development of glycogen synthesis and for its regulation by insulin, but not for its regulation by glucagon.

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Role of cortisol on the glycogenolytic effect of glucagon and on the glycogenic response to insulin in fetal hepatocyte culture.

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