Glycogenolytic Response to Glucagon of Cultured Fetal Hepatocytes

REFRACTORINESS FOLLOWING PRIOR EXPOSURE TO GLUCAGON

(Received for publication, December 16, 1974)

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SUMMARY

The glycogenolytic effect of glucagon has been studied in fetal hepatocytes cultured for 3 to 4 days in the presence of cortisol (10 μM). The hepatocytes, when transplanted from young fetuses (15-day-old), contain only minute amounts of glycogen, whereas when cultured 3 to 4 days in the presence of cortisol, they contain high levels of stored glycogen. Glucagon induced a rapid but partial mobilization of glycogen, which was maximal after 2 hours. The half-maximal response was observed with about 0.1 nM glucagon. The glycogenolytic effect of glucagon in fetal hepatocytes is probably mediated by cyclic adenosine 3':5'-monophosphate (cyclic AMP) as in adult liver. This effect was mimicked by cyclic AMP and N6,O2-dibutyryl cyclic AMP, (dibutyryl cyclic AMP), and potentiated by theophylline. Glucagon addition was followed by accumulation of cyclic AMP in the cells within 2 min.

Glucagon produces a marked stimulation of the rate of glycogen breakdown and an inhibition of the rate of incorporation of 14C-glucose into glycogen. The glycogenolytic effect of a single addition of glucagon was reversed within 4 hours. A second addition of glucagon at this time was unable to induce a new glycogenolytic response. A resistance to glucagon stimulation appeared in the cells after a first exposure to the hormone. This refractoriness was also shown by the loss of glucagon-dependent cyclic AMP accumulation and was not linked to the release by the cells of a "hormone antagonist" into the medium. The hepatocytes resistant to the action of glucagon retained their response to cyclic AMP, dibutyryl cyclic AMP, and norepinephrine. Finally, glycogenolytic concentrations of cyclic AMP and of its dibutyryl derivative failed to induce a refractoriness to glucagon.

Large stores of hepatic glycogen are built up during late gestation in the fetal rat under cortisol control (1-4). Immediately after birth, this glycogen is rapidly mobilized to supply the glucose needs of the newborn. Glucagon has been considered as a factor involved in this mobilization, both because premature depletion of glycogen is obtained after glucagon administration to the fetus (4-6), and because blood glucagon levels increase at birth (7, 8).

In a previous paper (9), an in vitro system has been described which permits the study of the development of glycogenesis and its regulation in hepatocytes. Primary cultures of hepatocytes were obtained from 15-day-old fetuses, i.e. 3 days before the stage where glycogen synthesis begins in vivo. When these hepatocytes, which contain negligible or very minute quantities of glycogen, are grown in the presence of cortisol, glycogen is actively synthesized and accumulated within 3 to 4 days of culture (9). The purpose of the present work has been to study the response of the hepatocyte to glucagon at the level of its glycogen pool.

In the adult, in vitro studies performed with perfused liver (10) and with surviving isolated hepatocytes (11-13) have shown that the glycogenolytic effect of glucagon is linked to cyclic AMP accumulation. In the cultured hepatocytes, glucagon has a glycogenolytic action which is also mediated by cyclic AMP. In addition, this study has provided new information concerning the rapid reversal of the glycogenolytic response which becomes resistant to a second addition of glucagon. Some characteristics of this refractoriness which exists at the level of both cyclic AMP production and glycogen degradation (i.e. the physiological response) are also described in this paper.

EXPERIMENTAL PROCEDURE

Culture Procedure—Primary cultures of hepatocytes were obtained from 15-day-old rat fetuses (Sprague-Dawley) by a method already described (9, 14) with minor modifications. Dissociation of the cells was performed by trypsin treatment for 3 hours at 4°C, and then for 10 min at 37°C, followed by manual shaking in the presence of 0.10-mm diameter glass beads in a culture medium containing fetal calf serum. Hepatocytes were separated from hematopoietic cells as previously described (9, 14), and grown for up to 4 days. The composition of the culture medium was as fol-

1 The abbreviations used are: cyclic AMP, cAMP, cyclic adenosine 3':5'-monophosphate; dibutyryl cAMP, N6,O2-dibutyryl cyclic adenosine 3':5'-monophosphate; ACTH, adrenocorticotropin hormone; LH, luteotropic hormone; PGF2, prostaglandin E2; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
performed on parallel control cultures. The medium was supplemented with Hepes buffer, pH 7.3 (10 mM), and cortisol (10 μM). The fresh culture medium contained 5 mM glucose, and was replaced every 24 hours. The addition of glycogenolytic agonists was performed far from the time of replacement of the medium, when the glucose concentration of the medium had decreased to about 4 mM. Enumeration of cells was performed by a nuclear-counting procedure (16), and the proportion of non-hepatic cells was estimated by the counting of isolated nuclei. After 3 to 4 days of culture in the presence of 10 μM cortisol, the primary cultures consisted essentially of typical hepatocyte clusters assembled via the bile canaliculi (17). Contamination by non-hepatic cells (fibroblastic cells, macrophages) accounted for 15 to 20% of the culture after 3 to 4 days. Most of the experiments presented in this paper were performed on 15-day-old hepatocytes containing no glycogen at the time of transplantation, but similar results were obtained with 16- and 17-day-old hepatocytes.

Glycogen—The collection of cells was performed by a method already described (9, 14). Glycogen was extracted and purified according to Van Handel (18), and was measured by the anthrone reaction (19). In experiments of incorporation of [14C]glucose into glycogen, [14C]glucose was added to the culture medium at the beginning of the culture. This assumption was confirmed by the fact that the specific activity of glucose units in the glycogen was labeled, since the size of the glycogen pool was very low at the time of transplantation, but the glycogenolytic effect on glycogen was measured after a 4-hour period of incubation. From the experiment with no glucagon addition.

Cyclic AMP—The culture medium was discarded and the cells were rapidly frozen on solid CO2. The cellular material was collected in ice-cold trichloroacetic acid at 6%. After centrifugation, the supernatant was extracted five times in 4 volumes of ethyl ether saturated with water. The aqueous phase was evaporated in an air stream, and the residue dissolved in 0.2 ml of 50 mM acetate buffer at pH 6.2. The concentration of cyclic AMP present in aliquots was estimated by the radioimmunoassay procedure of Steiner et al. (22).

Materials—Trypsin (TRSF) was obtained from Worthington, medium NCTC 109 from Difco, fetal calf serum from Sorga (batch 60), Hepes buffer from Calbiochem, and 3001 Petri dishes from Falcon Plastics. Pork and beef glucagon containing less than 10 ppm of insulin was kindly supplied by Dr. Schlichtkrull (NOVO Laboratories). Norepinephrine (Levophed) was a gift from Winthrop Laboratories. Cyclic AMP and dibutyryl cAMP were obtained from Boehringer, theophylline from Carlo Erba, hydrocortisone-21-sodium succinate from Sigma. Cyclic AMP antiserum and [3H]-noradrenaline cyclic AMP tyrosine methyl ester used in the radioimmunoassay of cyclic AMP were purchased from Institut Pasteur Production. [G-14C]Glucose was obtained from the Commissariat à l’Energie Atomique (Saclay).

RESULTS

Glycogenolytic Effect of Glucagon—After 4 days of culture in the presence of 10 μM cortisol, which, as previously shown (9), induces glycogen storage in primary cultures of fetal hepatocytes, hepatocyte glycogen content was high. Exposure to 10 nm glucagon for 4 hours produced a rapid depletion of glycogen from 30 μg to 11 μg/million hepatocytes (Fig. 1). Another experiment was performed, where the cells were grown for 4 days in the presence of [14C]glucose. At Day 4, the glycogen should be uniformly labeled, since the size of the glycogen pool was very low at the beginning of the culture. This assumption was confirmed by the fact that the specific activity of glucose units in the glycogen remained constant throughout the glycogen storage period (9).

![Fig. 1 (left)](http://www.jbc.org/) Glycogenolytic effect of glucagon on glycogen stored in the presence of cortisol. Hepatocytes were grown during 4 days in the presence of 10 μM cortisol, the medium being replaced every 24 hours. Addition of 10 nm glucagon was performed at Day 4, and the glycogen content of the cells was measured 4 hours later.

![Fig. 2 (center)](http://www.jbc.org/) Time course of the glycogenolytic effect of glucagon. Hepatocytes (15-day-old) were grown for 4 days in the presence of 10 μM cortisol and [14C]glucose. The culture medium was replaced every 24 hours. The specific activity of [14C]glucose in the fresh medium was 0.10 μCi/mg. In a first experiment, 10 nm glucagon was added (△) at Day 4, and the glycogen radioactivity was measured every hour for 4 hours. ●, control experiment with no addition of glucagon. In a second experiment, the medium was first replaced by a similarly conditioned medium containing no [14C]glucose, then 10 nm glucagon was added (△), and the glycogen radioactivity measured during 4 hours. ○, control experiment with no glucagon addition.

![Fig. 3 (right)](http://www.jbc.org/) Dose response relationship between glycogen synthesis and glucagon concentration. Hepatocytes (15-day-old) were grown for 4 days in the presence of 10 μM cortisol, the medium being replaced every 24 hours. At Day 4, 14C]glucose (1.25 μCi/mg) was added at zero time together with varying concentrations of glucagon to give the final concentrations indicated on the abscissa. Glycogen radioactivity was measured after a 4-hour period of incubation.
Fig. 2 shows that a decrease of glycogen radioactivity was obtained after addition of 10 nM glucagon. This decrease was considerable after 1 hour, maximal after 3 hours, and leveled off after this, 40% of the stored glycogen being resistant to glucagon-dependent degradation. Similar results were obtained using the same experimental conditions with the exception that glucagon addition was performed after transfer of the cells to an unlabeled medium. Under these conditions of labeling, a marked but again partial effect of glucagon on stored glycogen was demonstrated. On the contrary, when hepatocytes, after 4 days in the presence of cortisol, were incubated for only a 4-hour period with [14C]glucose, 60% of the labeled glycogen was degraded (see Fig. 4); i.e., a larger proportion than when glucagon was uniformly labeled. These results suggest that the last labeled glucosyl residues were the first to be removed by the action of glucagon. Thus, glucagon-dependent glycogenolysis seems to concern preferentially newly synthesized glycogen. The glycogenolytic response to glucagon was obtained at doses of glucagon which are in the range of the physiological blood concentrations in the adult and in the newborn rat (7, 8). Fig. 3 shows the dose response relationship for glucagon. The experiment was performed using a 4-hour [14C]glucose-labeling period in the presence of varying concentrations of glucagon. Half-maximal inhibition of [14C]glucose incorporation was observed for 0.1 nM glucagon. However, in different experiments, variations were observed in the concentration of hormone required to produce the half-maximal effect (3 pm to 0.1 nM).

**Effects of Glucagon on the Rate of Glycogen Degradation and on the Rate of Incorporation of [14C]Glucose into Glycogen**—To evaluate the effect of glucagon on the rate of glycogen breakdown, hepatocytes were cultured for 4 days in a medium containing 10 µM cortisol and unlabeled glucose. At Day 4, [14C]glucose was added for a 4-hour labeling period. The hepatocytes were then transferred to a medium containing unlabeled glucose, and 10 nM glucagon was added. The decrease in glycogen radioactivity was measured during a further 8-hour incubation period. Fig. 4A shows that glucagon induces a biphasic glycogenolytic response. A semilogarithmic plot of these results (Fig. 4B) enabled the calculation of the half-life of glycogen degradation. Immediately after glucagon addition, the half-life of glycogen was only 50 min, whereas 3 hours later, the rate of degradation was markedly reduced. However, the apparent half-life of glycogen (7 hours) during this second period still remained shorter than that measured in the absence of glucagon (12 to 14 hours). The biphasic glycogenolytic response to glucagon might be the result of uneven labeling of the glucosyl residues in which the last ones labeled were the first to be removed by the action of glucagon, as already suggested by the comparison of the results reported in Figs. 2 and 4. Another possibility might be that some time after glucagon addition, the ratio between the rate of degradation and the rate of synthesis decreases markedly, thus resulting in a slower apparent glycogen half-life.

The effect of glucagon on the rate of incorporation of [14C]glucose into glycogen was measured using hepatocytes grown for 4 days in the presence of cortisol; [14C]glucose was then added with or without glucagon (10 nM). Glycogen labeling was then measured every hour during a further 8-hour incubation period. No attempt was made, for theoretical reasons (23), to estimate the rate of glycogen synthesis immediately after glucagon addition, since the labeling time of glycogen (2 hours) is long with respect to the half-life of its degradation (50 min). However, 3 hours after glucagon addition (Fig. 5), the rate of labeling appeared clearly reduced when compared to assays performed in the absence of glucagon. After 4 to 5 hours, the rate of labeling accelerated and approached that measured in the absence of hormone.

Both of these results (Figs. 4 and 5) show, therefore, that after addition of glucagon, the rate of glycogen degradation was in-

![Fig. 4](image-url)

Fig. 4 (left). Effect of glucagon on the rate of glycogen degradation. Hepatocytes (15-day-old) were grown in the presence of 10 µM cortisol, the medium being changed every 24 hours. At Day 4, [14C]glucose was added (1.25 µCi/mg of glucose) and the hepatocytes grown for 4 hours in the labeled medium. After this pulse labeling (time zero of the experiment), the medium was replaced by a similarly conditioned medium containing no labeled glucose and 10 nM glucagon (Arrow 1) was added (A). ○, control experiment with no addition of glucagon. Glycogen radioactivity was measured during 8 hours. ■, culture which received a supplementary addition of 10 nM glucagon at time 4 hours (Arrow 2) (A). B, results in a semilogarithmic plot.

![Fig. 5](image-url)

Fig. 5 (right). Effect of glucagon on the rate of incorporation of [14C]glucose into glycogen. Hepatocytes (15-day-old) were grown in the presence of 10 µM cortisol, the medium being changed every 24 hours. At Day 4 (time zero of the experiment), [14C]glucose was added (1.25 µCi/mg of glucose) (Arrow 1) either with (A) or without (●) 10 nM glucagon. Glycogen radioactivity was measured every hour for 8 hours. ■, culture which received a second addition of 10 nM glucagon at time 4 hours (Arrow 2).
increased, whereas the rate of incorporation of [14C]glucose into glycogen was reduced, and that these changes were reversed after 4 hours. The glycogen content of the cells was also measured during the time course of the glycogenolytic effect of glucagon. The result represented in Fig. 1 shows that 4 hours after glucagon addition, glycogen content was reduced by 80%. After a further 4 hours of incubation (results not shown), the glycogen content increased to 80% of the initial value. Thus, the reversal of the glycogenolytic effect of glucagon was also observed at the level of the glycogen content.

**Evidence for Hepatocyte Refractoriness to Glucagon after a First Exposure to the Hormone**—The effect of a second addition of glucagon, 4 hours after the first addition, was examined. Figs. 4 and 5 show that no further response to glucagon could be observed. Glycogen labeling and degradation appeared to have become resistant to a second stimulation by the hormone. To confirm this point, experiments were designed (Fig. 6) whereby the effect of glucagon was estimated by measuring the inhibition of incorporation of [14C]glucose into glycogen. At Day 4 of the culture, [14C]glucose was introduced, and the glycogen radioactivity determined 4 hours later. This labeling was performed during the period either from 0 to 4 hours or from 4 to 8 hours after a first addition of 10 nM glucagon; a second addition of 10 nM glucagon was then performed in some cases, 4 hours after the initial one. The control experiment illustrated in Fig. 6A shows that in the absence of glucagon, the rate of glycogen labeling was identical during the two successive 4-hour periods. A single addition of 10 nM glucagon performed at zero time (Fig. 6B) or after 4 hours (Fig. 6C) was followed within 4 hours by a drastic inhibition of the glycogen labeling. Fig. 6B shows that 4 hours after a single glucagon addition, the rate of [14C] incorporation returned to normal, as expected from the results shown in Fig. 5. Fig. 6D shows that this restoration of high rates of glycogen synthesis was unaffected by a second addition of 10 nM glucagon. One possible explanation for these results is that the second dose of glucagon is rapidly inactivated when added to “conditioned” medium, i.e. to a medium where the hepatocytes have been cultured for 4 hours in the presence of the hormone. To test this hypothesis, the second glucagon addition was performed after removal of the conditioned medium and its replacement by a “nonconditioned” medium. A nonconditioned medium is one in which hepatocytes have been cultured in the absence of glucagon. Table I shows that in the presence of either conditioned or nonconditioned medium, a second addition of glucagon was ineffective. The same result was obtained when the cells were washed three times with fresh medium before replacement of the medium. Finally, supramaximal concentrations of glucagon (1 mM) failed to suppress this resistance to glucagon action. These results show that this refractoriness does not depend on the inactivation of the second dose of hormone by the conditioned medium. In addition, the fact that glucagon remains ineffective when added a second time together with nonconditioned medium suggests that the resistance to glucagon action is not linked to a modification appearing in the medium during the first incubation period performed in the presence of the first dose of glucagon. This modification might have resulted from the release by the cells of a diffusible inhibitory factor. If such an inhibitory factor were released by the cells, it should be present in the conditioned medium. Therefore, the

![Graph](image)

**FIG. 6.** Effect of two successive additions of glucagon on incorporation of [14C]glucose into glycogen. Hepatocytes (15-day-old) were grown for 4 days in the presence of 10 μM cortisol. At Day 4, two successive 4-hour-incubation periods in the presence of [14C]glucose were performed on parallel cultures; [14C]glucose was present either from 0 to 4 hours, or from 4 to 8 hours. At the end of each incubation, incorporation of [14C]glucose into glycogen was measured. Glucagon (10 nM) was added together with [14C]glucose (1.25 μCi/mg) at zero time (B), at 4 hours (C), and both at zero time and at 4 hours (D). A, control experiment with no addition of glucagon.

**TABLE I**

| Hepatocytes           | Addition at zero time | Addition at 4 hours | Glycogen cpm/10⁶ hepatocytes |
|-----------------------|-----------------------|---------------------|-----------------------------|
| Nontransferred        | None                  | None                | 10,730–9,680                |
|                      | 10 nM glucagon        | None                | 7,840–10,520                |
| Transferred           | None                  | 10 nM glucagon      | 10,340–10,030               |
|                       | 10 nM glucagon        | 10 nM glucagon      | 10,520                      |
| Washed and transferred| None                  | 10 nM glucagon      | 11,820–12,750–12,490*       |
|                       | 10 nM glucagon        | 10 nM glucagon      | 11,910–14,970–11,950        |

* One micromolar glucagon added at 4 hours.
The experimental protocol is as described in Fig. 6, except that at time 4 hours, the hepatocytes, previously grown in the absence of glucagon, were transferred to a conditioned medium, i.e., a medium in which hepatocytes had been grown for 4 hours in the presence of glucagon. Some of the cultures were washed three times with fresh medium before transfer. [14C]Glucose (1.25 μCi/mg) was then added together with 10 times with fresh medium before transfer. KJGlucose (1.25 medium in which hepatocytes had been grown for 4 hours in the presence of glucagon. Some of the cultures were washed three times with fresh medium before transfer. [14C]Glucose (1.25 μCi/mg) was then added together with 10 times with fresh medium before transfer. KJGlucose (1.25 medium in which hepatocytes had been grown for 4 hours in the presence of glucagon. Some of the cultures were washed three times with fresh medium before transfer. [14C]Glucose (1.25 μCi/mg) was then added together with 10 times with fresh medium before transfer. KJGlucose (1.25 μCi/mg) was then added together with 10

Table II

| Hepatocytes       | Addition at zero time | Addition at 4 hours | Glycogen (cpm/10^6 hepatocytes) |
|-------------------|-----------------------|---------------------|---------------------------------|
| Nontransferred    | None                  | None                | 10,720–9,680                    |
|                    | None                  | 10 nm glucagon       | 2,640–1,980                     |
| Transferred       | None                  | 10 nm glucagon       | 2,290–2,410                     |
| Washed and trans- | None                  | 10 nm glucagon       | 2,360–2,390                     |
| ferred            |                       |                     |                                 |

Fig. 7. Cyclic AMP levels after a first and a second exposure to glucagon. Hepatocytes (15-day-old) were grown for 4 days in the presence of 10 μM cortisol. At Day 4, 10 nm glucagon and 1 mM caffeine were added at zero time. The cultures were rapidly frozen at the time indicated on the abscissa and analyzed for cyclic AMP content as described under "Experimental Procedure" (A). The same experiment was repeated on cultures which had received a first dose of glucagon (10 nm) 4 hours before (B). Control cultures which were grown in the absence of glucagon are represented by O and △. Open symbols correspond to cultures which have been incubated with 1 mM caffeine alone added at zero time. Conditioned medium should transfer the resistant property to hepatocytes not previously exposed to glucagon. Table II shows that conditioned medium was ineffective in inducing resistance to glucagon stimulation in hepatocytes not previously exposed to glucagon.

Relationship between the Glycogenolytic Effect of Glucagon, Refractoriness to Glucagon, and Cyclic AMP—In the 15-day-old hepatocytes grown for 4 days in the presence of cortisol, a 10-fold increase of cyclic AMP levels was observed within 2 min after addition of 10 nm glucagon plus 1 mM caffeine (Fig. 7A). In addition, 0.5 mM dibutyryl CAMP produced a marked glycogenolytic effect. Fig. 8 shows the time course of the decay of glycogen radioactivity in the presence of 0.5 mM dibutyryl CAMP, which was similar to that observed with glucagon (Fig. 2). However, the concentration of dibutyryl CAMP used in this experiment was not sufficient to produce a glycogenolytic effect to the same extent as that of glucagon. Moreover the dose response relationship (Fig. 9) did not show saturation at higher doses, as in the case of glucagon. A nontoxic response was also obtained with 1 mM cyclic AMP (Table III) and with 1 mM theophylline, a well-known inhibitor of phosphodiesterases, either when used alone or in combination with submaximal concentrations of dibutyryl CAMP and glucagon (results not shown).

These data strongly suggest that glucagon exerts its glycogenolytic effect on fetal hepatocytes via the production of cyclic AMP, as in adult liver. A further experiment was performed to investigate whether the induction of the resistant property by glucagon was also mediated by cyclic AMP. Dibutyryl CAMP and cyclic AMP at glycogenolytic concentrations were added at zero time, and the effect of the addition of 10 nm glucagon was studied after 4 hours. Table III shows that, in spite of their clear glycogenolytic effects, dibutyryl CAMP (0.1 to 0.5 mM) and cyclic AMP (1 mM) did not render the hepatocytes resistant to glucagon, which exerted its typical glycogenolytic effect. In addition, Table III also shows that the glycogenolytic effects of dibutyryl CAMP and cyclic AMP were not completely reversed after 4 hours, as in the case of glucagon.

Another experiment was performed to test whether once the resistance to glucagon was established, i.e., 4 hours after a first exposure to this hormone, dibutyryl CAMP and cyclic AMP were still able to elicit a glycogenolytic response. Table IV shows that glycogenolytic effects of dibutyryl CAMP (0.3 to 0.5 mM) and cyclic AMP (1 mM) were still obtained under these conditions. Similar results were obtained using norepinephrine instead of cyclic AMP or its dibutyryl derivative. This agonist (10 nm to 0.1 μM) exerts a clear glycogenolytic effect, which was preserved in spite of the presence of resistance to glucagon stimulation (Table IV). These results suggest that the resistant property is located at the level of cyclic AMP production. This assumption is confirmed by the results shown in Fig. 7B. No increase in cyclic

Fig. 8 (left). Time course of the glycogenolytic effect of dibutyryl CAMP (Bt,cAMP). Hepatocytes (17-day-old) were grown for 3 days in the presence of 10 μM cortisol and [14C]glucose. The culture medium was replaced every 24 hours. The specific activity of [14C]glucose in the fresh medium was 0.10 μCi/mg. At Day 3, dibutyryl CAMP was added (●) and the glycogen radioactivity measured every hour for 4 hours. ○, control experiment without addition of dibutyryl CAMP. Fig. 9 (right). Dose response relationship between glycogen degradation and dibutyryl CAMP (Bt,cAMP) concentration. The culture conditions are those described in Fig. 4. At Day 3, varying concentrations of dibutyryl CAMP were added at zero time to give the final concentrations indicated on the abscissa. Glycogen radioactivity was measured after a 4-hour period of incubation.
**TABLE III**

**Glycogenolytic effect of a single addition of glucagon on hepatocytes previously exposed to dibutyryl cAMP and cyclic AMP**

The experimental protocol is as described in Fig. 6 except that at zero time, dibutyryl cAMP (Bt2cAMP) or cyclic AMP were added instead of glucagon; a single addition of 10 nm glucagon was then effected at time 4 hours. Labeling was performed either during the period from 0 to 4 hours or from 4 to 8 hours, the specific radioactivity of glucose being 1.25 μCi/mg.

| Addition at zero time | Addition at 4 hours | Time of [14C]glucose addition (and labeling period) | Glycogen cpm/10⁶ hepatocytes | Exp. 1 | Exp. 2 |
|-----------------------|---------------------|--------------------------------------------------|-----------------------------|-------|-------|
| None                  | None                | 0 (0 to 4 hr)                                     | 10,030                      | 14,910|
| 0.1 mM Bt2cAMP        | None                | 0 (0 to 4 hr)                                     | 6,250                       | 7,250 |
| 0.5 mM Bt2cAMP        | None                | 0 (0 to 4 hr)                                     | 3,360                       | 6,240 |
| 1 mM cAMP             | None                | 0 (0 to 4 hr)                                     | 4,190                       | 6,240 |
| None                  | None                | 4 hr (4 to 8 hr)                                  | 11,540                      | 10,200|
| 0.1 mM Bt2cAMP        | None                | 4 hr (4 to 8 hr)                                  | 7,220                       | ---   |
| 0.5 mM Bt2cAMP        | None                | 4 hr (4 to 8 hr)                                  | 4,350                       | 6,320 |
| 1 mM cAMP             | None                | 4 hr (4 to 8 hr)                                  | 4,780                       | 7,820 |
| None                  | 10 nm glucagon      | +                                                 | 2,810                       | 2,310 |
| 0.1 mM Bt2cAMP        | 10 nm glucagon      | +                                                 | 3,940                       | ---   |
| 0.5 mM Bt2cAMP        | 10 nm glucagon      | +                                                 | 3,290                       | 1,580 |
| 1 mM cAMP             | 10 nm glucagon      | +                                                 | 3,730                       | 2,100 |

AMP levels was obtained after addition of 10 nm glucagon plus 1 mM caffeine in hepatocytes which have been exposed to glucagon 4 hours before. It should be noted that the basal level of cyclic AMP in the resistant hepatocyte was higher (4-fold) than that in fresh cells.

**TABLE IV**

**Glycogenolytic effect of dibutyryl cAMP, cyclic AMP, and norepinephrine on hepatocytes previously exposed to glucagon**

The experimental protocol is as described in Fig. 6 except that at time 4 hours, dibutyryl cAMP (Bt2cAMP), cAMP and norepinephrine (norE) were added instead of glucagon, together with [14C]glucose (1.25 μCi/mg). Labeling was performed during the period from 4 to 8 hours.

| Addition at zero time | Exp. 1 | Exp. 2 |
|-----------------------|--------|--------|
| Addition at 4 hours   | Glycogen cpm/10⁶ hepatocytes | Glycogen cpm/10⁶ hepatocytes |
| None                  | 6,530  | None   |
| 0.3 mM Bt2cAMP        | 3,080  | 1 mM cAMP 4,190 |
| 0.5 mM Bt2cAMP        | 2,040  | 0.5 mM Bt2cAMP 3,360 |
| 0.1 μM norE           | 1,680  | 0.1 μM norE 3,700 |
| 1 μM norE             | 1,410  | 10 mM norE 7,580 |
| 10 nm glucagon        | 8,020  | None   |
| 0.3 mM Bt2cAMP        | 3,800  | 1 mM cAMP 6,080 |
| 0.5 mM Bt2cAMP        | 2,720  | 0.5 mM Bt2cAMP 5,320 |
| 0.1 μM norE           | 2,430  | 0.1 μM norE 4,540 |
| 1 μM norE             | 1,610  | 10 mM norE 8,100 |

AMP levels was obtained after addition of 10 nm glucagon plus 1 mM caffeine in hepatocytes which have been exposed to glucagon 4 hours before. It should be noted that the basal level of cyclic AMP in the resistant hepatocyte was higher (4-fold) than that in fresh cells.

**DISCUSSION**

In cultured fetal hepatocytes, addition of 10 nm glucagon produced a rapid mobilization of glycogen, which concerned 60% of the glycogen pool (Figs. 1 and 2). Norepinephrine (10 nm) was also found to have a marked glycogenolytic effect (Table IV). In explants from fetuses at term, after preincubation where spontaneous depletion of glycogen occurs, glycogenolytic effects of epinephrine (24) and glucagon (25) have also been observed. It appears, therefore, that both glucagon and catecholamines are likely glycogenolytic agents for the fetal liver.

Addition of glucagon was followed within 2 min by the accumulation of cyclic AMP (Fig. 7A). In addition, the glycogenolytic effect of glucagon was mimicked by cyclic AMP and its dibutyryl derivative, and potentiated by theophylline. In adult in vitro systems, perfused liver (10), and surviving isolated hepatocytes (11-13), glycogenolysis was always accompanied by a great increase in cyclic AMP levels. It may be postulated that in cultured fetal hepatocytes as in the adult systems, glucagon acts according to the scheme of Sutherland. The fixation of the hormone to a specific membrane site is accompanied by stimulation of a plasma membrane adenylate cyclase system (26-28). The cyclic AMP produced activates in turn a protein kinase (29, 30), which regulates via phosphorylation the activity of two key enzymes for glycogen metabolism: glycogen phosphorylase and glycogen synthetase (31). This assumption implies the presence of all of these steps in 15-day-old hepatocytes grown for 4 days in the presence of cortisol. Adenylate cyclase activity has been shown to be present and to respond to glucagon and epinephrine (36), and according to others, it did not (35). It should be pointed that the adenylate cyclase thus detected may belong at least partly to the hematopoietic cells, which represent, at these stages, 60% of the liver population (37, 38). The present work shows that glucagon stimulates the accumulation of cyclic AMP in 15-day-old hepatocytes grown for 4 days in the presence of cortisol. Plas and Nunez also suggest that this system is not induced by cortisol during the culture period, and that it is present before significant amounts of glycogen are stored.

In the adult liver, cyclic AMP produced after glucagon administration is responsible for both the activation of glycogen phosphorylase (39) and the inactivation of glycogen synthetase (39, 40). From in vivo studies, it has been postulated that the regulation by glucagon of these enzymatic activities appears around the time of birth (41). In explant liver cultures from term fetuses, glucagon was found to activate glycogen phosphorylase by some authors (42), but no effect was found by others (29). In cultured fetal hepatocytes, it is clear that cyclic AMP activates the glycogen degradation system (Fig. 5). In addition, this effect of glucagon was rapidly reversed (after 4 hours), suggesting a reversibility of the activation state of glycogen phosphorylase, probably via the action of phosphatases (43).

In fact, it was found that the reversal of the response to glucagon was not modified by a second addition of glucagon (Figs. 4 to 6). This second addition of glucagon was also ineffective after transfer of the hepatocytes to a fresh medium with or without repetitive washing of the cells (Table I). Consequently, the loss of the glycogenolytic response depends neither on a fast inactivation of the hormone in the conditioned medium, nor on the occupation of the receptor sites by inactivated glucagon. This latter possi-

2 Unpublished results.
ility is unlikely because it has been shown that the binding of glucagon and the inactivation of the hormone are clearly independent (27, 44). These results support the hypothesis that the cells really become resistant to a second addition of glucagon after a first exposure and response to the hormone. It should be emphasized that this resistance is expressed at the level of glucagon metabolism, i.e. the physiological response. However, it cannot be excluded that the receptor sites are occupied by irreversibly bound native glucagon or by some contaminant present in the glucagon solution.

The loss of response to glucagon implies a blocking of some step in the glucagon-dependent glycogenolytic pathway, which may be located before or after cyclic AMP production. Escape from blocking was obtained with maximal glycogenolytic concentrations of cyclic AMP and dibutyl cAMP (Table IV). Therefore, refractoriness does not occur, at least qualitatively, at the protein kinase activation step or later, but probably at the level of cyclic AMP production. This conclusion was confirmed by the fact that in the resistant hepatocytes, no cyclic AMP production was observed after a second glucagon addition. A similar loss of hormonal response at the level of cyclic AMP production has been shown in several in vitro systems after a first exposure to a hormone (45–51). For example, with adipose tissue, both in isolated cells (46, 47, 51) and in tissue explants (51), a lack of response to a second epinephrine or ACTH stimulation has been described. However, a relationship between the refractoriness in the production of cyclic AMP and the lipolytic response to epinephrine and ACTH was not demonstrated (51). On the other hand, in the present work, the fact that the glycogenolytic response to glucagon was rapidly reversed permitted the demonstration of refractoriness both for the production of cyclic AMP and for the physiological response. A glycogenolytic response to norepinephrine was observed even while the cells exhibited refractoriness to glucagon (Table IV). If the glycogenolytic effect of norepinephrine is mediated by cyclic AMP (10, 52), this result again suggests that the refractoriness to glucagon is not located at a step in the glycogenolytic response involving activation by cyclic AMP. In addition, refractoriness to glucagon seems specific, since norepinephrine was able to induce a glycogenolytic response in the hepatocytes resistant to glucagon. In adipose tissue, no specificity for the onset of refractoriness at the level of cyclic AMP production and the lipolytic response to epinephrine and ACTH was not demonstrated (51). On the other hand, in the present work, the fact that the glycogenolytic response to glucagon was rapidly reversed permitted the demonstration of refractoriness both for the production of cyclic AMP and for the physiological response. A glycogenolytic response to norepinephrine was observed even while the cells exhibited refractoriness to glucagon (Table IV). If the glycogenolytic effect of norepinephrine is mediated by cyclic AMP (10, 52), this result again suggests that the refractoriness to glucagon is not located at a step in the glycogenolytic response involving activation by cyclic AMP. In addition, refractoriness to glucagon seems specific, since norepinephrine was able to induce a glycogenolytic response in the hepatocytes resistant to glucagon. In adipose tissue, no specificity for the onset of refractoriness at the level of cyclic AMP production and the lipolytic response to epinephrine and ACTH was not demonstrated (51). On the contrary, in other in vitro systems, such as brain slices for epinephrine and histamine (45), ovarian follicles for LH and I-GE2 (49), and cyclic AMP release.

Another question is whether the onset of this autorefractory property induced by glucagon is mediated by cyclic AMP, as is the glycogenolytic response. Experiments summarized in Table III show clearly that glycogenolytic concentrations of cyclic AMP and dibutyl cAMP were unable to induce the resistant property to glucagon at the level of glycogenolytic response. This observation suggests that the appearance of refractoriness occurs via a mechanism where cyclic AMP is not involved. On the contrary, in isolated adipose cells, the production of the antagonist by epinephrine seems to be mediated by cyclic AMP (46). Data on the dose response of glucagon with regard to the refractoriness phenomenon will be necessary to know if the refractoriness is related to an interaction between glucagon and its receptor.

Finally, the fact that this hormonal autoregulation was shown to exist at the level of the glycogenolytic response might have a physiological significance by providing an additional regulatory mechanism for glycogenolysis. However, such a refractoriness toward glucagon has not been described in adult perfused liver (10), where continuous presence of this hormone was accompanied by a continuous production of cyclic AMP. It is not known at the present time if this refractoriness is specific to the fetal hepatocyte. In any way, this mechanism may be one factor contributing to the maintenance of high stores of hepatic glycogen before birth in spite of the presence in fetal blood, during late gestation, of pancreatic glucagon levels similar to those of adult rats (7, 53).

Acknowledgments—We are most grateful to Dr. André Sentenac for critical review of the manuscript, and to Claude Sais for her assistance with the preparation of the manuscript.

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J. Biol. Chem. 1975, 250:5304-5311.

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