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# BIOCHEMICAL EFFECTS OF PREDNISOLONE ON HUMAN LIVER CELLS IN TISSUE CULTURE*

ALEXANDER PIHL and PER EKER†

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway

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**Abstract**—The growth-inhibiting effect of prednisolone on human liver cells (Chang) grown in monolayer cultures, was most pronounced during the first hours of incubation. Prednisolone in concentrations (125 $\mu$M) which inhibited completely cell multiplication during the first 3 hr, had no effect on the concurrent incorporation of $^{14}C$ labeled thymidine into DNA and of $^{14}C$-methionine into protein. The incorporation of $^{14}C$-orotic acid into RNA was slightly reduced by higher concentrations (250 $\mu$M) of prednisolone.

The glycogen content of the cells was markedly increased after incubation with prednisoline for 20–30 hr. Experiments with labeled glucose indicated that the extra glycogen was not derived from added glucose. The incorporation of labeled pyruvate into glucose and glycogen was not affected by prednisolone, while the steroid increased the incorporation of radioactivity from labeled alanine.

PREDNISOLONE strongly increased glucose utilization of the liver cells. The effect was most pronounced during the early period of incubation and was abolished by actinomycin D and by puromycin. The lactate production of the liver cells was only slightly affected by prednisolone during the first hours of incubation, while after 24 hr the lactate production was increased to about the same extent as was the glucose utilization.

No effect of prednisolone on the activity levels of hexokinase, glucokinase, glucose-6-phosphatase and fructose-1,6-diphosphatase was found.

In previous studies on human liver cells grown in monolayer cultures[1, 2] it was found that the growth inhibiting effect of prednisolone is associated with pronounced effects on glucose metabolism. Thus, prednisolone increased markedly the glucose utilization and lactate production of the liver cells in contrast to the action of glucocorticoid steroids in other systems.[3–7] The data indicated that the effects of prednisolone on glucose metabolism and on growth were early and largely transient.[2]

In the present paper further studies on the biochemical effects of prednisolone on human liver cells are reported. In particular the action on glucose metabolism is studied in more detail. Part of this work has been reported in a preliminary communication.[8]

## MATERIALS AND METHODS

### *Cultivation of cells*

All experiments were carried out with human adult liver cells (Chang) grown in monolayer cultures under conditions described previously.[9, 10]

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† Fellow of the Norwegian Cancer Society.

*Analytical*

Cell counts were carried out with an automatic particle counter. Protein was determined by the Lowry method[11] as modified by Oyama and Eagle,[12] using crystalline bovine albumin as standard. Glucose was determined by the glucose oxidase method[13] and lactate by the method of Barker and Summerson, as modified by Ström.[14] Glycogen was isolated[15] and determined as glucose.

Hexokinase and glucokinase activities were determined according to the procedure of Salas *et al.*[16] on cell extracts. These were prepared as described by Katzen *et al.*[17], except that the cells were sonicated only for 1 min. For assay of glucose-6-phosphatase and fructose-1,6-diphosphatase, about $10^7$ cells were collected by trypsinization[10] and centrifugation, washed twice with 0·9% NaCl, and suspended in 1 ml of ice-cold 0·01 M glycine-NaOH buffer, pH 10·5. Homogenization was carried out for 90 sec in a small Potter-Elvehjem all-glass homogenizer, and the homogenate was used immediately for enzyme assay. The amounts of orthophosphate liberated from the substrates during incubation were taken as a measure of the enzyme activities. Incubations were carried out as follows: 300 $\mu$l of 0·2 M glycine-NaOH buffer, pH 10·5 and 100 $\mu$l of the homogenate (100–150 $\mu$g of protein) in flat bottomed glass tubes were equilibrated in a water bath at 37° for 5 min with continuous shaking. The reactions were started by adding 100 $\mu$l (10 $\mu$moles) of the substrate solutions. The reactions were stopped after 20 min by rapid cooling to 0° and addition of 50 $\mu$l of 100% (w/v) trichloroacetic acid. The protein precipitate was removed by centrifugation and orthophosphate in the supernatant was determined by the method of Fiske and Subba-

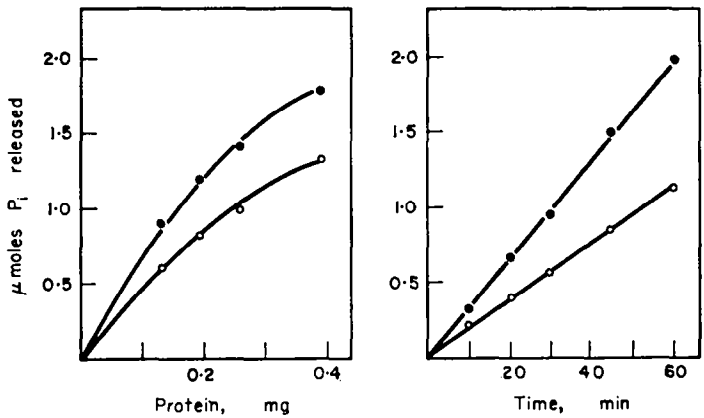


FIG. 1. Effect of protein concentration and time of incubation on dephosphorylation of fructose-1,6-diphosphate (●) and glucose-6-phosphate (○). Incubations were carried out as described under Materials and Methods except that the protein concentration of the incubation mixtures and the time of incubation were varied as indicated. The results are expressed in $\mu$moles of orthophosphate liberated in the reaction mixture.

Row.[18] The blanks contained all components except the substrate. Corrections were made for inorganic phosphate present in the substrate solutions. Under these conditions the amount of inorganic phosphate ($P_i$) released was proportional to the amount of protein used and to the time of incubation (Fig. 1).

*Reagents*

Prednisolone ($\Delta^1$-hydrocortisone) was obtained as the water-soluble phosphate ester, sodium *bis*(prednisolone-21)-phosphate (Glucortin) from Fredriksberg Chem. Fabr. A/S, Copenhagen, Denmark. Glucose oxidase was obtained from Boehringer & Soehne GmbH., Mannheim, Germany. Thymidine-2-$^{14}$C (specific activity 126 $\mu$c per mg) orotic acid-6-$^{14}$C (specific activity 187 $\mu$c per mg), L-alanine-$^{14}$C (U) (specific activity 47·5 $\mu$c per mg), L-methionine-methyl-$^{14}$C (specific activity 122 $\mu$c per mg), sodium pyruvate-$^{14}$C (U) (specific activity 19·5 $\mu$c per mg), and D-glucose-2-$^{14}$C (specific activity 173 $\mu$c per mg) were obtained from The Radiochemical Centre, Amersham, England.

Puromycin dihydrochloride was purchased from Nutritional Biochemicals. Actinomycin D was obtained from Merck, Sharp & Dohme. All other chemicals used were commercial products of the highest purity.

## RESULTS

Previous studies indicated[2] that prednisolone exerted its strongest growth inhibiting effect during the first 10 hr of incubation. Subsequently, the growth rate of the cells again increased, although it remained at a somewhat reduced level, compared to that of the untreated cells. The results in Fig. 2 demonstrate that the strongest growth

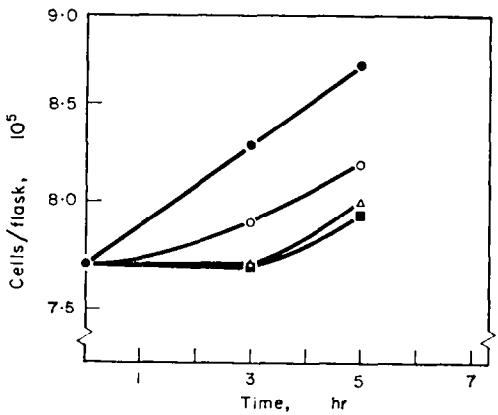


FIG. 2. Effect of prednisolone on the rate of multiplication of Chang liver cells. 12-ml samples (0·6 × $10^6$ cells) in glass bottles were flushed with 5 % $CO_2$ in air, and incubated at 37°. After 24 hr the medium was replaced by fresh medium containing increasing concentrations of prednisolone, and cell counts were performed at the times indicated. Each point represents the average of four flasks. Control (●), 10 $\mu$g/ml (○), 50 $\mu$g/ml (△) and 100 $\mu$g/ml (■).

inhibiting effect of prednisolone actually occurs during the first 3 hr of incubation. During this period the growth was completely inhibited by 50 $\mu$g of prednisolone per ml (125 $\mu$M). The previous observation that cells exposed for 24 hr to prednisolone become unresponsive to much higher steroid concentrations,[2] is confirmed in Fig. 3A. The results suggest that the cells somehow adapt themselves to the presence of the steroid.[2] In order to study whether such cells become permanently altered, cultures pretreated for 24 hr with steroid (25 $\mu$M) were allowed to grow for 24 hr in the absence of prednisolone and were then exposed to the original steroid concentration (Fig. 3B).

The results demonstrate that under these conditions the cells responded to the steroid in the same way as untreated cultures. The data support the view[2] that the unresponsiveness of pretreated cells to higher prednisolone concentrations can not be due to a selection of more resistant cells.

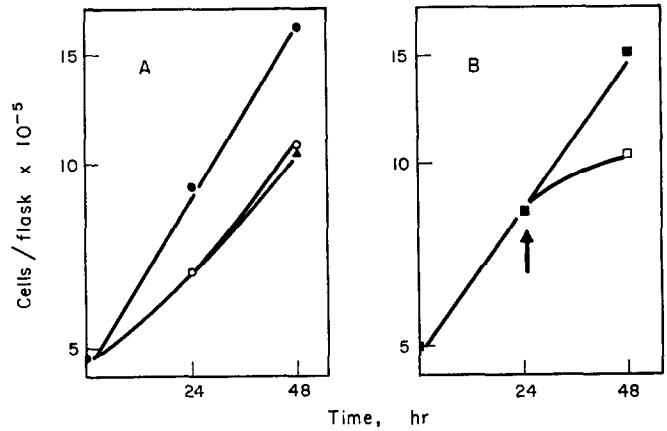


FIG. 3. Growth inhibiting effect of prednisolone on Chang liver cells previously exposed to the steroid. A: (●), no prednisolone (control); (○), 25 μM prednisolone; (△), medium replaced after 24 hr with fresh medium containing 250 μM prednisolone.
B: (■), cells preincubated for 24 hr with 25 μM prednisolone. At zero time the medium was replaced with fresh medium containing no prednisolone. At the time indicated by the arrow, the medium was replaced with medium containing 25 μM prednisolone (□).

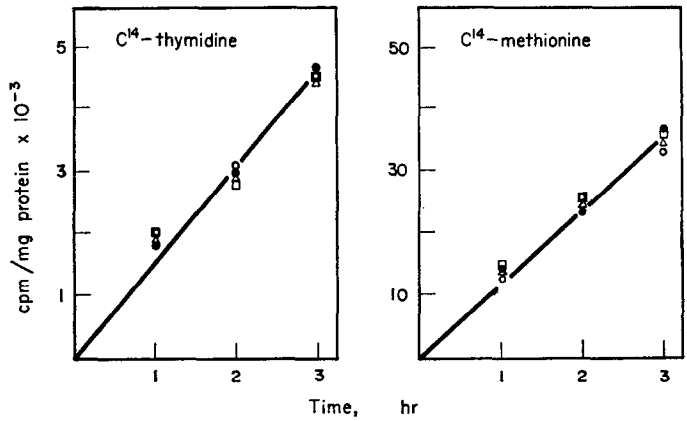


FIG. 4. Effect of prednisolone on incorporation of $^{14}$C-thymidine into DNA and of $^{14}$C-methionine into protein. Exponentially growing cultures were incubated under conditions as described in Fig. 2 in the presence of $^{14}$C-thymidine ($1{\cdot}3 \times 10^4$ counts/min per ml) and $^{14}$C-methionine ($2{\cdot}9 \times 10^4$ counts/min per ml), respectively. DNA[19] and protein[20] were isolated, and the radioactivity determined with a flow counter after plating in aluminium cup planchets. The results are expressed as counts per mg of cell protein. (●) control; (△) 10 μg/ml; (○) 50 μg/ml; (□) 100 μg/ml.

In attempts to throw light on the mechanism underlying the growth-inhibiting action of prednisolone its effect on nucleic acid and protein synthesis was investigated. It was found (Fig. 4) that prednisolone concentrations, which initially inhibited completely cell division (see Fig. 2), did not influence the rate of incorporation of labeled

thymidine and methionine into DNA and protein, respectively. On the other hand, the incorporation of $^{14}$C-orotic acid into RNA (Fig. 5) was moderately, but definitely inhibited by 100 $\mu$g (250 $\mu$M) prednisolone. Lower concentrations (25 and 125 $\mu$M) had no demonstrable effect. The results indicate that the growth inhibiting effect of prednisolone cannot be accounted for by a blocking of DNA or protein synthesis. The significance of the orotic acid data is not clear. Previous investigators have shown that glucocorticoid steroids increase the incorporation of labeled precursors into RNA of liver tissue.[22–24]

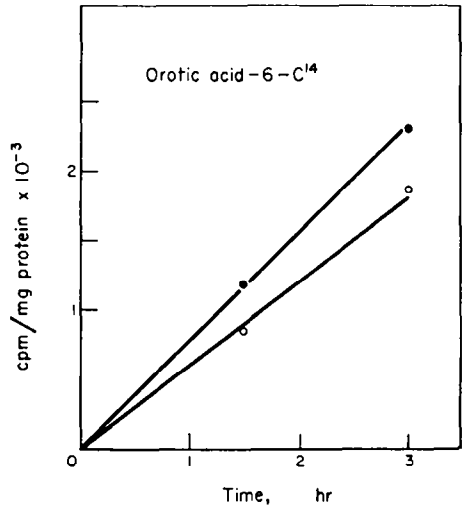


FIG. 5. Effect of prednisolone, 100 $\mu$g per ml (250 $\mu$M) on incorporation of $^{14}$C-orotic acid ($2{\cdot}2 \times 10^5$ counts/min per ml) into RNA. Conditions as in Fig. 4. RNA was isolated[21] and the radioactivity determined. (●), Control; (○), 100 $\mu$g/ml.

In Fig. 6 is shown the effect of increasing concentrations of prednisolone on glucose utilization and lactate production of liver cells, measured after 6 and 24 hr of incubation. The results confirm our previous finding[2] that in this system prednisolone surprisingly induces a strong enhancement in the glucose consumption and lactate production. It appears that the effect increased rapidly with the steroid concentration up to 25 $\mu$g per ml, and was considerably greater after 6 hr than after 24 hr of incubation. This finding indicates that the effect of prednisolone on glucose metabolism, like that on growth, is most pronounced during the initial phase of incubation. Together with our previous observations on glycolysis during the period 24–48 hr of incubation[2] the present results indicate that the enhanced glucolysis by prednisolone subsides in the course of 24 hr. Only a moderate effect on lactate production was found after 6 hr, while after 24 hr the lactate production was increased to about the same extent as was the glucose consumption.

From the data in Table 1 it can be seen that addition of puromycin to the culture medium prevented completely the ability of prednisolone to increase the glucose utilization of the liver cells. The concentration of the puromycin used ($10^{-4}$M) inhibits completely protein synthesis in Chang cells.[25] It appears that actinomycin D,

which is known to inhibit DNA-dependent RNA synthesis, similarly abolished the effect of the steroid. These results indicate that the effect of prednisolone on glucose metabolism of Chang cells is not due to an activation of pre-existing glycolytic enzymes, but is associated with synthesis *de novo* of such enzymes.

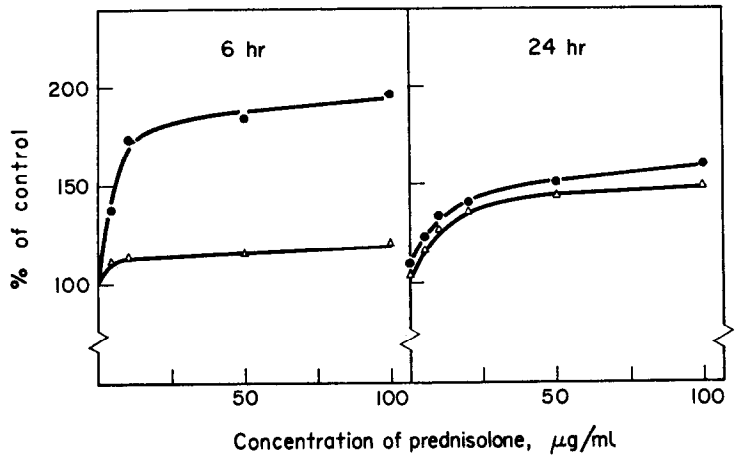


FIG. 6. Effect of increasing concentrations of prednisolone on glycolysis of Chang cells. Glucose utilization and lactate production per $10^6$ cells were determined[2] after incubation for 6 and for 24 hr. The values are expressed in per cent of those for untreated cells. (●), Glucose; (△), Lactate.

TABLE 1. ABILITY OF PUROMYCIN AND ACTINOMYCIN D TO ABOLISH THE ENHANCING EFFECT OF PREDNISOLONE ON GLUCOSE UTILIZATION OF CHANG LIVER CELLS

| Conditions | Glucose utilization | |
|---|---|---|
| | μg/$10^6$ cells/hr | % of control |
| No addition. Control. | 44·8 | 100 |
| Prednisolone (250 μM) | 79·3 | 177 |
| Prednisolone (250 μM), + puromycin ($10^{-4}$M) | 43·8 | 98 |
| Prednisolone (250 μM), + actinomycin D (0·5 ug/ml) | 42·6 | 95 |

Exponentially growing cultures were incubated for 6 hr under the conditions indicated and the glucose utilization determined.[2]

In order to investigate the mechanism of action of prednisolone on glycolysis, studies of its effect on enzymes involved in glucose metabolism were initiated. It appears (Table 2) that growth in the presence of prednisolone did not affect the cellular activity of hexokinase, glucokinase, glucose-6-phosphatase and fructose-1,6-diphosphatase.

A typical effect of glucocorticoid steroids is to increase the glycogen content of liver tissue.[3, 22, 26] Experiments were, therefore, carried out to investigate whether prednisolone could elicit a similar effect in our system of isolated liver cells. It is of interest that during the period 20–30 hr when small concentrations of prednisolone no longer are able to increase glycolysis[2] a clearly demonstrable increase in the glycogen

content was found (Table 3). After 48 hr the glycogen level was the same as in the untreated cells. Experiments with $^{14}C$-glucose demonstrated (Table 4) that the extra glycogen in the treated cells was not derived from added glucose. In fact, the incorporation of $^{14}C$ glucose into glycogen was somewhat lower in the presence of the steroid. The incorporation of labeled pyruvate into glucose and glycogen was not

TABLE 2. EFFECT OF PREDNISOLONE ON ACTIVITY OF ENZYMES INVOLVED IN GLUCOSE METABOLISM IN HUMAN LIVER CELLS

| Enzyme | Incubation time (hr) | Enzyme activity % of control |
|---|---|---|
| Hexokinase | 3 | 108 |
| | 18 | 109 |
| Glucokinase | 3 | 108 |
| | 18 | 106 |
| Glucose-6-phosphatase | 2 | 100 |
| | 4 | 102 |
| | 6 | 102 |
| Fructose-1,6-diphosphatase | 2 | 93 |
| | 4 | 100 |
| | 6 | 105 |

Cell cultures were grown in the absence and presence of prednisolone (250 $\mu$M). At the times indicated the cells were harvested and the enzyme activities determined.

TABLE 3. EFFECT OF PREDNISOLONE ON GLYCOGEN CONTENT OF CHANG LIVER CELLS

| Hr | Glycogen % of control |
|---|---|
| 20 | 145 |
| 30 | 178 |
| 48 | 106 |
| 72 | 76 |

Cell cultures were grown in the absence and presence of prednisolone (25 $\mu$M). At the times indicated the cultures were harvested and the glycogen content determined. The results represent the average of four independent experiments.

affected by prednisolone, while the steroid definitely increased the incorporation of radioactivity from alanine. These data are compatible with the view that the gluconeogenic effect of adrenal steroids is associated with an increased utilization of glycogenic amino acids.[3, 23]

## DISCUSSION

The typical effect of glucocorticoid steroids *in vivo* and *in vitro* is to inhibit glucose utilization and enhance gluconeogenesis. Recent work in several laboratories has demonstrated that the latter effect is associated with increased activity levels of enzymes exerting a rate limiting role in the production of glucose from lactate and

other gluconeogenic precursors.[27-31] In contrast, the data in the present and previous paper[2] establish that in rapidly growing human liver cells in culture, the hydrocortisone derivative, prednisolone, increases glycolysis. This effect has several notable features. It appears rapidly upon exposure of the cells to the steroid, it is of limited duration, and by and large it appears to coincide with the growth inhibiting effect of the steroid.

TABLE 4. EFFECT OF PREDNISOLONE ON INCORPORATION OF LABELED PRECURSORS INTO GLUCOSE AND GLYCOGEN OF CHANG LIVER CELLS

| Treatment | $^{14}C$ in glycogen from glucose counts/min/ $10^7$ cells | $^{14}C$ in glycogen and glucose from | |
|---|---|---|---|
| | | Pyruvate counts/min/ $10^7$ cells | Alanine counts/min/ $10^7$ cells |
| None (control) | 100 | 277 | 1530 |
| Prednisolone | 89 | 276 | 2000 |

Exponentially growing cultures were incubated in the presence of $^{14}C$-glucose ($3{\cdot}7 \times 10^4$ cpm per ml) for 30 hr, and in the presence of $^{14}C$-pyruvate ($1{\cdot}8 \times 10^4$ counts/min per ml) and $^{14}C$-alanine ($2{\cdot}3 \times 10^4$ counts/min per ml) for 24 hr. Prednisolone, when added, was present in a concentration of 25 $\mu$M. In the experiments with $^{14}C$-glucose the radioactivity was determed in the isolated glycogen. In the experiments with labeled pyruvate and alanine the cells were hydrolyzed and the radioactivity determined in glucose isolated as the glucosazon.[33]

The mechanism of the increased glycolysis in Chang cells is not revealed by the present data. Prednisolone did not increase the activity of glucokinase or hexokinase. Work is now in progress to investigate whether prednisolone will affect the activity of phosphofructokinase and pyruvate kinase, enzymes which also are important in channeling glucose into lactate.[31] It is of interest that no effect of prednisolone was found on the activities of glucose-6-phosphatase and on fructose-1,6-diphosphatase. These enzymes show increased activities under conditions when glucocorticoid steroids exhibit their typical gluconeogenic effect.[27, 28, 31]

The present results indicate that a glucocorticoid steroid may elicit opposite biochemical responses under different conditions. This seems to be of some general interest. The evidence is strong that the increased activity levels of gluconeogenic enzymes normally induced by glucocorticoid hormones is due to *de novo* formation of enzymes.[28, 29, 31, 32] Similarly, the present results with actinomycin D and puromycin suggest that under our conditions prednisolone induced the formation of enzymes in glycolysis. In terms of current views on the mechanism of regulation of protein synthesis, this suggests that a substance which normally induces one set of enzymes, may induce an alternative set of enzymes under other conditions, and hence that the action of an inducer may be modified by environmental factors.

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