Changes in the sensitivity of lipogenesis in rat hepatocytes to hormones and precursors over the diurnal cycle and during longer-term starvation of donor animals

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Abstract In rat hepatocytes freshly isolated from donor rats at different times of the day, the rates of lipogenesis (de novo fatty acid synthesis) varied with a diurnal periodicity. The maximal rate occurred approximately 5 hr after the end of the normal 8-hr feeding period and at this time was four- to fivefold higher than the minimum rate which occurred midway through the feeding period. A similar diurnal pattern of change persisted even when the supply of lipogenic substrate, present in the medium as pyruvate, was not limiting. Although insulin stimulated the basal rates of lipogenesis to different relative extents in hepatocytes isolated at different times of the day, in absolute terms the hormone had little effect on the overall pattern of change during the diurnal cycle. The presence of pyruvate protected lipogenesis against inhibition by glucagon. The degree of protection varied over the diurnal cycle. During the early stages of starvation (up to 24 hr) there was a continuous decline in the rate of hepatocyte lipogenesis, irrespective of whether insulin and/or lipogenic substrate (pyruvate) were available or not. After this time the decline in the rate of lipogenesis was much less rapid. Seventeen hr after removal of food from donor rats, a point was reached beyond which pyruvate was incapable of supporting the maximum basal rate of lipogenesis which occurred during the normal diurnal cycle of fed rats. After this time lipogenesis in the presence of pyruvate was inhibited by glucagon to a much greater relative extent than that observed during feeding. The results suggest that variations in the rate of lipogenesis over the diurnal cycle and during the first 24 hr of starvation could not be accounted for entirely by fluctuations in substrate availability. In contrast, changes which occurred subsequent to this (up to 43 hr of starvation) could be eliminated when lipogenic substrate was made more abundant. Longer periods of starvation were marked by a relative increase in the ability of glucagon to prevent the substrate-induced stimulation of lipogenesis.—**Gibbons, G. F., C. R. Pullinger, and 'O. G. Björnsson.** Changes in the sensitivity of lipogenesis in rat hepatocytes to hormones and precursors over the diurnal cycle and during longer-term starvation of donor animals. *J. Lipid Res.* 1984. **25:** 1358–1367.

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Diurnal changes in hepatic lipogenesis occur in experimental animals in vivo (1–3) and appear to be dependent upon the changing nutritional state of the animal over the 24-hr cycle. However, it is not known to what extent, if at all, these changes are due to variations in the availability of lipogenic substrates, or whether other factors such as a change in the activity of enzymes such as acetyl-CoA carboxylase (EC 6.4.1.2) are also involved. Diurnal variations also occur in the rate at which other metabolic processes such as glycogenesis, glycogenolysis (4, 5), and cholesterogenesis (6–8) operate in the liver in vivo. These variations are reflected in hepatocytes prepared from animals at the appropriate times of day (5, 9). It is not known whether the diurnal changes in hepatic lipogenesis which occur in vivo are similarly retained in hepatocytes in vitro. If so, such an in vitro system would permit investigations into the individual contribution of precursor supply, and other potentially important factors such as pancreatic hormones, to the diurnal lipogenic cycle in liver. This approach has been used extensively to investigate hepatic lipogenesis (e.g., 10–13) and has the obvious advantage that ambiguities that arise from concurrent effects in other organs in vivo are eliminated. In addition, the causes of the low rates of hepatic lipogenesis observed in hepatocytes from starved animals have recently been investigated (13) and it has been shown that rates characteristic of those in fed animals may be restored by suitable mixtures of lipogenic precursors. However, detailed studies of the time-dependent effects of starvation on hepatic lipogenesis have not yet been carried out. For instance, it is possible that the lipogenic response to hormones and precursors may vary as the liver continuously adapts to increasingly long periods of food

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deprivation. In this respect there are thought to be both short-term and long-term factors involved in the control of hepatic lipogenesis (12, 14) and it is important to identify the time-point at which the factors involved in the longer-term adaptation become of greater importance in determining the overall rate of lipogenesis during starvation. The experiments described in the present report were designed to answer the following questions. First, can the diurnal changes in hepatic lipogenesis observed in vivo be reproduced in isolated hepatocytes in vitro? Second, if so, to what extent and in what manner may those changes be influenced by potential regulatory factors such as substrate availability and pancreatic hormones? An answer to this question may help to clarify the nature of the diurnal changes in hepatic lipogenesis. Finally, we wished to know how rapidly rates of hepatic lipogenesis declined after removal of food and to what extent this decline was due to substrate scarcity after various periods of time. The overall objective, therefore, was to gain an insight into the means by which hepatic lipogenesis responds to short- and longer-term variations in the nutritional status of donor animals.

MATERIALS AND METHODS

Materials

Pancreatic insulin and glucagon derived from a mixture of bovine and porcine pancreas were obtained from Sigma (St. Louis, MO). Both were dissolved in a solution of sodium chloride (0.9%) before use. Sodium pyruvate and amino acids were also obtained from Sigma. Tritiated water and [1-^{14}C]oleic acid (sodium salt) were obtained from Amersham International Ltd. (Amersham, Bucks., England). Collagenase was obtained from the Boehringer Corporation (London) Ltd. (Sussex, England). The sources of all other materials have been described previously (15, 16).

Maintenance of animals

Male Wistar rats were placed two per cage in a room thermostatically controlled at 23°C and lit artificially between 2100 hr and 0900 hr. Food (Formula "PRD", Labsure, Poole, England) was available for the first 8 hr of the dark period (i.e., until 1700 hr). Water was available ad libitum. This regimen has been used previously for studying variations in hepatic lipogenesis, cholesterogenesis, and carbohydrate metabolism (1, 5, 17). The animals were acclimatized under these conditions for 2–4 weeks before use. The final weight of the animals was between 250 and 320 g and over a typical acclimatization period of 19 days the average increase in weight was 26.3 ± 1.7% (SE) of initial body weight. During this period the average food consumption per cage (two rats) was 51.2 ± 6.2 g (SE) per 8-hr feeding period.

Preparation of hepatocytes and incubation procedures

Hepatocytes were prepared from animals removed from their cages at six different times over the 24-hr period. These times were 0900 hr, 1200 hr, 1500 hr, 1700 hr, 2200 hr, and 0300 hr (subsequently referred to as D_0, D_3, D_6, D_8, L_1, and L_6 hepatocytes, respectively). In some cases, food that was normally offered at 0900 hr to donor animals was withheld and hepatocytes were prepared 3 hr, 6 hr, 8 hr, and 27 hr later. The standard procedures for preparation and incubation of the hepatocytes have been described previously (15, 16, 18). The only modification was that glucose (25 mM) was present in the perfusion medium to prevent glycogenolysis during cell preparation (11). The standard incubation medium consisted of Krebs-Henseleit bicarbonate buffer containing Ca^{2+} (2.5 mM), albumin (3.6%) and glucose (11.1 mM). However, in those cells prepared from donor animals that had been starved for 43 hr, glucose (4 mM) was present both during the perfusion and during incubation (19). In all cases an amino acid mixture including glutamine (0.4 mM) was present during the incubation period (20). Tritiated water (^3H_2O) was added immediately before the start of the incubation. Although the final specific radioactivity of the water in the medium varied slightly from one experiment to another, this was always within the range 60–80 dpm/nmol.

Measurement of the rate of fatty acid synthesis

After incubation the cell suspension was chilled on ice and the cell pellet was sedimented by centrifugation at 80 *g*. After washing with ice-cold Krebs-Henseleit buffer and resedimentation, the incorporation of ^3H into the saponifiable fraction of the cell was determined as described previously (18).

Statistical treatment of results

All values are presented as the means ± the standard error of several independent observations, the exact number of which (n) is given in parentheses. The significance of observed differences was tested using Student's *t* test.

RESULTS AND DISCUSSION

Relationship between lipogenesis and duration of incubation

As part of an attempt to identify optimum incubation conditions for hepatocyte lipogenesis, the relationship between lipogenic rates and the length of the incubation

period was studied. Hepatocytes were prepared from animals at six different times over a 24-hr period and incubated for periods of 1, 2, and 3 hr. At the end of each incubation period, the incorporation of ^3H_2O into fatty acids was determined. **Fig. 1** shows that, irrespective of the time of day at which the hepatocytes were prepared, upon subsequent incubation the rates of lipogenesis were linear with time for at least 2 hr. In all subsequent experiments, therefore, rates of lipogenesis were studied over a 2-hr period.

Dependence of rates of lipogenesis upon the concentrations of insulin, glucagon, and pyruvate

Another prerequisite for these studies was the determination of concentrations of insulin and of lipogenic substrate that were maximally effective in stimulating lipogenesis in hepatocytes. Because of the possibility that these optimal concentrations may vary from time to time over the diurnal cycle, these relationships were examined at two different times of the day. Similar experiments were carried out to establish the concentration of glucagon required for maximal inhibition of lipogenesis. Hepatocytes prepared from animals at 0900 hr, immediately before the start of the normal feeding period (D_0-hepatocytes) and at 1500 hr, 6 hr after food was offered (D_6-hepatocytes), were incubated for 2 hr with various concentrations of insulin, glucagon, and pyruvate. The amounts of fatty acid synthesized during this period was determined in each case (**Fig. 2**). In both types of hepatocyte preparation, glucagon maximally inhibited lipogenesis at a concentration of 10^{-7} M. The maximal stimulation by pyruvate occurred at a concentration of about 25 mM in the D_0-cells and at a somewhat lower concentration in the D_6-cells. In subsequent experiments, where appropriate, glucagon and pyruvate were used at concentrations of 10^{-7} M and 25 mM, respectively. The response of lipogenesis to insulin appeared to increase over the entire concentration range used (0–10^3 μU/ml or 0–6.8 × 10^{-9} M). However, in other experiments with D_6-hepatocytes, there was very little, if any, further increase at an insulin concentration of 10^4 μU/ml (6.8 × 10^{-8} M) (130.0 ± 6.6% of basal). In view of this, all subsequent effects of insulin on lipogenesis were determined at a concentration of 10^3 μU/ml (6.8 × 10^{-9} M). Although the basal (control) rates of lipogenesis were higher in hepatocytes prepared at 1500 hr rather than at 0900 hr (Fig. 1), in relative terms, both pyruvate and insulin stimulated lipogenesis to a greater extent in the latter preparation. A particularly interesting feature of this differential sensitivity was that, whilst 10^2 μU/ml (6.8 × 10^{-10} M) of insulin produced a significant increase ($P < 0.01$) in the cells prepared at 0900 hr, there was no significant change in those prepared at 1500 hr.

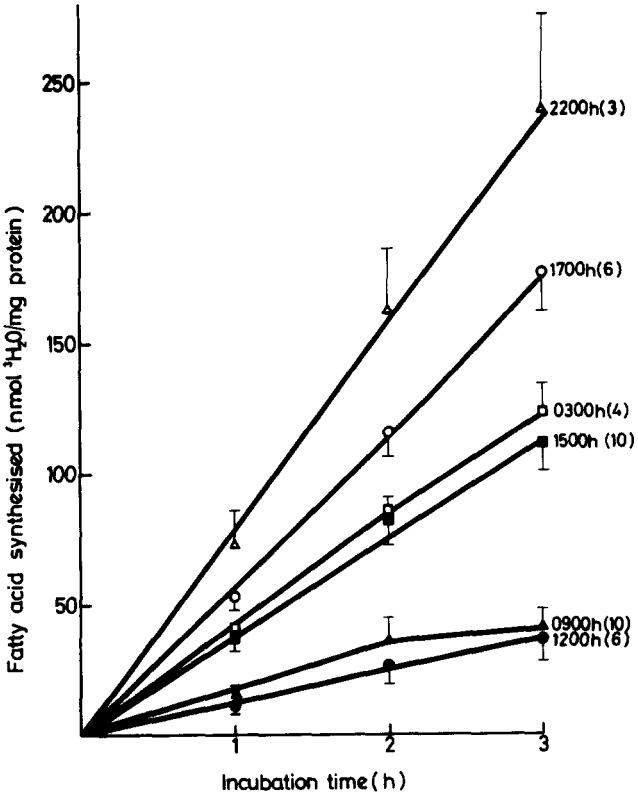

Fig. 1. Incubation time-dependence of lipogenesis in hepatocytes isolated at different times of the day. Hepatocytes were obtained from rats maintained on a strict lighting and feeding schedule and killed at the times shown. Each type of hepatocyte preparation was incubated at 37°C for periods of 1, 2, or 3 hr in the standard medium containing ^3H_2O. At the end of each incubation period the incorporation of ^3H into the total fatty acid-containing fraction was determined. Each point represents the mean ± SE from several independent hepatocyte preparations, the exact number of which at each time is shown in parentheses.

A further set of experiments was carried out to determine the precise effects of varying the pyruvate concentration on the increased rate of lipogenesis observed in the presence of 10^3 μU/ml of insulin (Fig. 2). In agreement with a previous report (13), the maximum rate of lipogenesis observed in the presence of substrate alone was unaltered by the additional presence of insulin. Despite this, in the present work insulin lowered the concentration of pyruvate required for maximal stimulation of lipogenesis from 25 mM to near 10 mM (Fig. 2). Comparison of the effects of insulin and pyruvate during different periods of incubation confirmed that neither of these substances, at the concentrations used, affected the linear relationship of lipogenesis with time, at least over a 2-hr incubation period (**Fig. 3**). However, the inhibitory effect of glucagon after 2 hr was somewhat greater than that observed after 1 hr (Fig. 3).

The concentrations of glucagon and pyruvate that were found to have maximal effect on the rates of

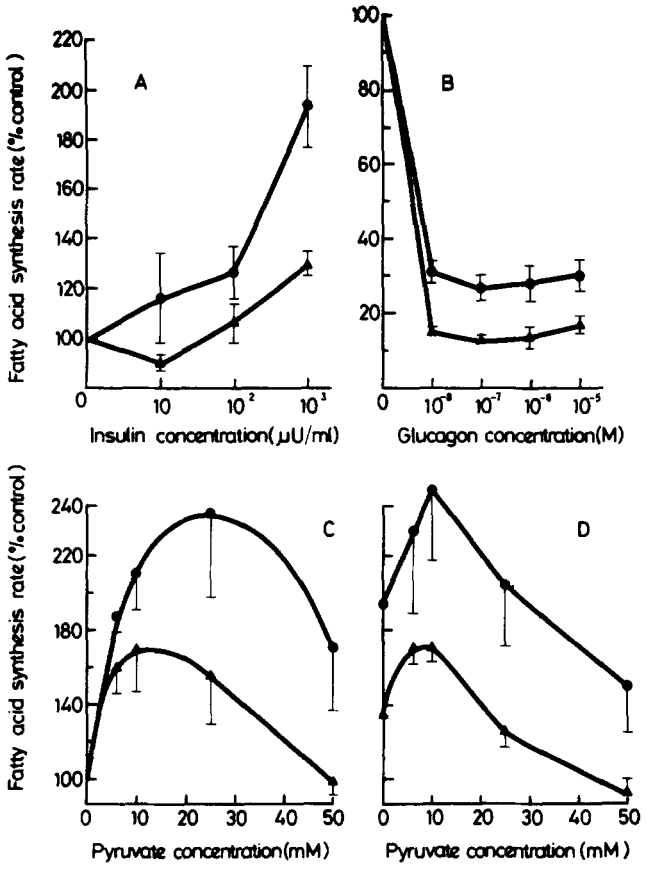

Fig. 2. Effects of insulin, glucagon, pyruvate and insulin + pyruvate on lipogenesis in hepatocytes. Hepatocytes were obtained from rats killed either at 0900 hr, immediately before the start of the normal feeding period (● —— ●), or at 1500 hr after they had been fed for 6 hr (▲ —— ▲). Each type of hepatocyte preparation was incubated in the standard medium for 2 hr either alone, in the presence of various concentrations of insulin (Fig. 2A), glucagon (Fig. 2B), pyruvate (Fig. 2C) or of pyruvate in the presence of insulin (10^3 μU/ml) (Fig. 2D). Tritiated water was also present in the incubation medium. In the absence of hormones the rates of fatty acid synthesis in the hepatocytes prepared at 0900 hr and 1500 hr corresponded to 43.8 ± 8.8 and 90.9 ± 8.3 nmol ^3H_2O incorporated per mg protein per 2 hr, respectively. Each point represents the mean ± SE of values obtained from six independent hepatocyte preparations.

hepatocyte lipogenesis were higher than those reported to occur in peripheral blood in vivo. In the case of glucagon this may have been due, at least partly, to the high rate of biodegradation of this compound by liver (21, 22). Since the objective of the present study was to determine whether these maximal responses were of sufficient magnitude to account for the changes in lipogenesis that occurred over the diurnal cycle and during starvation, these concentrations were used throughout this work. It was also for this reason that pyruvate, rather than lactate, was used as the lipogenic substrate. Thus pyruvate is more effective than lactate in stimulating pyruvate dehydrogenase (EC 1.2.4.1) in hepatocytes (23) and would be expected to more readily facilitate study of the behavior of lipogenesis when acetyl CoA is available in nonlimiting concentrations.

Diurnal variations in the rate of lipogenesis in hepatocytes

Hepatocytes isolated from animals at six different times over the 24-hr period were incubated for 2 hr alone or in the presence of insulin, glucagon, or pyruvate. Similar incubations were carried out in the presence of combinations of pyruvate and glucagon or pyruvate and insulin. In each case, the incorporation of ^3H_2O into fatty acids was determined (**Fig. 4**). The basal (control) rates of lipogenesis in the hepatocytes varied cyclically over the 24-hr period. The maximum observed rate corresponded to an incorporation of 162.2 ± 24.3 nmol of ^3H_2O per mg of cell protein per 2 hr and occurred at 2200 hr, 1 hr into the light phase (L_1) of the cycle and 5 hr after the donor animals had last had access to food. In contrast, the minimum observed value (31.5 ± 6.9 nmol of ^3H_2O per mg per 2 hr) occurred at 1200

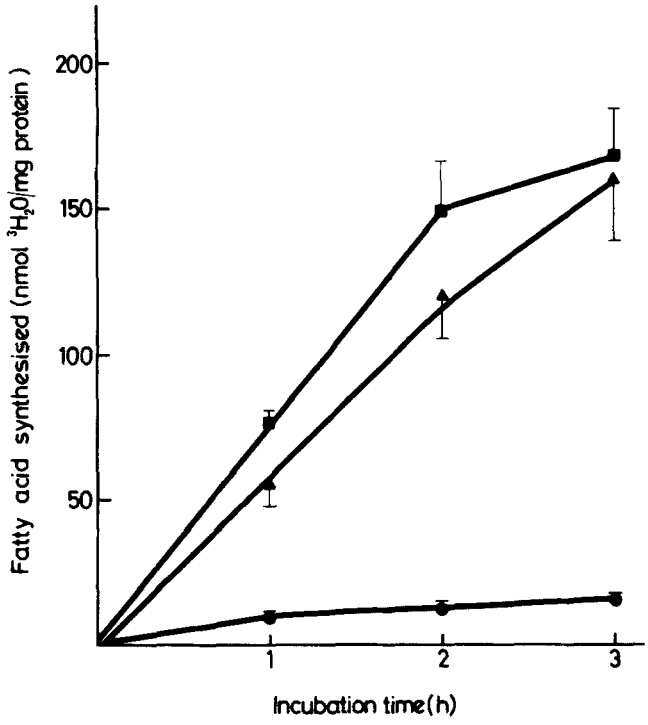

Fig. 3. Effect of glucagon, insulin, and pyruvate on the incubation time-dependence of lipogenesis in hepatocytes. Hepatocytes obtained from rats killed at 1500 hr were incubated in the standard medium containing ^3H_2O for periods of 1, 2, and 3 hr. In addition, either glucagon (10^{-7} M, ● —— ●), insulin (10^5 μU/ml, ▲ —— ▲), or pyruvate (25 mM, ■ —— ■) was also present. At the end of each incubation period the incorporation of ^3H into the fatty acid-containing fraction was determined. Each point represents the mean ± SE of values obtained from at least six independent hepatocyte preparations.

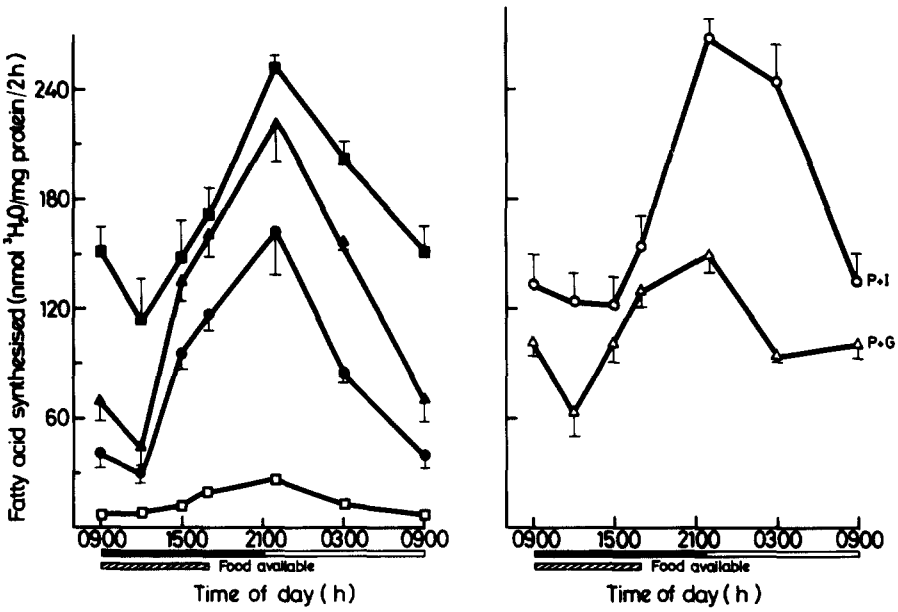

Fig. 4. Effects of insulin, glucagon, and pyruvate on lipogenesis in hepatocytes prepared at different times of the day. Hepatocytes were obtained from animals killed at 0900 hr (n = 14), 1200 hr (n = 6), 1500 hr (n = 14), 1700 hr (n = 6), 2200 hr (n = 3), and 0300 hr (n = 4). Each type of hepatocyte preparation was incubated in the standard medium containing ^3H_2O either alone (● —— ●), or in the presence of insulin (10^3 μU/ml; ▲ —— ▲), glucagon (10^{-7} M; □ —— □), pyruvate (25 mM; ■ —— ■) or of mixtures of insulin + pyruvate (○ —— ○) or glucagon + pyruvate (△ —— △). At the end of 2 hr, each incubation was stopped and the incorporation of ^3H into the fatty acid fraction of the cells was determined. Each point represents the mean ± SE of values obtained from several independent hepatocyte preparations (the exact number is shown given in parentheses above). The black and white rectangles alongside the time axis represent periods of darkness and light, respectively. The hatched rectangle refers to the period during which food was available.

hr (D_3) at which time food had been available to the donor animals for 3 hr. Similar studies using ^3H_2O in vivo have so far been conducted only on animals fed ad libitum (2, 3). Thus it may be inappropriate to make direct comparisons with the above observations made in hepatocytes from animals on a controlled feeding schedule. Nevertheless, in the in vivo studies, the maximum rate of hepatic lipogenesis occurred at or towards the end of the dark period (1–3) during which at some time the food intake is maximal. The present results are thus consistent with in vivo studies of hepatic lipogenesis and further suggest that hepatic lipogenesis continues to increase for some time after the point of maximum food intake. The latent period during which the rate of lipogenesis continues to decline for the first 3 hr of feeding (Fig. 4) may reflect the metabolic priority for hepatic glycogenesis, increased rates of which occur in hepatocytes during the first 3–4 hr of feeding of donor animals maintained on a controlled feeding schedule similar to that used in the present work (5).

Insulin stimulated the rate of lipogenesis in hepatocytes at all times of the cycle. Although this did not alter the overall pattern of change during the 24-hr period (there were no changes in the times of minimum and maximum lipogenic rate), the basal rates of lipogenesis were stimulated to different relative extents at different times. Thus the relative sensitivity of lipogenesis to insulin varied in a regular diurnal cycle (**Fig. 5**). This confirms the results of the concentration-dependent effects of insulin carried out at 0900 hr and 1500 hr and emphasizes the importance of maintaining strict control over the timing of experiments when investigating insulin effects on lipogenesis. The reason(s) for these variations is not immediately obvious. However, in this respect, it is well documented that the concentration of insulin receptors on the hepatocyte plasma membrane is inversely related to the external insulin concentration (24–26) which decreases during starvation (24, 27). Thus the increased sensitivity of lipogenesis to insulin during the latter part of the period when food was unavailable is consistent with an increase in the number of insulin receptors that would be expected to occur at these times.

At all times of the cycle pyruvate increased lipogenesis to a greater extent than did insulin (Fig. 4). However, this difference was less pronounced at those times when basal rates of lipogenesis were high. For instance, at 1700 hr and 2200 hr the rates in the presence of insulin although lower (94.2 ± 3.6% and 87.9 ± 7.7%, respectively) than those observed when pyruvate was present,

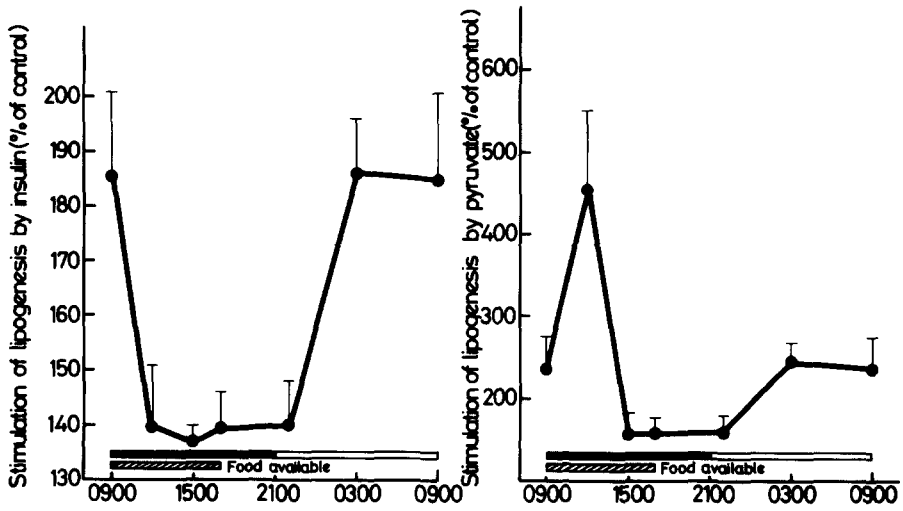

Fig. 5. Relative effects of pyruvate and insulin on lipogenesis at different times of the day. Hepatocytes were obtained from rats killed at various times over the diurnal cycle as described in the legend to Fig. 4. The cells were incubated in the standard medium for 2 hr either alone or in the presence of insulin (10^3 μU/ml) or pyruvate (25 mM). At the end of this period the incorporation of ^3H into the total fatty acid fraction was determined. The rates of lipogenesis in the presence of pyruvate or insulin in each type of hepatocyte preparation are expressed as a percentage of the rates observed in corresponding incubations carried out in the absence of these substances (i.e., as a percentage of the basal rates). Each point represents the mean ± SE of several independent hepatocyte preparations obtained at each time of day. The numbers of animals used at each time are shown in the legend to Fig. 4.

were not significantly so. Lactate at a concentration of 10 mM also produced a significantly greater increase in lipogenesis than did insulin. However, although the rate of hepatocyte lipogenesis at 0900 hr in the presence of insulin was only 47.7 ± 11.6% (n = 4) of that when lactate was present, at 1500 hr when the basal rate of lipogenesis was higher, this difference had narrowed to 72.8 ± 3.1% (n = 4).

Like insulin, pyruvate had very little overall effect on the pattern of change of lipogenesis during the diurnal cycle. Nevertheless, again, the basal rates were stimulated by pyruvate to different relative extents over the 24-hr period. In general, this resulted in more pronounced effects of pyruvate when the basal rates of lipogenesis were low (Fig. 5). It may be of interest to note that, although the profile of the diurnal variation in lipogenic rates occurred at a higher overall level in the presence of pyruvate, the amplitude of this diurnal change (i.e., the difference between the minimum and maximum rates of lipogenesis) was unaltered. This suggests that there is a basic underlying diurnal rhythm representing the cells' capacities for lipogenesis, the magnitude of which is unaffected by alteration in substrate availability. This interpretation is partly supported by the observation that, at certain times of the cycle (e.g., at 2200 hr), the basal rate of lipogenesis exceeded that which was reached at other times (e.g., at 1200 hr) under conditions of unrestricted substrate availability (Fig. 4). As was suggested by earlier experiments, addition of insulin to hepatocytes incubated in the presence of nonlimiting concentrations of substrate had very little, if any, further effect on lipogenesis at any point over the diurnal cycle (Fig. 4).

It has been reported previously that lipogenic substrates are capable of mitigating the inhibitory effects of glucagon on lipogenesis. Although the rate of lipogenesis observed in the presence of substrate alone was not achieved, with a combination of substrate and glucagon the original basal rate was restored, or even exceeded somewhat (11, 21, 22, 28). The present results are in agreement with these original findings and show that pyruvate protects lipogenesis against inhibition by glucagon (**Fig. 6**). Nevertheless, the results of the experiments reported in Fig. 6 show that the degree of protection by pyruvate varies according to the point in the diurnal cycle at which the hepatocytes were prepared. Thus at 1700 hr (D_8) glucagon was capable of inhibiting the pyruvate-stimulated rate of lipogenesis by only 23.6%, whilst at 0300 hr (L_6) the degree of inhibition had more than doubled, to 52.5%. It has previously been suggested (21, 22) that the extent to which glucagon inhibits lipogenesis in the presence of substrate reflects the extent to which glucagon directly affects lipogenic enzymes such as acetyl CoA carboxylase (EC 6.4.1.2) by means other than those involving changes in substrate availability. If this is the case then the present results suggest that the differing sensitivity of the pyruvate-stimulated rates of lipogenesis to glucagon reflects a

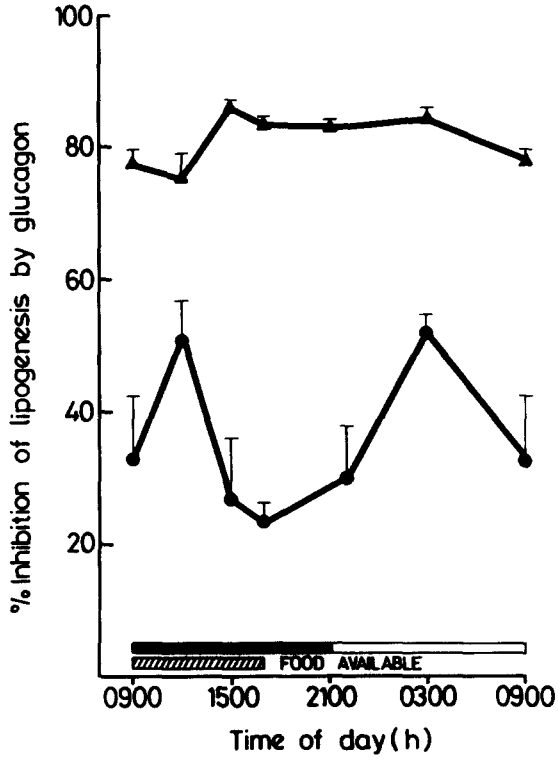

Fig. 6. Variations in the effects of glucagon on the basal- and pyruvate-stimulated rates of lipogenesis in hepatocytes prepared at different times of the day. Hepatocytes were prepared at various times over the diurnal cycle (see legend to Fig. 4) either alone or in the presence of either glucagon (10^{-7} M) or of a mixture of pyruvate (25 mM) and glucagon (10^{-7} M). Rates of lipogenesis were measured over a 2-hr incubation period in each case. The results are expressed in terms of the percentage by which glucagon inhibits either the basal rate of lipogenesis (▲——▲) or the rate of lipogenesis observed in the presence of pyruvate (●——●). Each point is the mean ± SE of values obtained from several independent hepatocyte preparations (see legend to Fig. 4).

diurnal change in the properties of acetyl CoA carboxylase.

Two other features of the changing pattern of lipogenesis over the diurnal cycle are worthy of note. First, when glucagon was simultaneously present with pyruvate, the amplitude of the lipogenic cycle was dampened (Fig. 4). Second, at certain times of the cycle, although the difference in the rates of lipogenesis in a particular batch of hepatocytes incubated in the presence either of insulin or of glucagon was very large, the presence of pyruvate in addition to each hormone almost eliminated this difference. This buffering effect of pyruvate was particularly noticeable in hepatocytes isolated towards the end of the feeding period. i.e., at 1500 hr and 1700 hr (Fig. 4).

Response of hepatic lipogenesis to increasing periods of starvation

In the experiments described above, food was available to animals only for an 8-hr period of each day from 0900 hr to 1700 hr. There was thus a 16-hr period inherent in the controlled 24-hr cycle during which the animals did not have access to food. The effects of longer periods of food deprivation (19 hr, 22 hr, 24 hr, and 43 hr) on lipogenesis in hepatocytes were investigated by withholding food normally given at 0900 hr and preparing hepatocytes at the appropriate times later.

As discussed above, compared to that observed at the end of the feeding period, 5 hr later the rate of lipogenesis was significantly higher. From this point on, however, increasing the length of the period of food deprivation was accompanied by a decrease in the rate of lipogenesis. The rate of decline appeared to be exponential up to 24 hr of starvation, whereafter little further change occurred between 24 and 43 hr (**Fig. 7**). The decline in the rates of lipogenesis observed during the light phase of the diurnal cycle in the presence of insulin or pyruvate continued during longer periods of starvation. Thus, even when lipogenic substrate availability was not rate-limiting, rates of lipogenesis continued to decline, a process which could not be reversed by the simultaneous presence of insulin except, perhaps, during a brief period from between 16 hr and 24 hr of starvation (**Fig. 8**). Nevertheless, in the presence of pyruvate, lipogenesis in hepatocytes from rats starved for increasing lengths of time declined more slowly than the basal rates. This resulted in an increased relative stimulation of lipogenesis by pyruvate with increasingly

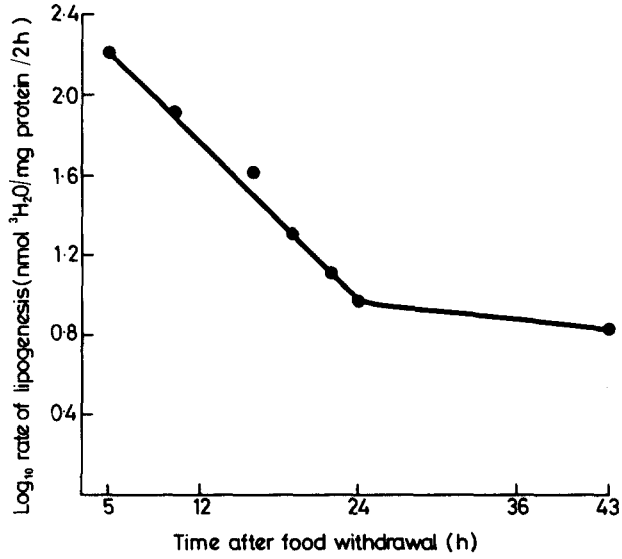

Fig. 7. Decline in the basal rates of lipogenesis in hepatocytes from rats starved for different periods of time. Hepatocytes were prepared from animals killed at the following times after removal of food: 5 hr (n = 3), 10 hr (n = 4), 16 hr (n = 14), 19 hr (n = 7), 22 hr (n = 8), 24 hr (n = 4), and 43 hr (n = 4). In each case the rates of lipogenesis were measured during a 2-hr incubation period. Each point is the mean of values obtained in hepatocytes prepared from several animals killed at each time (see above).

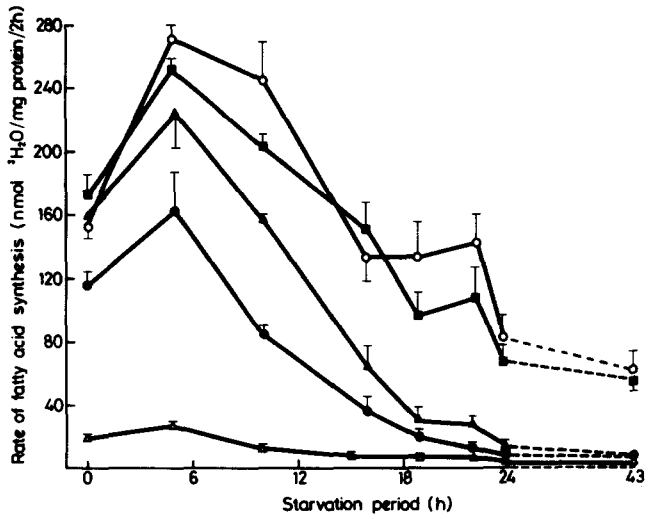

Fig. 8. Effects of glucagon, insulin, and pyruvate on the rates of lipogenesis in hepatocytes prepared from rats starved for different periods of time. Hepatocytes were prepared at the times of day shown in the legend to Fig. 7 and incubated either alone (● —— ●) or in the presence of glucagon (10^{-7} M) (Δ —— Δ), insulin (10^3 μU/ml) (▲ —— ▲), pyruvate (25 mM) (■ —— ■), or with a mixture of pyruvate + insulin (○ —— ○). In each case the rates of lipogenesis were determined over a 2-hr incubation period. Each point represents the mean ± SE of values obtained from several independent hepatocyte preparations at each time of day (see legend to Fig. 7).

long periods of starvation (**Fig. 9**). In contrast, although the relative stimulation of basal levels of lipogenesis by insulin increased during the latter part of the light phase (i.e., during the preprandial period), the relative sensitivity of lipogenesis to insulin remained unchanged during at least the next 8 hr of food deprivation (Fig. 9).

In general, the characteristics of lipogenesis between 24 hr and 43 hr of starvation were different from those observed during the earlier period up to 24 hr. Thus the relative stability of both the basal and stimulated rates, during the later period, is in contrast to the decline observed earlier. In addition, at some point between 24 hr and 43 hr of starvation, the basal level of lipogenesis became completely insensitive to stimulation by insulin (Fig. 9). As insulin receptors should be abundant in these cells (24–27) it is probable that this lack of response was due to some effect of starvation on events subsequent to insulin binding.

Contribution of substrate supply and of glucagon-related effects to changes in the rates of lipogenesis

It has been suggested that hepatic glycogen is the major source of carbon for lipogenesis in liver (10, 29). If this is the case it would be reasonable to expect that the availability of lipogenic substrate would vary over the 24-hr cycle as a result of diurnal changes in the rate of hepatic glycogenolysis (4, 5). On the other hand, lactate and pyruvate generated extra-hepatically may be important sources of fatty acid carbon (2). Irrespective of the origin of substrate it has been reported that, when hepatocytes from 18-hr starved rats were incubated in the presence of a suitable substrate mixture, the resultant rate of lipogenesis equalled or exceeded that observed in hepatocytes from fed animals (13). The present results confirm that, in hepatocytes from starved

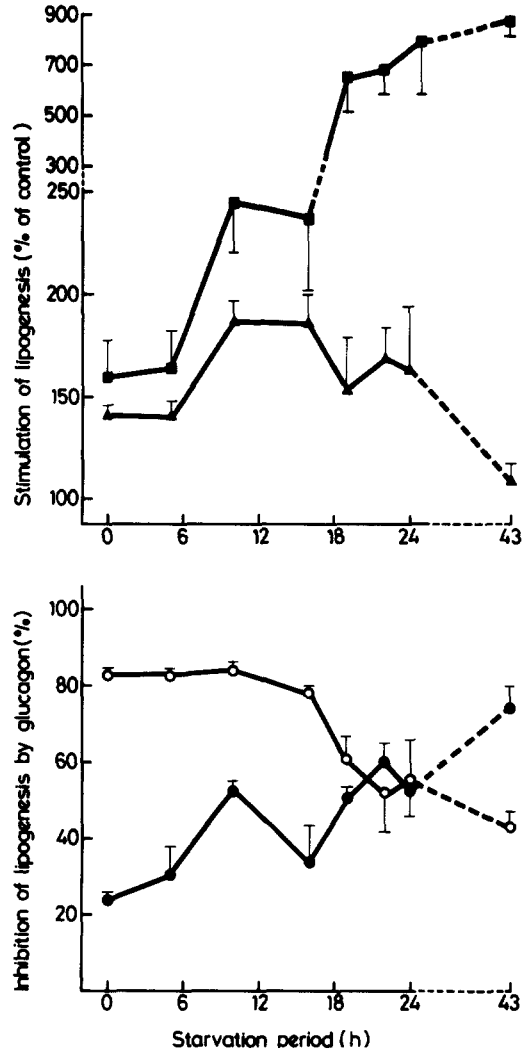

Fig. 9. Relative effects of glucagon, insulin, and pyruvate on lipogenesis in hepatocytes prepared from rats starved for different periods of time. Hepatocytes were prepared at different times of the day and incubated either alone or in the presence of glucagon (10^{-7} M), insulin (10^3 μU/ml), pyruvate (25 mM), or with a mixture of pyruvate + glucagon. The data are calculated from the experiments shown in Figs. 7 and 8. The effects of insulin (▲ —— ▲) and pyruvate (■ —— ■) are shown in terms of the % by which each of these substances stimulates the basal (i.e., control) rates of lipogenesis at each time. The effects of glucagon are shown as a % by which the hormone inhibits either the basal rate of lipogenesis (○ —— ○) or the pyruvate-stimulated rate of lipogenesis (● —— ●). Each point represents the mean ± SE of values obtained in several independent hepatocyte preparations at each time (see Fig. 7).

rats, added substrate is capable of restoring the rate of lipogenesis to the basal levels observed at certain times of the diurnal cycle in fed rats. Nevertheless, the patterns of change of lipogenesis reported here suggest that variations in the availability of lipogenic substrate are unable to account entirely for the changes in lipogenesis which occur over the diurnal period or during short-term (up to 24 hr) starvation. This conclusion is based upon the following evidence. First, if substrate supply were the only limiting factor, then basal rates of lipogenesis should fluctuate within a range, the highest point of which should not exceed any of the rates observed when substrate was present in unlimited supply. This was not the case. Thus during the diurnal cycle, at 1200 hr, even when maximally effective concentrations of pyruvate were present, the resulting rate of lipogenesis was exceeded by the basal rate observed at 2200 hr (Fig. 4). Again, the changing pattern of lipogenesis during starvation was such that, at times longer than 19 hr after removal of food, lipogenic substrate such as pyruvate was unable to support the maximal basal rate of lipogenesis observed during the normal diurnal cycle (Figs. 4 and 8). Thus, even with superphysiological concentrations of substrate, there were times when maximal basal rates of lipogenesis could not be achieved. These observations suggest that, under these circumstances, the decline in the rates of lipogenesis was due to factors in addition to changes in substrate availability and probably reflects a substrate-independent decline in the capacity of lipogenic enzymes such as acetyl CoA carboxylase. Second, even when substrate supply is not limiting, diurnal changes in the rates of lipogenesis persist with an amplitude similar to that observed for the basal rates. This suggests that, in addition to any changes in lipogenesis resulting from changes in substrate availability over the diurnal cycle and during short-term starvation, the capacity of certain lipogenic enzymes also changes. In this respect it is well documented that, under some circumstances, changes in the rate of lipogenesis occur concurrently with changes in the capacity of acetyl CoA carboxylase measured in cell-free extracts (22, 30–32). However, although the capacity of this enzyme decreases during starvation (33), corresponding diurnal changes have not yet been demonstrated. Finally, the basal rate of lipogenesis at 2200 hr was almost identical to the pyruvate-stimulated rate observed at 0900 hr. If the high basal rate at 2200 hr had been due entirely to an increase in substrate over and above that normally available at 0900 hr, then the extent of inhibition by glucagon of the 2200-hr basal rate and of the 0900-hr rate in the presence of pyruvate should have been identical; this was not the case—the former was inhibited by 82.7%, the latter by only 33.3% (Fig. 6).

At all times, apart perhaps from those times of the diurnal cycle at which basal levels of lipogenesis were high (i.e., 1700 hr and 2200 hr), insulin was less effective than was pyruvate in stimulating lipogenesis (Fig. 4). The present results do not clarify the means by which insulin stimulates lipogenesis, but the absence of any further effect of insulin in the presence of nonlimiting concentrations of lipogenic substrate would appear to rule out an increase in the activity of lipogenic enzymes, such as acetyl CoA carboxylase, by means other than increasing citrate levels. This latter effect could be achieved by accelerating substrate flux through glycolysis (22, 34).

The time-dependent decrease in the rate of lipogenesis during starvation was such that, at times longer than 19 hr after food removal, the basal rates were even lower than those reached in the presence of a maximally inhibitory concentration of glucagon in hepatocytes from fed rats killed at 2200 hr (Fig. 8). This suggests that the magnitude of the lipogenic response to concentrations of glucagon (or to increases in the glucagon/insulin ratio) which are probably higher than those encountered in vivo remains insufficient to account for the decrease in lipogenesis which occurs even during moderately short periods of starvation.

Short-term and long-term effects of starvation on lipogenesis

The characteristics of the changes in lipogenesis which occurred between 24 and 43 hr of starvation appeared to be different from those which were observed during the first 24 hr. First, the exponential decline in lipogenic activity was no longer apparent during the later period (Fig. 7). Second, lipogenesis became almost completely insensitive to stimulation by insulin. Finally, the decline in the maximum rates of lipogenesis in hepatocytes incubated in the presence of pyruvate was halted, although this substrate-dependent lipogenic activity was much more sensitive to inhibition by glucagon (Fig. 9). These combined observations suggest that between 24 and 43 hr of starvation there was no decline in the capacity of lipogenic enzymes such as acetyl CoA carboxylase and that the small decrease in the observed basal rates was due to a decrease in substrate availability. Nevertheless, during this latter period, there appeared to be a qualitative change in the enzyme(s) responsible for the control of lipogenesis in that, in the presence of glucagon, any increase in the availability of substrate was accompanied by a much smaller net increase in the observed rate of lipogenesis than was the case during shorter periods of starvation (Fig. 9). This may reflect a pattern of changing metabolic priorities in the liver such that, after increasingly long periods of starvation, when common metabolites eventually become available (e.g., during feeding) the hepatocyte ensures the preferential synthesis of substances (e.g., glycogen) other than fatty acids. In this respect it is noteworthy that

during the phase of the diurnal cycle when glycogenesis is high (during the first 3 hr of feeding) (5) glucagon is more effective in blocking the entry of pyruvate into the lipogenic pathway (Fig. 6) than at later times in the cycle when hepatic glycogen stores are high.

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