5) that lacks tyrosinase activity can develop protoperithecia under certain conditions.

Two wild strains of *N. crassa*, 5297a and 74A, and a mutant (ty-1) lacking tyrosinase activity were used. Westergaard and Mitchell's (*1*) crossing-media was used for maximum protoperithecial formation. However, the density of the hyphal growth on this medium prevented suitable photography of individual, developing protoperithecia. In contrast, they were readily discernible when the culture was initially grown in Petri plates on strips of sterile (autoclaved) Visking dialysis membrane cut to 4 by 1.5 cm and placed on top of Westergaard and Mitchell's medium for 4 to 6 days—that is, until protoperithecial development had been initiated. Subsequently, they were transferred to Petri plates containing 1.2 percent Bacto-agar. This procedure restricts hyphal growth sufficiently to permit accurate observation of the development and differentiation of individual protoperithecia on the agar, adjacent to the membrane, unobscured by heavy hyphal growth. Under these conditions, the protoperithecia are morphologically indistinguishable from those developing on Westergaard and Mitchell's medium under usual conditions and are normal as evidenced by mature perithecial formation and subsequent ascospore formation after fertilization.

In the wild type, about 76 hours after its transfer to the Bacto-agar, the developing protoperithecia were initially recognized by the extension of a hyphal filament to form a loop (Fig. 1a). The incipient protoperithecia usually, but not invariably, occur within a halo-like zone, and occasionally development appears to be preceded by the deposition of a granular material.

Subsequently, the hyphal loop extends to form numerous coils (Fig. 1, b and c) that become closely packed. The early protoperithecium then darkens, and hyphal filaments grow out from the organelle (Fig. 1d). The entire process takes about 12 to 18 hours.

Normally, on Westergaard and Mitchell's medium, the first protoperithecia begin to develop about 36 hours after inoculation at 25°C. Full development of a protoperithecium on Westergaard and Mitchell's medium also is complete within about 12 to 18 hours. The maximum number of protoperithecia is reached in about 6 days. At 14°C, development is normal but occurs more slowly, an 8 to 9 day lag occurring before initiation of protoperithecial formation.

Protoperithecia ordinarily do not develop at 35°C on Westergaard and Mitchell's medium. However, if wild type *N. crassa* is transferred to Bacto-agar medium by the method with Visking strips, protoperithecia will develop on the agar adjacent to the Visking membrane at 35°C after 12 to 20 days.

The female-sterile, tyrosinase deficient mutant, ty-1, has been described by Horowitz, *et al.* (*5*). Female sterility is not affected by the usual inducers and repressors of the tyrosinase. We have noted that ty-1 does develop to the coiling stage (Fig. 1, b and c) at both 14°C and 25°C on Bacto-agar during the usual period of incubation, and, furthermore, some of these structures do develop into mature protoperithecia if allowed to incubate for an extended period (25 to 35 days). With ty-1 at 35°C, neither the early nor late stages of development were noted on Westergaard and Mitchell's medium or on the Bacto-agar medium.

A variety of cross-feeding experiments with mixtures of both whole cells and extracts of wild type and ty-1 failed to provide an explanation for female sterility in terms of any readily detectable, diffusible inducers or repressors.

In summary, a new technique has permitted us to define more clearly the several stages of development of protoperithecia in *N. crassa* and to extend previous observations on the effects of temperature on the formation of this organelle. We feel this may be a useful procedure in future studies of the genetic and metabolic control of this reproductive structure.

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Feedback Inhibition of Key Glycolytic Enzymes in Liver: Action of Free Fatty Acids

Abstract. *Increasing concentrations of sodium octanoate were progressively inhibitory to the activities of glucokinase, hexokinase, phosphofructokinase, and pyruvate kinase. Glucose-6-phosphate and 6-phosphogluconate dehydrogenases were also markedly inhibited. Other enzymes of carbohydrate metabolism such as lactate dehydrogenase, phosphohexose isomerase, and fructose-1,6-diphosphatase were not decreased. Among the key glycolytic enzymes, the inhibition of pyruvate kinase by the fatty acid was most marked. The biological significance of the inhibition of the key glycolytic enzymes is interpreted as a feedback inhibitory mechanism in regulation of fatty acid biosynthesis. The mechanism may function for rapid adaptation by which the organism can use the fatty acid level as a metabolic directional switch in decreasing glycolysis and turning on gluconeogenesis.*

Metabolic regulation in the mammalian organism is exerted through mechanisms of acute and chronic adaptation. In the control of hepatic gluconeogenesis and glycolysis we emphasized that the dynamic balance of the key rate-limiting enzymes of glycolysis and gluconeogenesis may play a crucial role in determining the direction of overall pathways (*1*). Studies on chronic adaptation showed that insulin induced the biosynthesis of the key glycolytic enzymes, glucokinase (*1, 2*), phosphofructokinase (*1, 3*), and pyruvate kinase (*1, 3, 4*), whereas it suppressed the biosynthesis of the

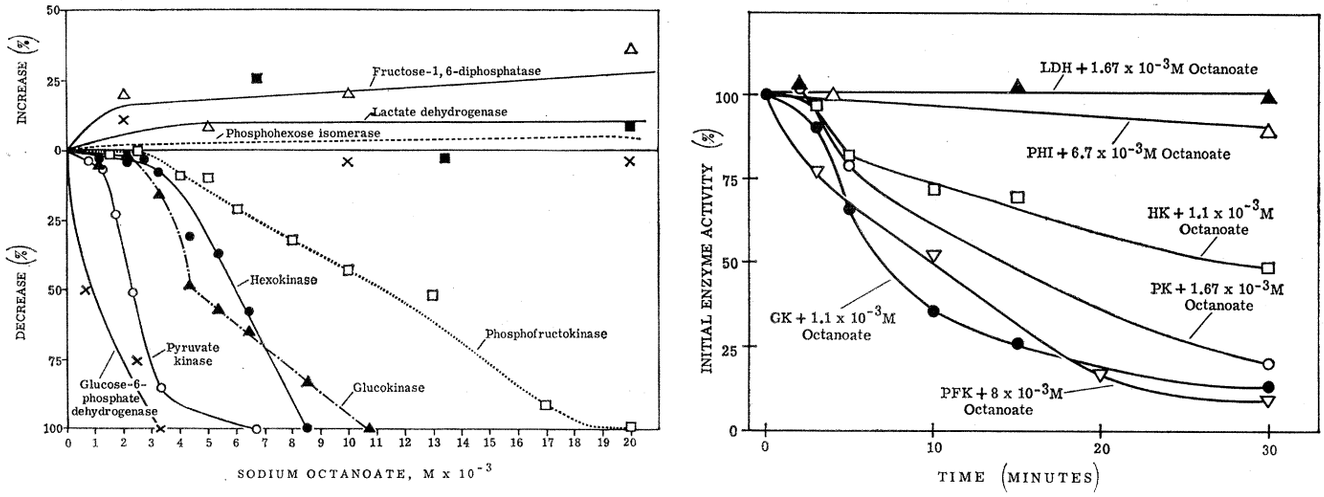

Fig. 1 (left). Dose-response effects of octanoate on hepatic enzyme activity. Various concentrations of sodium octanoate were incubated with liver supernatant at 37°C. Portions of this incubation mixture were tested for enzyme activities by our routine assays. In the case of glucokinase and hexokinase this prior incubation time was 3 minutes, and for all other enzymes it was 5 minutes. The control, indicated by the zero line, represents the activity in the supernatant, with distilled water used in the initial incubation period under identical conditions. Fig. 2 (right). Octanoate inhibition of liver enzymes: effect of the duration of the initial incubation period (abscissa). The supernatant from a liver homogenate was incubated with octanoate at 37°C. At subsequent intervals portions of this incubation mixture were taken and assayed for enzyme activities. *LDH*, lactate dehydrogenase; *PHI*, phosphohexose isomerase; *HK*, hexokinase; *GK*, glucokinase; *PK*, pyruvate kinase; *PFK*, phophofructokinase.

key gluconeogenic enzymes (*1*, *5*). However, such alterations in enzyme biosynthesis do not account for the rapid, acute changes in the direction of overall metabolic pathways in liver.

When considering the ratios of the key hepatic enzymes opposing each other in gluconeogenesis and glycolysis, we observed that the ratio of activity for glucose-6-phosphatase to that of glucokinase is 4.9, and the ratio for the activity of fructose-1,6-diphosphatase to that of phosphofructokinase is 2.6. However, the ratio of the sum of phosphoenolpyruvate carboxykinase plus pyruvate carboxylase to pyruvate kinase is 0.04 (*1*, *2a*). The significance of pyruvate kinase activity in opposing gluconeogenesis can be appreciated when the activity of this enzyme (5000 μmole/g per hour at 37°C) is contrasted with the activities of the two opposing gluconeogenic enzymes (about 20 μmole/g per hour). In view of this unfavorable ratio at the early step of gluconeogenesis, it appears necessary that a mechanism should operate in overcoming the pyruvate kinase activity barrier. In gluconeogenic states, such as diabetes or starvation, the activities of phosphoenolpyruvate carboxykinase and pyruvate carboxylase increase severalfold (*6*) and that of pyruvate kinase decreases (*1*, *3*, *4*, *7*). However, pyruvate kinase activity is still overwhelmingly more active than the sum of the opposing gluconeogenic enzymes. Accordingly, we assumed mechanisms must exist by which pyruvate kinase may be acutely inhibited, which would prevent recycling and permit the operation of the gluconeogenic pathway.

In a search for a physiological substance which could switch off pyruvate kinase activity and thus facilitate gluconeogenesis, our attention centered on recent reports that fatty acids promoted gluconeogenesis. Krebs *et al.* showed in kidney slices that addition of free fatty acids to the medium increased gluconeogenesis from lactate (*8*), and Haynes found that octanoate (3 m*M*) enhanced glucose production from alanine in liver slices (*9*). Herrera *et al.* reported that, in perfused preparations of rat liver, there was an increased conversion of alanine to glucose when linoleate was infused at a rate of 300 μmole/hr (*10*); in similar preparations Struck *et al.* observed that oleate markedly increased glucose formation from lactate (*11*). Williamson *et al.* demonstrated that addition of oleate to perfusate resulted in a rapid rise in the rate of glucose production from alanine, and it is relevant that they also observed a concomitant decrease in lactate and pyruvate production (*12*). Furthermore, Weinhouse and co-workers demonstrated that the injection of octanoate in rat resulted in a fivefold increase in gluconeogenesis in 1 hour (*13*).

From such demonstrations of the ability of fatty acids to produce a rapid increase in the rate of gluconeogenesis, we assumed that an acute adaptation took place involving an inhibition of activities of key enzymes of glycolysis. For the fatty acids to operate as an effective "metabolic directional switch" it was postulated that pyruvate kinase should be strongly inhibited, because without accomplishing this gluconeogenesis cannot effectively proceed. As an extension of the functional genic-unit concept (*1*), it was assumed that the key glycolytic enzymes might all have the same built-in regulatory site in their respective structures; thus, the activities of these enzymes might be inhibited simultaneously by the same regulatory molecule. Furthermore, since gluconeogenesis can operate only in the presence of active bifunctional (*1*) and gluconeogenic enzymes, it was postulated that such enzymes should not be inhibited by fatty acids. The experimental results were in accord with these predictions, and the data indicated that fatty acids can act as feedback inhibitors of the key hepatic glycolytic enzymes and thus facilitate the operation of gluconeogenesis.

Young male Wistar rats (100 to 200 g) were used. Liver homogenates were prepared in isotonic KCl, and the supernatant was obtained by centrifuging the homogenate for 30 minutes at 100,000*g* at 0°C. Glucokinase and hexokinase (*1*), phosphofructokinase

(*3*), pyruvate kinase (*4*), glucose-6-phosphate and 6-phosphogluconate dehydrogenases, phosphohexose, isomerase, lactate dehydrogenase, fructose-1,-6-diphosphatase, and glucose-6-phosphatase (*1*) were assayed as cited (*14*). The sodium octanoate was dissolved in distilled water and different concentrations were first incubated with the supernatant at 37°C. At the end of this prior incubation period, the mixture of supernatant and octanoate was added to the enzyme-assay reaction mixture, and the activity was determined (Fig. 1).

The activities of pyruvate kinase, glucokinase, hexokinase, and phosphofructokinase were inhibited (Fig. 1). Under these conditions, K_i (inhibition constant) for these enzymes were 2.5, 4.5, 5.8, and 13.0 $\times$ $10^{-3}M$. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were also strongly inhibited (K_i, 1.0 and 4.4 $\times$ $10^{-3}M$), but the curve for the latter is not shown. In contrast, the bifunctional enzymes, phosphohexose isomerase and lactate dehydrogenase, and the gluconeogenic enzyme, fructose-1,6-diphosphatase, were not affected.

The effect of different periods of the prior incubation is shown in Fig. 2. With longer periods, 1.67 $\times$ $10^{-3}M$ octanoate decreased pyruvate kinase activity to 17 percent of normal. The other key enzymes of glycolysis were also markedly inhibited with concentrations considerably lower than those used in short incubation periods. Lactate dehydrogenase, phosphohexose isomerase, and fructose-1,6-diphosphatase activities were not affected.

The experimental results presented indicate that the rapid action of fatty acids in promoting gluconeogenesis may be explained, in part at least, by their inhibitory effect on the key enzymes of glycolysis which antagonize the key enzymes of gluconeogenesis. The amount of fatty acid effective in this respect is of the order of magnitude of those amounts effective in slice and perfusion experiments (*8–12*), and the normal amounts occurring in the rat (*15*). Under conditions of gluconeogenesis, such as those induced by diabetes, starvation, or steroid treatment, the high rates of gluconeogenesis are associated also with large amounts of free fatty acid released by lipolytic hormones into the plasma. In consequence, fatty acids may function physiologically in acute adaptation as a "metabolic directional switch," acting

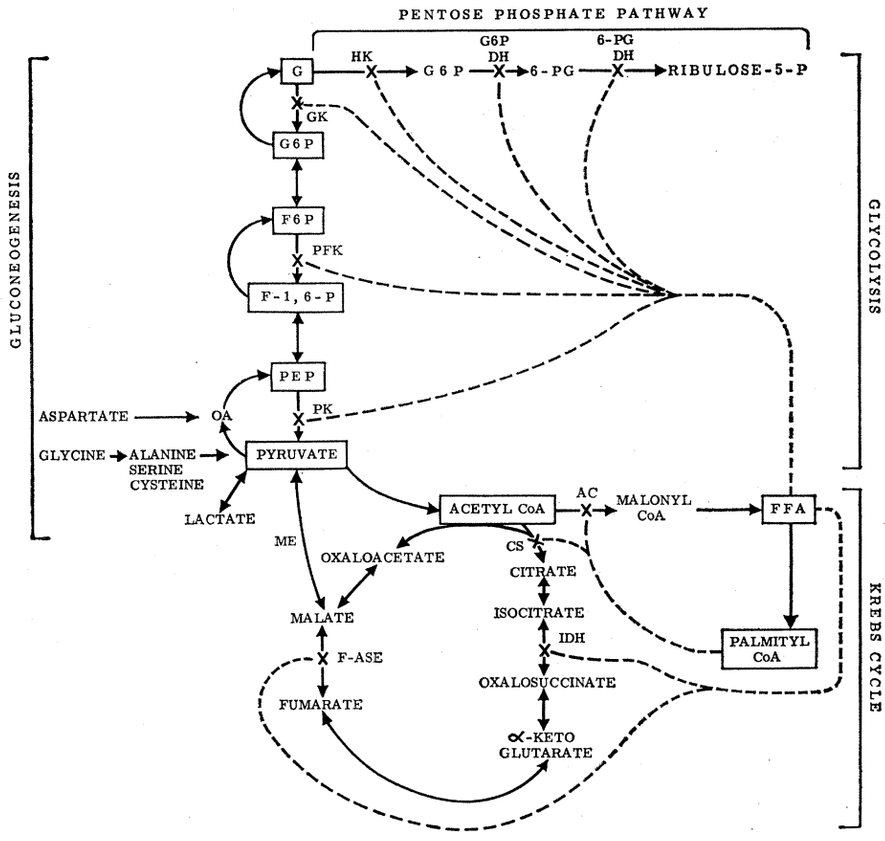

Fig. 3. Feedback inhibition of glucose-catabolizing enzymes in liver by fatty acids. *GK*, glucokinase; *PFK*, phosphofructokinase; *PK*, pyruvate kinase; *HK*, hexokinase; *G6P DH*, glucose 6-phosphate dehydrogenase; *6-PG DH*, 6-phosphogluconate dehydrogenase; *IDH*, isocitrate dehydrogenase; *F-ASE*, fumarase; *ME*, malic enzyme; *AC*, acetyl CoA carboxylase; *CS*, citrate synthase. The following enzymes are not inhibited: glucose 6-phosphatase, fructose-1,6-diphosphatase, phosphohexose isomerase, lactate dehydrogenase, the malic enzyme and malic dehydrogenase. The free fatty acids may be generated in the liver or may arrive from the periphery in gluconeogenic conditions, when insulin drops or when there is a preponderance of the lipolytic hormones, such as glucocorticoids, growth hormone, or glucagon.

through possible allosteric inhibitory mechanisms (*16, 17*) at the key glycolytic enzyme levels. This interpretation implies that octanoate and other fatty acids should be able to cut down the flow of glycolysis in liver and, in fact, the work of Williamson provides excellent evidence to this effect (*12*).

Fatty acids may be considered an end product of glucose metabolism. With respect to the metabolic flow of carbon to fatty acids, our data suggest that octanoate may function as a feedback inhibitor acting (i) on the key enzymes at the beginning of the reaction sequence (glucokinase, hexokinase) (ii) on phosphofructokinase, and (iii) especially on pyruvate kinase. The inhibition of the pyruvate kinase is probably most pertinent to the reversal of glycolysis, because it is the first step to be overcome in the operation of gluconeogenesis. A feedback inhibition is also exerted on the initial and rate-limiting enzyme of the direct oxidative pathway (glucose-6-phosphate dehydrogenase) and on the subsequent enzyme (6-phosphogluconate dehydrogenase) which are involved in the generation of TPNH (reduced triphosphopyridine nucleotide) utilized in the reductive biosynthesis of fatty acids. Through such a two-pronged feedback action on both the glycolytic and direct oxidative pathways, fatty acids could synchronously inhibit their own biosynthesis. This interpretation is in accord with the observations of others who showed that long-chain acyl-coenzyme A esters, which increase in liver during gluconeogenic conditions, inhibited enzymes that operate at later stages of lipogenesis, such as acetyl-CoA carboxylase, fatty acid synthetase, citrate synthase (*18*), and also the enzymes of the pentose phosphate pathway, glu-

cose-6-phosphate and 6-phosphogluconate dehydrogenases (*19, 20*). The enzyme selectivity shown by octanoate in our study is in line with the lack of inhibition described for lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-1,6-diphosphatase by palmityl-CoA (*20*). In addition, our studies showed that another gluconeogenic enzyme, glucose-6-phosphatase, was also not affected by octanoate under the conditions used in this study.

Further work also has demonstrated that the inhibition of glucokinase by octanoate can be prevented by glucose, and the effect was dependent on the glucose concentration (*2a*). However, phosphofructokinase is not protected by glucose, but is protected by its own substrate, fructose-6-phosphate. Glucose is not protective for pyruvate kinase, and this enzyme is not protected by its substrate, phosphoenolpyruvate. In a fortified system of supernatant from a rat-liver homogenate there was a dose-dependent inhibition by octanoate of the conversion of glucose to lactate. This confirms the findings that the key enzymes of glycolysis, glucokinase, hexokinase, phosphofructokinase, and pyruvate kinase, were inhibited by this fatty acid, and it indicates that these enzyme inhibitions were operative in decreasing overall glycolysis in vitro. The specificity of fatty acid action was further supported by our investigation of enzymes of the Krebs cycle. These data show that octanoate inhibited isocitrate dehydrogenase and fumarase from rat liver, whereas under the same conditions the activities of the malic enzyme and malic dehydrogenase (assayed from malate to oxaloacetate and also from oxaloacetate to malate) were not affected (Fig. 3). Our findings provide an explanation for the observation that fatty acids result in a decreased functioning of the Krebs cycle (*10*).

It is noteworthy and it is much in line with our proposed concept that the malic enzyme which provides the gluconeogenic pyruvate from the Krebs cycle intermediate malate is not inhibited by the fatty acid. Since it was shown that a number of free fatty acids were capable of inhibiting glucokinase and hexokinase (*21*) and blocking lipogenesis in a cell-free supernatant system (*22*) prepared from a rat-liver homogenate, it appears that fatty acids may function physiologically as feedback inhibitors of glycolysis, the direct oxidative pathway, and lipogenesis and may be capable of decreasing the activity of the Krebs cycle.

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Photoperiodism in Lemna: Reversal of Night-Interruption Depends on Color of the Main Photoperiod

Abstract. *With main photoperiods of red or white light, the inhibition of flowering in* Lemna perpusilla *6746 caused by interruptions of the night with red light cannot be reversed by far-red light, since far-red light itself is highly inhibitory. However, with a main photoperiod of blue light, far-red light is much less inhibitory and partially reverses the effect of red night-interruptions. If the main blue photoperiod is terminated by a brief red exposure, reversibility is abolished, as the far-red light is again fully inhibitory. This latter effect can be reversed by far-red light. These results add light quality to the already known characteristics of the main light period which affect reversibility in the dark period, and are consistent with the idea that the effects of blue light on photoperiodism in* L. perpusilla *are mediated exclusively by phytochrome.*

Demonstrations of the role of phytochrome in the photoperiodic control of flowering depend on the fact that effects caused by interruptions of the dark period with red light can be reversed by subsequent far-red illumination (for example, *1*). Exceptions to such reversibility, however, have been found in two photoperiodic plants, *Lemna perpusilla* 6746, a duckweed, and the Japanese morning glory, *Pharbitis nil* (*2, 3*). Partial reversibility has been obtained in *Pharbitis* by the use of very short intervals between the treatment with red light and the treatment with far-red (*4*), but this technique is hardly applicable to the *Lemna* system, in which the failure of reversal is due to the inhibitory action of far-red as a night-interruption (*3*).

A new approach to this problem was suggested by the fact that, though *L. perpusilla* 6746 is relatively indifferent to the day length under light schedules consisting of blue or far-red light alone (5), a brief red night-interruption in conjunction with a blue main