rum prepared in only one species is used. With the homologous antiserum only one line was revealed; this line was absent when the virus was tested with normal Balb/c serum, or homologous antiserums to normal spleen plus adjuvant or to adjuvant alone. Using the more potent monkey and guinea-pig antiserums, we detected more than one line of precipitation in virus from either the plasma or tissue culture. The monkey antiserum was capable of revealing at least two similar antigens in virus from fresh, unconcentrated plasma or spleen extract of infected mice.

A cross reaction with one antigen of the Rauscher virus was observed when Friend and Moloney leukemia viruses were tested with monkey antiserum to Rauscher virus. It does not necessarily follow that these antigens are identical, since tests with antiserums to Friend and Moloney viruses have not been made; but there is a suggestion of similarity of one Rauscher antigen to an antigen occurring in the other strains. It is odd, however, that, in six attempts, a cross-reaction between the Moloney and Rauscher antigens could not be demonstrated with guinea-pig antiserum which had a neutralizing antibody titer against Rauscher virus as high as that in the monkey, and which was capable of revealing as many or more antigens in the Rauscher plasma virus. Old (*12*) has postulated that the leukemic cells derived from mice infected with Friend, Moloney, or Rauscher viruses contain a common antigen. We have discussed certain possible antigenic relationships among these murine leukemia viruses, and our inability to show a cross reaction between the Moloney and Rauscher viruses except by immunofluorescence (*13*). We have interpreted these reactions as indicating antigen of viral rather than of cellular origin (*13*). Our findings reported herein, that ten-times-concentrated plasma preparations from leukemic mice infected with Moloney, Breyere-Moloney, Upton, or C-60 (Manaker) virus were negative—each of which preparations would be expected to contain an amount of cellular debris comparable to that present in any other similar leukemic plasma—strengthens the evidence that the reaction being depicted is truly one against a viral antigen, rather than against a nonviral antigen, of the leukemic cell.

The microscale Ouchterlony test, as described, is useful for virus detection and holds potential for elucidating further the antigenic structure of the murine leukemia viruses.

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31 August 1965

Acidosis: Effect on Lipolytic Activity of Norepinephrine in Isolated Fat Cells

Abstract: *Lowering of* p*H from 7.40 to 6.60 significantly decreased the rate of glycerol formation in fat cells incubated with either* l-*norepinephrine or theophylline. When these cells were incubated with both* l-*norepinephrine and theophylline, the glycerol formation proceeded at maximal rate and was quite similar at* p*H 7.40 and 6.60. These results indicate that the inhibitory effect of acidosis on the lipolytic action of* l-*norepinephrine is exerted on the process which activates lipase.*

Experiments in vivo and in vitro have indicated that the increases in free fatty acid (FFA) and glycerol concentrations, which follow epinephrine or norepinephrine administration, are significantly inhibited by acidosis (*1*). We now report on the mechanism of the inhibitory effect of acidosis on norepinephrine-induced lipolysis in vitro. Isolated fat cells were used for the study because the lipolytic activity in cell-free homogenates indicates a potential ability to release FFA (*2*) rather than the physiological level at which the lipolytic process is functioning (*3*). According to recent findings (*4*) the lipolytic process in the fat cell depends apparently upon the activity of at least three enzymes: adenyl cyclase, which forms cyclic 3′, 5′-adenylic acid (3′, 5′-AMP) (*5*) and is stimulated by catecholamines (*6*); phosphodiesterase, which inactivates cyclic 3′, 5′-AMP (*7*) and is inhibited by theophylline (*8*); and lipase, the activity of which depends upon the actual amount of cyclic 3′, 5′-AMP (*9*) (Fig. 1). The purpose of the present experiments was to determine whether it is lipase, or the process activating lipase, which is inhibited by acidosis. The rate of glycerol production was taken as an index of lipase activity.

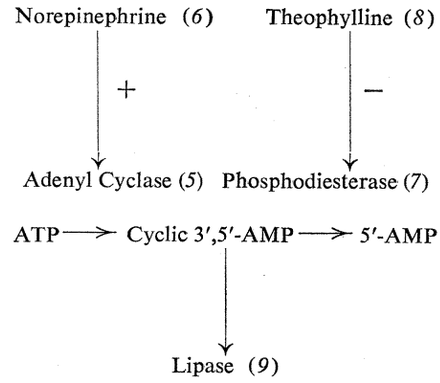

Fig. 1. Mechanism of lipase activation (this diagram does not include possible intermediate steps).

Epididymal fat pads were taken from male Sherman rats that had been permitted free access to food until they were decapitated. Isolated fat cells, prepared as described by Rodbell (*10*), were incubated at 37°C in a Krebs-Ringer phosphate buffer containing 5 percent bovine albumin, fraction V. The *p*H of this medium was adjusted either to *p*H 6.60 or to *p*H 7.40 (*11*), and the ratio of cells to medium was 1 ml of packed cells to 20 ml of medium. Suspensions of corresponding fat cells were incubated separately for each interval of time. Glycerol concentration (*12*) in the media was determined at the end of the incubation period (*13*).

In the first experiments the fat cells

were incubated for 30, 45, 60, and 75 minutes in media at *p*H 7.40 and *p*H 6.60 containing either *l*-norepinephrine ($1 \times 10^{-5} M$) or theophylline ($1 \times 10^{-2} M$) (*14*). The release of glycerol

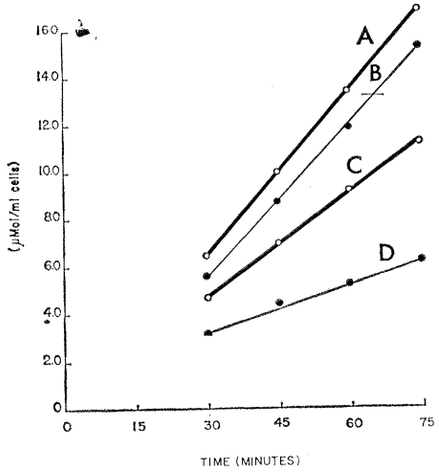

Fig. 2. Glycerol formation (μmole/ml cells plotted against time in minutes) in fat cells from rat epididymal pad incubated with: (*A*) theophylline, 1×10^{-2} mole at *p*H 7.4 (equation of the line is $y = -0.405 + 2.302\ x$); (*B*) *l*-norepinephrine, 1×10^{-5} mole at *p*H 7.4 ($y = -0.941 + 2.164\ x$); (*C*) theophylline, 1×10^{-2} mole at *p*H 6.6 ($y = 0.388 + 1.615\ x$); (*D*) *l*-norepinephrine, 1×10^{-5} mole at *p*H 6.6 ($y = 1.345 + 0.658\ x$). Each point represents the mean of five determinations.

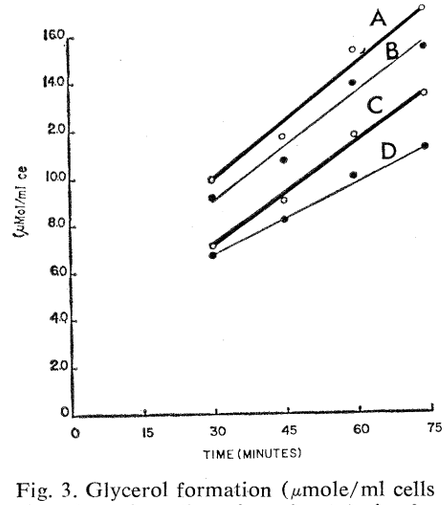

Fig. 3. Glycerol formation (μmole/ml cells plotted against time in minutes) in fat cells from rat epididymal pad incubated with: (*A*) theophylline, 1×10^{-2} mole, and *l*-norepinephrine, 1×10^{-5} mole, at *p*H 7.4 (equation of the line is $y = 4.62 + 1.682\ x$); (*B*) theophylline, 1×10^{-2} at *p*H 7.4 ($y = 4.54 + 1.479\ x$); (*C*) theophylline, 1×10^{-2} mole, and *l*-norepinephrine, 1×10^{-5} mole, at *p*H 6.6 ($y = 2.83 + 1.427\ x$); (*D*) theophylline, 1×10^{-2} mole, at *p*H 6.6 ($y = 3.67 + 1.016\ x$). Each point represents the mean of five determinations.

occurred at a quite constant rate (Fig. 2), and the slope of the regression lines calculated from the rate of glycerol production thus reflect the rate of lipolysis in the incubated fat cells. During incubation with *l*-norepinephrine, this rate should be dependent upon the activity of adenyl cyclase, phosphodiesterase, and lipase, whereas during incubation with theophylline the rate should depend upon the activity of adenyl cyclase and lipase since theophylline is a potent inhibitor of phosphodiesterase. The rate of lipolysis in both cases was lower at *p*H 6.60 than at *p*H 7.40. In other words, acidosis decreased the rate of lipolysis even when phosphodiesterase was inhibited. We may, therefore, assume that under these conditions phosphodiesterase is not responsible for the decreased rate of lipolysis at lower *p*H. The regression lines at *p*H 6.60 are not parallel, apparently because there was a different degree of lipase activation with theophylline or with *l*-norepinephrine present in the medium. However, at *p*H 7.40 the slope of the regression lines is parallel, which may indicate that at *p*H 7.40 complete activation of lipase was achieved in both cases.

In order to test this assumption, the dynamics of glycerol production by fat cells were followed in media at *p*H 7.40 containing theophylline, with or without the addition of *l*-norepinephrine at the concentrations indicated (Fig. 3). In both instances when phosphodiesterase was inhibited, whether or not adenyl cyclase was stimulated, the lipolytic activity was the same (the regression lines of glycerol production are parallel). It could be assumed that in both cases a sufficient amount of 3′, 5′-AMP was available to completely activate lipase, and in this case the rate of lipolysis was no longer dependent upon the actual activity of adenyl cyclase.

To distinguish whether lipase or the process which activates lipase (adenyl cyclase) is, at lower *p*H, the rate-limiting step of lipolysis stimulated by catecholamines, the ability of completely activated lipase to hydrolyze triglycerides was determined in media at *p*H 6.60 containing *l*-norepinephrine and theophylline. In this preparation, with stimulated adenyl cyclase and inhibited phosphodiesterase, the same amount of glycerol per milliliter of cells in the same time interval was produced at *p*H 6.60 as at *p*H 7.40. The regression lines have the same slope, indicating that in both cases lipase was completely activated. By contrast, at *p*H 6.60 complete activation of lipase was not achieved by inhibiting phosphodiesterase only, as it was at *p*H 7.40. Both stimulation of adenyl cyclase and inhibition of phosphodiesterase were necessary for complete activation of lipase at *p*H 6.60.

These results indicate that lipase, if completely activated, has the same lipolytic activity at *p*H 6.60 and *p*H 7.40 and, in this sense, is not responsible for the decreased lipid-mobilizing effect of catecholamines in intact fat cells at lower *p*H. However, the process, that is, the formation of cyclic 3′, 5′-AMP by adenyl cyclase (*15*) which activates lipase is *p*H-dependent. It is this formation which would be inhibited by acidosis and could account for the decrease in the mobilization of lipids by catecholamines at low *p*H.

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21 September 1965

Transhydrogenation in Root Tissue: Mediation by Carbon Dioxide

Abstract. *The $C^{14}O_2$ incorporated into organic acids by excised corn roots is released with an estimated half-time of 3 hours. Homogenates of root tissue can mediate these reactions by coupling, in series, phosphoenolpyruvate carboxylase, diphosphopyridine nucleotide malic dehydrogenase, and triphosphopyridine nucleotide malic dehydrogenase. An important consequence is the transfer of hydrogen from reduced diphosphopyridine nucleotide to triphosphopyridine nucleotide which is then reduced.*

During investigations concerning nonautotrophic CO_2 metabolism in succulent plants, we observed that the subsequent release of the incorporated CO_2 is relatively rapid (*1*). This finding, as well as our observations regarding the rapid loss of labeled malic acid after incubation in $C^{14}O_2$ (*2*), led us to postulate a CO_2 cycle which generates the reduced form of triphosphopyridine nucleotide (TPNH) by transhydrogenation from reduced diphosphopyridine nucleotide (DPNH) to TPN. The initial fixation of the CO_2 is by way of phosphoenolpyruvate (PEP) carboxylase, an essentially irreversible reaction (*3*), with formation of oxaloacetate. Nucleotide cofactors are not required, and the reaction generates inorganic phosphate (*3*). The oxaloacetate is immediately reduced to malate by DPN malic dehydrogenase (*4*) with the generation of DPN. Since the products of CO_2 fixation do not take part in the citric acid cycle (*5*) and the fixation reaction is not reversible, steady-state release in vivo is quite likely by way of TPN malic dehydrogenase. Under physiological conditions of malate concentration, the equilibrium of the TPN malic dehydrogenase is in favor of decarboxylation (*6*). We now present evidence that the capacity for such a cycle is present in corn roots and, in fact, that such a cycle probably is operative. The hypothesis is not entirely without precedent since Young *et al.* (*7*) reported the coupling of DPN malic dehydrogenase with TPN malic dehydrogenase in mammalian adipose and liver tissue and stressed its importance in lipogenesis. The proposed CO_2 cycle as envisioned for corn root tissue is shown in Fig. 1.

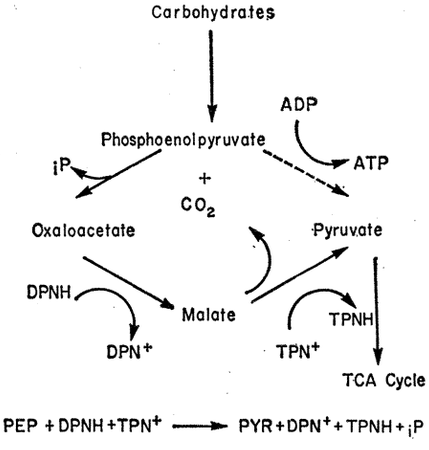

Fig. 1. The proposed cycle of the utilization of CO_2 with the net transfer of hydrogen from DPNH to TPN to form TPNH. The dashed arrow indicates the direct pathway for the utilization of PEP. TCA, tricarboxylic acid; iP, inorganic phosphorus.

About 0.3 g (from the first 2 cm) of 3-day-old corn roots (Burpee Golden Bantam) was incubated in 50 μc of $HC^{14}O_3^-$ (2.2 μc/μmole) in tris buffer (0.05*M*, *p*H 7.4) for 2 hours. After the incubation, the roots were quickly washed with distilled water and transferred to a 100-ml flask containing nonradioactive buffer. The flask was attached to a closed circulating system which was monitored with a 250-ml Cary-Tolbert ion chamber and Cary Model 31 vibrating-reed electrometer. Therefore continuous recording of the released $C^{14}O_2$ was obtained. Both the first and second incubations were conducted at 30°C. In agreement with our findings with *Opuntia* roots (*1*) and stem tissue (*2*), the release was biphasic and described adequately by two first-order equations (Fig. 2). The lower curve (phase 1) of Fig. 2 was obtained by extrapolating the linear portion of the upper curve (phase 2) to the vertical axis and subtracting the estimated values from the actual datum points. The first-order rate constant for phase 1 was 89.2 × 10^{-3} min^{-1} with a half-

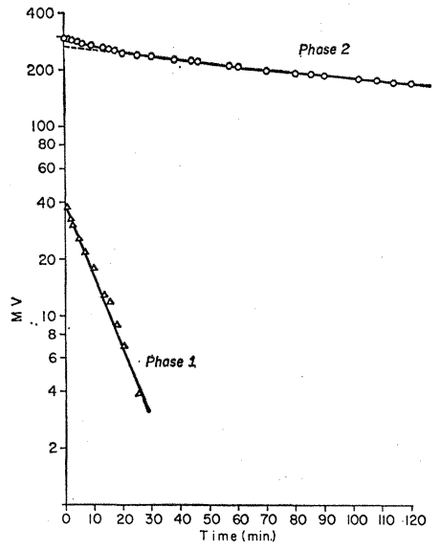

Fig. 2. Release of $C^{14}O_2$ by corn root tips after a 2-hour fixation period. Phase 2 represents steady state decarboxylation of the $C^{14}O_2$ fixation metabolites. MV, millivolts; 3300 dpm are equivalent to 1 mv.

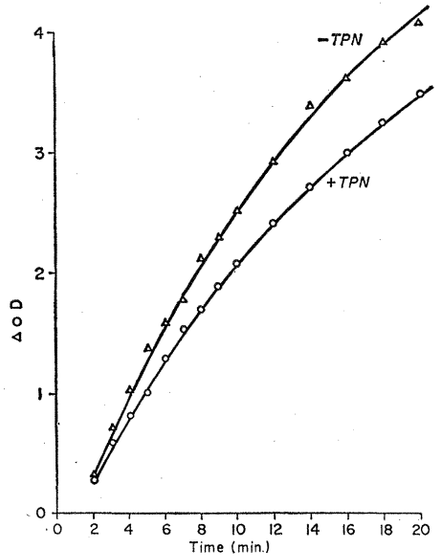

Fig. 3. Change in optical density (O.D.) per gram of tissue (fresh weight) for complete coupling reaction mixture (2 μmole PEP, 10 μmole $NaHCO_3$, 10 μmole $MgCl_2$, 0.256 μmole DPNH, 0.4 ml of enzyme preparation; with and without 0.37 μmole TPN). It was assumed that the slower apparent rate of DPNH oxidation in the presence of TPN was due to reduction of TPN. The reduction of TPN was due to the TPN-mediated enzymic decarboxylation of the initial product, malic acid, formed by PEP carboxylase coupled with DPN malic dehydrogenase.