trol DNA and that exposed to mitomycin. As discussed earlier (*1*), such scissions would free DNA fragments from their association with covalent cross-links.

V. N. IYER
Microbiology Research Institute, Canada Department of Agriculture, Ottawa

WACLAW SZYBALSKI
McArdle Memorial Laboratory, University of Wisconsin, Madison

References and Notes

1. V. N. Iyer and W. Szybalski, *Proc. Natl. Acad. Sci. U.S.* **50**, 355 (1963).
2. Abbreviations: MC, mitomycin C; TPN, DPN, TPNH, and DPNH, tri- and diphosphopyridine nucleotides in oxidized and in reduced forms; SSC, standard saline citrate (0.15*M* NaCl + 0.02*M* trisodium citrate; *p*H 7.7); DSC, SSC diluted 1:10; DNA, deoxyribonucleic acid; G, guanine; C, cytosine.
3. From Nutritional Biochemicals Corp., Cleveland 28, Ohio.
4. Kindly supplied by S. Fleischer and H. C. Pitot of the University of Wisconsin.
5. C. Martius, in *The Enzymes*, P. D. Boyer, H. Lardy, K. Myrbäck, Eds. (Academic Press, New York, 1963), vol. 7, p. 517.
6. H. S. Schwartz and F. S. Philips, *J. Pharmacol. Exptl. Therap.* **133**, 335 (1961); H. S. Schwartz, *ibid.* **136**, 250 (1962); H. S. Schwartz, J. E. Sodergren, F. S. Philips, *Science* **142**, 1181 (1963).
7. E. R. Garrett, *J. Med. Chem.* **6**, 488 (1963); J. S. Webb, D. B. Cosulich, J. H. Mowat, J. B. Patrick, R. W. Broschard, W. E. Meyer, R. P. Williams, C. F. Wolf, W. Fulmor, C. Pidacks, J. E. Lancaster, *J. Am. Chem. Soc.* **84**, 3185, 3186 (1962).
8. J. Epstein, R. W. Rosenthal, R. J. Ess, *Anal. Chem.* **27**, 1435 (1955).
9. This was first suggested to us by J. D. Scribner of the McArdle Memorial Laboratory.
10. The possibility of this reaction was first suggested to us by J. A. Miller of the McArdle Memorial Laboratory.
11. E. J. Conway, *Microdiffusion Analysis and Volumetric Error* (Lockwood, London, 1957), p. 246.
12. C. C. J. Culvenor, A. T. Dann, A. T. Dick, *Nature* **195**, 570 (1962). We are greatly indebted to J. A. Miller for pointing out this publication and the possibility of alkylating action by the substituted allylic esters.
13. K. V. Rao, K. Biemann, R. B. Woodward, *J. Am. Chem. Soc.* **85**, 2532 (1963).
14. Streptonigrin alone (1 to 100 μg/ml) or in the presence of a reducing agent (100 μg of $Na_2S_2O_4$ per milliliter) does not inactivate extracellular T1 phage or transforming DNA extracted from *B. subtilis* cells. Residual binding of tritiated streptonigrin to DNA is not increased by the presence of 100 μg of $NaBH_4$ per milliliter (Iyer and Szybalski, unpublished data).
15. O. M. Friedman, G. N. Mahapatra, R. Stevenson, *Biochim. Biophys. Acta* **68**, 144 (1963).
16. P. D. Lawley and P. Brookes, *Biochem. J.* **89**, 127 (1963); ———, *Exptl. Cell Res., Suppl.* **9**, 512 (1963).
17. J. Marmur and P. O. P. T'so, *Biochim. Biophys. Acta* **51**, 32 (1961).
18. Pancreatic ribonuclease, Worthington Co., heated to 95°C for 10 minutes.
19. Contribution No. 569 from the Microbiology Research Institute, Canada Department of Agriculture, Ottawa. Supported in part by USPHS grant CA-07175 and by NSF grant G-18165. We thank H. H. Muxfeldt, J. A. Miller, C. Heidelberger, and J. D. Scribner for helpful discussion; and W. Schroeder (Upjohn Co.), who supplied us with porfiromycin and the triethylamine salt of 7-hydroxyporfiromycin (U-12, 270E, lot No. 4702-WS-136.1); A. Weissbach (NIH) who provided a sample of mitomycin B; S. Wakaki (Kyowa Hakko Kogyo Co., Tokyo) who supplied us with mitomycins A, B, and C and others; and Mrs. V. G. Arneson, L. Fenton, and Miss S. Becker for technical assistance.

26 February 1964

Pyruvate Metabolism and Control: Factors Affecting Pyruvic Carboxylase Activity

Abstract. *Pyruvic carboxylase activated by acetyl coenzyme A is highly active in the mitochondria of rodent liver, and its activity is increased in fasting and alloxan diabetes. In conjunction with acyl carboxylase activated by di- and tricarboxylic acid, it forms a reciprocating control network. Analog models of similar networks tend to correct for perturbations, stabilizing the overall system.*

Utter and Keech have demonstrated pyruvic carboxylase (*1*) in avian and beef liver. This enzyme, activated by acetyl coenzyme A, carboxylates pyruvate to oxalacetate without utilization of the cofactor. Pyruvic carboxylase, therefore, provides a link between fat and carbohydrate metabolism in a manner similar to the activation of acetyl coenzyme A carboxylase by members of the tricarboxylic acid cycle (*2*).

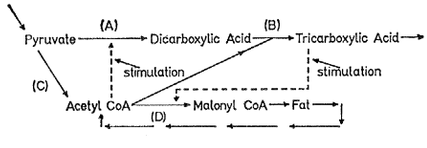


Fig. 1. Proposed control network having crossed over feedback loops.

Since this enzyme is a significant means of formation of dicarboxylic acid in the rat liver, we are reporting the results of food withdrawal and diabetes on its activity.

Several preparations were utilized in the data presented here. Powdered whole liver was prepared by homogenization of mouse or rat liver in 10 times its volume of cold acetone. Each homogenate was dried and, prior to use, was suspended in 50 times its weight of tris buffer. Fresh mitochondria were prepared by homogenization in 0.25*M* sucrose with a loose-fitting Teflon pestle. The debris was removed by centrifugation at 850*g* for 10 minutes; the mitochondria were obtained by centrifugation at 7000*g* for 10 minutes. Just before use they were suspended in an amount of tris buffer (*p*H 7.4) equivalent to the volume of liver from which the mitochondria were obtained. Powdered mitochondria were prepared by suspending the isolated mitochondria in 20 volumes of acetone, drying the precipitate, and grinding the dry powder in a volume of tris buffer (*p*H 7.4) equivalent to the volume of liver from which it was obtained. Reaction mixtures, made as indicated in Table 1, were incubated for 5 minutes at 30°C. The reactions were stopped by freezing the mixture in a bath cooled with solid carbon dioxide and alcohol. The protein was denatured by acid, the precipitate was discarded, and the supernatant was gassed vigorously with carbon dioxide gas. Radioactivity measurements were made on a Packard Tri-Carb scintillation counter.

Table 1. Comparison of enzyme preparations. One milliliter of incubation mixture contained protein obtained from 125 mg of fresh liver; 200 μmole of tris (*p*H 7.4); 2.5 μmole of adenosine triphosphate; 1.4 μmole of acetyl coenzyme A; 10 μmole of pyruvate; 9 μmole of Mg; 10 μmole of $NaHC^{14}O_3$. The mixture was incubated for 5 minutes at 30°C.

Preparation	$C^{14}O_2$ fixed * (mμmole)		
	Per g of liver	Per mg of liver protein	Per mg of mitochondrial protein
Powdered mitochondria	1013	6.0	69
850g supernatant	963	5.6	96
9000g supernatant	913	5.5	119
Fresh mitochondria	162	0.9	11.6

* In nonvolatile compounds.

In agreement with results (*1*) on avian and beef liver, and within the error of the method, all the pyruvic carboxylase activity is located in the mitochondrial fraction of rat and mouse liver cells, and the enzyme is biotin-dependent and avidin-sensitive.

Table 1 indicates the results from various types of enzyme preparations. A much higher degree of activity is present in the powders of mitochondria than in the equivalent amount of fresh mitochondria, thus a control mechanism exists whether it be selective membrane permeability, competing enzymatic reactions, or the interaction of two or more enzymatic sequences.

In preparations of whole liver powders, gradient elution (*3*) indicated that 80 percent of the radioactivity was in citrate, 10 percent in aspartate, 3 percent present in a form easily decarboxylated by $Al_2(SO_4)_3$ (presumably oxalacetate), and about 1 percent each in malate, glutamate, and α-ketoglutarate.

In order to determine whether acetyl coenzyme A activated the carboxylating enzyme or condensing enzyme, glutamic oxalacetic transaminase (Bohringer), glutamate and pyridoxal phosphate were added. Gradient elution now showed 35 to 40 percent of the radioactivity present as citrate, 50 to 55 percent as aspartate, and 7 to 8 percent was easily decarboxylated. It is apparent that activation occurred prior to condensation.

Investigation was undertaken to assess the response of pyruvic carboxylase to the fasting animals. One member each of several pairs of littermate rats (Sherman strain) was fasted for 24 to 48 hours. Fresh mitochondrial preparations from livers of fasted animals have pyruvic carboxylase activity that is 280 percent that of similar preparations from a fed animal, and powdered mitochondria from fasted animals show an activity that is 170 percent that from livers of fed animals. Since under fasting conditions changes in mitochondrial density per volume would be expected, the data were recalculated on the basis of activity per milligram of mitochondrial protein. The percentage increases were slightly smaller (265 and 165 percent, respectively), but were equally significant.

Sherman rats were made diabetic by alloxan. The diabetic state was manifest by polyuria, polydypsia, a blood sugar of over 300 mg per 100 ml, and glycosuria. Table 3 indicates that pyruvic carboxylase activity in both fresh mitochondrial preparations and powders prepared from mitochondria of diabetic animals was greater by 215 and 160 percent, respectively, than that of preparations from normal animals. This change was reversed by the administration of protamine-zinc insulin.

In rat liver mitochondria, acetyl coenzyme A—a precursor of fat—stimulates the carboxylation of pyruvate—a product of carbohydrate metabolism—thus interrelating these two metabolic sequences. A reciprocal of this stimulation of the activity of one sequence by a member of another, is the stimulation of fat synthesis (*2*) by citrate activation of transcarboxylation (*4*). These two points of activation form part of a complex network which is similar to a theoretical system which Spangler (*5*) studied both analytically and by analog computer model. In the analog computer system the substrate concentration shows a periodic oscillation about a mean value when plotted with respect to time. At some time in the periodic state, the concentration of a substrate (which is represented by the amplitude of the wave) is independent of the initial substrate concentration. Such a system, therefore, tends to compensate for momentary variations of substrate concentration if there is reasonable constancy of reaction rates. In this type of system in a multicellular organism, if phase synchronization does not exist, which is the usual case, oscillations would not be apparent because of averaging effects, and a relatively constant concentration would be expected.

Table 2. The effect of fasting. The incubation mixture as in Table 1. The $C^{14}O_2$ fixed is expressed as millimicromoles per gram of liver. Each experiment was done on rat littermates.

Expt.	Fresh mitochondria		Mitochondrial powder	
	Fed*	Fasted†	Fed	Fasted
I	129	338	1381	2130
II	91	269		
III	71	218		
IV	84	226		
V			1455	2740

* From livers of rats fed as desired on standard laboratory chow. † From livers of rats fasted 24 hours.

In Fig. 1 the control system we propose is represented; Fig. 1 demonstrates that, in a single metabolic state, the interactions are such that network stability would be expected. An arbitrary increase of any intermediary substrate (Fig. 1) would induce responses throughout the network which would tend to restore the pre-existing values of this substrate. Thus an increase in dicarboxylic acid would provide an increase in acceptor for acetyl coenzyme A and an increase in the condensation product of the two. This condensation product, citrate, by stimulating synthesis of malonyl coenzyme A, would decrease the available activator for reaction A, acetyl coenzyme A. Reaction A slows, tending to restore the steady state value of dicarboxylic acid. Similarly, an increase in the available acetyl coenzyme A would result in a chain of events tending to restore the previous values of this substrate. Thus, as in Spangler's model, perturbations would tend to be corrected.

In the model proposed by Spangler and Snell (*6*), the amplitude of oscillation was not a critical function of any single parameter. This is in contrast to the situation in the kinetics of linear sequences where substrate or reactant concentrations are clearly related to the parameters of the rate-limiting step. One would expect that when a tissue goes from one metabolic state to another, if the chemical definition of this state implies a complex network rather than a series of linear sequences, many parameters would be altered. The search for a single critical change may therefore, in fact, be illusory.

Two metabolic states that have a long history of chemical investigation are the fasting state and the diabetic state. We have previously shown that when food is withdrawn from a rat, the proportion of pyruvate entering the tricarboxylic acid cycle by carboxylation to a dicarboxylic acid increases markedly, whereas the percentage decarboxylated to acetyl coenzyme A decreases (*7*).

A reasonable explanation of our previous work can be found in the work reported here. Preparations from livers of fasted rats show a higher pyruvic carboxylase activity than those from normal rats fed on standard mouse pellets. Since it appears that in the rat liver other enzymes suggested for carboxylation of pyruvate are localized primarily in the 100,000*g* supernatant (*8*), pyruvic carboxylase remains the chief and perhaps the only mitochondrial means of forming dicarboxylic acids from pyruvate. Thus the formation of a dicarboxylic acid molecule within the mitochondrion, whether as part of a gluconeogenetic pathway or for maintenance of tricarboxylic acid cycle activity, is a function of pyruvic carboxylase. We suggest then, that among the changes known to occur in the fasting animal there is an increased activity of pyruvic carboxylase. The net effect of

Table 3. Effect of alloxan diabetes and insulin administration. The incubation mixture as in Table 1. The $C^{14}O_2$ fixed expressed as millimicromoles per gram of liver. Each experiment done on littermates.

No. of animals	Fresh mitochondria			Mitochondrial powders		
	Normal*	Diabetic†	Insulin‡	Normal*	Diabetic†	Insulin‡
			Experiment I			
6	129	330	107	1388	2217	1279
			Experiment II			
4	122	221		1420	2325	

* From livers of normal rats. † From livers of littermates made diabetic with alloxan. ‡ From livers of littermates made diabetic with alloxan and given 20 units protamine zinc insulin 24 hours before animals killed.

all parameter changes results in a new locus of stability.

The elevation of pyruvic carboxylase in alloxan diabetes may represent a mechanism whereby the concentration of intermediates of the tricarboxylic acid cycle is maintained despite the production by the liver of large amounts of glucose. It is noteworthy that the effect of the diabetic state on enzyme activity is reversed by the administration of protamine zinc insulin.

Note added in proof. After this manuscript was submitted for publication, a report was published by Wagle (*9*) on the effect of diabetes on pyruvic carboxylase activity which is in agreement with the data on diabetes presented here.

AARON D. FREEDMAN
LEONARD KOHN
Departments of Biochemistry and *Medicine, College of Physicians and Surgeons, Columbia University, New York*

References and Notes

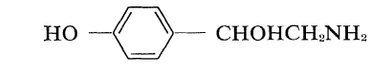
1. M. F. Utter and D. B. Keech, *J. Biol. Chem.* **235**, P.C. 17 (1960); **238**, 2603 (1963); D. B. Keech and M. F. Utter, *ibid.*, p. 2609.
2. D. B. Martin and P. R. Vagelos, *ibid.* **237**, 1787 (1962).
3. L. H. Weinstein, C. A. Porter, H. J. Laurencot, Jr., *Contrib. Boyce Thompson Inst.* **20**, 121 (1959).
4. M. White and S. J. Wakil, *J. Biol. Chem.* **238**, 81 (1963).
5. R. A. Spangler, in *Biomedical Applications of Analog Computation*, G. G. Moser, Ed. (Princeton Computation Center, Princeton, 1963).
6. R. A. Spangler and F. M. Snell, *Nature* **191**, 457 (1961).
7. A. D. Freedman, P. Rumsey, S. Graff, *J. Biol. Chem.* **235**, 1854 (1960).
8. E. Shrago, H. A. Lardy, R. C. Nordlie, D. O. Foster, *ibid.* **238**, 3188 (1963).
9. S. R. Wagle, *Biochem. Biophys. Res. Commun.* **14**, 533 (1964).
10. We thank Samuel Graff for laboratory facilities and advice. Work supported by USPHS grant CA-03141.

6 January 1964

l-Octopamine in Citrus: Isolation and Identification

Abstract. l-*Octopamine*, [l-p-*hydroxy-α-(aminomethyl)benzyl alcohol*], *has been isolated and identified from extracts of juice and leaves of the Meyer lemon. Identification was by chromatography, optical rotation, ultraviolet absorption curves, fluorescence spectra, and infrared spectroscopy.* l-*Octopamine has not previously been isolated and identified from plants.*

While making studies on the identification of *l*-synephrine in citrus (*1, 2*), a brown ninhydrin spot was observed on paper chromatograms of extracts of the juice and leaves of Meyer lemon (*Citrus spp.*) (*3*). After extraction and purification, this spot was identified by infrared analysis and optical rotation as *l*-octopamine, [*l-p*-hydroxy-α-(aminomethyl)benzyl alcohol], also called norsynephrine and *p*-hydroxymandelamine.



l-Octopamine was extracted from Meyer lemon leaves that had been frozen shortly after picking. The extraction procedure was similar to that used for *l*-synephrine (*1*), except that the solvent consisted of a mixture of 80 percent methanol and 20 percent water. After eluting the basic compounds from the ion-exchange column, the mixture was evaporated to a small volume under reduced pressure at 60°C. In some instances, a few milligrams of sodium metabisulfite was added to the extract as an antioxidant before extraction and after passage through the column. The eluate was diluted with methanol and treated directly with oxalic acid as previously described (*1*). A mixture of crystalline oxalates of several nitrogen compounds was thus obtained. Tyramine was identified by infrared analysis and *l*-synephrine by its paper chromatographic R_F values and by its characteristic reddish brown ninhydrin color.

l-Octopamine was separated from the other constituents by chromatography on a column (10 by 80 mm) of ion exchange resin (Dowex 50W-X8, 200–400 mesh in the ammonium form). The resin was prepared as previously described (*1*) except that it was washed until the eluate was neutral. The oxalate mixture was dissolved in water, placed on the column, and eluted with a gradient produced by slowly adding 3*N* NH_4OH to 1 liter of water. Fractions (10 ml) were collected and *l*-octopamine was eluted approximately after 300 ml of eluting solution was added as indicated by ultraviolet absorption at 290 mμ and paper chromatography. Fractions containing *l*-octopamine were taken to dryness and the residue was sublimed in a vacuum at 0.1 mm-Hg and 120°C. Authentic *dl*- or *l*-octopamine purified in this manner was not altered.

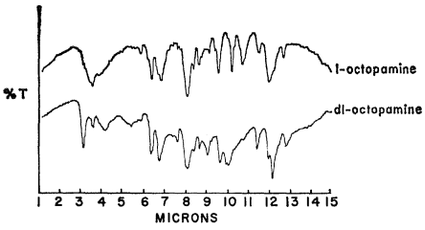


Fig. 1. Infrared absorption curves of *l*-octopamine and *dl*-octopamine (*%T*, percent transmission).

Several methods were used in identifying *l*-octopamine, including the aforementioned chromatographic column system in which the unknown separated into the same fractions as an authentic sample of *dl*-octopamine. Separation was made with paper chromatography with three solvent systems. *l*- and *dl*-Octopamine had an R_F of 0.37 with an EDTA-buffered paper chromatographic method (*4*). When a known sample of octopamine was cochromatographed with the unknown from citrus leaves, the R_F values and color were the same. Winitz (*5*) and others have pointed out that it is not rare for nitrogen compounds of varying structure to possess identical mobilities in a number of different solvent systems. For this reason, methods other than chromatography were also used for identification.

Ultraviolet absorption curves of *dl*-octopamine and the isolated compound were identical. In 0.1*N* HCl, absorption peaks were at 222 and 273 mμ; in phosphate buffer *p*H 12, peaks were at 241 and 290 mμ. Fluorescence spectra of *dl*-octopamine and the isolated compound in 0.1*N* HCl were also identical with the excitation peak at 270 mμ and the emission peak at 310 mμ. However, similarities in absorption and fluorescence characteristics of *l*-octopamine and *l*-synephrine precluded the use of such characteristics in a positive identification of *l*-octopamine. Infrared absorption curves were made of the extracted free base and compared with an authentic sample of *dl*-octopamine. The general shape of the curves was similar but there were distinct differences in the regions of 3, 6.5–7.2, and 9.5–10.5 μ (Fig. 1). Similar differences between the infrared curves of *l*-synephrine and the racemic form were reported previously (*1*). Finally, a synthetic sample of *l*-octopamine was compared with the naturally occurring compound and the infrared absorption curves were identical in all regions. *l*-Octopamine and the compound isolated from citrus decomposed at 167° to 168°C when heated at the rate of approximately 3°C per minute.

The octopamine extracted from citrus leaves was the levoisomer with an optical rotation in 0.5*N* HCl $[\alpha]_D^{25}$ of −41. The optical rotation of naturally