of histamine formation invites speculation. In the first place, the time course of increased histamine excretion parallels the one previously found to occur in the pregnant rat(8); in both species the onset of elevated HFC is at a late stage of gestation, and the rate of formation falls to about the non-pregnant level just prior to, or at term. This fact may suggest involvement in the complex machinery which controls the contractility of the uterus. It should be recalled here that, in the rat, histamine inhibits uterine contractility and thus appears to contribute to maintaining the uterus quiet under the mechanical stimulus of the growing foetuses. Studies on the effect of histamine on the uterus of the pregnant hamster are in progress. However, a clear answer seems to be remote due to experimental difficulties. Lastly, the possibility of the high placental HFC being part of a specific metabolic function must be considered. The placenta of mammals has been shown to be endowed with an impressively wide range of enzymatic capabilities(9).

Summary. In the hamster the histamine forming capacity (HFC) of various tissues was determined in the non-pregnant and pregnant states. In pregnancy, the HFC, as reflected in urinary histamine, was greatly elevated during the last 4-5 days of pregnancy. The placenta, and furthermore certain layers therein, were recognized as the sites of the increased rate of histamine formation.

1. Rosengren, E., J. Physiol., 1963, v169, 499.
2. Guggenheim, M., Löffler, W., Biochem. Z., 1916, v72, 303.
3. Reuse, J. J., Brit. J. Pharmacol., 1948, v3, 174.
4. White, T., J. Physiol., 1959, v149, 34.
5. Kahlson, G., Rosengren, E., Thunberg, R., ibid., 1963, v169, 467.
6. Schayer, R. W., Kennedy, J., Smiley, R. L., J. Biol. Chem., 1953, v205, 739.
7. Ward-Orsini, M., Am. J. Anat., 1954, v94, 273.
8. Kahlson, G., Rosengren, E., Westling, H., J. Physiol., 1958, v143, 91.
9. Hagerman, D. D., Fed. Proc., 1964, v23, 785.

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Incorporation of Tritium from Succinate-2,3-H^3 into Long Chain Fatty Acids by Aortic Mitochondria.* (29999)

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It was observed in this laboratory that long chain fatty acid synthesis from acetate in rabbit aortic intima-media strips was greatly stimulated by succinate(1). In fact, in the intact tissue, succinate was a greater stimulant on a molar basis than citrate.

Citrate and isocitrate are the most potent stimulants of fatty acid synthesis by the non-mitochondrial or "malonyl CoA pathway" in liver, adipose tissue, and mammary gland (2,3,4). The mechanism of stimulation by these Krebs Cycle intermediates is still uncertain, and the best available evidence indicates that citrate somehow causes a direct "activation" of the enzyme acetyl CoA carboxylase(5).

The mitochondrion from rabbit aorta is the subcellular unit in which most fatty acid synthesis occurs in this tissue. In isolated, washed aortic mitochondria citrate is a more potent stimulant of fatty acid synthesis than succinate. However, succinate does stimulate by itself and a combination of succinate and citrate will stimulate to a greater extent than equimolar quantities of either separately. Hülsmann(6) has described isocitrate and succinate stimulation of fatty acid synthesis

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by heart sarcosomes. He believes the pathway in sarcosomes involves malonyl CoA and is therefore a different system. Although it has been suggested that succinate might serve as a source of protons for fatty acid synthesis, it has never been demonstrated. To learn more about the mechanism of Krebs Cycle acid stimulation of mitochondrial fatty acid synthesis, this report is concerned with testing the latter hypothesis.

Methods. The tissue studied was aorta from male, white New Zealand rabbits weighing between 5-8 lb. The rabbits had been on a diet of Purina rabbit chow. The tissue was prepared by stripping with scalpel and scissors the intima and media away from the adventitia as cleanly and rapidly as possible. The entire aorta from 1 cm distal to the aortic valves to just proximal to the iliac bifurcation was used. In most experiments the aortic preparations from 3 rabbits were pooled to give sufficient material. For cell fractionation studies, homogenates were prepared by first mincing the tissue with scissors and then homogenizing in an all-glass Tenboeck homogenizer by hand. Whole homogenates consisted of the supernatant from a 3 minute 800 $\times$ *g* spin in a refrigerated Sorvall centrifuge. Mitochondria were sedimented, washed and resedimented by centrifugation at 10,000 $\times$ *g* for 10 minutes.

All the incubations were carried out in phosphate buffer at pH 7.4 using air as the gas phase. Incubations were performed in a Dubnoff metabolic shaker at 37°C for 2 hours. The added cofactors and succinate-2,3-H^3 are listed in the individual tables.

After completion of the incubation, the entire incubation flask contents were handled as follows. The flask contents were hydrolyzed in methanolic-NaOH overnight at 60°. Non-saponifiable lipid was extracted with ether. The alkaline hydrolysis mixture was then acidified and extracted with hexane 3 times. The combined hexane extracts were freed of radioactive succinate by addition of unlabeled succinate followed by exhaustive extraction with water. The hexane extract was finally run through a silicic acid column and succinate contamination of product was entirely eliminated. A portion of the hexane extract containing the fatty acids was assayed for tritium. The remainder of the fatty acids was esterified with diazomethane and chromatographed by gas-liquid chromatography. The instrument used was a Burrell Kromotog K-5 with a 6-foot column. The stationary phase was 17% ethylene glycol succinate by weight on Gas-Chrom P, 80-100 mesh, obtained from Applied Science Laboratories. The instrument was calibrated with a methyl ester standard provided by National Institutes of Health. The effluent vapors from the column were collected and counted in a Packard Tricarb Liquid Scintillation Spectrometer.

TABLE I. Incorporation of H^3 from Succinate Labeled in 2,3 Position into Fatty Acids by Subcellular Components of Rabbit Aorta.

The reaction mixture contained: 10 μc succinate-2,3-H^3 (0.087 μmole), 16 μmoles ATP, 1.6 μmoles $MgCl_2$, 6.4 μmoles nicotinamide, 0.32 μmole CoA, 26 μmoles citrate, 1.02 μmole $NADH_2$, 64 μmoles potassium phosphate buffer (pH 7.4) in a total volume of 2.0 ml.

The mitochondrial suspension contained 8.3 mg protein; supernatant 6.2 mg protein, the microsomal 6.5 mg protein. 20 μmoles $KHCO_3$ and 1 μmole $NADPH_2$ were added to the reaction mixture containing the supernatant and microsomal fractions. All incubations were at 37° for 2 hours.

Fractions	$\mu\mu$moles H^3 incorporated into fatty acids per mg protein
Mitochondria	30.3
Soluble supernatant	18.0
Microsomes	17.0

Results. We have found that the mitochondria of the aortic wall are responsible for most of the fatty acid synthesis from acetate in this tissue. This is in contrast to the greater lipogenic activity of the supernatant system in most tissues such as liver, adipose tissue and mammary gland.

The transfer of tritium from succinate to fatty acids occurs principally in the mitochondria (Table I). Inasmuch as succinic dehydrogenase is probably exclusively a mitochondrial enzyme, it is not surprising that this phenomenon has similar localization. This tissue has extraordinary cohesive strength and homogenization is accomplished by subjecting the tissue to much more trauma and greater frictional forces than conventionally used for cellular fractionation. It is reasonable to presume that many mitochon-

TABLE II. Cofactor Requirements for Incorporation of Tritium from Succinate into Long-Chain Fatty Acids by Rabbit Aortic Mitochondria.

The complete system contained the following components except where indicated: 10 μc succinate-2,3-H^3 (87 m μmoles), 4 μmoles nicotinamide, 5 μmoles ATP, 0.56 μmole $NADH_2$, 5 μmoles acetate, 0.2 μmole CoA, 1 μmole $MgCl_2$, 4 μmoles citrate, 20 μmoles $KHCO_3$, 64 μmoles potassium phosphate buffer (pH 7.4) in a total volume of 2.0 ml. These are representative data from one of 4 experiments showing similar requirements.

	Atoms tritium incorporated per mg mitochondrial protein $H^3 \times 10^{-3}$
Complete system	123.0
—ATP	31.1
—$NADH_2$	62.2
—Acetate	112.8
—CoA	46.7
—$MgCl_2$	20.3
—Citrate	68.6
—HCO_3^-	119.8

dria are fragmented to such an extent that the tritium incorporation occurring in the "supernatant" or "microsomes" is due to mitochondrial fragments. To rule out the possibility that the tritium was passively being incorporated along with succinate carbon, cellular fractions were prepared in similar fashion and incubated with succinate-2,3-C^{14}. No significant C^{14} from succinate was incorporated into the fatty acids.

The co-factor requirements are ATP, $NADH_2$, CoA, Mg^{++} and citrate (Table II). The needed co-factors for maximal incorporation of tritium into fatty acids are identical to those for maximal fatty acid synthesis from acetate in this tissue. It should be noted that added bicarbonate is not a requirement.

Comparing adenine dinucleotide requirements (Table III) it is noteworthy that the maximal incorporation occurs in the presence of $NADH_2$. On a molar basis, $NADH_2$ produced greater tritium incorporation than $NADPH_2$ or either of the oxidized adenine dinucleotides. Similarly we have found that acetate-1-C^{14} incorporation into fatty acids in mitochondria from this tissue is greater with $NADH_2$ than $NADPH_2$.

Increased amounts of isotopic succinate yield greater tritium incorporation. Increasing the concentration of non-isotopic succinate causes a proportional reduction in tritium incorporation into fatty acid (Table IV). Incorporation is inhibited by malonate and cyanide. The cyanide inhibition can be overcome by supplying additional ATP. Avidin does not inhibit. After incubation in a medium containing twice as many microcuries of tritiated water as could come from succinate, there was no tritium incorporation into fatty acid (Table IV).

We performed a series of experiments to determine if the tritium from succinate and the C^{14} from acetate were incorporated into the same fatty acids by the mitochondria

TABLE III. Comparison of Adenine Dinucleotide Requirements for Incorporation of Tritium from Succinate-2,3-H^3 into Long-Chain Fatty Acids by Rabbit Aortic Mitochondria.

The reaction mixture contained the following components: 10 μc succinate-2,3-H^3 (87 m μmoles), 4 μmoles nicotinamide, 5 μmoles ATP, 0.2 μmole CoA, 1.0 μmole $MgCl_2$, 4 μmoles citrate, 64 μmoles potassium phosphate buffer (pH 7.4) in a total volume of 2.0 ml. Adenine dinucleotides were added as indicated below. Mean values from 5 experiments.

Additions	Atoms tritium incorporated per mg mitochondrial protein $H^3 \times 10^{-3}$
1.0 μmole NAD	105.0
1.0 " $NADH_2$	133.1
1.0 " NADP	98.1
1.0 " $NADPH_2$	81.2

TABLE IV. Effect of Various Substances on Tritium Incorporation from Succinate-2,3-H^3 into Long-Chain Fatty Acids in Rabbit Aortic Mitochondria.

The reaction mixture was the same as in Table III except for addition of 1.02 μmole $NADH_2$.

Additions	Atoms tritium incorporated per mg mitochondrial protein $H^3 \times 10^{-3}$
No further additions	138.0
5 μmoles succinate	71.0
20 " succinate	18.3
10 " malonate	10.0
1.0 " cyanide (minus ATP)	0.0
1.0 " cyanide (plus 10 μmoles ATP)	150.5
100 μg avidin*	144.9
50 μc tritiated water (tritiated succinate omitted)	0.0

* The avidin was incubated at 36° for 10 minutes with the mitochondrial suspension before the cofactors and isotope were added.

TABLE V. Distribution of Tritium and C^{14} Among Fatty Acids Synthesized from Succinate-2,3-H^3 and Acetate-1-C^{14} by Rabbit Aortic Mitochondria.

The co-factors present were the same as in Table I. 250 μc succinate-2,3-H^3 (2.2 μmoles) and 5 μc acetate-1-C^{14} (2.5 μmoles) were added to each pool of 3 aortas. The data are average values from 4 pools of aortic mitochondria, 3 aortas used for each pool (*i.e.*, total of 12 aortas). Details are described in text. The "percent" figures did not vary from one pool to another by more than 4%.

	H^3		C^{14}	
Fatty acids	$\mu\mu$moles tritium incorporated	% of total recovered radioactivity	$\mu\mu$moles acetate incorporated	% total recovered radioactivity
<14:0	1.66	1.9	0	
14:0 + 14:1	2.97	3.3	0	
16:0	10.69	12.1	30.00	10.7
16:1	2.65	3.0	11.50	4.1
18:0	5.71	6.4	38.50	13.7
18:1	4.24	4.8	26.50	9.4
18:2	11.30	12.8	7.50	2.7
18:3 + 20:0	9.06	10.2	57.00	20.3
>20:0	40.35	45.5	110.00	39.1

from rabbit aorta. Since there is some variation in the pattern of newly synthesized fatty acids in aortic mitochondria from individual animals, experiments were done on mitochondria pooled from at least 3 rabbits at a time. Table V illustrates data obtained in 2 ways from a pool of 12 animals. In one series of experiments, the mitochondria were incubated with succinate-2,3-H^3 and acetate-1-C^{14} simultaneously. The subsequently isolated and identified fatty acids were subjected to simultaneous assay of tritium and C^{14} by the simultaneous equation method(7). This assured against overlap of counts (H^3 + C^{14}) from fatty acids imperfectly separated due to unsatisfactory chromatographic resolution. In the other series of experiments aliquots of mitochondria from the same pool were incubated separately with acetate-1-C^{14} plus unlabeled succinate or with tritiated succinate plus unlabeled acetate. In the latter case, the isotopes were assayed individually and we did not have to be concerned about overlap of isotope counting due to imperfect discrimination by the scintillation counter. The amount and distribution of isotopic material (both H^3 and C^{14}) was the same whether assayed individually or simultaneously. Except for notable differences in distribution in $C_{18:0}$, $C_{18:2}$, $C_{18:3}$, the relative incorporation of tritium is similar to the relative incorporation of C^{14}. This suggests that tritium, for the most part, is incorporated into newly formed or newly-elongated fatty acids. The data are given as $\mu\mu$moles of isotope incorporated and per cent of recovered counts in adjacent columns. Since the values ($\mu\mu$moles or per cent) in each column are from the same sample or identical sample sizes, the specific activity of fatty acids within each column is the same. The quantitative distribution of fatty acids is not changed by incubation with succinate nor is there any suggestion from the data that the hydrogens (or tritium) are used predominantly to saturate enoic or polyenoic acids.

Discussion. The mechanisms that regulate the rate of fatty acid synthesis are of considerable current biochemical interest. Since a source of protons is essential for fatty acid synthesis, it has been speculated by many workers that availability of $NADPH_2$ might serve to regulate the rate(8). It seemed hopeful that this hypothesis could be tested in the diabetic animal and possibly explain the well-known defect in fatty acid synthesis in diabetes. The evidence at present would favor the view that $NADPH_2$ is *not* the limiting factor in fatty acid synthesis in diabetes(9). However, it is nevertheless very likely that the availability of protons at the site of synthesis probably influences the rate. The fact that tritium (but not carbon) from succinate can be incorporated into newly synthesized fatty acid suggests that succinate could be a source of available protons and might thereby influence the rate of synthesis.

Several features of the system should be emphasized. This is a mitochondrial system and not the palmitate synthetase system

which accounts for most fatty acid synthesis in adipose tissue, liver and mammary gland. Palmitate is not the principal product, HCO_3^- is not required, avidin is not inhibitory and consequently a malonyl CoA intermediate is probably not involved. The fatty acids newly formed from acetate (and which are also recipients of tritium) are mostly 22 and 24 carbons in length, and therefore probably are formed chiefly by addition of acetyl CoA's to preformed 16 and 18 carbon chains *via* the "elongation pathway"(10).

Moreover, $NADH_2$ is the optimal adenine dinucleotide co-factor. This is in contrast to other fatty acid synthesizing systems in which there is either an obligate $NADPH_2$ requirement or in which optimal rates cannot be achieved without it.

The proposed mechanism for the proton incorporation from succinate is as follows:

1) Removal of protons from succinate by succinic dehydrogenase.

2) A transfer of proton from the flavin involved to NAD or NADP by a reversal of oxidative phosphorylation(11).

3) Transfer of proton from $NADH_2$ to the fatty acid at either of the reductive steps (a. beta-hydroxyl acyl dehydrogenase; b. enoyl reductase).

The transfer of tritium from succinate to NAD has recently been demonstrated in a Slater-Keilin-Hartree preparation by Gawron *et al*(12) with an efficiency of 25% to 35%. This transfer requires ATP.

The stimulation of tritium incorporation by $NADH_2$ is of interest. The reduced coenzyme at the site of synthesis (intra-mitochondrial) undoubtedly influences fatty acid synthesis and tritium incorporation. It is likely that added $NADH_2$ does not participate in the reductive steps since it would be expected to "dilute" the tritium incorporation, which is does not. Instead, it is probably oxidized by $NADH_2$ dehydrogenase thus resulting in additional phosphorylation of ADP to form intra-mitochondrial ATP, which in turn, can enhance the reversal of electron flow.

Summary. The mechanism of succinate stimulation of fatty acid synthesis by aortic mitochondria was studied. Tritium, but not carbon, from succinate labeled in positions 2- and 3- is incorporated into long-chain fatty acids in this system. The relative incorporation of tritium in various fatty acids is similar to that of C^{14} from acetate. Succinate can, therefore, provide protons for the reductive steps. We believe this is a demonstration of energy-requiring reversal of electron flow participating in a synthetic process and another way in which Krebs Cycle acids can influence fatty acid synthesis.

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1. Whereat, A. F., J. Atherosclerosis Res., 1964, v4, 272.

2. Brady, R. O., Gurin, S., J. Biol. Chem., 1952, v199, 421.

3. Bucher, N. L. R., J. Am. Chem. Soc., 1953, v75, 498.

4. Popjak, G., Tietz, A., Biochem. J., 1954, v56, 46.

5. Martin, D. B., Vagelos, P. R., J. Biol. Chem., 1962, v237, 1787.

6. Hülsmann, W. C., Biochim. Biophys. Acta, 1962, v58, 417.

7. Okita, G. T., Kabara, J. J., Richardson, F., LeRoy, G. V., Nucleonics, 1957, v15, III.

8. Langdon, R. G., J. Biol. Chem., 1957, v226, 615.

9. Abraham, S., Matthes, K. J., Chaikoff, I. L., ibid., 1960, v235, 2251.

10. Wakil, S. J., J. Lipid Research, 1961, v2, 1.

11. Chance, B., Hollunger, G., J. Biol. Chem., 1961, v236, 1534.

12. Gawron, O., Glaid, A. J., Nobel, S., Gan, M., Biochem. Biophys. Res. Comm., 1964, v16, 432.

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