acid after one to three months of the deficiency and on the relation of calorie restriction to these reproductive upsets on the SST-diet are in progress.

Summary. Adult rats maintained on a purified diet containing succinylsulfathiazole and supplemented with all the known vitamins except pteroylglutamic acid from one to 3 months before breeding showed impaired reproduction.

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Effect of Estrogens on Malic Dehydrogenase of Rat Liver.*

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The natural and synthetic estrogens are important in controlling the function of reproductive tissues, and they are also known to affect the metabolism and function of other tissues not directly concerned with reproduction. It is probable that these changes depend basically on the part played by the estrogens in certain of the enzymatic processes concerned with cellular function. As a means of obtaining information on these basic cellular processes McShan and Meyer[1] studied the effect of the estrogens on the succinoxidase system of liver and pituitary tissues. It was found that both natural and synthetic estrogens were effective inhibitors of this system, and that the inhibition was mediated through the action of the estrogens with the cytochrome oxidase of the system. This work has been extended to the study of the effect of the estrogens on the malic dehydrogenase system using the method reported by Potter[2] for the determination of this dehydrogenase. In addition results are given on the activity of malic dehydrogenase when the cytochrome c of the system is replaced by brilliant cresyl blue.

Experimental. Male and female rats of the Sprague-Dawley strain which were three to four months old were used in these experiments.

The rats were killed by decapitation and the tissues were removed immediately, weighed and placed in sharp-pointed homogenizing tubes containing 0.1 ml of glass-distilled water. These homogenizing tubes containing the tissues were kept in an ice bath during homogenization and until the tissues were placed in the Warburg flasks.

The method of Potter[2] for the determination of malic dehydrogenase was followed in these experiments. Two and one-half per cent water homogenates of the liver tissue were used throughout. Conventional Warburg flasks without side arms were employed. The main compartment of the flasks contained, in addition to the desired amount of liver homogenate, 0.3 ml of 0.5 M l-malic acid, 0.6 ml of 0.5 M glutamic acid, 0.6 ml of 0.1 M nicotinamide, 0.3 ml of a 0.5% (5 mg per ml) solution of coenzyme I (diphosphopyridine nucleotide), 0.3 ml of 4 x 10^{-4} M cytochrome c, 0.4 ml of 0.2 M phosphate buffer pH 7.4, and enough water to make a final reaction volume of 3.0 ml. The center wells of the flasks contained 0.1 ml of 2N NaOH. All solutions were made with glass-distilled water.

The cytochrome c was made from beef heart muscle according to the method of Keilin and Hartree[3] except that the final

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1 McShan, W. H., and Meyer, R. K., *Arch. Biochem.*, 1946, **9**, 165.

2 Potter, V. R., *J. Biol. Chem.*, 1946, **165**, 311.

3 Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. (London)*, 1937, **122**B, 298.

TABLE I.
Malic Dehydrogenase Activity of Liver Homogenate.

ml of 2.5% homogenate				
0.15	0.2	0.25	0.3	0.4
		Q_{O_2}		
	59.0	—	59.0	57.4
	81.6	—	78.8	
65.4	72.1	67.6	—	
	59.7	60.0	57.0	
	53.7	49.8	45.7	
64.4	60.9	—	—	
	106.6	101.3	—	
	90.6	93.0	—	

product was dialyzed against glass-distilled water instead of 1% sodium chloride solution. The coenzyme I was prepared in our laboratory according to the LePage modification of the Williamson and Green[4] method of preparation. Both Eimer and Amend, and Eastman 1-malic acids were used, and values obtained with each were found to be in close agreement.

Approximately 20 minutes elapsed between the time the animals were killed and the placing of the flasks in the Warburg bath at 38°C. They were allowed to equilibrate for 6 minutes, the manometer stopcocks were closed and readings were taken at 10, 20 and 30 minutes. Q_{O_2} values were based on the average of these 3 readings.

Results and Discussion. A series of determinations was made to ascertain whether the oxygen uptake was proportional to the amount of liver homogenate used. In these runs, quantities of liver varying from 0.15 to 0.4 ml of 2.5% homogenate were used. The data presented in Table I show that the oxygen uptake of the malic dehydrogenase system is essentially proportional to the amount of liver tissue used within a narrow range of wet weight of tissue. There were several determinations made, however, in which an exact proportionality was not obtained.

Several studies were made employing various estrogenic compounds and sodium androsterone sulfate† as *in vitro* inhibitors of malic dehydrogenase activity of rat liver. The test system contained 0.25 ml of a 2.5% liver homogenate and 0.15 ml of 2 x 10^{-3} M solution of the estrogenic compound. The estrogens employed were diethylstilbestrol, hexestrol, dienestrol and disodium 3,4-diphenylhexane-p,p′-dioxyacetate and benzestrol estrogenic compounds No. 020 (HO-C_6H_4-CH_2-CHC_2H_5-CH_2-C_6H_4-OH), No. 103 (HO-C_6H_4-$CHCH_3$-CH_2-CHC_3H_7-C_6H_4-OH) and No. 221B-2 (HO-C_6H_4-CHC_2H_5-CHC_2H_5-$CHCH_3$-C_6H_4-OH). These inhibitors, with the exception of disodium 3,4-diphenylhexane-p,p′-dioxyacetate, which is water soluble, were dissolved in 0.5 ml water that contained 0.03 ml of 2N NaOH after which sufficient glass-distilled water was added to make 5 ml. Control flasks were run that contained 0.15 ml of a solution containing 0.03 ml of 2N NaOH per 5 ml, and the addition of this weakly basic solution had no effect on the malic dehydrogenase values.

The data presented in Table II indicate that the estrogenic compounds are efficient inhibitors of the malic dehydrogenase activity of rat liver *in vitro*. The 8 compounds used are listed in the order of increasing inhibitory power: Androsterone sulfate 2% inhibition, disodium-3,4-diphenylhexane - p,p′- dioxyacetate, 6%, No. 020 (benzestrol) 22%, dienestrol 36%, diethylstilbestrol 44%, hexestrol 75%, No. 221B2 (benzestrol) 90%, and No. 103 (benzestrol) 93%.

To determine whether malic dehydrogenase itself was inhibited, or whether the inhibition was effected through inhibition of cytochrome oxidase, cytochrome c was replaced by brilliant cresyl blue. Preliminary results indicated that the cytochrome c of the malic dehydrogenase system can be completely substituted for by brilliant cresyl blue. On the basis of these data and before doing further work on the inhibition of this system by the estrogens, it was decided to determine the amount of the dye required for optimum activity of malic

[4] Williamson, S., and Green, D. E., *J. Biol. Chem.*, 1940, **135**, 345.

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TABLE II.
Malic Dehydrogenase Activity of Liver with Estrogenic and Androgenic Inhibitors *in Vitro*.

Q_{O_2}			
Control	Inhibitor Final molarity 10^{-4} M	Inhibition, %	Avg, %
	Diethylstilbestrol.		
55.9	22.5	60	44
79.7	46.9	42	
82.6	57.3	30	
	Hexestrol.		
71.8	21.3	70	75
75.3	26.6	81	
	Dienestrol.		
90.7	60.3	34	
90.8	34.2	63	
64.0	42.7	33	
65.8	38.4	32	36
79.2	47.4	40	
80.6	74.8	7	
80.6	48.2	40	
	Disodium 3.4-diphenylhexane-p,p'-dioxyacetate.		
64.0	61.4	4	
65.8	59.7	9	6
76.5	77.5	0	
85.4	76.2	11	
	No. 020.		
84.2	65.2	23	22
78.3	62.6	20	
	No. 103.		
84.2	4.8	94	93
78.3	6.1	92	
	No. 221B2.		
90.7	10.4	89	90
90.8	8.0	91	
	Na Androsterone SO_4.		
79.2	75.0	6	2
80.6	80.9	0	
80.6	80.9	0	

dehydrogenase. From the data of Table III it can be seen that there is a range of optimum dye concentration, above and below which the oxygen uptake of 0.25 ml of liver homogenate begins to decrease. This optimum range is from 0.125 to 0.75 mg of brilliant cresyl blue per flask contents contained in a total volume of 3 ml. As a result 0.5 mg of the dye was used per flask in the following experiments.

Using an amount of brilliant cresyl blue which gives maximum activity (0.1 ml of a 0.5% solution) varying amounts of tissue (from 0.05 ml to 0.35 ml) were used in an effort to determine whether the oxygen uptake was proportional to the concentration of tissue. As can be seen from the data given in Table IV, the Q_{O_2} decreases fairly rapidly with increasing amounts of liver homogenate which shows that the oxygen uptake was not directly proportional to the amount of tissue reacting. Furthermore, when each of the reagents was increased in turn to determine whether there was an insufficient amount of one of the constituents, a direct proportionality between oxygen uptake and the amount of tissue used was not obtained.

The inhibiting power of diethylstilbestrol on the malic dehydrogenase activity of liver was tested using cytochrome c alone, brilliant cresyl blue alone and a combination of this dye and cytochrome c. A final concentration of 1×10^{-4} M diethylstilbestrol was used in these *in vitro* studies. The data of Table V indicate that brilliant cresyl blue can adequately take the place of cytochrome c in the malic dehydrogenase system in that under the conditions used the oxygen uptake is as high in the presence of the dye as in the presence of cytochrome c. This is in contrast

TABLE III.
Malic Dehydrogenase Activity of Liver with Various Concentrations of Brilliant Cresyl Blue.

Brilliant cresyl blue, mg/flask	Q_{O_2}					
	Run I	II	III	IV	V	Avg
—	3.9	0.0	4.7	6.2		4
.0125	25.6	—	—	—		26
.025	52.8	—	—	—		53
.0375	62.1	—	—	—		62
.05	64.4	—	—	—		64
.0625	76.0	61.3	66.7	—		68
.125	76.0	77.6	72.9	—		76
.25	72.9	77.6	71.4	77.6		75
.325	71.4	69.1	77.6	—		73
.5	77.6	77.6	76.0	77.6	74.5	77
.75	76.0	77.6	69.8	71.4	—	74
1.0	68.3	55.1	77.6	62.9	55.9	64
2.0	57.4	62.9	60.5	59.8		60
3.0	66.0	59.8	65.2	53.5		61
4.0	49.7	77.6	62.9	43.5		58

TABLE IV.
Malic Dehydrogenase Activity with Brilliant Cresyl Blue* and Varying Concentrations of Liver Homogenate.

	Q_{O_2}						
	0.05 ml	0.1 ml	0.15 ml	0.2 ml	0.25 ml	0.3 ml	0.35 ml†
	91.7	92.4	85.7	77.0	77.0	74.1	
	—	74.2	74.6	—	71.5	66.4	57.5
	106.7	90.6	108.1	82.0	66.4	—	
	—	—	85.6	80.9	71.2	—	
	—	88.9	74.2	85.0	71.0	—	
	71.2	87.6	73.9	71.6	60.7	—	
	71.2	68.2	68.4	58.5	57.3	60.9	
	106.9	74.1	62.4	67.8	68.0	56.1	
Avg	89.5	82.3	79.1	74.7	67.9	64.4	57.5

* Concentration of brilliant cresyl blue was 0.5 mg per flask.
† Liver homogenate was 2.5%.

to the succinoxidase system in which the activity is decreased considerably when the dye is substituted for cytochrome c.[5,1] The fact that this dye is autoxidizable in the presence of oxygen permits the malic dehydrogenase system to function without the action of cytochrome oxidase. Thus, if the estrogen inhibition is taking place by way of cytochrome oxidase, the addition of brilliant cresyl blue to the system should result in the malic dehydrogenase being essentially as active in the presence as in the absence of the inhibitor. The data of Table V show that this is the case. Diethylstilbestrol in a final concentration of 1×10^{-4} M is an effective inhibitor of the malic dehydrogenase system when cytochrome c is used, but when it is replaced with brilliant cresyl blue there is little inhibition, and there is even less when both cytochrome c and dye are present. These results support the concept that the estrogens inhibit the malic dehydrogenase system through action with the cytochrome oxidase of the system and not by direct action with the malic dehydrogenase.

Furthermore, only those compounds that have phenolic groups inhibit the malic dehydrogenase system, and when these groups are replaced by oxyacetate groups little, if any, inhibition occurs. Also sodium androsterone sulphate which contains an alcoholic in place of a phenolic group does not show inhibitory

[5] Weil-Malherbe, H., *Biochem. J.*, 1937, **31**, 299.

TABLE V.
Inhibition by Diethylstilbestrol of Malic Dehydrogenase of Liver Using Cytochrome c and Brilliant Cresyl Blue.

	Q_{O_2}					
	Cytochrome c		Cresyl blue		Cytochrome c-Cresyl blue	
	Control	Inhibitor*	Control	Inhibitor*	Control	Inhibitor*
	84.3	34.4	82.2	62.9	—	—
	62.9	3.5	69.0	61.6	—	—
	70.0	13.0	73.9	71.0	77.5	71.3
	75.3	27.2	78.7	75.6	71.3	68.9
	71.2	5.9	67.9	59.8	64.0	61.9
	71.2	7.1	69.2	58.3	64.0	60.4
	74.8	11.8	75.1	63.0	70.1	67.8
Avg	72.8	14.7	73.7	64.6	69.4	66.1

* Diethylstilbestrol, final concentration 1 x 10^{-4} M.

action. This does not seem unreasonable as cytochrome oxidase, through which the inhibition was shown to be effected, is known to catalyze the oxidation of certain phenolic compounds. It is suggested that there is an affinity, therefore, between the active centers of the enzyme and phenolic groups. Thus, it appears that the phenolic groups of the estrogenic compounds combine with the active centers and remain attached to the enzyme which prevents the latter from acting. This mechanism was shown previously to account for the inhibition of the succinoxidase system by estrogens.[1]

Summary. The malic dehydrogenase activity of liver homogenate was shown to be proportional to the amount of tissue used.

It was found that the cytochrome c of the malic dehydrogenase system could be replaced by brilliant cresyl blue. The maximum activity of the dehydrogenase was obtained when the concentration of the dye ranged from 0.125 mg to 0.75 mg per flask.

The inhibitory action of one androgenic and 7 estrogenic compounds was tested on liver malic dehydrogenase *in vitro*. Stilbestrol and benzestrol estrogens are effective inhibitors, but little inhibition occurs when the phenolic groups of the stilbestrol type compounds are replaced by oxyacetate groups as for example in disodium 3,4-diphenylhexane-p,p′-dioxyacetate. In addition sodium androsterone sulphate which contains an alcoholic in place of a phenolic group shows very little inhibitory action. It appears that the presence of phenolic groups are necessary for inhibition to take place.

It was concluded also from inhibitor studies with diethylstilbestrol, in which brilliant cresyl blue was substituted for cytochrome c, that diethylstilbestrol exerts its inhibitory action on cytochrome oxidase rather than malic dehydrogenase.