[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, ST. LOUIS UNIVERSITY SCHOOL OF MEDICINE]

Choline Metabolism. VI. Hemorrhagic Degeneration and the Labile Methyl Supply[1]

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Methylation has long been recognized as an important biological process. In 1894 Hofmeister[2] investigated the formation *in vivo* of methyl selenides and tellurides and postulated the transfer of methyl groups in metabolism. In 1917 Thompson[3] concluded that methyl transfer occurred in the synthesis of creatine in the animal body, a result in agreement with the experiments of Bloch and Schoenheimer,[4] and of du Vigneaud, Chandler, Cohn and Brown.[5] Transmethylation has been demonstrated by the latter workers who found deuterium in tissue choline and creatine following the administration in rats of methionine containing deuterium in the sulfur methyl. Furthermore, du Vigneaud, Chandler, Moyer and Keppel[6] have reported the availability for the growth of rats of homocystine in a methionine-free diet containing choline or betaine and have suggested that these compounds furnished the methyl required for the formation of methionine from homocysteine. du Vigneaud[5] has also suggested that utilizable methyl groups may be essential in the diet.

The dietary importance of choline as a lipotropic substance in adult rats was noted by Best, Hershey and Huntsman[7] following the feeding of low choline diets which were high in fat (40%) and low in protein. Best and Huntsman[8] also reported the choline-like action of betaine and Tucker and Eckstein[9] observed that methionine was similarly protective.

The significance of these studies on older rats fed the high fat, low protein ration was increased by the recognition by Griffith and Wade[10] of a pathological state, hemorrhagic degeneration, in young rats fed food mixtures which were adequate in protein and either high or low in fat. This abnormal condition which was prevented by choline and by methionine[11] was characterized by severely acute renal and ocular hemorrhage as well as by excessive deposition of liver fat. Its occurrence in rats on a diet which has been commonly used in nutritional investigations as an apparently adequate food mixture[12] emphasized the nutritive importance of choline and of substances having choline-like action, such as methionine. Hemorrhagic degeneration was aggravated by cystine[11] as is the deposition of liver fat in older animals.[13]

The demonstrations of methyl transfer and of the dietary significance of methionine, betaine and choline, cited above, have emphasized the importance of those groups which du Vigneaud has designated utilizable methyl groups. The present study deals with the relative effectiveness of methionine, betaine and choline in the prevention of hemorrhagic degeneration, a condition which may be the result of a deficiency of the indispensable labile methyl supply. In addition, the influence of the sulfur compounds, cysteine, homocystine, glutathionine and taurine has been investigated.

Young male rats, 38 to 42 g. in weight and twenty-one to twenty-six days of age were fed the experimental diets during an eight-day period. The occurrence and severity of hemorrhagic degeneration were determined by noting the appearance and weight of the kidneys, the weight of the liver and the weight of liver fat (total chloroform-soluble substances). The average weight of the kidneys of normal young rats in this colony is 1.0 to 1.2% of the body weight and the normal liver fat is 4.3% of the liver weight. The basal diet, AC 50, consisted of purified casein 18%, lard 20, sucrose 48.9, calcium carbonate 1, salt mixture[14] 4, agar 2, powdered brewer's yeast 6 (Anheuser–Busch, Strain G) and fortified fish liver oil (Natola) 0.1%, respectively. Commercial casein was purified by 20 extractions with water in a motor-driven rotary churn followed by 3 washings with 95% ethanol. Diets AC 118 and AC 51 were the same except that 0.1% and 0.3% of cystine, respectively, were added. Diet AC 84 was the same as AC 51 except that the protein fraction consisted of casein 9, fibrin 3 (Difco desiccated blood fibrin),

(1) Presented in part before the Division of Biological Chemistry of the American Chemical Society in Detroit, Sept. 12, 1940; and, in part, before the Society for Experimental Biology and Medicine in St. Louis, Oct. 9, 1940.

(2) Hofmeister, *Arch. expil. Path. Pharmakol.*, **33**, 198 (1894).

(3) Thompson, *J. Physiol.*, **51**, 347 (1917).

(4) Bloch and Schoenheimer, *J. Biol. Chem.*, **134**, 785 (1940).

(5) du Vigneaud, Chandler, Cohn and Brown, *ibid.*, **134**, 787 (1940).

(6) du Vigneaud, Chandler, Moyer and Keppel, *ibid.*, **131**, 57 (1939).

(7) Best, Hershey and Huntsman, *Am. J. Physiol.*, **101**, 7 (1932).

(8) Best and Huntsman, *J. Physiol.*, **75**, 405 (1932).

(9) Tucker and Eckstein, *J. Biol. Chem.*, **121**, 479 (1937).

(10) Griffith and Wade, *ibid.*, **131**, 567 (1939).

(11) Griffith and Wade, *ibid.*, **132**, 627 (1940).

(12) Griffith, *J. Nutrition*, **19**, 437 (1940).

(13) Beeston and Channon, *Biochem. J.*, **30**, 280 (1936).

(14) Hawk and Oser, *Science*, **74**, 369 (1931).

lactalbumin 3 (Labco, #7HAAX), and gelatin 3% (Difco Bacto-Gelatin), respectively. The food intake varied from 4.5 to 5.5 g. per day.

Earlier observations[12] suggested the possibility of using the response of experimental rats to the feeding of choline in the bioassay of choline and of choline-like substances. In order to determine more definitely the quantitative nature of this response, seven groups of rats were fed, during the eight-day period, a low choline diet (AC 51) supplemented with choline varying from 0 to 1.5 mg. of choline chloride per g. of food. The results, which are shown in Fig. 1, indicate that the choline-like potency of test materials may be estimated by comparison with the effect of four levels of choline; the two which partially and completely prevent renal lesions (or abnormal increases in kidney weight), and the two which partially and completely prevent the deposition of excess liver fat. On diet AC 51 these four levels are 0.5–1.5, 2, 2–4 and 6 mg. of choline per day, respectively (Fig. 1). A detailed study is being made of the accuracy of assays at two of these levels, the one limiting the incidence of renal hemorrhage to 40 to 50% of the group and the other limiting the fatty liver to 10 to 15% of fat per liver. In Fig. 1, these amounts correspond to choline intakes of 1 and 3 mg. per day, respectively. Liver fat values in rats showing severe renal lesions in over 60% of the group are not reliable for assay purposes because of the poor nutritive state of such animals.

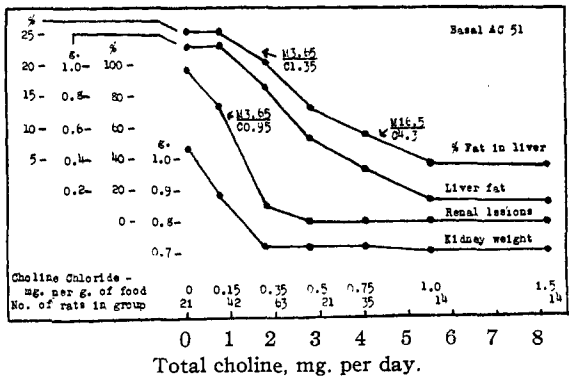

Fig. 1.—The deposition of liver fat and the severity of renal damage during an 8 day period in groups of rats fed a low choline diet (AC 51) supplemented with 0 to 1.5 mg. of choline chloride per g. of food. The experimental data are plotted according to the actual daily intake of choline chloride. Figures are included to illustrate the method of estimation of the effectiveness of methionine as a substitute for choline (Table I). The M:C ratios show the mg. of methionine (M) and of choline (C) per day which have equal effects in partially preventing the fatty liver and the renal hemorrhage.

Table I shows the results of preliminary tests designed to measure the effectiveness of methionine and betaine as substitutes for choline. The amounts of these compounds equivalent in effect to that of one mg. of choline are estimated from the data of Table I and from curves such as those for diet AC 51 (Fig. 1). For instance, 0.75 and 3.0 mg. of added methionine per g. of food (Diet AC 51, Table I), or 3.6 and 16.5 mg. per day, approximate the effect of 1 and 4 mg. of choline per day (Fig. 1). On the basis of a theoretical transfer of methyl groups, 3.2 mg. of methionine and 1.1 mg. of betaine hydrochloride are required to furnish the methyl supplied by 1 mg. of choline chloride. Experimentally, 3 to 4 mg. of methionine and of betaine are necessary to equal the effect of 1 mg. of choline. On the assumption that choline formation is possible through a transfer of methyl groups, these results support the conclusion that the methionine methyl is efficiently utilized and that either the three betaine methyls are poorly utilized or that only one of the three is a labile methyl.

TABLE I

COMPARISON OF METHIONINE, BETAINE AND CHOLINE

Diet	Supplement, mg. per g. of food		No. of rats	Liver fat, % of liver	Renal lesions, % of rats	Mg. of supplement equivalent to one mg. of choline chloride
AC 84	Choline chloride	0.25	42	28.1	83	
		0.45	21	26.3	52	
	dl-Methionine	1.0	21	28.0	86	3.5 to 4.0
		2.0	21	23.7•	43	3.5 to 4.0
AC 51	Choline chloride	0.15	21	23.4	81	
		0.75	35	9.3	0	
	dl-Methionine	0.75	42	22.3	64	3.0 to 3.5
		3.0	21	8.0	0	3.5 to 4.0
	Betaine hydrochloride					
		0.5	49	23.7	87	2.5 to 3.5
		2.0	43	10.8	0	3.0 to 4.5
AC 50	Choline chloride	0.2	42	19.9	52	
		0.4	56	12.5	4	
	Betaine hydrochloride					
		1.0	42	17.9	24	3.5 to 4.5
		2.0	75	8.7	0	3.5 to 4.5

In contrast to the high degree of protection afforded by methionine and betaine, supplements of creatine decrease the severity of but do not prevent hemorrhagic degeneration (Table II). Furthermore, this slight but definite degree of protection is not increased by larger supplements of creatine. The influence of creatine is not marked enough to affect the deposition of liver fat except

in the presence of a partially protective supplement of choline. In view of these results it is suggested that dietary creatine does not supply methyl for choline formation but that it may spare the labile methyl supply of the body so that those methyl groups become available which otherwise would be used in the synthesis of required creatine.

TABLE II

EFFECT OF CREATINE

Diet	Creatine mg. per g. of food	No. of rats[a]	Body wt., g.	Kidney wt., % of body wt.	Liver fat, % of liver	Renal lesions, % of rats
Basal AC 118	0	88-78	54	1.86	21.5	88
	2	42-34	60	1.58	21.1	79
	5	42-38	58	1.52	21.3	81
Basal AC 50	0	88-71	50	1.94	19.9	95
	2	42-39	58	1.43	20.6	74
	5	58-56	57	1.42	17.5	67
	10	21-21	61	1.41	18.0	67
+ Choline chloride 0.4 mg. per g. of food	0	56-56	61	1.05	12.5	4
+ Choline chloride 0.4 mg. per g. of food	5	21-21	61	1.00	7.7	0

[a] Second figure shows the number of surviving animals.

TABLE III

EFFECT OF SULFUR COMPOUNDS

Diet	Choline chloride mg. per g. of food	No. of rats[a]	Body wt., g.	Kidney wt., % of body wt.	Liver fat, % of liver	Renal lesions, % of rats
Basal AC 50	0	88-71	50	1.94	19.9	95
+ Taurine, 0.1%	0	96-77	52	1.99	19.0	95
+ Cysteine hydrochloride, 0.13%	0	46-39	49	2.08	20.9	100
+ Cystine, 0.1%	0	46-39	51	1.89	21.0	89
Basal AC 50	0.4	56-56	61	1.05	12.5	4
+ Taurine, 0.1%	.4	56-56	61	1.09	11.5	11
+ Cysteine hydrochloride, 0.13%	.4	56-56	61	1.15	16.1	18
+ Cystine, 0.1%	.4	56-56	61	1.18	17.2	32
+ Glutathione (G-SH), 0.26%	.4	23-23	64	1.07	21.1	22

[a] Second figure shows the number of surviving animals.

Table III shows the effect of supplements of taurine, cysteine and cystine in a low choline diet and in the same diet plus the addition of a partially protective level of choline. The results illustrate the fact that differences between food mixtures may not be evident if the choline deficiency is so marked that hemorrhagic degeneration of maximum severity occurs. In the same diets, supplemented with choline, the addition of cysteine or of cystine exaggerates the deficiency whereas taurine is without effect. Values for liver fat are considered of greater significance than the incidence of renal lesions on diets, such as these, in which the hemorrhagic state is too moderate to cause an appreciable increase in kidney weight. The results with glutathione are unusual in that the liver fat is excessive for animals with kidneys of normal weight.

Table IV shows the effect of homocystine[15] compared with that of cystine and of methionine. Homocystine does not show methionine-like activity even in the presence of a partially protective level of betaine. In this experiment in which rats were on a diet deficient in an adequate labile methyl supply the effect of homocystine resembles that of cystine rather than that of methionine.

TABLE IV

EFFECT OF HOMOCYSTINE

Diet	No. of rats[a]	Body wt., g.	Kidney wt., % of body wt.	Liver fat, % of liver	Renal lesions, % of rats
Basal AC 50	21–21	62	1.42	20.5	62
+ Cystine, 0.1%	42–39	57	1.82	22.0	86
+ Homocystine, 0.11%	41–38	59	1.51	22.8	83
+ Methionine, 0.1%	21–21	65	1.17	17.6	24
+ Betaine hydrochloride, 0.05%	42–42	65	1.26	19.7	43
+ Betaine hydrochloride, 0.05% and Homocystine, 0.11%	28–28	60	1.43	19.4	64

[a] Second figure shows the number of surviving animals.

Discussion

The occurrence of hemorrhagic degeneration in young rats is evidence of a deficiency of choline and of the labile methyl supply to which choline, methionine and betaine contribute. Creatine spares the supply but its methyl does not appear to be available for choline formation. The prevention of hemorrhagic degeneration provides a relatively sensitive method for the detection of sources of labile methyl which is required for the synthesis of other important metabolites in addition to choline. This procedure is being used not only in the study of the maintenance of the methyl reserve by dietary means but also in the study of the specific role of individual methylated compounds in the prevention of the various aspects of hemorrhagic degeneration. The question of the lability of methyls, bound other than by sulfur and by quaternary nitrogen, is also being examined.

The observations in this paper are in agreement with other reports in which different experimental procedures were used in the investigation of betaine,[8] of creatine[16] and of sulfur compounds.[17,18]

(15) We are indebted to Dr. V. du Vigneaud for the homocystine used in these experiments.

(16) du Vigneaud, personal communication.

(17) Singal and Eckstein, *Proc. Soc. Exptl. Biol. and Med.*, **41**, 512 (1939).

(18) Channon, Manifold and Platt, *Biochem. J.*, **34**, 866 (1940).

Of particular interest are the experiments of du Vigneaud who has reported that in young rats the methionine methyl may be transferred to choline and to creatine[5] and that the choline[6] and betaine,[19] but not the creatine,[16] methyls may be transferred to homocystine.

Acknowledgments.—We wish to acknowledge financial assistance from the Theelin Fund administered by the Committee on Grants for Research of St. Louis University. We are also indebted to Merck and Co., Inc., for a supply of choline chloride and to Parke, Davis and Co., Inc., for a supply of Natola.

(19) Chandler and du Vigneaud, *J. Biol. Chem.*, **135**, 223 (1940).

Summary

1. Hemorrhagic degeneration is the result of a dietary deficiency of choline and of the labile methyl supply.

2. Betaine, like methionine, contributes to the labile methyl supply of the body and may be substituted for choline. The effectiveness of betaine corresponds to the utilization of only one of the three methyl groups.

3. Creatine does not contribute to but does spare the labile methyl supply.

4. Cysteine, homocystine and glutathione, but not taurine, increase the severity of hemorrhagic degeneration.

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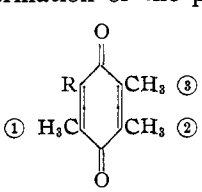
[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

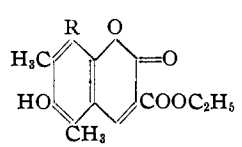
The Reaction between Quinones and Metallic Enolates. XIII. Trimethylethylquinone and Sodium Malonic Ester[1]

BY LEE IRVIN SMITH AND J. W. OPIE[2]

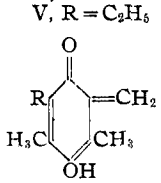
Trimethylbromoquinone (I) reacts with sodium malonic ester to produce 3-carbethoxy-6-hydroxy-5,7-dimethyl-8-bromocoumarin (II).[3] This coumarin is the only product of the reaction; the bromine atom is unaffected by the enolate, but it does exert a strong directing influence upon the formation of the pentad-enol system (III) from



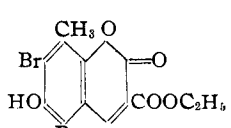
I, R = Br
V, R = C_2H_5



II, R = Br



III, R = Br



IV

I, since of the three methyl groups, the one meta to the bromine atom reacts exclusively. In the previous paper[1] it was shown that while no product resulting from attack of sodium malonic ester upon the bromine atoms of dibromo-*m*-xyloquinone could be isolated, the reaction leading to the coumarin (IV) was difficult to control and, moreover, the coumarin IV differed from all its analogs previously studied in the great ease with which the heterocyclic ring could be opened. In dibromo-*m*-xyloquinone, there is no methyl group meta to either of the bromine atoms, and it appeared, when the results obtained with this quinone were compared those obtained with the monobromoquinone I, that the nature as well as the orientation of the substituents in the quinones exert a pronounced effect not only upon the course of the reaction, but also upon the ease with which it occurs and upon the stability of the heterocyclic ring in the coumarin formed.

In order to explore these effects further, a study has been made of the reaction between trimethylethylquinone (V) and sodium malonic ester. Having shown that tetraethylquinone was inert toward the enolate, it was assumed that the ethyl group of V would not react, and that the reaction with sodium malonic ester would be confined to the methyl groups. Each of the three methyl groups thus became a potential point of entry for the malonic ester residue, and three isomeric coumarins could result, depending upon the direction and magnitude of the orienting effect of the ethyl

(1) Paper XII, THIS JOURNAL, **63**, 612 (1941).

(2) Abstracted from a thesis by Joseph W. Opie, presented to the Graduate Faculty of the University of Minnesota, in partial fulfillment of the requirements for the Ph.D. degree, July, 1940.

(3) Smith and Johnson, THIS JOURNAL, **59**, 673 (1937).