SUMMARY

1. Inositol exerts a limited but clear-cut lipotropic effect when added to a hypolipotropic diet devoid of fat.

2. Addition to the same diet of a saturated fat fraction from beef dripping, of a hydrogenated vegetable fat (Crisco) or of an unsaturated fat (corn oil, Mazola brand) abolishes the effect noted above. Thus it appears that inositol exerts either no, or at most a slight and variable, lipotropic effect in diets containing fat.

3. The great variation in individual liver lipid values in both basal and treated groups makes it impossible to interpret the significance of small differences in mean values unless individual analyses from a large number of animals are available for statistical assessment.

4. The importance of using animals of one sex in

lipotropic studies is stressed. The tendency for liver lipids to be higher and more variable in females (70–100 g.) is most apparent in the rats consuming the basal diets.

5. A highly purified preparation of inositol did not differ in lipotropic properties from that available commercially.

6. Inositol injected subcutaneously into rats consuming a fat-free diet exhibits practically the same lipotropic activity as when ingested; injected inositol shows no such activity when the diet contains fat. Thus obliteration of the lipotropic effect of inositol by dietary fat cannot be explained by interference of fat with absorption of inositol since injected material is equally ineffective.

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The Rates of Lipotropic Action of Choline and Inositol under Special Dietary Conditions*

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Five substances possessing lipotropic properties are known to occur in nature, namely choline, betaine, methionine, inositol and β -propiethetin. The last named has been found in a seaweed, *Polysiphonia fastigiata* (Challenger & Simpson, 1947), but its presence in materials commonly consumed as food has not been established. The other four substances occur widely distributed and are present in many dietary components. The relative lipotropic potencies of these four compounds have been tested in rats

under different nutritional conditions, and the results of some preliminary dose-response studies are now in the press (Best, Lucas, Ridout & Patterson, 1950). In the case of inositol, observations made in our laboratory agree in fact with those reported by McHenry and his colleagues (Gavin, Patterson & McHenry, 1943; MacFarland & McHenry, 1945, 1948), but different interpretations have been made of the findings. Persons unfamiliar with the details of the experimental conditions under which the observations were made find these discrepancies in the literature confusing. MacFarland & McHenry (1945) state quite definitely that inositol alone

* A brief report of part of this work was presented before the American Society of Biological Chemists at Atlantic City in March, 1950 (Best, Lucas, Patterson & Ridout, 1950).

possesses certain lipotropic properties, although their experiments were performed in the presence of a large amount of choline, as was pointed out shortly afterwards (Best, Lucas, Patterson & Ridout, 1946*a*). Their most recent report (MacFarland & McHenry, 1948) admits the presence of choline, but continues to convey the idea that inositol possesses unique lipotropic properties not possessed by choline. To secure further data concerning the importance of the special conditions under which their experiments were conducted, the following study of the role of inositol in lipotropic phenomena was undertaken.

Gavin & McHenry (1941*a*) first observed the lipotropic activity of inositol. This new nutritional role of inositol was not recognized at the time as being the discovery of a supplementary effect. Even to-day few realize that the lipotropic effects ascribed then and more recently to inositol by McHenry and his colleagues are those of inositol in the presence of a maximally effective dose of choline (Gavin & McHenry, 1941*b*; MacFarland & McHenry, 1945, 1948). In the few experiments in which choline was not present, the results agree exactly with our findings, but these limited effects of inositol alone have never been featured by McHenry. Engel (1942) and Forbes (1943) confirmed the supplementary lipotropic effect of inositol, and other laboratories have extended the early findings (Beveridge, 1944; Handler, 1946*a*).

Beveridge (1944) was apparently the first to report that inositol acting alone, i.e. without choline, exerts a lipotropic effect when conventional feeding techniques (without previous depletion) are adopted. During the course of the work he noted that corn oil interferes with this effect. The interference of corn oil has been studied further (Beveridge & Lucas, 1945; Handler, 1946*a*) and confirmed. Subsequent studies have indicated that under the conditions used in our laboratory inositol exerts a significant lipotropic action in fat-free diets only, whether or not the depletion technique is followed, although a pseudo-lipotropic effect is sometimes observed when female rats fed diets containing fat are used in the tests (Best, Ridout, Patterson & Lucas, 1950). In rats fed fat-free diets for 3 weeks (without preliminary depletion) the lipotropic effect of inositol alone is much less than that of choline. Using a hypolipotropic basal diet which causes deposition of about 22–25 % of total lipids in the liver, a maximally effective dose of inositol reduces the value to about 13–17 %; with choline the value falls usually within the range 5–7 % and with the two agents acting together, total liver lipids are generally reduced to 4–5 %.

As MacFarland & McHenry (1948) point out in their most recent communication, they have always tested the lipotropic properties of inositol in rats

maintained under special conditions, namely, a preliminary 3-week 'depletion' period on a fat-free, low-protein diet devoid of B vitamins followed by a 7-day feeding period during which supplements of crystalline B vitamins were given subcutaneously and a special dietary supplement such as an ox-liver fraction rich in B vitamins (and which, incidentally, contains much choline) or a mixture to simulate it. Because of the failure to determine the effect of inositol by itself, an erroneous impression has been given of the relative lipotropic potency of inositol and of choline.

Data reported about 4 years ago by Handler (1946*a*), and unpublished results of the authors, indicated the importance of the time factor in lipotropic studies following the depletion procedure. The 7-day test period is unsuitable because the liver lipids are changing rapidly in quantity and composition at this time. It should be emphasized that the fatty liver resistant to the action of choline (McHenry & Patterson, 1944; Gavin & McHenry, 1941*b*; Gavin *et al.* 1943; MacFarland & McHenry, 1945, 1948) is mainly an artifact resulting from an inappropriately chosen time of making the observations. It is our contention that comparisons of lipotropic potency should be made only when the liver lipids are at or near equilibrium values characteristic of the lipotropic supplements in the diet.

To clarify the two main uncertainties in this literature, namely, the lipotropic potency of inositol by itself in rats following depletion of vitamins and the effect of the duration of the experimental period, these points have been studied using diets containing fat and diets free from fat. The effect on liver lipids of inositol alone, of choline alone and of the two together has been determined when animals were transferred from the depletion diet to similar diets containing an abundance of the B vitamins, and were fed the supplemented diets for different periods from 3 to 28 days.

EXPERIMENTAL

In the first experiment fat-free diets were used throughout the depletion and subsequent test periods. The percentage composition of the basal diet fed during the depletion period was casein, 10; arachin, 2; fibrin, 2; salt mixture (Beveridge & Lucas, 1945), 4; cellulose, 2; sucrose, 80; cod liver oil concentrate (Ayerst, McKenna & Harrison; 50,000 i.u. vitamin D/g., and 200,000 i.u. vitamin A/g.), 0.015. Diets containing 10 % casein as the only protein, formerly used by investigators in the field, are possibly somewhat inadequate (for young rats) in four or five essential amino-acids. The addition of 2 % arachin and 2 % fibrin improves this situation considerably, while keeping the methionine intake reasonably low.

Two hundred male rats of the Wistar strain (weighing 75–95 g.) were placed in individual cages and fed the depletion diet *ad lib.* for 21 days. The loss in weight during this period was much less than in previous experiments in which

10% casein alone supplied the protein. The average daily food consumption of the rats during the depletion period dropped from almost 9 g. to less than 2 g. to give an over-all average of about 5.5 g., and the average weight loss was about 8 g., as contrasted with 25–30 g. when the diet contained 10% casein as the only protein. Several rats were sacrificed at the end of the depletion period to obtain some idea of the initial liver lipid values. The animals were killed by stunning and exsanguination; livers were analysed individually for total lipids and for free and total cholesterol by methods previously described (Best *et al.* 1946*b*). The remaining rats were divided into twenty-four groups—eleven groups of 6 rats each and thirteen containing 10 rats each—and the vitamins shown in the upper part of Table 1 were added to the food offered all groups. The lipotropic supplements shown in the lower part of the table were added to the diets given certain groups, and these variously supplemented diets were fed for periods of 3, 5, 7, 9, 14, 21 and 28 days, respectively.

Table 1. *Supplements to basal ration*

Extra vitamins in all diets (per 10 g. food)

	μg.
Biotin	15
Folic acid	25
2-Methyl-1:4-naphthaquinone	50
Pyridoxine HCl	100
Riboflavin	125
Aneurin HCl	250
Calcium pantothenate	500
Nicotinic acid	500
p-Aminobenzoic acid	5000
α-Tocopherol acetate	1000

Lipotropic supplements (per 10 g. food)

Diet		mg.
1	(Basal)	None
2	Inositol	35
3	Choline chloride	35
4	{ Choline chloride	35
	{ Inositol	35

Later, an essentially identical experiment was performed, the only difference being the substitution of 12% fat (10% beef fat, 2% corn oil) for an equal weight of sucrose in the food during both the depletion and test periods.

RESULTS

With restoration of B vitamins to the diet, in amounts about five times those usually considered adequate for rats, there was a sudden increase in food consumption from about 2 to 8 g./day. This increased further within the next few days to about 12–15 g. The daily food intakes shown in Table 2 (column 3) do not reveal these sudden increases because the values have been averaged. During this period of excessive food intake an equally sudden increase in liver lipids may be noted in the rats given the basal diets (Figs. 1 and 3). After a week or 10 days, as the food intake declined and became relatively constant at 10–12 g., the liver lipids dropped

slowly from the peak values observed on the seventh day.

Some of the rats consuming the basal and inositol-supplemented (fat-free) diets developed haemorrhagic kidneys, and several in the 21- and 28-day groups died. This left these groups very small. To augment their numbers, therefore, forty-five slightly

Fig. 1. Changes in lipid content of livers of rats consuming fat-free diets. A, basal diet; B, supplementary inositol (0.35%); C, supplementary choline chloride (0.35%); D, supplements of both inositol (0.35%) and choline chloride (0.35%).

Fig. 2. Changes in cholesteryl ester content of livers of rats consuming fat-free diets. Dietary supplements as in Fig. 1.

older rats (males, 90–120 g.) were placed on the same fat-free depletion diet for 3 weeks. Five were killed at the end of this period and liver lipids were determined. Fifteen depleted rats were placed on the ration supplemented with B vitamins only (diet 1), fifteen were given supplementary vitamins plus inositol (diet 2) and ten were given vitamins plus choline (diet 3). These rations were fed for 28 days. In each case the liver lipid values were almost identical with those obtained in the original experiment and have been included in the data presented in Figs. 1 and 2 and Table 2.

Table 2 presents, in condensed form, the effects of inositol, of choline and of the two together upon the free and total cholesterol in the livers of the rats consuming the fat-free diets. The constancy of the

Table 2. *Effect of inositol and choline upon free and total cholesterol in liver*

(Rats depleted of B vitamins for 3 weeks and then fed, *ad lib.*, fat-free diets supplemented as shown for periods indicated in column 1. Mean, standard error and range are shown in columns 6 and 7.)

Time on diet (days)	Survivors/ starters	Average food intake (g./day)	Average weight change (g.)	Average liver weight (g.)	Liver cholesterol (mg./100 g. liver)	
					Free	Total
End of depletion period						
0	7/7*	5.5	- 8	4.4	203 ± 10 (173-244)	349 ± 50 (225-519)
Basal ration (diet 1)						
3	6/6	7.2	13	7.5	145 ± 8 (123-158)	373 ± 22 (295-440)
5	5/6	9.0	21	8.8	146 ± 5 (130-162)	677 ± 81 (527-918)
7	10/10	10.2	30	10.2	156 ± 5 (135-190)	836 ± 107 (406-1571)
14	9/10	9.2	39	9.4	166 ± 4 (149-185)	873 ± 78 (536-1286)
21	3/6	10.1	63	10.8	175 ± 5 (165-183)	972 ± 188 (759-1346)
28	16/21*	11.4	73	10.0	170 ± 3 (134-190)	822 ± 110 (286-1695)
Inositol (diet 2)						
3	6/6	7.1	16	7.4	139 ± 5 (127-155)	390 ± 75 (230-714)
5	6/6	8.9	19	6.6	168 ± 8 (144-188)	422 ± 40 (300-597)
7	10/10	9.8	28	6.9	191 ± 9 (139-241)	590 ± 88 (323-1324)
14	10/10	9.2	44	8.1	179 ± 9 (150-239)	563 ± 55 (325-907)
21	9/10	8.8	49	8.6	174 ± 5 (155-198)	616 ± 69 (281-909)
28	18/21*	11.0	73	9.1	175 ± 5 (122-217)	537 ± 54 (304-1083)
Choline (diet 3)						
3	6/6	6.2	14	7.2	146 ± 5 (130-159)	296 ± 14 (245-327)
5	6/6	8.7	22	6.9	153 ± 9 (117-175)	493 ± 56 (333-696)
7	10/10	9.0	29	7.5	186 ± 5 (162-221)	598 ± 58 (471-869)
9	8/10	10.2	41	8.4	169 ± 5 (148-189)	540 ± 76 (266-786)
14	10/10	10.1	55	7.6	178 ± 6 (146-219)	457 ± 56 (250-748)
21	6/6	11.0	80	8.5	175 ± 6 (154-191)	474 ± 94 (304-790)
28	16/16*	12.6	96	8.9	184 ± 4 (156-212)	404 ± 30 (269-675)
Inositol plus choline (diet 4)						
3	6/6	6.5	14	7.0	143 ± 5 (127-157)	256 ± 17 (220-315)
5	6/6	9.0	23	6.8	174 ± 3 (168-184)	402 ± 48 (247-540)
7	10/10	9.3	31	6.1	173 ± 7 (132-219)	289 ± 30 (164-442)
9	10/10	9.8	38	7.2	169 ± 7 (124-190)	289 ± 38 (162-563)
14	10/10	10.3	55	7.3	181 ± 7 (165-240)	316 ± 48 (228-617)
28	5/6	12.9	114	9.9	160 ± 6 (142-181)	258 ± 15 (228-314)

* As explained in the text (p. 454), extra rats were started later and incorporated in these groups to give a reasonable number of survivors.

free cholesterol values, regardless of the supplements in the diet or of the period during which the diets had been consumed by the rats is remarkable. Since the values for free cholesterol remain essentially constant throughout these procedures, it is obvious that

Fig. 3. Changes in lipid content of livers of rats consuming diets containing 12% fat. Units and supplements same as in Fig. 1.

Fig. 4. Changes in cholesteryl ester content of livers of rats consuming diets containing 12% fat. Units and supplements same as in Fig. 2.

the changing values for total cholesterol (the form in which the data are reported in the papers from McHenry's laboratory) represent variations in the amount bound. In the present studies this portion has been calculated as cholesteryl oleate (bound cholesterol $\times 1.68$) and is plotted as such in Figs. 2 and 4.

DISCUSSION

Inositol alone appears to be about as effective as choline in holding the liver lipids of depleted rats on fat-free diets at moderate levels during the first few days after restoration of B vitamins. After about 10–14 days the liver lipids in the rats receiving choline dropped below 10%, while in those getting inositol the lipids remained above 15% for several

weeks, falling slowly to about 12% after 28 days. Rats on the basal diet, i.e. receiving no choline, exhibited a slow steady fall in both total lipids and cholesterol during this same period. Thus as equilibrium is approached, inositol exerts an increasingly limited lipotropic action. The strong lipotropic effect of inositol on the fifth and seventh days is, however, of great interest and is so far unexplained. It may be that inositol can delay the rise in liver lipids, but cannot aid the fall.

On the first few days after restoration of vitamins to the fat-free diet the increase in food consumption, although abrupt, is not abnormal. During this period choline is able to effect a slight decrease in liver lipids (total lipids only 6.7% on the third day), but as the food consumption becomes excessive, the lipids rise in spite of the presence of choline. However, it is important to note that they reach scarcely one-half the values attained without choline. Only by examining the liver at the critical 7-day period and then only when the basal value is neglected may the impression be gained that the situation does not respond to choline. It is not generally appreciated that the 7-day data published by McHenry and his colleagues include nothing corresponding to points on either of the two upper curves in Figs. 1 or 2, i.e. to a basal value or to a value for inositol alone at 7 days. The figures presented as basal values (MacFarland & McHenry, 1948) are those obtained when several times the maximally effective dose of choline was present. Additional choline could scarcely be expected to produce any further lipotropic effect. A value corresponding to the 7-day point on the lowest curve (Fig. 1) has been erroneously described as the effect of inositol alone (MacFarland & McHenry, 1945), although the difference between curves C and D is obviously the supplementary effect of inositol. Choline and inositol acting together exert a distinctly more rapid action and a slightly greater equilibrium effect than choline alone in rats on the fat-free diet. Inositol alone does not exhibit any unique or specific effect upon either glycerides or free or bound cholesterol, at 7 days or at any other time.

When the diet contains fat no clear-cut lipotropic effect of inositol is obtained even in the early phases of the experiment. The slight decreases in liver fat seen in Figs. 3 and 4 at 7, 14, 21 and 28 days are of dubious significance. When the 't' test was applied to the differences observed between the results for rats consuming the basal and inositol-supplemented diets, *P* values from 0.1 to 0.6 were obtained. Thus there is no justification for claiming a lipotropic effect from inositol under these conditions when fat is present in the diet. The delay that occurs before choline is able to exert its maximal effect in fat-free diets is almost absent when the diet contains fat. Apparently, the ability to handle fat efficiently is

more seriously impaired when both fat and vitamins are removed from the diet than when vitamins only are withheld. Choline under these circumstances prevents the early rise in liver lipids that occurs when the diet is free from fat, and very quickly reduces the liver lipids to low levels (on the seventh day 9.1 %, on the fourteenth day 7.3 %). Choline plus inositol produced no significantly greater reduction in liver lipids in these fat-containing diets. The apparent difference on the fifth day is of questionable statistical significance ($P=0.06$).

A more rapid and excessive accumulation of cholesteryl esters was observed in the livers of the rats consuming the fat-free high carbohydrate basal diet than in those ingesting the diet containing fat, a finding similar to that recorded by Chanutin & Ludewig (1933) that carbohydrates accelerate and fat inhibits the deposition of cholesterol in the liver of the rat. On the fat-free diet values for cholesteryl esters reached their maximum (almost 1200 mg./100 g. of liver) within a week; on the diet containing fat, 3 weeks were required to reach the considerably lower maximal value of about 800 mg. Because the total liver lipids were higher in the groups consuming the fat-containing diet, the possibility had to be considered that these percentage values might be giving an erroneous impression because of the greater dilution of cholesterol by the larger amount of glyceride present in the latter group. However, a comparison of the absolute weight of cholesteryl esters per liver revealed a significantly greater amount at all times in the livers of the rats consuming the fat-free diet than in those ingesting some fat (e.g. compare 120 mg. with 51 mg. at 7 days, and 112 mg. with 71 mg. at 14 days).

It may be of physiological significance that the maximal levels of cholesteryl esters attained in the presence of inositol are almost the same under both dietary conditions. The greater lipotropic effect of choline than inositol on the esters is evident in Figs. 2 and 4. For reasons not as yet understood, choline reduces the esters to a considerably lower level (about 100 mg./100 g. liver in 28 days) in the presence of dietary fat than in its absence (esters over 300 mg.).

There never has been any question as to the supplementary effect in fat-free diets of the two lipotropic agents when used together. This effect is clearly shown by the data in Figs. 1 and 2. But even when the potency of the two agents is compared from this aspect, choline can be seen to be the more active; supplementary choline produces a much greater decrease from the level of liver lipids (or cholesteryl esters) resulting from inositol alone than supplementary inositol does from the lipid (or cholesteryl ester) level established by choline alone.

Thus inositol is less effective than choline either in direct or supplementary action in reducing either

total lipids or total (or more correctly, ester) cholesterol. This statement applies not only to conventionally fed rats, but also to those maintained under the special conditions (restoration of B vitamins to low protein, fat-free diets fed for 7 days only, following 3 weeks depletion of B vitamins), laid down (MacFarland & McHenry, 1948) as essential for the demonstration of the alleged effects of inositol. It is obvious, therefore, that inositol does not possess the unique lipotropic characteristics which have erroneously been attributed to it.

There is, of course, a nutritional need for inositol as a member of the vitamin B complex. When one considers the data in this present communication in conjunction with that in the related study by Best, Ridout, Patterson & Lucas (1950), one is tempted to question whether (provided that this small amount is supplied) there is ever, under any conditions of normal nutrition a 'lipotropic need' for further inositol. Although the activity of inositol when added to fat-free diets and the mechanism of this action is of great interest and potential importance, it is our opinion that inositol should not be regarded, at present, as an essential lipotropic agent. The absence of inositol from a diet produces none of the signs characteristic of the absence of the lipotropic factors, i.e. of choline and its precursors methionine and betaine. Choline added to a hypolipotropic diet fed to weanling rats consistently prevents the haemorrhagic kidney lesions, but when inositol alone is added these pathological changes are usually aggravated (Best *et al.* 1946a; Handler, 1946b).

SUMMARY

1. Young white rats that had subsisted for a preliminary period of 3 weeks on a diet devoid of vitamins of the B complex, when transferred to the same hypolipotropic ration containing an abundance of all commercially available B vitamins, exhibited an abrupt increase in food intake and in total lipids in the liver. The ability of maximally effective doses of inositol, of choline and of the two together to affect the rate of change in amount and composition of hepatic lipids under these special conditions has been determined.

2. Following the abrupt increase in liver lipids which reached a maximum after about 7 days, a slow spontaneous fall occurred even in the absence of supplementary choline or inositol.

3. Inositol appeared to be about as effective as choline in preventing the early increase in the total lipids seen in rats on the fat-free ration during the first 7 days.

4. As food consumption returned to normal and equilibrium conditions were re-established, inositol exerted a strictly limited lipotropic effect in fat-free diets and a negligible effect upon glycerides or total cholesterol when the diet contained fat.

5. Choline was more effective than inositol in reducing total lipids and total cholesterol in the liver, in both fat-free and fat-containing diets.

6. Choline exerted a stronger supplementary lipotropic effect than did inositol on both total lipids and total cholesterol.

7. No data were noted that would support the statement that inositol possesses unique lipotropic

properties, either under these special conditions (following depletion of B vitamins) or under any other conditions.

8. Reasons for the existing confusion in the literature as to the real lipotropic potency of inositol have been discussed.

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The Stabilization of D-Amino-acid Oxidase by Flavin-adenine Dinucleotide, Substrates and Competitive Inhibitors

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The stabilization of an enzyme by its substrate has been little studied since the effect was described by O'Sullivan & Tomson (1890). Delory & King (1943) concluded that the stabilization of alkaline phosphatase by different substrates increases with the rate of hydrolysis. Kunitz & McDonald (1946) could not correlate the substrate stabilization of yeast hexokinase with the rates at which the sugars react in the hexokinase system.

The stabilization of D-amino-acid oxidase of sheep kidney has now been studied and compared with the kinetics at the same temperature and pH.

EXPERIMENTAL

D-Amino-acid oxidase was prepared by the method of Negelein & Bromel (1939). The precipitate obtained after removal of the flavin-adenine dinucleotide (FAD) by acidifi-

cation in $(\text{NH}_4)_2\text{SO}_4$ solution at 0° , was washed in 16% $(\text{NH}_4)_2\text{SO}_4$ solution and suspended in 0.056M-pyrophosphate buffer pH 8.3 (250 ml./100 g. acetone-dried kidney) and the pH adjusted to 6.5.

The same preparation of oxidase was used for all the experiments reported in this paper except those connected with Table 3, for which several different preparations were used. The solution of oxidase was stored at -10° . Small batches were thawed and subsequently stored at 0° for use as required. Each sample was rejected when 0.20 ml. failed to catalyse the uptake of 110 $\mu\text{l. O}_2$ /20 min. in the activity test. (With no added FAD, about 10 $\mu\text{l. O}_2$ were consumed in 20 min.)

Catalase was prepared according to Keilin & Hartree (1945). After the first precipitation by $(\text{NH}_4)_2\text{SO}_4$, the catalase was dialysed until free from ammonium ions.

Flavin-adenine dinucleotide (FAD). The procedure of Warburg & Christian (1938) was followed to obtain a crude Ag salt of FAD from baker's yeast. The salt was suspended in water and decomposed by a method introduced by Dr M. Dixon: KCl was added to dissolve the suspended Ag salt and 2N-HCl was added drop by drop until there was no further precipitate. The suspension was centrifuged and the pre-

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