# A Statistical Evaluation of the Lipotropic Action of Inositol

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Investigators experienced in the field of lipotropic phenomena are aware that large, and as yet unexplained, variations may occur in the amount of fat deposited in the livers of rats consuming hypolipotropic diets. One of the earliest papers on this subject (Best, Hershey & Huntsman, 1932) reported a wide range of values (8–30 %) for the crude fatty acids in livers of rats on a mixed grain, high fat diet. Later, Best & Huntsman (1935) presented a graphic representation of the variations encountered in some of their experiments, and Beveridge & Lucas (1945) used the same technique to emphasize the great variability in the amount of fat accumulating in the livers of animals receiving hypolipotropic diets supplemented with inositol.

In spite of this well known variability, reports of lipotropic studies may be found in the literature in which only a small number of animals have been fed the test rations. Some workers have routinely used as few as five, or even three, animals on each diet. Where very large differences in liver fat are observed consistently between the test and control groups, the results may be accepted without hesitation, but frequently the differences are much too small to warrant the conclusions reached. Even with groups consisting of ten, fifteen or twenty rats, numbers that are commonly used in this laboratory, it has sometimes been difficult to decide whether or not the results of a particular treatment had any physiological significance because the differences in liver fat content between the controls and treated groups were small and erratic. Because extraction and analysis of large numbers of individual livers is so time-consuming, pooling of the livers of each group, before extraction, has been frequently practised in this and other laboratories. The mean values may thus be obtained with a minimum of labour, but then no data on the variability are available. One cannot assess the statistical significance of the differences between the means of the different groups without knowing the standard deviations. This matter has been of special interest to us in connexion with the assessment of the lipotropic activity of inositol in diets containing different kinds and amounts of fat.

The lipotropic effect of inositol observed in fat-free diets appears to be interfered with by the presence of corn (maize) oil (Beveridge, 1944; Beveridge & Lucas, 1945; Handler, 1946). Although Beveridge & Lucas (1945) had used twenty rats per group, unfortunately the livers of the rats given the diets containing saturated fats were pooled before analysis, and a statistical test of the significance of the differences between the means could not be applied. For reasons which have been discussed elsewhere (Best, Lucas, Patterson & Ridout, 1946; Ridout, Lucas, Patterson & Best, 1946) over fifty separate experiments have been conducted in recent years, using from ten to thirty rats per group, in attempts to clarify the situation with respect to the lipotropic action of inositol. In some of these experiments different kinds and amounts of fat were included in the diets, and it became increasingly clear to us that the lipotropic activity of inositol was interfered with not only by corn oil, but by all dietary fats studied. Statistical support for this impression was lacking, however, since the livers in each group had been pooled for analysis.

Later, several experiments with inositol were conducted in which individual livers were analysed. The wide variations observed in individual values for total liver lipids of rats on the inositol-supplemented rations, as well as in those of rats on the basal diet, aroused curiosity as to the actual significance of the differences observed not only by Beveridge & Lucas, but in all the previous experiments. The data accumulated up to this point made it obvious that a satisfactory interpretation could be expected only if a large number of individual livers were examined and the results were subjected to statistical analysis. Our findings confirm the necessity for statistical treatment of the data obtained with inositol because of the small and erratic changes which it produces in total liver lipids.

Several possibilities which might account for the extreme variability of the results were investigated and may be mentioned briefly. One of these was the unlikely possibility that commercial inositol may contain an unevenly distributed lipotropically-active contaminant. Finally, the interference of dietary fat either with the bacterial synthesis or absorption of inositol was considered. These several experiments, which have been conducted during the past 4 years, are now presented collectively, since the data may be of interest to others working in this field.

## EXPERIMENTAL

For the statistical assessment of the lipotropic activity of inositol 180 white rats of the Wistar strain, weighing 75±10 g., were fed the same type of ration as was used by Beveridge & Lucas (1945), namely, casein, 8; gelatin, 12; sucrose, 72; salt mixture, 5; celluflour, 2; 'vitamin powder', 1; cod liver oil concentrate, 0·015. (For details see Best, Lucas, Ridout & Patterson, 1950.) The rats were divided into three comparable groups. The first group (i.e. 60 rats) received the fat-free diet, the second was given the same diet to which had been added 2% of a saturated triglyceride fraction ($I_2$ val. = 2) isolated from beef fat by repeated fractional crystallization from acetone, and the third group was given the same basal diet to which had been added 2% of corn oil (Mazola brand). One half of the animals in each of the above groups (i.e. 30 rats) was given a dietary supplement of 0·1% inositol. The diets were fed for 3 weeks, without the preliminary period of depletion adopted by some investigators (Gavin & McHenry, 1941; MacFarland & McHenry, 1945, 1948; Handler, 1946). The animals were kept in individual cages and 'group pair-fed' as described elsewhere (Best *et al.* 1946). Extraction of total liver lipids with hot ethanol, rectification of the crude lipid residue with light petroleum, and analytical details are described in the same publication.

Smaller groups of rats (8 males, 8 females, 100–130 g.) were used to test the lipotropic activity of inositol (0·3%) in diets containing: (1) 10% of a moderately saturated fat (hydrogenated cotton-seed oil, Crisco), (2) mixed fats (Crisco 10% and corn oil 2%).

Commercial inositol (General Biochemicals, Inc.) was used for most of the work, but for the study of the possible presence of a lipotropic contaminant a sample of very highly purified inositol was obtained from Prof. H. O. L. Fischer, to whom we are most grateful. This material was prepared by several recrystallizations of a 'pure' commercial sample of the free hexitol from water, then from glacial acetic acid, conversion to hexapropionic ester, fractional distillation of the latter in high vacuum, hydrolysis and further recrystallization of the free hexitol. The lipotropic potency of this material was compared with that of the commercial inositol at a dietary level of 0·15% in rats (7 males and 8 females, 100–150 g. per group) on the fat-free diet.

Finally, variable absorption of inositol was considered as a possible cause of the spread in individual values. Since interference of fat with the absorption of inositol might possibly explain its lack of lipotropic effect in diets containing fat, the lipotropic potency of inositol when injected subcutaneously was compared with that of equal quantities ingested with fat-free and fat-containing diets. Three groups of 10 rats each (5 males, 5 females, 80–100 g.) were fed the usual fat-free diet. One group served as a control. The second group received 0·16% inositol in the diet and the third group (started 3 days later) was injected subcutaneously with a solution of inositol containing 16 mg./ml. of physiological saline. These animals were group pair-fed with the second group, and the amount of inositol to inject was calculated from the amount of diet consumed by the former group of rats. Three other comparable groups of rats were fed the same basal diet in which 12% fat (10% beef fat and 2% corn oil) was substituted for an equal quantity of sucrose. These rats were treated in a similar fashion to the rats on the fat-free diet.

## RESULTS

The results of the statistical assessment are shown graphically in Fig. 1, where total liver lipids as percentage of wet liver weight are given for individual rats. The effects of sex and of pooling of values may be seen at a glance. These data are condensed and analysed statistically in Table 1. Data from individual rats will be supplied upon request in a form enabling the results to be calculated to any desired basis.

Attention is drawn to the difference in response of males and females to the basal diets. The significance of a sex difference in the response of older rats to hypolipotropic diets has not been generally recognized.

*Fat-free diets.* The lipotropic effect of inositol in a fat-free diet was confirmed (cf. groups 1 and 2 in Table 1). The difference between the mean values for total liver lipids of all surviving rats in these two groups (20·1–15·1 = 5·0) is statistically significant ($P = 0·002$); essentially the same values and conclusions are reached when data from all animals (from various experiments) fed a fat-free diet are pooled (see groups *A* and *B* in Table 1).

*Diets containing fat.* When 2% of an almost completely saturated, naturally occurring glyceride fraction from beef fat was added to the basal diet, no lipotropic effect of inositol could be detected (cf. groups 3 and 4 in Table 1). The lipotropic effect of inositol, when 2% of corn oil is included in the diet, remains questionable, since the interpretation of these data for all rats (i.e. males and females) is ambiguous (groups 5 and 6, Table 1). The difference (3·1) observed between the means could occur by chance once in twenty trials. In diets containing 10% of a hydrogenated vegetable fat (Crisco) inositol exhibited no lipotropic effect whatever (groups 7 and 8). The addition of 2% corn oil to diets containing 10% Crisco did not appreciably alter the picture (groups 9 and 10).

*Sex difference in lipotropic response.* The data have been re-examined to find out whether there is any difference between male and female rats in their response to inositol, and if so, to assess the significance of any apparent differences. Cols. 5 and 7 in Table 1, giving the means with standard errors and numbers of test animals, supply the data necessary to make such comparisons and the other columns (4, 6, 8 and 9) give the probability of the differences observed being due to chance (sampling error). The data pooled under the headings groups *A* and *B* indicate that inositol exerts a small but statistically significant lipotropic effect in both male and female rats fed fat-free diets.

In the case of diets containing the saturated fat (groups 3 and 4) no lipotropic action in the males was observed. In fact, the liver fat appeared to be in-

creased slightly, although the reality of this increase is doubtful ($P = 0{\cdot}05$). In the case of the females, a small decrease in the mean liver fat was noted, but the difference is not significant. Male rats on diets containing 2% corn oil (groups 5 and 6) showed no lipotropic response whatever to inositol; however, in the female rats inositol caused a decrease in the liver lipids which was moderately significant ($P = 0{\cdot}03$). In the diets containing Crisco (groups 7 and 8) or Crisco and corn oil (groups 9 and 10) no lipotropic effect of inositol in either males or females was observed.

When differences in the amount of liver fat between males and females on the same diets were compared, highly significant differences were apparent in two cases (groups 3 and 5), and it is interesting to note that the sex difference appeared in the rats consuming the basal diets and not in those consuming diets supplemented with inositol. Both of these basal diets contained fat, and the data suggest that female rats fed fat-containing diets

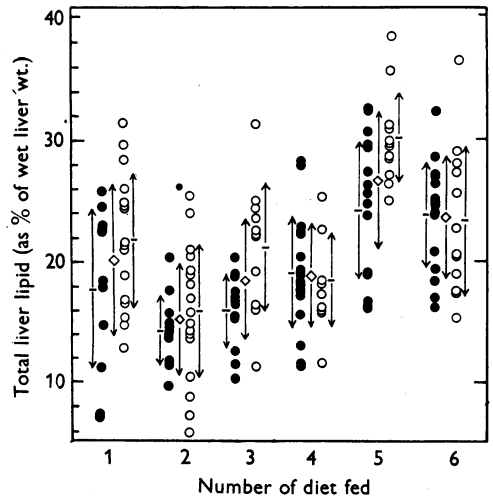


Fig. 1. Total liver lipids of rats on various diets. ●, Individual males; ○, individual females. ←|→, mean ± standard deviation for males and females on each diet. ←◇→, mean ± standard deviation for all rats on each diet.

Table 1. *Assessment of lipotropic activity of inositol*

(The average daily food consumption of the rats in groups 1–3 was 7·2 g., in group 4 it was 7·8, in groups 5 and 6 it was 8·0, in group 7 it was 9·4, and in groups 8–10 it was 10·1 g.)

| | | Total liver lipids (% wet weight) and their significance | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Group no. | Type of diet | All rats | *P* | Males | *P* | Females | *P* | *P* of difference between sexes being due to chance |
| 1 | Fat-free | 20·1±1·21 (27)* (7·2–31·5) | 0·002 | 17·7±2·0 (11) (7·2–25·7) | 0·1 | 21·8±1·4 (16) (12·8–31·5) | 0·001 | *P* = 0·1 |
| 2 | Fat-free + inositol (0·3%) | 15·1±0·87 (29) (5·8–25·4) | | 14·2±0·84 (12) (9·7–20·3) | | 15·8±1·4 (17) (5·8–25·4) | | *P* = 0·8 |
| *A*† | Fat-free | 19·4±0·71 (80) | 0·001 | 18·5±1·0 (38) | 0·001 | 20·2±0·98 (42) | 0·015 | *P* = 0·25 |
| *B*† | Fat-free + inositol | 15·2±0·62 (69) | | 13·9±0·72 (36) | | 16·7±0·99 (33) | | *P* = 0·03 |
| 3 | Saturated fat 2% | 18·4±1·1 (23) (10·2–31·4) | 0·8 | 15·9±0·89 (12) (10·2–20·3) | 0·05 | 21·2±1·6 (11) (11·2–31·4) | 0·2 | *P* = 0·01 |
| 4 | Saturated fat + inositol (0·3%) | 18·8±0·82 (29) (11·3–28·3) | | 19·0±1·1 (18) (11·3–28·3) | | 18·4±1·2 (11) (11·5–25·3) | | *P* = 0·75 |
| 5 | Corn oil (2%) | 26·7±1·1 (29) (16·2–38·4) | 0·05 | 24·2±1·4 (17) (16·2–32·6) | 0·8 | 30·2±1·1 (12) (25·1–38·4) | 0·03 | *P* = 0·01 |
| 6 | Corn oil + inositol (0·3%) | 23·6±1·0 (28) (15·3–36·4) | | 23·8±1·1 (16) (16·2–32·4) | | 23·3±1·8 (12) (15·3–36·4) | | *P* = 0·8 |
| 7 | Crisco (10%) | 24·0±1·1 (15) (17·2–29·2) | 0·4 | 23·0±1·2 (8) (18·8–29·2) | 0·7 | 25·1±1·0 (7) (17·2–28·9) | 0·15 | *P* = 0·25 |
| 8 | Crisco + inositol (0·3%) | 22·5±1·3 (15) (13·2–31·1) | | 23·8±1·2 (8) (18·7–31·1) | | 21·0±2·1 (7) (13·2–27·9) | | *P* = 0·3 |
| 9 | Crisco 10% + corn oil 2% | 26·5±1·3 (15) (16·1–39·3) | 0·6 | 26·6±1·2 (7) (23·8–30·7) | 0·2 | 26·5±2·4 (8) (16·1–39·3) | 0·9 | *P* = 0·9 |
| 10 | Crisco 10% + inositol (0·3%) | 25·2±1·9 (16) (15·1–37·0) | | 22·9±1·8 (8) (15·5–32·1) | | 27·4±3·3 (8) (15·1–37·0) | | *P* = 0·25 |

* Mean with standard error; range is shown below; number of survivors in parenthesis.
† All animals (from various experiments) fed fat-free diets.

tend to deposit more fat in their livers than do the male rats. Consideration of all of the data in columns 5 and 7 indicates that this is generally true, although, as shown in column 9, the differences are in most cases not sufficiently great to be of much statistical significance. Such comparisons reveal that any 'lipotropic' effect of inositol, if observed in female rats consuming diets containing both fat and inositol, is a peculiar artifact. The data indicate a tendency for female rats on these basal diets lacking inositol to deposit somewhat more fat in their livers than do the males. The apparent lipotropic effect of inositol in females consuming hypolipotropic diets containing fat is therefore merely the influence of inositol in bringing the excessively fatty liver of these female rats back into line with that of the males (which exhibit no, or at most a negligible, response to inositol when the diet contains fat). This difference between the sexes in response to inositol deficiency appears to be real since in both basal groups, i.e. those ingesting saturated fats (group 3) and those getting corn oil (group 5), the differences observed between the means (5·3 and 6·0, respectively) would not occur by chance once in a hundred times.

*Purified inositol.* The comparison of the purified specimen with commercial inositol did not reveal any difference in lipotropic activity. The percentage of lipids in the livers (pooled) of the rats on the fat-free basal diet was 26·0. The supplement of purified inositol reduced the mean liver fat to 18·2% with a standard error of ±2·8 (range 6·0–29·9), whilst the commercial product gave a mean value of 19·9% ±1·7 (range 10·4–30·6). The '$t$' test showed that there was no significant difference in the response to the two products ($P = 0·6$).

*Effect of fat on absorption of inositol.* The livers of the rats used in the comparison of the effectiveness of dietary and injected inositol were pooled before analysis. In the three groups consuming fat-free diets the liver lipids were (1) basal, 20·9%, (2) ingested inositol, 13·7%, (3) injected inositol, 14·5%. A marked lipotropic effect is obvious in both cases. However, when the diets contained fat the results are less clear cut, especially in the light of our subsequent observation of the influence of the sex of the rat on apparent lipotropic effect of inositol: (4) basal 30·2%, (5) ingested inositol 23·6%, (6) injected inositol 26·6%. Since the injected inositol was no more (and apparently less) effective than ingested inositol in diets containing fat, interference with absorption of inositol by dietary fat appears to be excluded as an explanation for the obliteration of the lipotropic effect of inositol by the fat in these diets.

## DISCUSSION

It should be emphasized that in all of these experiments healthy rats not subjected to any preliminary dietary depletion were used, and that the rations were fed for a period (3 weeks), which previous experience had shown permitted establishment of a state of equilibrium with respect to deposition of liver lipids.

The livers of young rats (70–100 g.) consuming these hypolipotropic diets exhibit an extremely variable lipid content (Fig. 1). The livers of the females contain consistently more lipid material and display a much greater variability than do those of comparable males. These variations are essentially as great whether the results are expressed as absolute weight of liver lipids, percentage of wet liver weight, or percentage of dry, fat-free liver residue. The advisability of using, preferably, animals of one sex for lipotropic studies is obvious from a glance at Fig. 1. If this is not feasible, equal numbers of animals of each sex should be used. Furthermore, the data illustrate clearly the importance of using large numbers of animals in experiments with inositol, where the variations encountered in both basal and treated groups are so large that the small effects to be expected may easily be submerged by biological variation. It is obvious that pooling of the livers before analysis should be avoided, since under such conditions no assessment of the significance of even moderate differences is possible.

Fig. 1 presents data which illustrate, in graphic form, the small but definite lipotropic effect which inositol exerts when diets devoid of fat are used. Table 1 shows that the differences observed between the liver lipids of groups 1 and 2 (and of $A$ and $B$) are highly significant. Consideration of the data from all the remaining groups, i.e. from rats ingesting highly saturated fats (groups 3 and 4), moderately saturated fats (groups 7 and 8), unsaturated fats (groups 5 and 6) or mixed fats (groups 9 and 10) reveals no clear-cut evidence of a lipotropic effect of inositol.

The ambiguity of the effect of inositol in diets containing fat raises a question as to the correct interpretation of the observations reported by Beveridge & Lucas (1945). The obliteration of the lipotropic action of inositol by corn oil is confirmed by the present investigations. This effect is not specific to corn oil, however, since all of the dietary fats tested appear to block the action in a similar fashion. The apparent lipotropic effect observed by Beveridge & Lucas in the presence of saturated fat was small; in the light of the data presented in the present paper the difference observed was almost certainly an expression of biological variation. If Beveridge & Lucas had not pooled the livers of their animals of series $B$ before analysis, they would doubtless have seen the variability of the values and probably would have concluded, as we have, that the saturated fat fraction is just as inhibitory as the corn oil, i.e. that saturated as well as unsaturated fats interfere with the lipotropic action of inositol.

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-propiothetin. 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).