can be sufficiently strong to react with the small number of the more polarizable excited molecules in solution. These excited molecules are present in extremely small concentration, but they alone are responsible for fluorescence.

We thus arrive at the conclusion that, despite apparent discrepancies, the quenching effects of purines are due to the same intermolecular forces as are responsible for their solubilizing effect and the formation of molecular compounds.

SUMMARY

1. The fluorescence of polycyclic aromatic hydrocarbons in aqueous caffeine solution is quenched by acidification. The quenching is reversible on neutralization. The active quenching agent is the caffeine ion, not the H-ion.

2. Other purine bases, such as hypoxanthine, xanthine and *N*-methylxanthines, have an effect similar to that of caffeine. Uric acids quench even in pure water or aqueous ethanol, and their quenching effect is not appreciably affected by changes in the pH of the medium. A third group of purines, comprising compounds with electro-negative substituents other than uric acids, and including adenine and guanine, occupy an inter-

mediate position in that they quench without acidification, but their quenching effect is somewhat intensified in acid solution. Substances without the 4:5-double bond or the double bond adjacent to C₈ do not quench.

3. The quenching effects are strongest in water and decrease in less polar solvents.

4. The quenching is due to a reversible photochemical reaction and not to absorption of either primary or secondary radiation.

5. The quenching effects were also observed with non-hydrocarbons, such as quinine sulphate, acridine sulphate, quinaldine sulphates, thiochrome, riboflavin, fluorescein. Neutral caffeine solutions sometimes exert a quenching effect, e.g. on riboflavin, and sometimes they cause an intensification of fluorescence, e.g. with thiochrome, mepacrine or eosin.

6. The results are discussed, and it is concluded that, despite apparent discrepancies, the quenching effects of purines are due to the same intermolecular forces as are responsible for the solubilizing effect and the formation of molecular compounds.

I am greatly indebted to Dr J. Weiss for help with the discussion part of this paper.

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The Influence of Biotin upon the Relative Lipotropic Effects of Choline and Inositol

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(Received 4 February 1946)

It has been reported recently from this department (Beveridge & Lucas, 1945) that choline was more effective than inositol in decreasing the cholesteryl esters in the livers of rats which had been fed on a diet low in the lipotropic factors. This incidental finding was difficult to reconcile with the repeated claims of McHenry and his collaborators (Gavin & McHenry, 1941*a, b*; Gavin, Patterson & McHenry, 1943) that 'inositol is effective under conditions unfavourable for the action of choline particularly when large amounts of cholesterol are present in the liver' (McHenry & Patterson, 1944, p. 159). A more extensive comparison of the relative lipotropic effects of choline and inositol was therefore under-

taken and the results of a number of studies in which biotin was fed are reported in this paper. No evidence has been secured to support McHenry's contentions (*a*) that biotin produces a fatty liver characterized by a high content of cholesterol, or (*b*) that the fatty liver observed when biotin is fed is 'definitely resistant to the action of choline' (McHenry & Patterson, 1944, p. 136). Recently MacFarland & McHenry (1945) have modified the original statement from their laboratory and state that the fatty liver produced by administering biotin is partially responsive to choline. They now report that both choline and inositol exert an effect upon the cholesterol content of the liver. While our

experimental results are in agreement with this latter observation, they demand a much more extensive revision of McHenry's original interpretation than has as yet been made. Our results lend no support to the idea still prevalent in the literature that biotin interferes, in any way, with the lipotropic action of choline or that inositol has a selective effect upon the fatty liver observed in experiments in which biotin is administered.

An attempt has been made to determine whether the preliminary period of vitamin depletion used by other workers produces an accelerated deposition of liver lipids during the test period as a specific response to the avitaminosis or as a result of the decreased food intake which is inevitable under these conditions.

METHODS

General. For each experiment about 180 young rats (60–120 g.) of the Wistar strain were placed on depletion diets for 3 weeks (see below). At the end of this period the animals were divided into 17 groups with similar weight and sex distribution and one group was killed to determine the condition of the livers. The remaining 16 groups were then given the test diets as described below. Each diet was given to two groups. One group was killed after 1 week and the other after 3 weeks. Throughout the experiment the rats were housed in individual cages with a false bottom of coarse wire screen. Water was available at all times. Weighed amounts of diet were placed each morning in galvanized iron food trays, designed to minimize spilling. The amount left over and the scattered material were weighed daily, from which individual food consumptions could be calculated.

The animals were killed by a blow on the head. Livers were removed immediately and weighed. When small portions were taken for histological examination, allowance was made for the weights removed.

Extraction and analysis of liver fat. The pooled livers were covered with freshly redistilled acetone and disintegrated in a Waring blender. The tissue brei was transferred quantitatively to large centrifuge pots and acetone added, equal to five times the weight of the livers. After about 1 hr. the mixture was centrifuged and the acetone extract decanted into a large distilling flask. The residue was extracted at least four times with hot absolute ethanol (3 ml./g. of wet liver) under reflux. In the case of very fatty livers as many as seven extractions have been used. The hot extracts, separated by centrifuging, were added to the flask containing the acetone. The extracted tissue residue was transferred quantitatively to a Büchner funnel with hot ethanol. It was sucked as dry as possible, dried further in a current of warm air, transferred to a

vacuum desiccator and brought to constant weight over P_2O_5 . Completeness of extraction was verified by saponification of the dry tissue residue with hot alkali, acidification and extraction with light petroleum. Usually the fatty acids thus obtained weighed less than 0.1% of the total lipid extracted.

The combined extracts were taken to dryness *in vacuo* (bath temperature about 45°) under N_2 or CO_2 . The fatty residue was dehydrated by adding 25–100 ml. of a benzene-ethanol mixture (4:1) and again distilling off the solvent *in vacuo*. This was repeated if necessary. The lipids in the residue were taken up in a number of small portions of light petroleum (b.p. 30–60°). These were centrifuged at high speed, giving a crystal-clear solution of the lipids which was made to volume at 2° and stored in the refrigerator at this temperature.

Samples were removed (in the refrigerator) on which total lipids, phosphorus and cholesterol (free and total) were determined in duplicate. Phosphorus was measured by the method of King (1932) and cholesterol by a slight modification of the Schoenheimer-Sperry procedure (Sperry & Brand, 1943). Phospholipins were calculated by multiplying the phosphorus content by the factor 25.2; the content of bound cholesterol multiplied by 1.68 was assumed to give the weight of cholesteryl esters; the undetermined residue, calculated by difference, has been referred to as glyceride.

Beveridge & Lucas (1945) have discussed briefly the different methods of expressing results of liver-fat determinations. For ease of comparison with values in the literature, total lipid figures are presented in Tables 1 and 2 as percentage of wet liver weight, and also as mg./liver. From these figures and the other data in the tables, the percentage of wet weight or of dry residue weight for any component may be calculated.

Diets (Depletion Period). In Exp. I, during the 3-week depletion period, a fat-free diet of the following percentage composition was fed *ad lib.*: casein 10, sucrose 84, salt mixture no. 185 (McCollum & Simmonds, 1918) 4, cellulflour 2, and cod-liver oil concentrate 0.015 (Ayerst, McKenna & Harrison; 50,000 i.u. vitamin D/g., and 200,000 i.u. vitamin A/g.). This diet, lacking all B vitamins, is essentially the same as that used by McHenry and his collaborators (Gavin & McHenry, 1940) during the depletion period common to most of their studies in this field. Cellulflour, which has no lipotropic activity, was substituted for the agar used by McHenry. The food consumption of the animals on this deficient diet steadily decreased from almost 10 g. daily at the beginning to about 3–4 g. at the end of the depletion period.

In Exp. II, the same basal diet was fed during the depletion period, but supplements of both choline and inositol were included (20 mg. of each/day in

8 g. of food; choline was given as chloride, 23 mg./day). The following B vitamins were injected daily in 0.5 ml. physiological saline solution: aneurin hydrochloride 25 µg., pyridoxin 20 µg., riboflavin 20 µg., nicotinic acid 100 µg., calcium pantothenate 100 µg. The rats also received daily injections of a liver concentrate stated to contain 5 µg. biotin (liver concentrate stated to contain 20 µg. biotin/ml. (S.M.A. Corporation); each rat received 0.25 ml.) During each week of the depletion period the animals in Exp. II were fed the same average amount of food consumed by the rats of Exp. I during the corresponding week. Thus, in this experiment, during the depletion period, the diet was adequate with respect to vitamins but the caloric intake was limited to correspond to that in Exp. I.

Test period. All diets, freshly prepared each week, were stored in tightly closed tin cans in a refrigerator at about 2°. In Exp. I the basal ration during the

test period was the same as that used during the depletion period, but was supplemented by injections of the following B vitamins: aneurin, pyridoxin, riboflavin, nicotinic acid and calcium pantothenate in the amounts described above under Exp. II. The basal ration supplemented with these vitamins is referred to as the control diet. The diets during the test period of Exp. II were the same as the test diets of Exp. I. One half of the rats in each experiment also received 5 µg. crystalline biotin daily by injection, and lipotropic factors were added to the diet as shown in the tables.

During the test periods the animals in each group were 'group pair-fed' with those on the control ration (Diet I), i.e. the average food consumed/rat/day was determined for group 1 of each experiment and this quantity (plus a small amount to allow for scatter) was offered to each rat in groups 2-8 on the following day. The actual food intakes are given in Tables 1 and 2.

Table 1. *Effects of biotin, choline and inositol on rats after a period of depletion of B vitamins*

(Average initial weight of rats was 90 g. (range 62-199 g.))

Group	Addition to diet		Survivors Starters	Av. food intake (g./day)	Wt. change (%)	Liver			Composition of liver lipids (mg./liver)					
						Av. wet wt. (g.)	Av. dry residue weight (g.)	Total lipids (% wet wt.)	Cholesterol					Ester (calc. as Glyc- eride)
	Substance	Amount (mg./day)							Total lipids	Phospho- lipins	Free	Total	oleate	
0	None (depletion period)		8/8	5.4	-21	3.64	0.811	8.28	301	134	7.5	11.9	7.5	152
Test diets fed for 1 week														
(a) Diet contains B vitamins but no biotin														
1	None	—	9/9	7.0	+15	5.34	1.154	13.74	734	116	8.9	21.7	21.6	588
2	Choline	20	9/9	7.6	25	4.78	1.044	8.65	414	137	9.8	18.7	15.0	252
3	Inositol	20	9/9	7.8	22	4.75	0.978	10.48	499	140	9.8	17.8	13.5	336
4	{ Choline + inositol	20 20	9/9	7.9	24	4.34	0.960	6.80	295	144	8.9	13.9	8.5	134
(b) Diet contains B vitamins plus 5 µg. of biotin														
5	None	—	9/9	7.9	17	5.96	1.058	19.36	1156	119	8.9	29.5	34.7	993
6	Choline	20	9/9	7.6	25	4.51	0.956	8.38	378	137	9.8	17.8	13.5	218
7	Inositol	20	9/9	7.5	19	4.22	0.876	10.70	452	116	8.4	16.1	13.0	315
8	{ Choline + inositol	20 20	9/9	7.5	21	3.96	0.881	6.30	249	130	8.4	11.7	5.6	105
Test diets fed for 3 weeks														
(a) Diet contains B vitamins but no biotin														
1	None	—	7/9	8.2	+45	6.02	1.210	15.17	913	158	11.4	36.4	42.1	702
2	Choline	20	8/9	7.9	49	5.33	1.287	7.99	426	134	9.1	20.0	18.4	264
3	Inositol	20	9/9	7.7	36	5.69	1.144	10.09	575	136	10.0	27.8	30.0	399
4	{ Choline + inositol	20 20	9/9	8.1	51	5.36	1.232	5.78	311	150	9.8	13.9	6.9	144
(b) Diet contains B vitamins plus 5 µg. of biotin														
5	None	—	9/9	8.1	43	6.67	1.193	22.95	1530	150	11.5	54.4	74.3	1294
6	Choline	20	8/9	7.9	49	5.10	1.194	7.84	399	139	9.5	19.0	16.0	234
7	Inositol	20	9/9	8.1	43	5.41	1.106	13.46	728	130	10.0	27.2	29.0	559
8	{ Choline + inositol	20 20	9/9	7.9	49	4.86	1.140	6.04	295	133	8.9	12.2	5.7	147

Table 2. *Effects of biotin, choline and inositol on rats after caloric restriction but without vitamin depletion*

(Average initial weight of rats was 76 g. (range 57–101 g.))

Group	Addition to diet		Survivors	Av. food intake (g./day)	Wt. change (%)	Liver			Composition of liver lipids (mg./liver)					
						Av. wet wt. (g.)	Av. dry residue weight (g.)	Total lipids (% wet liver wt.)	Cholesterol					Glyc- eride
	Substance	Amount (mg./day)							Total lipids	Phospho- lipins	Free	Total	Ester (calc. as oleate)	
0	B vitamins, biotin, choline and inositol (see text)		10/10	5.8	-6.4	4.55	1.032	5.09	232	126	8.8	10.5	2.9	94
Test diets fed for 1 week														
(a) Diet contains B vitamins but no biotin														
1	None	—	9/9	7.5	+13	5.41	1.166	14.28	772	129	10.0	23.3	22.5	610
2	Choline	20	9/9	7.6	17	4.79	1.177	6.60	316	123	9.1	13.9	8.1	176
3	Inositol	20	9/9	7.3	17	5.39	1.127	12.28	662	133	10.6	22.2	19.5	499
4	{ Choline + inositol	20	8/8	7.7	20	4.60	1.119	5.40	248	122	8.1	11.9	6.2	112
(b) Diet contains B vitamins plus 5 µg. of biotin														
5	None	—	9/9	7.6	15	5.74	1.203	16.36	940	140	10.3	28.9	31.3	758
6	Choline	20	8/9	7.7	17	4.91	1.140	7.63	374	134	9.4	16.3	11.6	219
7	Inositol	20	9/9	7.8	16	5.12	1.082	12.64	648	126	9.4	21.7	20.7	492
8	{ Choline + inositol	20	7/8	7.7	17	4.58	1.070	5.33	244	130	9.0	12.1	5.2	100
Test diets fed for 3 weeks														
(a) Diet contains B vitamins but no biotin														
1	None	—	9/9	7.8	+29	6.31	1.418	16.99	1050	144	11.5	38.4	45.3	849
2	Choline	20	8/9	7.8	42	5.54	1.132	7.47	419	158	10.6	20.0	15.8	235
3	Inositol	20	9/9	7.8	37	5.47	1.212	8.48	464	144	10.3	24.4	23.8	286
4	{ Choline + inositol	20	8/8	7.8	44	5.24	1.206	6.17	324	165	9.8	13.8	6.8	142
(b) Diet contains B vitamins plus 5 µg. of biotin														
5	None	—	9/9	7.3	25	5.98	1.128	17.42	1042	140	11.1	41.7	51.5	839
6	Choline	20	9/9	7.8	40	5.09	1.152	7.42	378	154	10.0	17.2	12.2	202
7	Inositol	20	9/9	7.9	31	5.50	1.146	12.54	690	154	11.1	28.3	29.2	496
8	{ Choline + inositol	20	7/8	7.9	39	5.06	1.179	5.73	290	149	9.7	13.6	6.6	125

RESULTS

The results are given in Tables 1 and 2. The data for Fig. 1 are based on those in Table 1.

DISCUSSION

The increased deposition of liver lipids which has been reported (McHenry & Patterson, 1944) to occur following the injection of biotin, has been observed in some but not in all of our experiments. This increase is clearly demonstrated by the data in Table 1 (cf. groups 1 and 5). This effect of biotin is also seen but to a lesser extent (Table 2) when, as in Exp. II, there was no preliminary 3-week period of vitamin depletion. In this second experiment the food intake was, however, restricted during the 3-week preliminary period to correspond to that consumed by the rats in the first experiment when

Fig. 1. Relation between cholesteryl esters and glycerides in liver lipids.

the latter were on the diet lacking the B vitamins. Vitamin depletion during the preliminary period is

thus not necessary to produce a rapid deposition of liver lipids, but a lowered caloric intake may be. We have also encountered conditions under which biotin has little if any effect upon the quantity of lipids deposited in the liver: (a) after the preliminary period of depletion of B vitamins, when a moderately large amount of fat (20 % beef dripping) was added to the control diet in the test period, and (b) in one experiment when no fat was added to the test diet (i.e. under conditions similar to those of Exp. I), but in which the control group of rats developed particularly fatty livers.

The data presented in Tables 1 and 2 indicate that the fatty liver produced by giving biotin is not characterized by a selective deposition of cholesteryl esters. This finding has been confirmed in other experiments not reported here. Numerous scatter diagrams were prepared to illustrate more clearly the relationship of the levels of cholesteryl esters to those of glycerides in liver lipids of rats on various diets. Because of their essential similarity, only one of these diagrams is presented. In Fig. 1, which is based on data in Table 1, an almost linear increase of cholesteryl esters is seen to occur as the glycerides accumulate. The points lie on the same line whether or not biotin is present. Thus there is no support for the statement that the so-called biotin fatty liver is characterized by an excessive accumulation of bound cholesterol.

The lipotropic effect of choline upon the total liver lipids is invariably greater than is that of inositol, whether biotin is present or not. The data in Tables 1 and 2 show that both glycerides and steryl esters were reduced by choline to essentially the same levels whether or not biotin was given to the rats. Fatty livers produced by injections of 5 μ g. crystalline biotin (following a 3-week period of vitamin or caloric depletion) obviously do not show any specific resistance to the action of choline.

Neither the specific lipotropic effect of inositol upon 'biotin fatty livers' nor its specific effect upon cholesteryl esters, originally claimed by McHenry, was noted in any of some twenty experiments. Those in which cholesterol was fed will be reported in a later communication. In short-term experiments (1 week) choline was at least as effective as inositol in reducing the bound cholesterol content of liver lipids. The presence of biotin did not affect this result. In more prolonged experiments (3 weeks) choline was more active than inositol in this respect and again the presence of biotin did not alter the result in any way.

The synergistic effect of a combination of choline and inositol is definitely shown in Tables 1 and 2. Both glycerides and bound sterols are reduced to a significantly lower level by the combination of these two lipotropic factors than by either one alone.

Biotin does not interfere with the lipotropic effect of the combination.

It may be mentioned that there is no evidence of any effect of choline, inositol or biotin on the absolute amount of phospholipin or free cholesterol in the livers (Tables 1 and 2). The kidneys of all the rats used in these experiments were analyzed and no effect of these supplements on any component of the lipids was observed.

A number of deductions have been made by McHenry and his collaborators which have been based on the now untenable conclusion that 'choline has no appreciable effect in preventing biotin fatty livers' (Gavin *et al.* 1943, p. 278). Two of these may be mentioned. McHenry & Cornett (1944, p. 9) state that they have found that the addition of biotin to high carbohydrate diets containing pyridoxin and aneurin quickly causes the production of fatty livers containing large amounts of cholesterol but that it fails to do so with protein-rich diets containing the same supplements. On the basis of these results they suggest that the pathway for the synthesis of fat from protein does not proceed by way of carbohydrate. Their evidence does not support this suggestion, since it is probable that the high protein diets provided methionine for the formation of choline, which would presumably inhibit the development of fatty livers under their experimental conditions. McHenry (1941, p. 189) has also speculated that 'Practically all of the existing evidence can be reconciled if we postulate that the development of a fatty liver in the depancreatized dog is due to the positive action of biotin, supplied in the meat of the diet, plus a failure of digestion which prevents the liberation of a lipotropic factor so that it can be absorbed'. The second part of this suggestion does not concern us here. The first part relating to biotin is now pointless in view of the absence of proof of any specific effect of biotin in the production of a particular type of fatty liver in any species. There would appear to be no justification for retaining the term 'biotin fatty liver'.

SUMMARY

1. No evidence has been obtained to support the claims (a) that biotin produces a selective deposition of cholesteryl esters in liver, (b) that inositol has a specific effect on bound cholesterol, and (c) that the fatty liver observed when biotin is administered is particularly resistant to the action of choline.

2. Evidence has been secured that under these experimental conditions, the accumulation of cholesteryl esters in liver bears a remarkably constant relationship to the deposition of glyceride in this organ. The administration of biotin does not affect this relationship.

3. The synergistic effect of choline and inositol on liver lipids has been confirmed.

4. No evidence of any effect of choline, inositol or biotin upon the absolute amount of phospholipin or of free cholesterol in the liver has been obtained in this series of experiments, nor has any effect upon kidney lipids been observed.

5. The absence of a specific effect of biotin on the deposition of liver fat necessitates a revision of certain theories and deductions, two of which have been discussed. It is recommended that the term 'biotin fatty liver' be abandoned.

This work has been financed, in part, by a grant from the Banting Research Foundation.

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The Antagonism of some Antimalarial Drugs by Riboflavin

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(Received 31 December 1945)

An approach to the chemotherapy of malaria from the point of view of antagonism of essential growth factors presents the difficulty that the requirements of essential factors by the malaria parasites are unknown. However, as a large number of drugs has been tested for antimalarial activity, it was thought that a study of the essential metabolites antagonized by them, with reference to an organism more easily handled *in vitro* than the malaria parasite, might throw some light on the mode of action of antimalarial drugs.

Riboflavin was selected for initial work as bearing some structural resemblance to the acridine antimalarials (Fig. 1), and *Lactobacillus casei* ϵ as the test organism because it requires an extraneous supply of riboflavin for its growth. Of a number of substances tested as riboflavin inhibitors, only those which are antimalarials have now been shown to be antagonized by riboflavin.

EXPERIMENTAL

Organism. *Lactobacillus casei* ϵ is known to require an extraneous source of riboflavin for its growth and was used as test organism.

Medium. The medium used was essentially that described by Snell & Strong (1939) for the quantita-

tative assay of riboflavin. It contains: photolyzed NaOH-treated peptone 0.5%, glucose 1%, Na acetate 0.6%, cystine 0.01%, inorganic salts and a riboflavin-free yeast supplement.

In the present work Evans's peptone (Evans, Sons, Lescher and Webb) was used instead of Difco Bactopeptone, and the riboflavin-free yeast supplement prepared as follows: Marmite (100 g.) in water (150 ml.) was treated with basic Pb acetate (250 ml. 10%) and filtered. The clear filtrate was made alkaline (pH 10) with ammonia and the resulting precipitate discarded. The Pb salts were removed with H₂S, the PbS filtered and the H₂S removed by a current of air. Five ml. of this clear solution were used for each 250 ml. medium.

The medium prepared in this way produces a very slight, but definite, growth of *L. casei*. Addition of riboflavin up to 0.05 μ g./ml. caused an increase of growth, with no further increase with larger quantities of riboflavin. The growth was measured turbidimetrically.

Method for observing the effect of riboflavin on the growth inhibitory action of some drugs

Double-strength medium, prepared according to Snell & Strong (1939), was adjusted to pH 6.8. Two parts of this medium were mixed with one part of