The Influence of Pyridoxine on Fat Metabolism in the Rat

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The role of pyridoxine in fat metabolism has been the subject of a number of investigations. McHenry & Gavin (1938) reported that supplementation of a synthetic diet with a concentrate of rice polishings together with vitamin B₁, riboflavin and choline resulted in a greater deposition of body fat than occurred with vitamin B₁ and riboflavin alone. The inclusion of rice concentrate or rice polishings provided a good supply of pyridoxine. Each of these factors appeared to contribute to the increase of body fat, the effect being, at least in part, independent of appetite stimulation. In later work these authors (McHenry & Gavin, 1941) observed that rats on a basal diet containing 96% caseinogen developed more body fat when receiving supplements of vitamin B₁, riboflavin, pantothenic acid, choline, nicotinamide and pyridoxine than when pyridoxine was omitted. They interpreted their results on the hypothesis that pyridoxine is required for the synthesis of fat from protein, and suggested that it is necessary for such stages of protein metabolism as may be essential for the conversion of protein into carbohydrate. Sure & Easterling (1949) compared the body fat of groups of rats maintained for 26–63 days on diets with and without pyridoxine with that of weanling animals fed for 1 week on a stock diet. A paired-feeding technique was employed in these experiments. During the experimental period the pyridoxine-deficient animals suffered a mean loss of 0.24 g. fat, whereas those receiving pyridoxine showed a mean gain of 8.12 g. fat.

Many factors are known to influence the level of fat in the liver. Halliday (1938) claimed that there was an accumulation of fat in the livers of rats rendered deficient in pyridoxine. The vitamin supplements used in these experiments were, however, incomplete, and the food intakes of the normal and deficient animals were not controlled. Engel (1942) found that, with rats subsisting on an unrestricted diet, deprivation of pyridoxine for a period of 6 weeks was without effect on the liver fat. If, however, the deficiency were prolonged up to 8–24 weeks, the deficient animals had a liver-fat content approximately twice that of the controls. The use of dry ethyl ether in these experiments for extraction of the fat is open to objection and may have been responsible for unduly low values. Thus, Erlandsen (1907) pointed out that ether is an inefficient solvent for tissue lipids unless the tissue

had undergone preliminary treatment with ethanol. Engel's findings are further complicated by a coincident deficiency in the diets used of inositol and biotin, both of which are known to influence the level of liver fat. On the other hand, Gavin & McHenry (1940) have concluded that pyridoxine has no supplementary action with choline in preventing the deposition of fat in the liver which occurs when vitamin B₁ is administered. The importance of controlling the food intake so as to eliminate the effects of variations of appetite was emphasized by Quackenbush, Steenbock & Platz (1942). These workers observed that, in acrodynic rats deficient in pyridoxine and pantothenic acid, the addition of rice-bran concentrate supplying both these factors induced a marked increase of liver and body fat if the basal diet was supplied *ad lib*. If, however, the body weights of the control animals were maintained constant by restriction of food intake, no increase of fat occurred as a result of supplementation with rice-bran.

In the present experiments the neutral fat, phospholipin and total cholesterol content of the liver, and the total body lipids have been determined in rats subjected to uncomplicated deficiency of pyridoxine. In addition, observations are reported on the oxidation of fatty acids by liver tissue, and on the faecal excretion of fat. The food intake of control animals has been equated to that of the deficient animals by a paired-feeding technique. The food consumption of each deficient animal was determined daily. This amount of food was fed to the corresponding control receiving pyridoxine in the succeeding 24 hr. In one series, animals fed by this technique were compared with normal and deficient animals which were fed *ad lib*.

METHODS

Diet. Two types of basal diet, of low and high fat content respectively, have been employed. These diets had the following composition:

	Low-fat diet (g.)	High-fat diet (g.)
Casein	18	20
Sucrose	63.5	50
Salt mixture	4.5	5
Cod liver oil	4	5
Water	10	—
Margarine	—	20

Casein used in these diets was twice extracted with 50 % ethanol, followed by two extractions with 96 % ethanol, over a period of a week with frequent stirring. The salt mixture had the following composition: NaCl, 4.69 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 7.22 g.; Na_2HPO_4 , 9.21 g.; KH_2PO_4 , 25.82 g.; $\text{Ca}_3(\text{PO}_4)_2$, 14.62 g.; calcium lactate, 35.22 g.; ferric ammonium citrate, 3.15 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05 g.; KI, 0.01 g. Food consumption of the deficient animals was determined daily and the controls were pair-fed. All rats received daily supplements of vitamin B₁, 20 μg .; riboflavin, 40 μg .; nicotinamide, 100 μg .; calcium D-pantothenate, 240 μg .; *p*-aminobenzoic acid, 250 μg .; biotin, 2.5 μg .; inositol, 500 μg .; choline chloride, 1 mg. and arachis oil, 3 drops. Riboflavin, calcium D-pantothenate, inositol, *p*-aminobenzoic acid, nicotinamide and biotin from Roche Products Ltd. The controls received in addition pyridoxine hydrochloride 40 μg . daily. The animals were killed by stunning and decapitation when typical signs of dermatitis were manifest.

Fat estimations. Total body lipids were estimated by refluxing the whole carcass with 30 % (w/v) ethanolic KOH for 6 hr. After cooling, the solution was transferred to a 500 ml. flask and made up to volume with 96 % (v/v) ethanol. Duplicate 50 ml. portions were pipetted into evaporating dishes, acidified with conc. HCl and evaporated to dryness on a water bath. The residues were transferred to Soxhlet thimbles and extracted twice with ethyl ether for 72 hr. The second extraction yielded negligible amounts of fat. The ethereal extracts were evaporated to dryness, shaken with CHCl_3 and filtered into weighed glass dishes. After evaporation to dryness in air, the CHCl_3 -soluble material was dried in a vacuum desiccator to constant weight.

The following procedure was adopted for the determination of the liver lipids and cholesterol. The liver was weighed and a portion reserved for determination of dry weight. The remainder was transferred to a mortar containing about 5 vol. 97 % (v/v) ethanol, minced with scissors and ground with sand. The supernatant was separated by centrifuging, and the residue re-extracted with about 50 vol. of a mixture at boiling temperature containing 97 % (v/v) ethanol 2 parts and ethyl ether 1 part, used in eight portions. The final residue was continuously extracted in a Soxhlet apparatus for 6–8 hr. with ethyl ether. The combined extracts were evaporated nearly to dryness under reduced pressure below 50°. The residue was taken up in 40 ml. boiling light petroleum (A.R., b.p. 40–60°) in four or five portions and centrifuged at 4000 rev./min. The supernatant was decanted into a standard flask and made up to volume. The completeness of the extraction may be assessed from the fact that further extraction of the residue for 6 hr. with the ethanol-ether mixture yielded an additional 1–2 % of light petroleum-soluble material.

The phosphorus content of the light petroleum extract was estimated by the method of Berenblum & Chain (1938). In calculating the phospholipin content, it was assumed that the phospholipins of rat liver contain 4 % P.

Neutral fat was estimated by the dichromate oxidation method of Bloor (1928, 1929). Phospholipin was precipitated from a portion of the light petroleum extract with acetone and MgCl_2 . The completeness of the precipitation was found to vary between 88 and 100 % with a mean value of 96 %. Thus, the acetone-soluble fraction contains, in addition to

neutral fat and cholesterol, a small variable proportion of phospholipin. In order to determine the oxidation equivalent of neutral fat in terms of standard dichromate solution it is therefore necessary to make a correction for the oxidation equivalents of cholesterol and acetone-soluble phospholipin present in the acetone-soluble extracts.

The cholesterol content of the acetone-soluble fraction was estimated as described below, and Bloor's conversion coefficient (3.92) used in calculating its oxidation equivalent.

The acetone-soluble phospholipin was determined by estimation of phospholipin P as described above. In agreement with the results of Gortner (1945), the ratio phospholipin P/oxidation equivalent was found to vary in different experiments. In consequence, this ratio was determined in each experiment. The acetone-insoluble fraction was used for this purpose, and the assumption has been made that the acetone-soluble phospholipin is a representative sample of the phospholipin present in the original light petroleum extract.

In calculating the neutral fat, Bloor's conversion coefficient (3.60) was used. It was shown that, after addition of neutral fat to the light petroleum extract, a 97 % recovery was obtained in the acetone-soluble fraction.

Cholesterol was estimated by the Liebermann-Burchard reaction, using the method of Sackett (1925), except that the reaction mixture was incubated in the dark for exactly 60 min. Standard solutions of cholesterol were estimated simultaneously, using a Spekker absorptiometer. The acetone-soluble fraction was used in these estimations. This procedure has two advantages. First, the cholesterol content of this fraction is required in calculating the neutral fat value. Secondly, it was found that the brown colour which occasionally interferes with the estimation of cholesterol by the Liebermann-Burchard method (Sperry & Brand, 1943) does not develop if the acetone-soluble fraction is used instead of the original light petroleum extract containing the bulk of the phospholipins. The recovery of added cholesterol averaged 99 % when estimated colorimetrically, and 95 % by the oxidation method. Prior hydrolysis of cholesteryl esters, as recommended by Sperry & Brand (1943), was found not to influence the results and was therefore omitted. This omission is not necessarily justified under experimental and dietary conditions differing from those adopted in the present experiments.

For the estimation of faecal fat, the faeces were collected over an 8-day period and allowed to dry in air at room temperature. The dried material was then ground to a fine powder and continuously extracted with ethyl ether in a Soxhlet apparatus for 10 hr. The ethereal extract was evaporated to dryness and the residue taken up in CHCl_3 . The CHCl_3 extract was filtered, evaporated to dryness and dried in a desiccator to constant weight.

Oxidation of fatty acids by liver. The oxidation of sodium butyrate and sodium octanoate was studied by the Warburg technique, using liver slices from animals fasted overnight. The slices, having a thickness of 0.2–0.4 mm., were cut with a razor blade and triplicate samples transferred to Warburg bottles containing sodium β -glycerophosphate buffer (Jowett & Quastel, 1935) at pH 7.4 in the main chamber. The substrate, dissolved in buffer to give a final concentration of 0.02-M after tipping, was placed in the side arm. O_2 consumption was measured in an atmosphere of O_2 over a period of 90 min.

RESULTS

Effect of deprivation of pyridoxine on total body lipids

Table 1 records the values for total body lipids of normal and pyridoxine-deficient rats fed on basal diets having a low and high fat content respectively. The data are calculated on the basis of the lipid content/100 g. body weight. The deficient animals were deprived of pyridoxine for periods varying from 46 to 183 days in individual cases, and at the time when they were killed well marked signs of dermatitis were manifest. The data in Table 1 indicate that, in animals maintained on a diet low in fat, deficiency of pyridoxine results in a marked reduction of body fat as compared with the positive controls. This is the case whether the results are calculated on a percentage basis or whether the absolute levels of body fat are compared. This observation is in contrast to the results obtained

with animals whose diet contained 20 % fat (groups *C* and *D*). In this case the concentration of total body lipids of the deficient animals was not significantly less than that of animals receiving pyridoxine. When comparison is made between groups *A* and *C* it is evident that both the absolute amount and the concentration of body fat of deficient animals on the high-fat diet is significantly greater than that of deficient animals on the low-fat diet. On the other hand, the control groups in both series which received pyridoxine have a concentration of body fat which is independent of the level of preformed fat in the diet.

Effect of deprivation of pyridoxine on liver lipids

Table 2 summarizes the results of estimations of neutral fat, phospholipins and total sterols of pyridoxine-deficient rats and controls at low and high

Table 1. *Total body lipid content of rats deficient in pyridoxine*

Groups	Diet	No. of animals	Mean final body wt. (g. $\pm$ S.E.)	Mean total body lipids (g./100 g. body wt. $\pm$ S.E.)
<i>A</i>	Low-fat	5	75 $\pm$ 5	2.32 $\pm$ 0.55
<i>B</i>	Low-fat + pyridoxine 40 μ g.	5	114 $\pm$ 13	8.55 $\pm$ 1.20
<i>C</i>	High-fat	7	80 $\pm$ 7	5.17 $\pm$ 1.02
<i>D</i>	High-fat + pyridoxine 40 μ g.	7	122 $\pm$ 9	7.95 $\pm$ 1.18

Statistical analysis of results

	<i>AB</i>	<i>AC</i>	<i>BD</i>	<i>CD</i>
<i>t</i>	4.70*	2.40*	0.35	1.78
<i>P</i>	<0.01	<0.05	0.8	0.1

* Denotes a significant difference.

Table 2. *Neutral fat, phospholipin and total sterol content of livers of pyridoxine-deficient and control rats*

Group	Diet	No. of animals	Final body wt. (g.)	Liver wet wt. (g.)	Liver water (g./100 g. liver)	(g./100 g. liver wet wt. $\pm$ S.E.)		
						Neutral fat	Phospholipins	Total sterols
<i>E</i>	Low-fat; pair-fed	12	88	3.97	71.2	0.63 $\pm$ 0.11	3.66 $\pm$ 0.19	0.28 $\pm$ 0.03
<i>F</i>	Low-fat; pair-fed + pyridoxine 40 μ g.	12	142	5.92	68.8	1.27 $\pm$ 0.15	3.73 $\pm$ 0.21	0.31 $\pm$ 0.03
<i>G</i>	Low-fat; pair-fed	6	92	4.08	71.1	0.64 $\pm$ 0.10	3.64 $\pm$ 0.20	0.31 $\pm$ 0.04
<i>H</i>	Low-fat; pair-fed + pyridoxine 40 μ g.	6	141	5.55	69.6	1.31 $\pm$ 0.18	4.00 $\pm$ 0.15	0.31 $\pm$ 0.04
<i>I</i>	Low-fat; fed <i>ad lib.</i> + pyridoxine 40 μ g.	6	175	7.94	70.2	1.46 $\pm$ 0.30	3.67 $\pm$ 0.19	0.28 $\pm$ 0.03
<i>J</i>	High-fat; pair-fed	3	107	4.53	68.9	0.93 $\pm$ 0.14	3.87 $\pm$ 0.08	0.31 $\pm$ 0.03
<i>K</i>	High-fat; pair-fed + pyridoxine 40 μ g.	3	151	4.93	68.7	1.99 $\pm$ 0.20	3.33 $\pm$ 0.26	0.31 $\pm$ 0.02

Statistical analysis of results

	<i>EF</i>		<i>GH</i>		<i>GI</i>		<i>HI</i>		<i>JK</i>	
	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
Neutral	3.44*	<0.01	3.22*	<0.05	2.58*	>0.01	0.43	0.7	4.35*	<0.05
Phospholipin	0.25	>0.05	1.44	>0.05	0.11	>0.05	1.37	>0.05	1.98	>0.05
Sterol	0.71	>0.05	0.00	>0.05	0.60	>0.05	0.60	>0.05	0.00	>0.05

* Denotes a significant difference.

Table 3. *Faecal excretion of nitrogen in pyridoxine deficiency*

No. of observations	Mean nitrogen intake (mg./day)	Mean faecal nitrogen excretion (mg./day $\pm$ S.E.)		<i>t</i>	<i>P</i>
		Pyridoxine-deficient	Control		
27	148.6	9.3 $\pm$ 0.43	8.7 $\pm$ 0.54	0.87	<0.01

levels of dietary fat. In groups *G*, *H* and *I*, comparison is made between the liver lipids of deficient animals, pair-fed controls and controls fed *ad lib*.

The data for groups *E* and *F* and for groups *G* and *H* indicate that the percentage of neutral fat present in the livers of deficient animals on the low-fat diet is only about half that in the livers of the pair-fed controls. In the case of groups *J* and *K* relating to animals fed on the high-fat diet a similar difference in neutral fat is found for the livers of deficient and control animals. This result may be contrasted with the influence of preformed dietary fat on the total body lipid content of deficient animals already referred to. The percentages of neutral fat in the livers of animals fed on the high-fat diet are somewhat higher than those of the corresponding groups on the low-fat diet, but the differences are not significant. When comparison is made between pair-fed animals and controls fed *ad lib*. (groups *G*, *H* and *I*) it will be seen that the percentage of neutral fat in the livers of the latter is not significantly different from that of the pair-fed control, though in both cases the values are higher than that for the deficient group *G*.

Consideration of the values obtained for phospholipins and total sterols leads to the conclusion that the concentration of these constituents in the liver is not influenced by the absence of pyridoxine in the diet.

Effect of deprivation of pyridoxine on the absorptive function of the intestine

In the course of a study of the nitrogen balance in pyridoxine deficiency carried out for another purpose, estimations of faecal fat and faecal nitrogen were performed. The results, in so far as they relate to the problem of fat absorption in this condition, are as follows. In seven observations on animals on

the high-fat diet, the mean total lipid content of the faeces in pyridoxine deficiency was 0.019 g./day compared with 0.017 g./day for control animals, the mean dietary intake of lipid being 0.89 g./day. The results indicate that there is no impairment of fat absorption as a result of deficiency of pyridoxine. Table 3 records data on the excretion of faecal nitrogen in normal and deficient animals receiving controlled intakes of protein. The balances were carried out over 8-day periods on animals at varying stages of depletion. The methods employed in these experiments will be described more fully in a later paper. Table 3 indicates that the difference in the mean values for faecal nitrogen between the deficient animals and controls was less than 1% of the nitrogen intake and was not significant. We are

Table 4. *Absorption of glucose by loops of jejunum and ileum in pyridoxine deficiency*

Exp. no.		Absorption of glucose (mg./cm. intestine/hr.)	
		Pyridoxine-deficient	Control
1	Jejunum	3.3	2.3
	Ileum	2.7	1.3
2	Jejunum	2.3	2.0
	Ileum	1.5	1.0

indebted to Dr R. B. Fisher and Dr D. S. Parsons for permission to include in Table 4 the results of experiments on the absorption of glucose by loops of small intestine from two pairs of rats by means of their *in vitro* perfusion technique (Fisher & Parsons, 1949). After making allowance for differences in length of the small intestine, the total absorptions of glucose per hr. by the deficient animals were 205 and 184 mg., whereas the corresponding values for

Table 5. *Oxidation of sodium butyrate and sodium octanoate by liver slices from normal and pyridoxine-deficient rats*

(Warburg bottles contained 2.5 ml. sodium β -glycerophosphate buffer pH 7.4 with 40–50 mg. liver slices in the main chamber and 0.5 ml. 0.12 M-substrate in the side arm. In measuring the residual respiration the substrate was replaced by 0.5 ml. buffer. The bottles were filled with O_2 and shaken at 38° for 90 min. CO_2 was absorbed by filter paper moistened with 30% (w/v) KOH in the centre well.)

No. of observations		Substrate	Final body wt.	Liver wet wt.	O_2 uptake	<i>t</i>	<i>P</i>
			(g. $\pm$ S.E.)	(g. $\pm$ S.E.)	(μ l./hr./g. liver wet wt. $\pm$ S.E.)		
5	Pyridoxine-deficient	Sodium butyrate	84 $\pm$ 15	3.48 $\pm$ 0.2	64 $\pm$ 22.3	6.0	<0.01
	Control	Sodium butyrate	110 $\pm$ 17	4.87 $\pm$ 1.6	309 $\pm$ 34.3	—	—
5	Pyridoxine-deficient	Sodium octanoate	59 $\pm$ 5	2.23 $\pm$ 0.03	207 $\pm$ 128	2.3	0.05
	Control	Sodium octanoate	104 $\pm$ 12	4.23 $\pm$ 0.39	696 $\pm$ 167	—	—

the controls were 209 and 206 mg. respectively. The results indicate that there is no overall impairment in the absorption of glucose by the pyridoxine-deficient rat.

Effect of deprivation of pyridoxine on the oxidation of sodium butyrate and sodium octanoate by liver slices

Table 5 summarizes data relating to the respiration of liver slices from pyridoxine-deficient and control animals fed on the low-fat diet with sodium butyrate and sodium octanoate as substrates. With both substrates, after correcting for the residual respiration, there is a significant reduction in oxygen uptake with liver slices from the pyridoxine-deficient group.

DISCUSSION

The failure of rats fed on a low-fat diet deficient in pyridoxine to deposit fat in the body, which is indicated in the present work, confirms the observation of Sure & Easterling (1949) who also adopted a paired-feeding technique in their experiments. The reduction of total body fat is associated with a lower value of the neutral fat content of the liver, though without change in the phospholipin and sterol fractions. Pyridoxine cannot, therefore, be regarded as a lipotropic agent. The absence of fatty livers cannot be due to lack of available fat to infiltrate the organ, since this condition was not observed even when the diet contained 20 % margarine. The reason for the fatty livers reported by Halliday (1938) and by Engel (1942) is uncertain; it may be noted that the degree of infiltration observed by these authors was comparatively slight, and that the diets employed were lacking in certain factors which are now believed to influence the content of fat in the liver. Our observations are in accord with those of Deane & Shaw (1947) who found that the livers of rats depleted of pyridoxine 'were uniformly free of histologically demonstrable lipid'.

The explanation for the low values of body fat in pyridoxine deficiency has not been hitherto examined. We have considered that there may be some failure in the absorptive function of the small intestine. Our evidence does not support this possibility and there seems, therefore, no reason to believe that disturbance in digestion or absorption of foodstuffs can account for the relative absence of body fat in this condition.

The reduction in the fatty acid oxidase activity of liver slices from pyridoxine-deficient rats, taken in conjunction with the reduction in substrate concentration revealed by the analyses of liver lipids, disposes of the possibility that a low value of body fat is a consequence of accelerated oxidation of fat.

There remains the hypothesis that in pyridoxine deficiency there is a failure of fat synthesis. Stetten &

Boxer (1944) have demonstrated that in the well nourished rat 30 % of the ingested carbohydrate is stored as fat and only about 3 % as glycogen. In this connexion the determinations of body fat in animals fed on the high-fat diet are of interest. In the case of animals receiving pyridoxine the level of body fat is unaffected by the partial replacement of carbohydrate by fat in the diet. The fat present in the depots of such animals, and representing a mixture of preformed dietary fat and fat newly synthesized from carbohydrate, is fairly constant in relation to body weight. On the other hand, if pyridoxine deficiency involves an impairment in the synthesis of fat from carbohydrate, the provision of preformed fat in the diet might be expected to raise the level of body fat to that found in the animals receiving pyridoxine. The data recorded in Table 1 indicate that this is the case. It may be noted, however, that the level of neutral fat in the liver is not restored; this finding might be anticipated in the light of the observation of Frazer (1943) that dietary glyceride is transported to the depots rather than to the liver. The synthesis of fat in the animal body may, however, depend on the availability of other nutritional factors in addition to pyridoxine. Thus, Quackenbush & Steenbock (1942) observed that the depletion of liver and body lipids which occurred on a diet deficient both in pyridoxine and pantothenic acid was not restored on addition of pyridoxine alone to the diet. With both supplements, on the other hand, there was a threefold increase in body lipid and a marked rise in body weight. While the data presented in this paper are consistent with the hypothesis of a failure in fat synthesis as a result of pyridoxine deficiency, there is at present no evidence to elucidate the relevant enzyme systems which are affected.

SUMMARY

1. Deficiency of pyridoxine in the rat involves a lowering in the total lipid content of the body and a diminution in the neutral fat content of the liver. There is no change in the phospholipin or sterol fractions of the liver lipids in this condition. Inclusion of 20 % fat in the diet restores the total body lipid content to the level of that found in animals receiving an adequate intake of pyridoxine.

2. A decreased rate of oxidation of sodium butyrate and sodium octanoate by liver slices from pyridoxine-deficient rats is reported.

3. There is no impairment in the absorption of carbohydrate, fat or the products of protein digestion in pyridoxine deficiency.

4. The observations reported are consistent with the view that deficiency of pyridoxine involves a failure in the ability of the rat to synthesize fat from carbohydrate.

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REFERENCES

- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
 Bloor, W. R. (1928). *J. biol. Chem.* **77**, 53.
 Bloor, W. R. (1929). *J. biol. Chem.* **82**, 273.
 Deane, H. W. & Shaw, J. H. (1947). *J. Nutrit.* **34**, 1.
 Engel, R. W. (1942). *J. Nutrit.* **24**, 175.
 Erlandsen, A. (1907). *Hoppe-Seyl. Z.* **51**, 71.
 Fisher, R. B. & Parsons, D. S. (1949). *J. Physiol.* **110**, 281.
 Frazer, A. C. (1943). *J. Physiol.* **102**, 306.
 Gavin, G. & McHenry, E. W. (1940). *J. biol. Chem.* **132**, 41.
 Gortner, W. A. (1945). *J. biol. Chem.* **159**, 97.
 Halliday, N. (1938). *J. Nutrit.* **16**, 285.
 Jowett, M. & Quastel, J. H. (1935). *Biochem. J.* **29**, 2143.
 McHenry, E. W. & Gavin, G. (1938). *J. biol. Chem.* **125**, 653.
 McHenry, E. W. & Gavin, G. (1941). *J. biol. Chem.* **138**, 471.
 Quackenbush, F. W. & Steenbock, H. (1942). *J. Nutrit.* **24**, 393.
 Quackenbush, F. W., Steenbock, H. & Platz, B. R. (1942). *J. biol. Chem.* **145**, 163.
 Sackett, G. E. (1925). *J. biol. Chem.* **64**, 203.
 Sperry W. M. & Brand, F. C. (1943). *J. biol. Chem.* **150**, 315.
 Stetten, DeW. & Boxer, G. E. (1944). *J. biol. Chem.* **155**, 231.
 Sure, B. & Easterling, L. (1949). *J. Nutrit.* **39**, 393.

Studies on the Analysis of Vitamins D

4. STUDIES ON THE IRRADIATION OF ERGOSTEROL AND 7-DEHYDROCHOLESTEROL AND THE ANALYSIS OF THE PRODUCTS FOR CALCIFEROL, VITAMIN D₃, AND COMPONENT STEROLS

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The chemical analysis of vitamins D in synthetic irradiation products is of considerable importance and offers more promise of success than attempts on fish-liver oils. There are, however, a number of difficulties to be met which have rendered most chemical methods of analysis unreliable. A review of published methods of analysis has been made and the difficulties discussed in earlier papers (Parts 1, 2 and 3; Green, 1951*a, b, c*).

In the experimental work to be described, analytical methods for the determination of vitamins D and related compounds have been evolved, with consideration of three factors. First, although it is realized that the irradiation products are of widely varying composition (depending mainly on the extent of photochemical reaction), the influence of this fact on the problems of chemical analysis has usually been neglected. Thus, many methods, based on examination of highly standardized types of industrial product fail in the hands of workers investigating materials of varying composition. Adequate study of the composition of the irradiation products is a prerequisite of success in vitamin D determination. Secondly, biological assay control must be rigorously applied at all stages of evolution of a chemical

method for vitamin D. Thirdly, agreement between chemical and biological assays should not necessarily be accepted as evidence for quantitative determination in the absence of recovery experiments.

The ultimate means of measurement have been the iodine trichloride titration method, described by Green (1951*a*) and the antimony trichloride-acetyl chloride colorimetric method of Nield, Russell & Zimmerli (1940).

EXPERIMENTAL

Materials and apparatus

Sterols (for specification, see Green, 1951*a*). *Calciferol and vitamin D₃*. For recovery experiments only, the International Standard preparations (see note in text). *Floridin earth*. Prepared for chromatography, British Drug House Ltd. Sieved to remove particles finer than 160 mesh/in. *Antimony trichloride*. A.R. grade. *Acetyl chloride*. A.R. grade. *Chloroform*. Purified as described by Nield *et al.* (1940). *Other reagents*. (For specification, see Green, 1951*a, b, c*).

ICl₃ titrations were carried out on the Spekker micro-absorptiometer, using spectrum green 604 filters. SbCl₃-acetyl chloride colorimetric measurements were made on the same micro-absorptiometer, using spectrum blue-green 603 filters, and on the Uvispek spectrophotometer.