was employed for the purpose of sterilizing certain preparations of possible growth stimulants. It was found that Ca^{++} and Fe^{+++} were extracted from filter pads in a single filtration. These ions were growth stimulants for the acid-fast organisms under the conditions used, and their presence in filtrates accounted for anomalous results obtained with some preparations.

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The Mechanism of Coprosterol Formation *in vivo*

2. ITS INHIBITION BY SUCCINYL SULPHATHIAZOLE AND BY CARBARSONE

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The discovery that brain contains a substance, probably identical with or allied to the cerebroside phrenosin, which enables the organism to convert large amounts of cholesterol, added to the diet, into coprosterol [Rosenheim & Webster, 1941], offers a convenient experimental method for investigating the effect on coprosterol formation of drugs which act specifically on the intestinal flora or fauna. In view of the surprising lack of evidence for the theory, advanced on purely theoretical considerations by the early investigators [Bondzynski & Humnicki, 1896; Müller, 1900], and held ever since, that putrefactive intestinal bacteria are responsible for the conversion of cholesterol into coprosterol by direct hydrogenation, we selected in the first instance the sulphonamide derivative succinyl sulphathiazole (S.S.) for investigation. This drug exerts a powerful bacteriostatic action on *Escherichia coli* and other coliform bacteria of the intestine and has the additional advantage of low toxicity, owing to its poor absorption from the alimentary tract.

As the outcome of this investigation, together with other experimental evidence, made it improbable that intestinal bacteria have a share in coprosterol formation, the possibility was considered whether the fauna, and especially the protozoan population, of the intestine rather than its flora was concerned in the process. This possibility was investigated by examining the coprosterol excretion of rats treated with the amoebicidal drug carbarsone (*p*-carbamino phenylarsonic acid). This arsenical also frees the intestinal tract from the flagellate *Trichomonas*, a non-pathogenic parasite occurring in the intestinal canal of man and animals. A drug specifically acting on *Trichomonas* was chosen for the test, since it has been claimed that cholesterol is an essential growth factor for four species of *Trichomonas* [Cailleau, 1937; 1939].

EXPERIMENTAL

Methods

The technique of the animal experiments, the composition of the diet, and the analytical methods used have previously been described [Rosenheim & Webster, 1941]. S.S. was administered by intimately mixing the drug with the diet to the amount of 5% [Welch, Mattes & Latven, 1942]. The S.S. contents of the faeces were determined by the method of Bratton & Marshall [1939]. Carbarsone was given in the drinking water, which consisted of a 0·5% solution of the drug in a 1% solution of sodium hydrogen carbonate in distilled water [Gabaldson, 1936]. *Esch. coli* counts were made by the usual method, using poured MacConkey's plates. The faeces and intestinal contents were examined for *Trichomonas* microscopically and by cultivation in the medium of Dobell & Laidlaw [1926]. The protozoological examinations were kindly made by our colleague, Dr Clifford Dobell.

Experiments with succinyl sulphathiazole

In a preliminary experiment two groups each of three male rats were used. During a period of 3 days they were fed on the stock diet, the experimental group receiving S.S. as well. Bacteriological examination of the faeces on the fourth day showed that *Esch. coli* had been almost eliminated in the experimental group, whilst the faeces of the control group contained 50×10^6 organisms/g.

In the subsequent period both groups were kept for 4 days on a diet of steamed brain (15 g./day/rat), the experimental group receiving S.S. as before. The faeces of this group were again found to be free from *Esch. coli*, the control group containing 80×10^6 organisms/g. Both groups lost in weight, the experimental group losing twice as much as the control groups. The analytical results are summarized in Table 1.

Table 1. *Influence of the addition of succinyl sulphathiazole to a diet of brain, on the faecal excretion of coprosterol and of* Esch. coli, *by the rat*

Period	Duration of period (days)	Total wt. of food consumed (g./day)	Total body weight: Initial (g.)	Final (g.)	Loss (g./day)	Total wt. of moist faeces (g.)	Faecal excretion, during last 24 hr. of period, of: *Esch. coli* (no./g. of moist faeces)	Succinyl sulphathiazole: (g./100g. of moist faeces)	(g./100g. of dry faeces)	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol: (g.)	(% of total)
Control	3	45	568	509	19·7	40·7	80×10^6	None	None	3·39	2·54	74·9
Experimental	3	45	534	418	38·7	42·7	1×10^3	4·6	13·6	2·34	Trace	<1

The addition of S.S. to the brain diet produced almost complete inhibition of coprosterol formation, whilst the coprosterol excretion of the control group on brain diet alone was of the same order as in our previous experiments [Rosenheim & Webster, 1941]. Pure cholesterol, m.p. 148–149°, was recovered from the digitonides obtained in the estimation of the sterols in the faeces of the experimental group, whilst those of the control group yielded coprosterol, m.p. 100–101°, in typical needles.

The administration of S.S. was stopped on the fifth day and the animals were put on a diet of brain alone, for 4 days. Only one of the rats survived this period, the high mortality rate probably being due to the increased toxicity of the drug when given in a diet low in protein [cf. Smith, Lillie & Stohlman, 1941; Kapnick, Lyons & Stewart, 1942]. Examination of the faeces of the surviving animal showed, however, that in spite of the return, in enormously greater numbers, of coliform bacteria (100×10^6/g.), the coprosterol content amounted to only 7 % of the total sterols.

In the next experiment the high mortality rate was avoided by substituting for brain the standard diet supplemented by cholesterol (160 mg./rat/day) and cholesterol-free brain powder (1·6 g./rat/day). The fact that the specimen of brain powder [Rosenheim & Webster, 1941] used had been prepared 6 years ago and had retained its full activity, incidentally proves the stability of the factor contained in it.

Two groups of three male rats each were used, the experimental group receiving S.S. for a preparatory period of 3 days. The experimental period, with the additional supplement of brain powder and cholesterol + S.S., lasted 4 days, the faeces of the last 3 days being collected for analysis. During this and part of the subsequent period the faeces were semifluid and unavoidable losses occurred in their collection. After 4 days the administration of S.S. was stopped and the animals were kept on the standard diet and brain powder + cholesterol for 7 days.

The results (Table 2) were practically identical with those of the previous experiment, in which brain was the sole food. The animals remained in good health, however, and during the short experimental period they actually increased in weight at the same rate as the control rats. In this connexion it is significant that the S.S. content of the faeces

Table 2. *Influence of the addition of succinyl sulphathiazole to a normal diet supplemented with cholesterol and brain powder, on the faecal excretion of coprosterol and of* Esch. coli, *by the rat*

Period	Duration of period (days)	Rats in group	Total wt. of food consumed (g./day)	Total body weight: Initial (g.)	Final (g.)	Increase (g./day)	Total wt. of moist faeces (g.)	Faecal excretion, during last 24 hr. of period, of: *Esch. coli* (no./g. of moist faeces)	Succinyl sulphathiazole: (g./100g. of moist faeces)	(g./100g. of dry faeces)	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol: (g.)	(% of total)
Control	3	3	34	486	505	6·3	13·0	17×10^6	None	None	1·02	0·77	75·5
Experimental	3	3	31	463	480	5·7	16·6	1×10^3	7·5	27·7	0·62	Trace	<1
Post-experimental	6	3	33	480	518	6·3	16·1	32×10^6	None	None	1·05	Trace	<1

on the last day of the experimental period was nearly double that of the faeces on the corresponding day in the preceding experiment (see Table 1), in which brain was the exclusive diet. A greater absorption of the drug from the intestinal tract may thus explain the higher mortality on the brain diet.

The persistence of the inhibitory effect on coprosterol formation was again shown, in spite of the return of *Esch. coli* in the faeces after the withdrawal of the drug. The duration of this inhibitory period has not yet been studied in detail, but in one experiment it was found that the coprosterol excretion was still *c.* 15% below the normal level after 3 weeks on the standard diet.

Experiments with carbarsone

In a preliminary experiment a male rat, weighing 220 g., was taken at random from the Institute's colony. On microscopical examination of its faeces, the animal was found to be heavily infected with *Trichomonas muris*. A feeding test with steamed sheep's brain showed a coprosterol excretion of 78% of total sterols. The rat then received 45 mg./day of carbarsone whilst on the standard diet for 2 days. During the next 5 days the standard diet was replaced by steamed sheep's brain (25 g.) containing bone meal (1 g.) and the dosing with carbarsone was continued. The rat received 210 mg. of carbarsone, i.e. 42 mg./day. At the end of this period the faeces were free from *Trichomonas*, but showed a high count of *Esch. coli* (100×10^6/g.). The faeces of the last 4 days were collected and analyzed. The sterols consisted of practically pure cholesterol containing only traces of coprosterol.

This striking inhibitory effect of carbarsone on coprosterol formation was confirmed by the subsequent experiments, which were carried out on individual animals in order to ensure adequate control in the collection of faeces for bacterial and protozoological examination. Male rats of the average weight of 270 g. were kept in separate cages with coarse wire-grid bottoms. For the first 5 days their diet consisted of a mixture, in equal parts, of the standard diet and steamed sheep's brain. All the rats were found to be infected with *Trichomonas* and bacteriological examination of the faeces showed the usual high count of *Esch. coli* at the end of this period. During the carbarsone period the drug was administered for 1 day in the drinking water, whilst the rats were on the standard diet. During the following 5 days they received known amounts of carbarsone (see Table 3) in their food, consisting of the mixture of steamed brain and standard diet described above. At the end of the experimental period the rats were in good condition. The faeces were found to be free from *Trichomonas*, whilst the *Esch. coli* counts were as high as before. The results of four experiments of this type, in which the faeces of the last 3 days were collected and analyzed, are given in Table 3.

The figures in the last column of Table 3 demonstrate conclusively the inhibitory effect of carbarsone on coprosterol excretion, which has been reduced to the merest traces even in the rat in which, before the administration of carbarsone, coprosterol accounted for as much as 90% of the total sterols excreted.

Attempted bacterial hydrogenation of cholesterol and cholestenone

The possibility that the failure of previous attempts to effect coprosterol formation by intestinal bacteria *in vitro* may have been due to the absence in the media used of an essential growth factor, led us to investigate their action on cholesterol and cholestenone in the presence of brain, which favours coprosterol formation *in vivo*.

(1) *Cholesterol.* (*a*) Flasks containing minced sheep's brain (10 g.) inoculated with faeces (1 g.) from rats fed on brain, were incubated in anaerobic jars at 37° for 8 and 16 days respectively. The unsaponifiable matter was prepared in the usual

Table 3. *Influence of the addition of carbarsone to a normal diet supplemented with brain, on the excretion of coprosterol by the rat*

Period	Duration of period (days)	Rat no.	Carbarsone intake (mg./day)	Total wt. of moist faeces (g.)	*Trichomonas* infection	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol (g.)	Coprosterol (% of total)
Control	3	1	0	10·0	+ + +	0·83	0·62	74·7
	3	2	0	6·2	+ + +	0·81	0·68	83·9
	3	3	0	7·7	+ + +	0·82	0·74	90·3
	3	4	0	6·1	+ + +	0·65	0·52	80·0
							Average	82·2
Experimental	3	1	69	10·5	None	0·73	Trace	<1
	3	2	64	8·4	None	0·69	Trace	<1
	3	3	63	12·1	None	0·88	Trace	<1
	3	4	60	7·6	—*	0·64	Trace	<1

* Not examined.

way for the estimation of total and saturated sterols by the gravimetric digitonin method. The cholesterol content of the brain was 2·6 % and the mixture in the control flask contained 37 mg. of coprosterol, derived from the added faeces. At the end of the experiment 36 mg. (± 7) of coprosterol were found in the incubated mixtures, showing that no conversion of cholesterol into coprosterol had taken place. (*b*) In another set of experiments 10 g. portions of minced sheep's brain, ground up with 20 ml. of glucose broth, were sterilized and inoculated with 5 ml. of a 24-hr. culture of mixed anaerobic bacteria. This culture was obtained from the caecal contents of three rats excreting large quantities of coprosterol on a diet of sheep's brain. After 17 days' incubation under anaerobic conditions the brain mixtures yielded 2·55 % and the control 2·65 % of cholesterol. No coprosterol had been produced.

(2) *Cholestenone.* (*a*) Suspensions of finely powdered cholestenone (200 mg.) in glucose broth (50 ml.) were sterilized and inoculated with a fresh mixed culture of intestinal bacteria (see above). No sterols precipitable by digitonin were obtained from these mixtures after anaerobic incubation for 17 days. It has previously been shown [Mamoli, Koch & Teschen, 1939] that cholestenone was not attacked by a pure culture of *B. putrificus* under the conditions in which the keto group and ethenoid linkage of testosterone, androstenedione, etc. were reduced. (*b*) Flasks containing 10 g. portions of sheep's brain, inoculated with faeces as above, and intimately mixed with a solution of 0·2 g. cholestenone in cetyl alcohol, were incubated anaerobically for 8 and 16 days respectively. The average amount of coprosterol recovered, 33 mg. (± 1), was slightly less than the amount (37 mg.) derived from the added faeces. These negative results agree with those of similar experiments by Diels [1937].

DISCUSSION

It is tempting to associate the striking inhibitory effect of succinyl sulphathiazole on coprosterol formation with the simultaneous disappearance, caused by the drug, of *Esch. coli* and coliform bacteria from the intestine. The conclusion that these bacteria are therefore directly responsible for coprosterol formation is, however, fallacious and disproved by the fact that the inhibitory effect persists in spite of the immediate return, in enormously increased numbers, of *Esch. coli* and coliform bacteria after the withdrawal of the drug.

The concept that coprosterol originates in the intestine by bacterial hydrogenation of cholesterol arose at a time when the stereochemical differences in the constitution of the reduction products of cholesterol were entirely unknown. Since then it has been recognized that, under all known conditions, the hydrogenation of the Δ^5 ethenoid linkage of cholesterol leads to the *trans*-decalin derivative (*β*)-cholestanol and that coprosterol, the corresponding *cis*-decalin derivative, can be obtained only by indirect methods. In ignorance of these facts, the early investigators linked up coprosterol formation in the intestine with the activity of 'putrefactive' bacteria, an apparently obvious interpretation of the facts known to them. Although this simple solution of a complex problem has not been supported by any convincing experimental evidence, it is still current. As an argument against the bacterial origin of coprosterol the fact may be adduced that bacteria are devoid of cholesterol, a sterol found in animal cells only. Moreover, bacteria and the primitive unicellular algae, Myxophyceae [Carter, Heilbron & Lythgoe, 1939], are the only cellular organisms in which sterols of any kind are absent.

Evidence is accumulating in favour of the view that the chemical changes occurring in coprosterol formation proceed in two stages, consisting in a primary oxidation of cholesterol to cholestenone followed by reduction of the latter to the *cis*-decalin derivative coprosterol [Rosenheim & Webster, 1943]. Whether these reactions are brought about by the enzymic activity of the intestinal mucosa or by other agencies is unknown. Our results show that intestinal bacteria in any case are unable to reduce cholestenone to coprosterol *in vitro*, even in the presence of the hypothetical growth factors, contained in brain, which favour coprosterol formation *in vivo*. In this connexion the recent work on the bacterial reduction and oxidation of steroid sex hormones, carried out mainly in Butenandt's laboratory, acquires particular importance. Some of the incidental observations recorded in these researches have a direct bearing on the coprosterol problem, since they led to the conclusion that the susceptibility of steroids to bacterial attack is dependent on the length of the side chain: cholesterol, cholestenone and cholestanone, possessing the intact side chain of eight carbon atoms, are recovered unchanged when subjected to the action of certain dehydrogenating or hydrogenating bacteria respectively, under the same conditions which bring about the oxidation or reduction of the functional groupings of steroid sex hormones possessing a shorter or no side chain [Mamoli & Schramm, 1938; Mamoli, 1938; Mamoli *et al.* 1939]. It is therefore not surprising that all attempts to convert cholesterol into coprosterol by bacterial action *in vitro* should have failed, and it appears unlikely that cholesterol is concerned in bacterial metabolism.

If we accept the view that bacteria have no share in coprosterol formation, it seems attractive to consider as an alternative the possible role of the intestinal fauna, and especially of protozoa, in this

process. Various species of flagellates belonging to the genus *Trichomonas* are non-pathogenic inhabitants of the intestinal tract of man and animals, so that the caecums and intestines of rats 'are often swarming with *Trichomonas*' [Wenyon, 1926]. A possible connexion between these protozoa and cholesterol metabolism is suggested not only by the fact that four species of *Trichomonas* require cholesterol as an essential growth factor [Cailleau, 1937; 1939], but also by various other considerations. Thus, infection with *Trichomonas* of babies up to 1 year is rare [Faust & Headlee, 1936] and their faeces, like the meconium, are devoid of coprosterol [Flint, 1862; Müller, 1900; Fox & Gardner, 1925]. When the consumption of a mixed diet at later stages of their development favours the chances of infection by the flagellates, coprosterol appears in their excreta. Further, an exclusive milk or meat diet reduces both the trichomonad infection [Hegner, 1923; 1924] and coprosterol excretion [Müller, 1900; Dorée & Gardner, 1908].

Attractive as this suggested correlation between protozoa and coprosterol formation seemed to be, and in spite of its apparent confirmation by the results of the above-described experiments with the arsenical drug carbarsone, such a correlation appeared to be fortuitous. On further investigation, it was found that rats, freed from *Trichomonas* by carbarsone treatment, excreted a considerable amount of coprosterol in the period following the stoppage of the drug. On the other hand, administration of succinyl sulphathiazole caused the evacuation of large numbers of *Trichomonas* in the faeces without freeing the rats from the infection, but they excreted only small amounts of coprosterol for some time after cessation of the treatment. It is realized that our exploratory experiments in this direction are liable to various interpretations. The results so far obtained, however, indicate that the participation of protozoa in coprosterol formation is at least as unlikely as that of bacteria.

It is equally difficult to relate the interference with cholesterol metabolism exhibited by carbarsone and succinyl sulphathiazole with the inhibitory effect of the latter drug on the synthesis of the essential vitamins, biotin and folic acid, by intestinal bacteria [Nielsen & Elvehjem, 1942; Welsh & Wright, 1943], especially as a possibly similar effect of carbarsone has not yet been investigated.

The unexpected fact thus emerges that two drugs of dissimilar chemical constitution, one of which acts specifically on *Esch. coli* and not on protozoa and the other on Protozoa but not on *Esch. coli*, have in common an inhibitory effect on coprosterol formation, which does not seem to be due to their direct action on either the bacteria or protozoa concerned. The mechanism of this inhibitory action remains so far unexplained.

SUMMARY

1. The bacteriostatic action of succinyl sulphathiazole on intestinal coliform bacteria was found to be concurrent with a complete inhibition of coprosterol formation. The apparent correlation, however, between *Esch. coli* and coprosterol formation is fortuitous and the available evidence makes it improbable that intestinal bacteria have any share in the process.

2. The possibility that the fauna, and especially the protozoan population, of the intestine rather than its flora is concerned in coprosterol formation was investigated by examining the coprosterol excretion of rats treated with carbarsone (*p*-carbamino phenylarsonic acid). This amoebicidal drug frees rats from *Trichomonas muris* infection and was found also completely to inhibit coprosterol formation. Exploratory experiments, however, indicate the absence of a relationship between protozoa and coprosterol formation.

3. The complexity of the problem is made apparent by this interference with cholesterol metabolism, shared by an antibacterial and an amoebicidal drug, which seems to be unconnected with their action on either bacteria or protozoa and which remains so far unexplained.

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A Micro-method for the Estimation of Vitamin B_1

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A micro-method for the estimation of vitamin B_1 has been described by Atkin, Schultz & Frey [1939]. It is based on the measurement of the rate of fermentation of sugar by baker's yeast, using a Warburg or similar apparatus, and is sensitive to 0·005 μg. of vitamin B_1. The method described below is based on the thiochrome technique and is of similar sensitivity. It was developed during work on the distribution of vitamin B_1 in the wheat grain and has been applied successfully to small quantities (1–50 mg.) of cereal products [Hinton, 1942].

EXPERIMENTAL

The method is based upon the optical design of the Spekker Photoelectric Fluorimeter. In this instrument, an image of the mercury discharge tube is brought to a focus at a point opposite the photo-cell, the focused beam normally passing through the test solution contained in a large glass cell. In the micro-method here described, the glass cell is replaced by a suitable holder by means of which a capillary tube containing the test solution is placed in the image, which measures 2 × 20 mm. approximately in the focal plane, and thus receives the full irradiation transmitted by the system (Fig. 1).

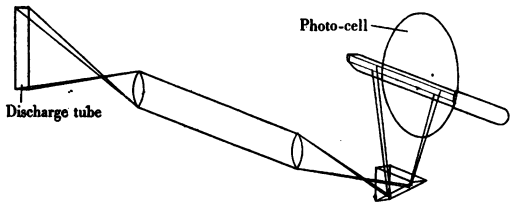

Fig. 1. Diagram showing optical design of the Spekker Photoelectric Fluorimeter and position of capillary-tube test cell used in the micro-method.

The Spekker Fluorimeter was used in conjunction with a Cambridge Short-Period Galvanometer of sensitivity 3000 mm./μA., and resistance 196 ohms, which was used at full sensitivity.

Procedure

The strength of ferricyanide to produce maximum fluorescence in the test solution is first ascertained by the procedure summarized below. With our cereal extracts this was found by testing a range of strengths from 0·05 to 0·5 %, in steps of 0·05 %. For standard aneurin solutions the best concentration was 0·005 %.

The pipette to be used for the test solution is first washed out with the solution, in readiness. 0·1 ml. of 30 % NaOH is measured into a 2 × $\frac{3}{8}$ in. tube, 0·02 ml. of the appropriate ferricyanide solution run in on top and left undisturbed. 0·1 ml. of the test solution is then measured out and, immediately before this is added to the tube, the NaOH and ferricyanide are mixed by shaking. The test solution is run in and the whole again mixed by shaking. Oxidation is carried out in this way with each tube in a series which, in order to check the calibration curve, includes three standard aneurin solutions of different strengths. 0·2 ml. of water-saturated isobutanol is then added to each tube, and extraction carried out by stirring for 30 sec. The tubes are allowed to stand for 10–15 min. to allow separation of the layers, those containing cereal extracts being centrifuged for 3–4 min. at 1500 r.p.m. to improve the separation. The isobutanol layer is drawn up directly into the capillary tube for measurement in the fluorimeter. A no. 39 Wratten filter was used in front of the right-hand photo-cell (Fig. 1).

The calibration curve was prepared by the same procedure, using solutions containing from 0·001–0·2 μg. aneurin/0·1 ml.