REDUCTION OF HELIOTRINE BY A RUMEN MICROORGANISM

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Summary

A small Gram-negative coccus, which brings about the reductive fission of the hepatotoxic pyrrolizidine alkaloids, has been isolated from the rumen contents of Australian Merino sheep. The organism was cultured in a partially defined medium, without rumen fluid, but containing yeast extract. Strictly anaerobic conditions and the presence of carbon dioxide were essential for growth. With limiting quantities of yeast extract, growth was greatly stimulated by any of several carbohydrates or moderately stimulated by the alkaloid heliotrine plus hydrogen. The products of the reductive cleavage of heliotrine were heliotric acid and 7α-hydroxy-1-methylene-8α-pyrrolizidine. One mole of hydrogen reduced one mole of heliotrine to produce one mole each of the two products, accounting completely for the substrate consumed. Formate in stoichiometric quantities could replace molecular hydrogen as hydrogen donor in the reaction in which heliotrine acted as hydrogen acceptor. With [^{14}C]formate as hydrogen donor $^{14}CO_2$ was evolved in stoichiometric amounts. The additional growth brought about by the degradation of heliotrine was proportional to the amount of heliotrine degraded, the organism appearing to derive energy from the reaction. Under strictly anaerobic conditions neither growth of the organism nor degradation of heliotrine was dependent on or stimulated by the presence of vitamin B_{12} in the medium. A method is described for the estimation of heliotrine.

I. Introduction

Sheep grazing summer pastures in Australia may sometimes ingest *Heliotropium europaeum* L., an annual plant containing several pyrrolizidine alkaloids, including heliotrine (Culvenor, Drummond, and Price 1954). In some circumstances the intake of these alkaloids may be sufficient to cause chronic liver disease (Bull *et al.* 1956). It has been shown that heliotrine may be reduced in the sheep's rumen to the non-hepatotoxic 7α-hydroxy-1-methylene-8α-pyrrolizidine (Culvenor, Dann, and Dick 1962). Experiments *in vitro* (Dick *et al.* 1963) showed that the residual microorganisms in strained rumen fluid were also capable of degrading some of the added heliotrine, when incubated with carbon dioxide. It was found that complete degradation of heliotrine could be similarly effected using lightly centrifuged rumen fluid if 1 μg/ml of vitamin B_{12} was added. The present work was undertaken to examine more closely the nature of the reactions leading to degradation of heliotrine by pure cultures of the microorganisms concerned, and to examine the nature of the vitamin B_{12} effect. Using rumen contents of Merino sheep which had been grazing on dry summer pastures, or irrigated ryegrass–white clover pastures (both of which were free of *H. europaeum*), we were unable to demonstrate an effect of vitamin B_{12} on

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the degradation of heliotrine *in vitro*. In such incubations heliotrine was degraded fairly rapidly after a lag period of up to 40 hr, and neither the lag period nor the subsequent rate of degradation was affected by vitamin B_{12}. From such cultures a microorganism has been isolated that brings about the reductive cleavage of heliotrine, using either molecular hydrogen or formate as hydrogen donor. The stoichiometry of this reaction has been examined and it has been shown that the substrates are not incorporated into bacterial substance, but the reaction appears to serve as an energy source. It is suggested that the stimulation in rate by vitamin B_{12} observed by Dick *et al.* (1963) may have been due to its known capacity to catalyse the oxidation of thiols by molecular oxygen and thus to remove traces of oxygen from solution (Peel 1963).

II. Materials and Methods

(*a*) *Culture Methods*

The anaerobic techniques of Hungate (1950) were used throughout, but with agar slopes substituted for roll tubes. Routine inoculations of 0·1–1 ml were made in final volumes of 10 ml liquid medium and shaken at 140 oscillations/min at 39°C, while clamped vertically. All media and equipment were autoclaved for 15 min at 15 lb/in^2 pressure before use.

(*b*) *Isolation of the Organism*

The presence of the organism in rumen liquor from Australian Merino wethers was detected by the method of Dick *et al.* (1963), but without prior centrifugation of the fluid. The particular isolate studied was obtained from fluid collected through a rumen fistula from a yearling wether depastured on green ryegrass–white clover. It was isolated in the presence of vitamin B_{12}. Five further isolates were obtained in the absence of added vitamin B_{12} from rumen liquor collected at slaughter from wethers on dry summer pastures. None of the pastures contained *H. europaeum*.

The strained rumen liquor (100 ml) was adjusted to pH 6·6 by addition of 20% (w/v) Na_2CO_3 solution while bubbling with oxygen-low CO_2 (Meites and Meites 1948). Heliotrine (final concentration 0·5 mg/ml as neutral hydrochloride) and vitamin B_{12} (5 μg/ml) were added before incubating at 39°C whilst bubbling continuously with CO_2. After 48 hr, samples were examined for degradation of heliotrine by thin-layer chromatography. When degradation was established, 10% subcultures were made in a lactate enrichment medium and incubated for 48 hr when the heliotrine was completely degraded. The enrichment medium (Table 1), referred to in the remainder of this paper as medium 1, was derived from the synthetic medium of Caldwell and Bryant (1966). After eight successive subcultures in medium 1, a 5% inoculum degraded the 5 mg of heliotrine present in 24 hr, and isolation was attempted by dilution culture on agar slopes. The same medium was used for agar slopes, but with 1·5% agar, and incubations were for 4–14 days at 39°C. After eight successive dilutions, colonies found to degrade heliotrine always contained at least two organisms, a large and a small coccus.

The large coccus was isolated and propagated in the lactate medium, but it did not degrade heliotrine. Superficial similarities to *Peptostreptococcus elsdenii* suggested that the apparent symbiosis between the organisms in bringing about degradation of heliotrine may have been due to production of formate or hydrogen by the large coccus. Accordingly, 0·2% formate was added to medium 1, and with an atmosphere of CO_2–H_2 (1 : 1 by vol.), it was possible to isolate the small coccus by dilution and cause it to degrade heliotrine in liquid culture. It was found later that either formate or hydrogen would support the degradation of heliotrine, and that vitamin B_{12} was not required. The five further isolates made were obtained using medium 3, a modification of medium 2 (Table 1) made by adjusting the pH to 6·9 with Na_2CO_3 and by omitting vitamin B_{12}. Of the five later isolates, one degraded heliotrine at a much slower rate than the others, and, after several subcultures, grew well but did not degrade heliotrine. Morphologically all five of the later isolates seemed to be identical with the first (Fig. 2).

(c) *Media*

Medium 1 (Table 1) was used only during the first isolation of the organism, but this particular isolate was used in all the experiments reported. Some of these experiments were conducted using medium 2 (Table 1) in which formate replaced the mixture of volatile acids in medium 1, but in most of the reported work vitamin B_{12} was also omitted and the pH was increased to 6·9 (medium 3). A medium low in vitamin B_{12} was prepared from medium 3 by the method of Davis and Chow (1952), after it was found that all of several preparations of yeast extract available contained about 10^{-3} ng vitamin B_{12} activity/mg dry solids, as estimated by the *Lactobacillus leichmannii* procedure (Dawbarn and Hine 1954). This vitamin B_{12} activity was probably due to deoxyribonucleosides in the yeast extracts (Smith 1960). Vitamin B_{12} added to medium 3 at 0·04 ng/ml was completely removed by the above procedure. Medium 3 was treated similarly and used to determine the effects of the absence of vitamin B_{12} on the degradation of heliotrine by the isolated organism.

TABLE 1

COMPOSITION OF MEDIA 1 AND 2

Medium 1*		Medium 2†	
Component	Concentration	Component	Concentration
DL-Lactate	0·05%	$Na_2S.9H_2O$	0·025%
$Na_2S.9H_2O$	0·025%	Cysteine.HCl.H_2O	0·025%
Cysteine.HCl.H_2O	0·025%	Resazurin	1 μg/ml
Resazurin	1 μg/ml	Na_2CO_3	1·06%
Na_2CO_3	0·53%	Difco yeast extract	0·05%
Difco yeast extract	0·05%	Agar	0·1%
Agar	0·1%	Heliotrine	0·5 mg/ml
Heliotrine	0·5 mg/ml	Vitamin B_{12}	5 μg/ml
Vitamin B_{12}	5 μg/ml	KH_2PO_4	$1·3\times10^{-3}$M
KH_2PO_4	$1·3\times10^{-3}$M	NaCl	$7·6\times10^{-4}$M
NaCl	$7·6\times10^{-4}$M	$(NH_4)_2SO_4$	$3·4\times10^{-3}$M
$(NH_4)_2SO_4$	$3·4\times10^{-3}$M	K_2HPO_4	$1·7\times10^{-3}$M
K_2HPO_4	$1·7\times10^{-3}$M	$CaCl_2$	$4·1\times10^{-4}$M
$CaCl_2$	$4·1\times10^{-4}$M	$MgSO_4$	$3·8\times10^{-4}$M
$MgSO_4$	$3·8\times10^{-4}$M	Formic acid	0·5%
Acetic acid	$2·9\times10^{-2}$M		
Propionic acid	$8·0\times10^{-3}$M		
Butyric acid	$4·3\times10^{-3}$M		
Isobutyric acid	$1·1\times10^{-4}$M		
n-Valeric acid	9×10^{-4}M		
Isovaleric acid	9×10^{-4}M		

* In equilibrium with an atmosphere of 100% CO_2 at pH 6·6.

† In equilibrium with an atmosphere of CO_2, and pH adjusted to 6·6.

The rumen fluid medium used to determine growth and degradation of heliotrine by the isolated organism (Fig. 5) was prepared from the rumen contents of a Merino ewe on dry summer pasture. After filtering through cheesecloth the fluid was autoclaved for 15 min at 15 lb/in² pressure before centrifuging for 1 hr at 15,000 *g*. To the supernatant were added heliotrine (0·5 mg/ml), formate (0·065%), Na_2S (0·025%), and cysteine (0·025%), and the solution was adjusted to pH 6·9 with Na_2CO_3 whilst bubbling with CO_2. The prepared medium was then dispensed in sealed tubes in the usual way and autoclaved before use.

In experiments where consumption of formate or hydrogen was related to consumption of heliotrine, a modified form of medium 3 was used in which 0·5% acetate replaced 0·5% formate.

(d) Reduction of Heliotrine with Formate

[^{14}C]Formate (obtained from the Radiochemical Centre, Amersham, England) was used to measure the stoichiometric relationship between heliotrine reduced and formate consumed. Medium 3 was used at pH 6·9 but with acetate instead of formate as buffer and with 1 mg/ml of heliotrine in 10-ml volumes. Equimolar amounts of [^{14}C]formate (32 μmoles, 0·3 nCi/μmole) were introduced before sterilization, and a formate-free inoculum (grown with hydrogen) was employed. After incubation for 63 hr, 0·5 ml of 10N NaOH was injected through the stopper and the solution shaken to trap labelled CO_2. A sample was taken for paper chromatographic estimation of 7α-hydroxy-1-methylene-8α-pyrrolizidine. The remaining tube contents were then bubbled with nitrogen in the apparatus of Phares (1951). After acidification with 2 ml of 10N HCl, $^{14}CO_2$ was recovered in two consecutive 3-ml traps of ethanolamine in methyl cellosolve (Jeffay and Alvarez 1961) by bubbling for 45 min. Formic acid was not distilled. Aliquots (1 ml) of the trapped $^{14}CO_2$ were counted in toluene phosphor (Jeffay and Alvarez 1961) in a Packard Tricarb scintillation counter model 3001 (Packard Instrument Co., Downers Grove, Illinois), with and without internal standards of [^{14}C]toluene (Radiochemical Centre, Amersham). After counting, small aliquots (1 nCi) of [^{14}C]formate were added to each tube and recounted to allow direct comparisons of counts as [^{14}C]formate and $^{14}CO_2$.

The acidic residue after removal of $^{14}CO_2$ was extracted five times with equal volumes of freshly distilled diethyl ether. The combined ether extracts, taken to dryness, constituted the crude heliotric acid fraction, which was weighed, titrated, and chromatographed. After removal of heliotric acid the aqueous phase was made alkaline with 10N NaOH and extracted with an equal volume of n-butanol, followed by three similar extractions with chloroform. The combined extracts were evaporated to dryness, taken up in ethanol, and precipitated with a few drops of ethanolic picric acid solution. The picrate was recrystallized and identified by melting point and by paper chromatography of the regenerated free base.

(e) Reduction of Heliotrine with Hydrogen

Measured quantities of sterile hydrogen gas were introduced in calibrated glass ampoules made from thin-walled capillary tubing. These were inserted into the incubation tubes at the time of inoculation, the tubes sealed, and the capillaries broken by mechanical vibration of the tubes. Each incubation tube contained a short piece of heavy glass rod to break the capillary. Immediately before insertion, the capillaries, open at one end, were filled in an enclosed jet of oxygen-low hydrogen (Meites and Meites 1948), sealed with paraffin wax under hydrogen at atmospheric pressure, and rapidly inserted into the tubes. After correction for temperature and pressure, the number of μmoles of hydrogen introduced was calculated. The tubes were incubated for 64 hr while inverted to prevent migration of hydrogen into the stoppers. The residual heliotrine was then estimated as described below and heliotrine consumption related to hydrogen added.

(f) Estimation of Heliotrine

This was a quantitative adaption of the method used by Chalmers, Culvenor, and Smith (1965) to detect pyrrolizidine alkaloids on thin-layer chromatography plates. It is based on the capacity of many nitrogenous compounds to form addition complexes with iodine vapour (Brante 1949). Such complexes may accrete many iodine atoms for each nitrogen atom and are often of uncertain constitution. In order to estimate the nitrogenous substance by the amount of iodine taken up it is necessary to use strictly standardized conditions of time and temperature. The following procedure was found to give reproducible results.

Whatman No. 1 paper was pre-washed (descending) in water–n-butanol–acetic acid (100 : 5 : 5, v/v/v). After drying, aliquots of heliotrine in ethanol were spotted at 5-cm intervals, 2 cm from one edge of the paper, and chromatographed overnight, ascending 26 cm. The solvent was n-butanol saturated by shaking with an equal volume of 5% (v/v) acetic acid (Chalmers, Culvenor, and Smith 1965). After drying for 8 hr in a stream of air the spots were visualized by exposure for 30 sec in a tank of iodine vapour and marked with pencil. Pieces of paper were then cut out to include the spot and a similar adjacent area of the same R_F. The excised paper pieces were then folded in half to include the spot in one half and suspended from one corner by cotton

thread in individual large weighing bottles containing air saturated with iodine vapour at 0°C in the dark. After standing 17 hr at 0°C an asymptotic value of iodine accretion was reached. The paper was then removed in the cold room, the visible spot cut out with a 0·5-cm margin, and the excised spot dropped immediately into a tube containing 10 ml of 0·1M KI solution. After standing for 1 hr the tube was shaken and the iodine in solution determined by absorption at 360 mμ against 0·1M KI solution in 1-cm cuvettes (Beckman model D.U. spectrophotometer). Blank areas of paper identical to the spots were also cut out from the iodine-treated samples, eluted similarly, and net optical density due to heliotrine calculated. The standard curve with heliotrine is shown in Figure 1. Residual heliotrine in culture tubes was estimated in triplicate. A sample of the spent medium (0·1 ml) was acidified with an equal volume of 1N HCl and dried in a vacuum desiccator at 1 mmHg pressure. The residue was made alkaline with 0·1 ml of 0·2N Na_2CO_3 and redried. The residue was then extracted three times, each with three drops of ethanol, the combined extract spotted onto the paper, and heliotrine estimated as described.

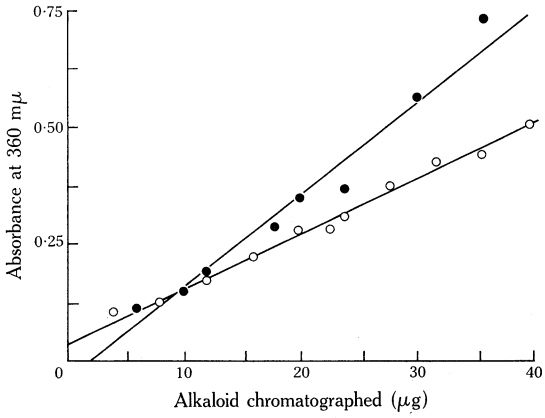


Fig. 1.—Standard curves for chromatographic estimation of alkaloids by accretion of iodine on paper chromatograms. Aliquots of the pure alkaloids were chromatographed and their uptake of iodine estimated as described in the text. ○ Heliotrine. ● 7α-hydroxy-1-methylene-8α-pyrrolizidine.

It was also possible to estimate 7α-hydroxy-1-methylene-8α-pyrrolizidine by this method (Fig. 1), although with considerably greater error.

(*g*) *Thin-layer Chromatography*

Cultures were routinely examined for heliotrine degradation by thin-layer chromatography (Chalmers, Culvenor, and Smith 1965). Samples were prepared as described above but extracted with chloroform for spotting on the plate.

(*h*) *Measurement of Bacterial Growth*

In agar-free media growth was followed variously by measuring turbidity with an EEL nephelometer (Evans Electro Selenium Ltd., Halstead, Essex), by measuring absorption at 650 mμ directly in the anaerobic tube (Coleman model 11 spectrophotometer, Coleman Electric Co. Inc., Maywood, Illinois), or by estimation of the bacterial protein produced. For protein estimation cells were recovered by centrifuging for 15 min at 3000 *g* in conical tubes, resuspending in 10 ml of washing fluid (medium 2 without yeast extract, heliotrine, or vitamin B_{12}), and recentrifuging. The protein in the washed sediment was dissolved by rubbing in 0·2 ml 1N NaOH and standing at room temperature for 30 min. After adding 0·2 ml water, protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine albumin (Nutritional Biochemicals

Inc., Cleveland, Ohio) as a standard. If only part of the contents of a culture tube was used, the bacteria were suspended by mechanical shaking (Whirlimixer, Scientific Industries International Inc. U.K. Ltd., London) before the sample was withdrawn.

(*i*) *Chemicals*

Heliotrine isolated from *H. europaeum* was used (Culvenor, Drummond, and Price 1954). Heliotric acid was prepared by alkaline hydrolysis of heliotrine (Culvenor, Drummond, and Price 1954). 7α-hydroxy-1-methylene-8α-pyrrolizidine was recovered from collected spent cultures of the isolated organism in which some degradation of heliotrine had occurred. It was identified by comparison with an authentic sample (Culvenor and Smith 1961). Both the isolated material and the authentic sample had an R_F of 0·29 on paper chromatography by the method described for estimation of heliotrine. Titration with standard acid indicated an equivalent weight of 159 (theoretical 154).

Vitamin B_{12} was obtained from Glaxo-Allenburys (Aust.) Pty. Ltd., Melbourne. Factor B was prepared from vitamin B_{12} by the method of Brown *et al.* (1955) and purified by chromatography on Whatman 3MM paper by the method of Ford, Holdsworth, and Kon (1955). The band with R_F twice that of vitamin B_{12} was eluted in ethanol and dried. The spectrum of this material agreed with that reported by Armitage *et al.* (1953) for factor B.

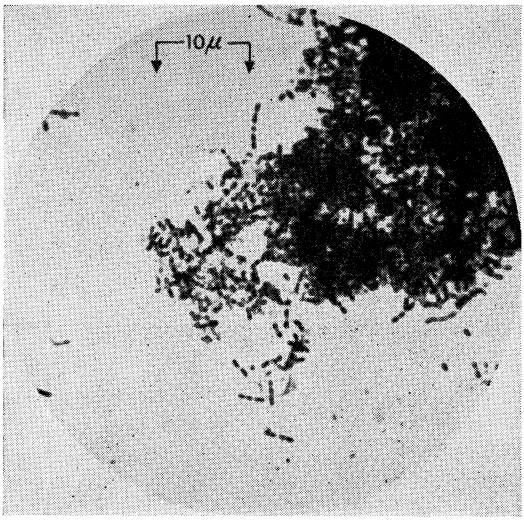


Fig. 2.—Photomicrograph of small coccus, showing characteristic growth of cells in pairs and short chains, forming clumps. Culture grown on medium 3 and stained with Hucker's ammonium oxalate–crystal violet.

III. Results

(*a*) *Description of Organism*

In agar slopes after growth for 2 weeks the organism grew in diffuse spherical white colonies, about 0·5 mm in diameter. Inoculation into 0·1% agar medium produced a culture which also tended to form small discrete colonies suspended in the medium. In liquid medium, in the absence of agar, the bacteria sedimented as a flocculent mass at the bottom of the tube, despite constant shaking, but could be suspended by vigorous mechanical shaking with the Whirlimixer. The organism was a strict anaerobe, and did not grow unless the resazurin indicator was colourless [$E_0 < -0{\cdot}042$V (Twigg 1945)]. As cultured the isolate was Gram-negative, and, when stained with Hucker's ammonium oxalate–crystal violet, was seen microscopically to be a micrococcus, with ovoids 0·6 μ in length, growing in pairs, short chains, and clumps (Fig. 2).

(*b*) *Requirements for Growth and Degradation of Heliotrine*

In medium 3, in the absence of heliotrine, yeast extract was found to be both essential and sufficient for growth, 1 mg of yeast extract permitting synthesis of 4 μg of bacterial protein. With limiting quantities of yeast extract (0·05%), additional growth was stimulated by heliotrine (Fig. 3), but in the absence of yeast extract

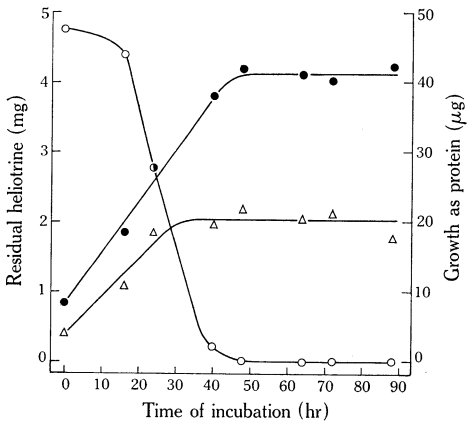


Fig. 3.—Growth of the organism in 10-ml culture tubes related to degradation of heliotrine. The difference between the asymptotes of the two growth curves shows the growth response accompanying complete degradation of heliotrine. ○ Chromatographic estimation of residual heliotrine in the medium. △ Protein production in control tubes with no heliotrine. ● Protein production in tubes containing 5 mg of heliotrine.

heliotrine did not support growth and was not degraded. In medium 3 degradation of 1 μmole of heliotrine gave rise to an additional 1·2 μg of bacterial protein. Under these conditions bacterial protein was shown to constitute about 60% of total dry weight of the washed organisms. Attempts to replace yeast extract with synthetic mixtures of amino acids and vitamins were not successful. The organism also failed to grow on the yeast medium if sodium carbonate and carbon dioxide were replaced by phosphate buffer and nitrogen respectively.

In medium 3 the maximum rate of degradation of heliotrine occurred at pH 6·9. Two further pyrrolizidine alkaloids, lasiocarpine and supinine (Culvenor, Drummond, and Price 1954), were also degraded by the isolate when substituted for heliotrine in medium 3. Degradation was established by thin-layer chromatography but products were not identified.

(*c*) *Conditions for Growth in the Absence of Heliotrine*

The following growth measurements were carried out using medium 3, but omitting heliotrine and substituting 0·5% yeast extract where no other growth substrate was included. The optimum pH for growth was 6·0–6·5. No growth occurred at pH 5·5 or 7·2. At pH 6·9, optimum temperature for growth was 39°C. After incubation for 82 hr no growth occurred at 26 or 50°C, while at 30°C growth

was about 60% of the maximum. No growth occurred with 2% NaCl in the medium. Nitrate was reduced to nitrite, ammonia was produced from arginine, gelatin was not liquefied, and starch was hydrolysed. In the presence of 0·05% yeast extract, the organism showed substantial additional growth on the following: mannose, fructose, galactose, xylan, xylose, starch, maltose, cellobiose, lactose, mannitol, sucrose, raffinose, melibiose, melizitose. Growth was not supported by arabinose, rhamnose, glucose, glycerol, sorbitol, dulcitol, or ribose.

(*d*) *Growth and Degradation of Heliotrine in the Presence and Absence of Vitamin B_{12} and Factor B*

Medium 3 contained sufficient yeast extract to give an apparent vitamin B_{12} content of 10^{-6} ng/ml, but this was reduced by the method of Davis and Chow (1952) to a point where the *L. leichmannii* assay (Dawbarn and Hine 1954) was insufficiently sensitive to detect any residual vitamin B_{12}. An initial inoculum containing 10^{-6} ng/ml of vitamin B_{12} activity was serially subcultured seven times at the 1% level in the vitamin B_{12}-low medium. In all these cultures the organism grew normally and reduced heliotrine as rapidly as in the presence of 5 μg/ml of vitamin B_{12}. The mean apparent vitamin B_{12} content of the spent cultures in vitamin B_{12}-low medium was 0·02 ng/ml. These are considered to be negligible levels and probably represent deoxyribonucleosides.

An attempt was then made to repeat the observed effect of vitamin B_{12} (Dick *et al.* 1963) with the supernatant fluid from lightly centrifuged rumen contents. Factor B has been found to give an enhanced Peel effect (Peel 1963), and so was compared with vitamin B_{12}.

Replicate flasks were set up, each containing 100 ml of lightly centrifuged rumen liquor, adjusted to pH 6·9 under CO_2, and 0·5 mg/ml of added heliotrine. One flask was untreated, one contained 2·0 μg/ml vitamin B_{12}, and the third an equivalent amount of factor B (1·3 μg/ml). A stream of CO_2 gas (which in this experiment was not treated to remove traces of oxygen) was bubbled through each flask while incubating at 39°C, and samples of the fluid taken for analysis at intervals over 72 hr. The results of the estimations of residual heliotrine are given in Figure 4. Estimation of heliotrine in rumen fluid was subject to greater apparent errors than in synthetic media, but it is clear that there was no effect of either vitamin B_{12} or factor B on the course of heliotrine degradation. Similar results were obtained in a second experiment.

(*e*) *Reduction of Heliotrine in a Rumen Liquor Medium*

The lag period before heliotrine began to be degraded by the rumen fluid supernatant (Fig. 4) suggests that adaptation of the organism to heliotrine may have occurred. Once degradation began it proceeded rapidly to completion. The result of inoculating sterile rumen fluid with the previously adapted organism is shown in Figure 5. The rumen fluid was supplemented with formate (0·065%) and prepared as described in Section II. When incubated at 39°C, and gassed continuously with CO_2, a 1% inoculum led to rapid growth of the organism and only a short lag

period occurred before heliotrine degradation commenced. If formate was omitted, heliotrine was degraded much more slowly and not to completion, but rapid growth of the organism still occurred.

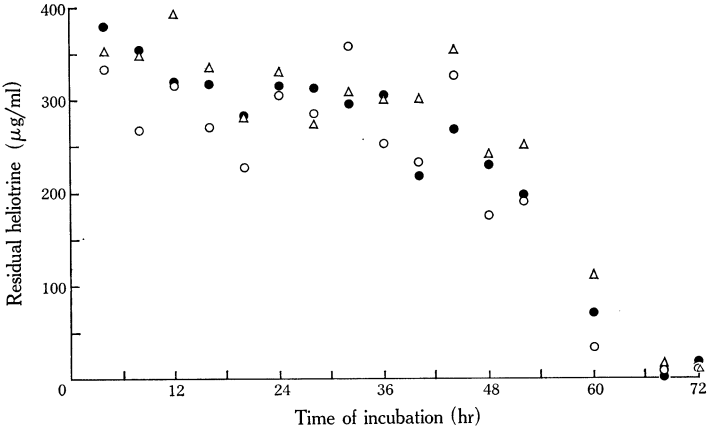


Fig. 4.—Degradation of added heliotrine by lightly centrifuged rumen fluid at pH 6·9, incubated at 39°C under CO_2 atmosphere. There was no increase in rate of degradation when either vitamin B_{12} or factor B was added to the medium. ○ Rumen fluid. △ Rumen fluid with added vitamin B_{12} (2·0 μg/ml). ● Rumen fluid with added factor B (1·3 μg/ml).

To test the possibility that the organism had to be adapted to degrade heliotrine, the isolate was maintained through 10 subcultures in medium 3 and also in the same medium with added yeast extract replacing heliotrine. These two cultures were

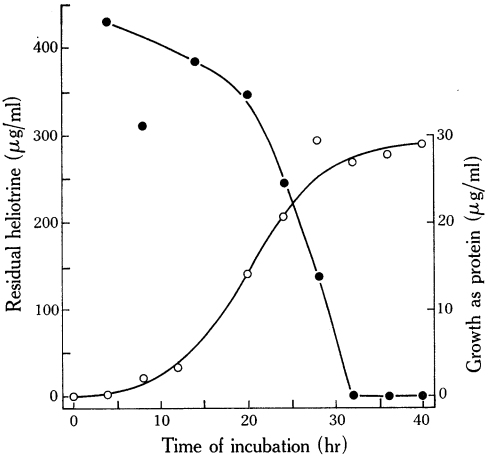


Fig. 5.—Growth of the organism related to heliotrine degradation when inoculated into sterilized and centrifuged rumen fluid medium ● Residual heliotrine in medium. ○ Protein produced.

then inoculated at the same level into medium 3. With the inoculum grown on yeast alone, heliotrine degradation commenced after incubation for 20 hr and was complete in 33 hr. With the inoculum grown in the presence of heliotrine, heliotrine degradation commenced at 12 hr and was complete in 26 hr.

These two inocula were similarly incubated in medium 3 in which heliotrine was replaced by lasiocarpine or supinine, two other alkaloids present in *H. europaeum* (Culvenor, Drummond, and Price 1954). The rate of degradation of these alkaloids was identical with both inocula; degradation commenced after incubation for 20 hr and was complete in 33 hr.

(*f*) *Reduction of Heliotrine with Formate*

Preliminary experiments showed that whereas in the presence of heliotrine [^{14}C]formate was converted to $^{14}CO_2$ in molecular proportions equivalent to the heliotrine degraded, when heliotrine was absent similar quantities of [^{14}C]formate were only partly (33%) converted to $^{14}CO_2$. An experiment was then conducted to measure the complete stoichiometry of heliotrine reduction by [^{14}C]formate. Five replicate incubation tubes contained 32 μmoles each of heliotrine and [^{14}C]formate, while three control tubes contained heliotrine but no formate. Tubes were inoculated and incubated at 39°C for 63 hr. In the absence of formate heliotrine was not degraded, whereas in tubes containing equimolar quantities of formate and heliotrine there was no measurable residual heliotrine at 63 hr. The mean recovery of products from the five replicate tubes that degraded heliotrine were: $^{14}CO_2$, 94% of [^{14}C]formate added; residual formate, nil; heliotric acid, 98% of heliotrine degraded; 7α-hydroxy-1-methylene-8α-pyrrolizidine, 108% of heliotrine degraded.

Recovery of heliotric acid was based on weight. By titration of the combined weighed extracts it was estimated that 92% of this material was heliotric acid (molecular weight 160). A sample of the combined extracts was chromatographed on Whatman No. 1 paper together with authentic heliotric acid. The two substances gave identical R_F values in the following solvents: (1) n-butanol saturated with 1·5N NH_4OH, ascending chromatography; single spot R_F 0·43 (Reid and Lederer 1952); (2) isoamyl alcohol saturated with 4N formic acid (Flavin and Ochoa 1957); major spot R_F 0·91 with trace spot R_F 0·69 in both standard and unknown. The remaining dry extract was then sublimed to yield white crystalline needles with m.p. 92°C. Mixed m.p. with authentic heliotric acid was also 92°C.

The 7α-hydroxy-1-methylene-8α-pyrrolizidine was identified by thin-layer chromatography in methanol, giving R_F 0·17, and by paper chromatography in n-butanol–5% acetic acid (1 : 1, v/v), giving R_F 0·29. The free base was converted to the picrate as described in Section II. Constant m.p. 172°C was obtained after recrystallizing three times from ethanol.

(*g*) *Reduction of Heliotrine with Molecular Hydrogen*

After it had been established that either hydrogen or formate could serve to bring about reductive cleavage of heliotrine, an experiment was conducted to compare the relative effectiveness of the two substrates. Using medium 3, with formic acid replaced by acetic acid, measured quantities of formate or hydrogen were added to

the medium, and the resulting degradation of heliotrine estimated after incubation for 64 hr. The results are given in the following tabulation:

	Control	Hydrogen					Formate	
Concn. of reducing agent (μmoles)	—	2·1	4·1	8·2	10·3	14·8	8·0	16·0
Amount of heliotrine reduced (μmoles)	0	3·5	4·2	9·4	10·4	13·2	9·4	16·0

Either formate or hydrogen quantitatively limited the amount of heliotrine reduced and it may be inferred that both were quantitatively consumed. Estimation of the additional growth brought about by reduction of heliotrine in a medium in which yeast extract was limiting, showed that both formate and molecular hydrogen resulted in about 1·2 μg additional protein being synthesized per μmole of heliotrine reduced.

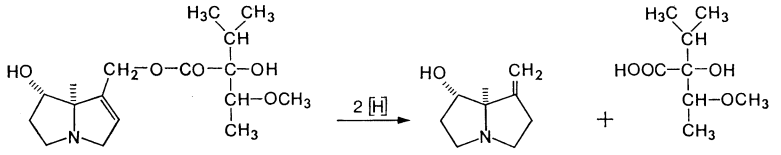
IV. Discussion

The presence in rumen contents of an organism that degrades heliotrine provides a rational explanation for the relative resistance of animals consuming *H. europaeum* to the toxic effects of the alkaloid (Bull *et al.* 1956). One of the products of the bacterial degradation, 7α-hydroxy-1-methylene-8α-pyrrolizidine, has been identified in the rumen contents of sheep fed *H. europaeum* (Culvenor, Dann, and Dick 1962), together with other degradation products. During the present work, other products which were stained by iodine and which had low R_F values on thin-layer or paper chromatograms were often observed when heliotrine was degraded by cultures of mixed rumen organisms. These never appeared as primary products of heliotrine degradation in the absence of 7α-hydroxy-1-methylene-8α-pyrrolizidine, however, but often appeared only after the heliotrine was almost exhausted and the methylene derivative the chief visible precursor. As isolation of the small coccus proceeded, these products ceased to form, and it is tentatively concluded that they were secondary degradation products of 7α-hydroxy-1-methylene-8α-pyrrolizidine formed by other rumen organisms. It is probable that the methylene derivative is also the primary degradation product in the intact rumen.

A stimulatory effect of vitamin B_{12} on the rate of degradation of heliotrine by organisms in rumen fluid (Dick *et al.* 1963) was not observed under the present conditions. Although it is possible that an entirely different organism was responsible for the reductive cleavage in the present case, the general similarities in relation to product and lag period suggest that this was not the case. The isolation of apparently identical organisms from the rumens of animals on two widely different pasture types from separate localities suggests that the small coccus isolated is commonly present in the rumen, while the relatively simple nutritional requirements and broad specificity for carbohydrates indicate its competence in the ruminal environment. The organism was found to be a strict anaerobe, and it is possible that the effect of vitamin B_{12} observed by Dick *et al.* (1963) was related to the achievement of anaerobiosis by the Peel effect (Peel 1963). Factor B was shown to be 4000 times as effective as vitamin

B_{12} in catalysing the oxidation of thiols by molecular oxygen (Peel 1963), but in the present experiments neither vitamin B_{12} nor factor B had any effect on the rate of degradation of heliotrine. There was no demonstrable requirement of the isolated organism for vitamin B_{12} in the culture medium and negligible quantities of B_{12}-active substances were present after heliotrine was degraded.

The course of heliotrine degradation by the small coccus is shown in the following scheme:



(I) (II) (III)

Heliotrine (I) acted as hydrogen acceptor in a reaction in which 1 mole of hydrogen gas led to reductive cleavage of 1 mole of heliotrine to produce 1 mole each of 7α-hydroxy-1-methylene-8α-pyrrolizidine (II) and heliotric acid (III). Hydrogen could be replaced by formate in which case 1 mole of carbon dioxide was evolved. As both heliotrine and the hydrogen source consumed were completely recovered in the cleavage products, they therefore cannot have been incorporated into the bacteria. Nevertheless, the bacteria showed a reproducible growth proportional to alkaloid degraded, and it may be inferred that an energy yield was derived from the reaction.

The maximum rate at which heliotrine was degraded was quite high in terms of bacterial protein present. A value of about 0·5 μmoles/mg bacterial protein/min may be derived from the data in Figure 3. On the other hand, the magnitude of the net growth response coupled to the reductive cleavage was surprisingly small. Organisms grown in the presence of heliotrine contained about 60% of their dry matter as protein. The net protein yield of 1·2 μg protein per μmole of heliotrine degraded therefore represents about 2 μg of dry matter per μmole of heliotrine. It has been shown (Bauchop and Elsden 1960) that bacterial dry matter generally represents a growth yield of about 10 μg of dry matter per μmole of ATP derived from the prevailing energy-yielding reactions. Assuming only one ATP to have been produced from ADP per mole of hydrogen accepted by heliotrine, the growth yield in the present case was only 20% of this value. This apparent inefficiency may have been an expression of toxicity of the alkaloid.

The organism used molecular hydrogen to reduce heliotrine with surprisingly high efficiency, degradation of heliotrine approaching closely the amount of hydrogen added in the incubation time allowed. Similar results were obtained with formate, and the growth yield was practically identical in both cases. When formate was added in the absence of heliotrine only part of the formate was converted to carbon dioxide, and the intervention of the enzyme system "formate–hydrogen lyase" seems possible. This system was shown to be reversible (Woods 1936), and in the presence of an excess of carbon dioxide may have catalysed an equilibrium between formate and hydrogen. In the experiments reported, carbon dioxide was always present in excess and so it cannot be decided from the results whether formate or

molecular hydrogen was the immediate electron donor in the reaction sequence leading to reduction of heliotrine. Both substances, however, are found in the rumen (Hungate 1966).

The relatively long lag period before heliotrine was degraded by the mixed organisms in rumen fluid suggests the occurrence of an adaptive process, and this is supported to some extent by the much shorter lag period when pure cultures were grown in a rumen fluid medium after previous culture in the presence of heliotrine. The rumen fluid medium, however, contained both additional formate and additional reducing agents. Although the latter were not capable of supporting reduction of heliotrine they may have hastened the establishment of anaerobiosis and so contributed to a shorter lag period. When inoculated into medium 3, the difference in lag period between inoculum cultured on heliotrine medium, and that grown on yeast extract, was only 8 hr. Thus, for the isolate, the apparent time required for adaptation was not great when considered in practical terms of sheep ingesting the alkaloid.

It is possible that in the rumen the small coccus isolated brings about the reduction by molecular hydrogen of other unsaturated substrates. Much of the hydrogen produced in the rumen is consumed again by processes that are not understood (Hungate 1966), although Kemp and White (1968) recently reported the isolation from a sheep's rumen of three unidentified microorganisms capable of hydrogenating linolenic and linoleic acids.

The nutritional requirements of the small coccus appear to be adequately met in the environment of the normal rumen, and the occurrence of the organism in the rumen of sheep consuming heliotrine may be expected to lead to degradation of the alkaloid.

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