STUDIES ON ENDEMIC GOITRE

II. THE BEHAVIOUR OF 3-METHYLSULPHONYLPROPYL ISOTHIOCYANATE (CHEIROLIN) AND OTHER ISOTHIOCYANATES IN BOVINE RUMEN LIQUOR

By H. S. BACHELARD* and V. M. TRIKOJUS*

[*Manuscript received October* 10, 1962]

Summary

When incubated with bovine rumen liquor cheirolin (3-methylsulphonylpropyl isothiocyanate) has been shown to be converted into the disubstituted thiourea, dicheirolin thiourea, with traces of other identified compounds. At the pH tested, only one other isothiocyanate was found to undergo a similar conversion. The reaction is not enzyme dependent but appears to be catalysed by a metal ion. During studies of the behaviour of thioglucosides in rumen liquor the presence of thioglucosidases could not be demonstrated in this medium.

I. INTRODUCTION

The fruit and leaves of *Rapistrum rugosum* (Queensland turnip weed), the major pasture contaminant of the goitrous area of Warwick, Qld., have been shown to contain relatively large amounts of glucocheirolin, with only a trace of another unidentified thioglucoside (Bachelard and Trikojus 1960, 1963). In the preliminary report (Bachelard and Trikojus 1960) it was observed that the corresponding aglucone cheirolin (3-methylsulphonylpropyl isothiocyanate), when incubated with bovine rumen liquor, was transformed into *N*,*N*′-di(3-methylsulphonylpropyl)thiourea (dicheirolin thiourea) which was characterized by isolation and comparison with an authentic sample. A trace of monocheirolin thiourea was also produced. Unlike cheirolin, which markedly depressed the uptake of ^{131}I by the rat thyroid in acute tests, dicheirolin thiourea proved to be, unexpectedly, only weakly goitrogenic.

The experiments described in the present communication were designed to study the mechanism of the transformation of cheirolin in rumen liquor and to ascertain to what extent the reaction is applicable to other isothiocyanates. Observations on the goitrogenic activities of a range of isothiocyanates and substituted thioureas are reported in Part III of this series (Bachelard, McQuillan, and Trikojus 1963).

II. MATERIALS AND METHODS

(*a*) *General*

Methods and some of the solvent systems used in paper chromatography are as described in Part I of the series (Bachelard and Trikojus 1963). Another solvent system containing n-propanol–water (4 : 1 v/v) (referred to as PW) was also used.

* The Russell Grimwade School of Biochemistry, University of Melbourne.

Ninhydrin spray contained 0·2% ninhydrin in n-butanol–water (9 : 1 v/v), the colour being developed by heating at 80°C for 15 min. Myrosinase was prepared according to Sandberg and Holly (1932). pH was determined with the glass-electrode.

(*b*) *Isothiocyanates*

Cheirolin was prepared from 3-methylsulphonylpropylamine and thiophosgene as described in Part I and crystallized to constant melting point before use. Allyl, ethyl, isopropyl, and s-butyl isothiocyanates and the corresponding *N*-mono- and *N*,*N*′-disubstituted thioureas were donated by Imperial Chemical Industries of Australia and New Zealand Ltd. 3-Methylthiopropyl isothiocyanate was prepared from 3-methylthiopropylamine by reaction with thiophosgene following the conditions used for cheirolin; the isothiocyanate had b.p. 119°C, 11 mm (Kjaer, Gmelin, and Larsen (1955) recorded b.p. 120–1°C, 12 mm). Attempts to synthesize 3-methylsulphinylpropyl isothiocyanate by the method described by Karrer, Scheitlin, and Siegrist (1950) were unsuccessful. This isothiocyanate was prepared from the thioglucoside, glucoiberin (California Corporation for Biochemical Research) by the action of myrosinase. 4-Methylsulphonyl-n-butyl isothiocyanate (erysolin) and the corresponding amine were donated by Professor A. Kjaer. Glucocheirolin, glucotropaeolin, and sinigrin were prepared as described in the previous paper. Progoitrin was a gift from Professor M. A. Greer.

(*c*) *Rumen Liquor*

Rumen liquor was taken from freshly slaughtered cattle at the abattoirs, brought immediately to the laboratory, filtered in the cold room through butter muslin, and the filtrate used directly.

III. Experimental and Results

In the preliminary report (Bachelard and Trikojus 1960) cheirolin was incubated with rumen liquor in the presence of added urea to produce levels of ammonium ion comparable with those attained in animals grazing during the flush spring growth. In the following experiment cheirolin was incubated in the presence and absence of urea. The effect of incubation on the monocheirolin derivative of thiourea was also studied. Incubation was carried out in tightly stoppered tubes for 3 hr at 37°C. The tube contents were then freeze-dried, the residues extracted with chloroform (30+20 ml), and the extracts concentrated under reduced pressure to 5 ml. Aliquots were then applied to paper, chromatographed in the solvent system chloroform–water (CW), and the paper sprayed with Grote's reagent (Grote 1931). The results are given in Table 1 and show that monocheirolin thiourea is not an intermediate in the conversion by rumen liquor of cheirolin to dicheirolin thiourea and that urea is not required in the process.

In other experiments the conversion was shown to occur regularly with rumen liquor collected at various times throughout the year and to be unaffected when the incubations were carried out under aerobic conditions. The reaction appeared to reach a maximum after about 2 hr.

(a) Spectrophotometric Assay of Thioureas Formed

A solution of cheirolin (10 mg) in 0·1M phosphate buffer (2 ml), pH 6·9, was incubated with rumen liquor (5 ml) for 3 hr at 37°C. Cheirolin (10 mg) in buffer (7 ml) served as control. Following incubation, freeze-drying, and extraction of the product into chloroform (2×10 ml) the extract was concentrated and made up to 2 ml. (Paper chromatography of an aqueous solution of the chloroform-insoluble residue showed that no thioureas were present.) Duplicate aliquots (0·048 ml) of the chloroform extract were applied as lines (2 mm wide) on Whatman No. 3 paper previously washed (Connell, Dixon, and Hanes 1955). The chromatograms were developed in descending CW until the solvent had reached the bottom of the papers.

TABLE I

INCUBATION OF CHEIROLIN (WITH AND WITHOUT ADDED UREA) AND MONOCHEIROLIN THIOUREA IN RUMEN LIQUOR FOR 3 HR AT 37°C

Chromatographic solvent system : chloroform–water (CW)

Tube	Buffer* (ml)	Urea (mg)	Cheirolin (mg)	Mono-cheirolin Thiourea (mg)	Rumen Liquor (ml)	Grote-positive Spots†		
						R_F = 0·12	R_F = 0·02	R_F = 0·98
1	35	60	50	—	—	+/−	−	++++
2	35	—	50	—	—	+/−	−	++++
3	10	60	50	—	25	++++	+	−
4	10	—	50	—	25	++++	+	−
5	10	60	—	—	25	−	−	−
6	10	60	—	50	25	−	++++	−

* 0·1M phosphate, pH 6·4.

† i.e. R_F values in CW of dicheirolin thiourea, monocheirolin thiourea, and cheirolin respectively. ++++ strong; + weak; +/− trace.

Strips containing detection spots were cut out and developed with Grote's reagent; the chromatograms were then reconstituted and areas corresponding to R_F 0·12 (dicheirolin thiourea) and R_F 0·02 (monocheirolin thiourea) cut out, divided into small pieces, and extracted three times with boiling methanol. The combined filtered extracts were concentrated *in vacuo*, made up to 3·0 ml with methanol, and the optical densities read at 243 mμ in a Unicam SP-500 spectrophotometer. The control incubation mixture was treated in duplicate in a similar manner—a trace spot at R_F 0·12 was the only Grote-positive constituent. Pure samples of both thioureas in methanol showed $\lambda_{max.}$ 243 mμ, the optical density being proportional to concentration up to 0·125M (ϵ = 11,500 for dicheirolin thiourea and 12,250 for monocheirolin thiourea). Blank values for the paper were obtained by cutting out and extracting with methanol pieces of the same size below the areas of R_F 0·12 in the main chromatograms. The optical densities observed and the calculated results, indicating a 31% conversion of cheirolin to dicheirolin thiourea, are given in Table 2. Duplicate

test runs with a solution of dicheirolin thiourea (3·5mM) after chromatography and elution as described gave recoveries of 90–95%.

TABLE 2

PERCENTAGE CHEIROLIN TRANSFORMED ON INCUBATION WITH RUMEN LIQUOR*

Treatment	R_F Area Eluted	Net Optical Density (minus blank)	Concentration in Extract (mM)	Yield (mg)	Cheirolin Converted (%)
Cheirolin (10 mg)	0·12†	0·805	0·070	2·75	31
plus rumen liquor	0·02‡	0·19	0·0155	0·38	3
Buffer control	0·12	0·155	0·0135	0·53	6

* For conditions see text.

† Dicheirolin thiourea.

‡ Monocheirolin thiourea.

(*b*) *Non-enzymic Nature of the Reaction*

Cheirolin was incubated with rumen liquor and with the same sample after boiling for 15 min and after filtration through a Seitz filter. The results are set out

TABLE 3

INCUBATION OF CHEIROLIN WITH NORMAL, BOILED, AND SEITZ-FILTERED RUMEN LIQUOR

Incubation period 3 hr at 37°C

Tube	Buffer* (ml)	Cheirolin (mg)	Rumen Liquor (ml)	Grote-positive Spots†		
				R_F = 0·12	R_F = 0·02	R_F = 0·98
1	2	10	5	++++	+	+
2	2	10	5‡	++++	+/−	+
3	2	10	5§	++++	+	+
4	2	—	5‡	−	−	−
5	2	—	5§	−	−	−
6	7	10	—	+/−	−	++++

* 0·1M phosphate buffer, pH 6·8.

† For relevant details of chromatography and notations used see Table 1.

‡ Rumen liquor previously boiled for 15 min.

§ Seitz-filtered rumen liquor.

in Table 3. The conclusion from this experiment is that either a heat-stable extracellular enzyme is involved or that the reaction is non-enzymic. Therefore, the effect of rumen-liquor dialysate was investigated. Rumen liquor was heated to

90°C for 7 min, cooled to 5°C, centrifuged, and the supernatant (40 ml) dialysed against water (400 ml) for 18 hr. The dialysate was freeze-dried and made up to the original volume (40 ml) with water. The activities of the original rumen liquor (after heating and centrifuging), the non-dialysable components, and the dialysate were compared as set out in Table 4. The results show that the factor in rumen liquor responsible for the conversion of cheirolin to dicheirolin thiourea is dialysable (Table 3); however, the usual weak spot due to monocheirolin thiourea (R_F 0·02) did not appear under these conditions.

TABLE 4

INCUBATION OF CHEIROLIN WITH HEATED RUMEN LIQUOR AND WITH ITS DIALYSABLE AND NON-DIALYSABLE FRACTIONS

Tube	Buffer* (ml)	Cheirolin (mg)	Rumen Liquor and Fractions (ml)	Grote-positive Spots†		
				R_F = 0·12	R_F = 0·02	R_F = 0·98
1	2	10	5	++++	+	+
2	2	10	5‡	+/-	—	++++
3	2	10	5§	++++	—	+
4	7	10	—	+/-	—	++++

* 0·1M phosphate buffer, pH 6·8.

† For relevant details of chromatography and notations used see Table 1.

‡ Non-dialysable fractions.

§ Dialysate.

(c) *Mechanism of the Reaction in Rumen Liquor*

It seems clear from the above experiments that the conversion of cheirolin to dicheirolin thiourea by rumen liquor is a non-enzymic process. Hydrogen sulphide is known (Sell and Proskauer 1876; Anschütz 1910) to convert isothiocyanates to disubstituted thioureas:

$$2RNCS + H_2S \longrightarrow (RNH)_2CS + CS_2; \quad (1)$$

however, any H_2S originally present in the rumen liquor would have been removed by boiling or during freeze-drying of the rumen liquor dialysate. Alternatively, the isothiocyanate could be partially converted to the primary amine which would readily condense with unchanged isothiocyanate:

$$RNH_2 + RNCS \longrightarrow (RNH)_2CS. \quad (2)$$

Schneider (1910) observed that cheirolin is hydrolysed by warm dilute acid or alkali to yield the amine quantitatively with liberation of CO_2 and H_2S:

$$RNCS + 2H_2O \xrightarrow{H^+ \text{ or } OH^-} RNH_2 + CO_2 + H_2S. \quad (3)$$

However, the conversion of cheirolin by buffered rumen liquor takes place at pH values close to 7, whereas in control tests with buffer alone it remains virtually unchanged (Tables 1–4).

In order to test whether the corresponding amine is formed, cheirolin was incubated for 0·75 and 3 hr at pH 6·8 (0·1M phosphate) and 37°C with rumen liquor dialysate or buffer only (Table 5). The contents of the tubes were freeze-dried, extracted into chloroform, and concentrated as described previously. Aliquots were applied to paper, and chromatographed in four solvent systems. After developing with ninhydrin, a strong and a trace spot appeared, corresponding to the products

TABLE 5
DETECTION OF 3-METHYLSULPHONYLPROPYLAMINE IN RUMEN LIQUOR DIALYSATE INCUBATED WITH CHEIROLIN

Tube	Cheirolin (mg)	Rumen Liquor Dialysate (ml)	0·1M Phosphate Buffer, pH 6·8 (ml)	Incubation Time (hr) at 37°C	Amine Detected*
1	10	5	2	0·75	+
2	10	—	7	0·75	—
3	10	5	2	3	+
4	10	—	7	3	—
5	—	5	2	3	—

* Ninhydrin spray. For chromatographic solvent systems see text.

in tubes 1 and 3 (Table 5), the former having the same R_F values as 3-methylsulphonylpropylamine in n-butanol–acetic acid–water (4:1:1 by vol.; R_F 0·12), n-butanol–acetic acid–water 4:1:3 by vol. (BAW), R_F 0·37), n-butanol–ethanol–water 4:1:4 by vol. (BEW), R_F 0·16), and PW (R_F 0·35). There was no difference in the intensity of this spot between the 0·75-hr and 3-hr incubation times. The second unidentified spot had R_F 0·85 (PW) and R_F 0·87 (BEW). In view of these results it is most likely that the conversion of cheirolin to the disubstituted thiourea occurs by a combination of reactions (3) and (2) above.

Compounds, in particular reducing agents, which could possibly promote the formation of amine and hence of the thiourea transformation product were incubated with dialysed (inactive) rumen liquor (5 ml) and cheirolin (10 mg) in 0·1M phosphate buffer (pH 6·9; 2 ml) for 3 hr at 37°C. The mixtures were extracted and chromatographed on paper in the usual manner. Incorporation in the incubation mixture of sodium ascorbate (30 mg), sodium formate (5, 15, and 30 mg), cysteine hydrochloride (30 mg), sodium lactate (20 mg), and sodium sulphide (15 mg) or ammonium chloride (15 mg) had no effect. Thioureas which appeared were no stronger than the barely discernible trace spots which were usually present in controls.

(d) Effects of Metal Ions in Rumen Liquor on the Transformation of Cheirolin to Dicheirolin Thiourea

In view of the negative results with reducing agents, the possibility of metal-ion catalysis was investigated. The filtrate (100 ml) of rumen liquor was dialysed at 3°C for 24 hr against water (1 l.). The dialysate was freeze-dried and made up to 100 ml with water. A portion (40 ml) was stored in the frozen state for use in control incubations while the remainder was divided (2 × 30 ml) and freeze-dried. One portion was heated at 600°C in a crucible in a muffle furnace until no carbon remained. The pale green residue (approx. 0·2 g) was dissolved in 10N H_2SO_4 (0·5 ml), adjusted to pH 6·7 with 1N NaOH, and diluted to 30 ml with 0·1M phosphate buffer, pH 6·8

TABLE 6

INCUBATION OF CHEIROLIN WITH RUMEN LIQUOR DIALYSATE (WITH AND WITHOUT EDTA), AND THE EFFECTS OF ASHING OF THE DIALYSATE

Incubation period 3hr at 37°C

Tube*	Further Additions	Grote-positive Spots†		
		R_F = 0·12	R_F = 0·02	R_F = 0·98
1	Dialysate	++++	—	+
2	Dialysate + EDTA (100 mM)	+/−	—	++++
3	Control (buffer alone)	+/−	—	++++
4	Ash solution A (see text)	—	—	++++
5	Ash solution B (see text)	++	—	++

* Each tube contained 7 ml buffered solutions (0·1M phosphate, pH 6·8) and 10 mg cheirolin.

† For relevant details of chromatography and notations used see Table 1.

("ash solution A"). The second portion was moistened with 10N H_2SO_4 (0·3 ml), dried by cautious heating, and then heated at 800°C in a muffle furnace until no carbon remained. The sulphate ash was diluted to 30 ml with 0·1M phosphate buffer pH 6·8 ("ash solution B"). The results of tests in which cheirolin was incubated at 37°C for 3 hr with the two ash solutions, the original rumen liquor dialysate with and without EDTA (100 mM), and buffer alone are given in Table 6, the incubated solutions being dried, extracted, and chromatographed in the usual manner. The results show that EDTA inhibits the reaction and that the sulphate ash ("ash solution B") is moderately catalytic.

Examination of three rumen liquor dialysates by atomic absorption spectroscopy* gave the results set out in Table 7. Of the constituents analysed copper ion appeared as a likely catalyst in view of its reported ability to promote the hydrolysis of esters (Wagner-Jauregg *et al.* 1955). However, when added to dialysed (inactive)

* By courtesy of Dr. A. Walsh and Dr. J. B Willis, Division of Chemical Physics, C.S.I.R.O., Melbourne.

rumen liquor to give a concentration of 20 p.p.m., the solution was unable to catalyse the conversion of cheirolin to its disubstituted thiourea. Further investigations along these lines have not yet been carried out.

TABLE 7

CONCENTRATION (p.p.m.)* OF METAL IONS IN THREE RUMEN LIQUOR DIALYSATES (I, II, III) AS DETERMINED BY ATOMIC ABSORPTION SPECTROSCOPY

n. d., not detectable

Dialysate No.	Na	K	Ca	Mg	Cu	Fe	Mn	Zn
I	2600	600	10	3	10	<1	<0·5	<0·5
II	2800	900	26	25	1	n.d.	n.d.	<0·5
III	3300	900	16	27	1	n.d.	n.d.	<0·5

* Based on original rumen liquor filtrates.

(e) *Effect of Incubation with Rumen Liquor on Other Isothiocyanates*

The incubation procedure followed that described for the incubation of cheirolin with freshly drawn ruminal contents. Control tubes contained the isothiocyanate

TABLE 8

INCUBATION OF VARIOUS ISOTHIOCYANATES WITH RUMEN LIQUOR*

Incubation period 3 hr at 37°C in 0·1M phosphate buffer, pH 6·8.
+, trace spot on chromatogram; —, not detected

Isothiocyanate	*N,N'*-Disubstituted Thiourea	Monothiourea	Amine
Allyl	—	+	—
Ethyl	—	—	—
isoPropyl	—	—	—
s-Butyl	—	—	—
3-Methylthiopropyl	—	—	+
3-Methylsulphinylpropyl	—	+	—
4-Methylsulphonylbutyl	—	+	—

* For chromatographic system see text.

without rumen liquor, while tests with cheirolin were included to ensure that active rumen liquor had been obtained. The preparation of the extracts for chromatography in the solvent system CW was as described above for cheirolin. Duplicate papers were sprayed with Grote's reagent and ninhydrin. If spots appeared, the extracts were again chromatographed with relevant marker spots. The substances tested and the results are presented in Table 8. Surprisingly, none of the isothiocyanates

TABLE 9

INCUBATION OF THIOGLUCOSIDES WITH RUMEN LIQUOR

Incubation at 37°C for 5 hr in 0·1M phosphate buffer, pH 6·8. Chromatography in chloroform–water

Tube*	Gluco-cheirolin (mg)	Gluco-tropaeolin (mg)	Pro-goitrin (mg)	Sinigrin (mg)	Rumen Liquor (ml)	0·1M Phosphate Buffer (ml)	Grote-positive Spots		
							R_F	Intensity†	Compound
1	25	—	—	—	5	2	0·12	++++	Dicheirolin thiourea
							0·02	+/–	Monocheirolin thiourea
2	25	—	—	—	—	7	0·98	++++	Cheirolin
3	—	25	—	—	5	2	0·96	+	Dibenzyl thiourea
4	—	25	—	—	—	7	—	—	—
5	—	—	25	—	5	2	0·88	++++	Goitrin
6	—	—	25	—	—	7	0·88	++++	Goitrin
7	—	—	—	25	5	2	—	—	—
8	—	—	—	25	—	7	—	—	—

* Each tube contained myrosinase (5 mg) and ascorbic acid (10^{-6}M).

† ++++, strong; +, weak; +/–, trace.

tested was found to be converted to a disubstituted thiourea, although the list included three substances (compounds 5–7, Table 8) closely related to cheirolin in structure. Each isothiocyanate remained almost completely unchanged. Trace spots indicated a slight formation of monothioureas in these cases, while in one amine formation was detected. Any amine formed from the first four isothiocyanates would possibly have volatilized during the preparation for chromatography.

Certain thioglucosides were also incubated with rumen liquor in order to study any conversion of aglucones at the moment of liberation, in particular the water-insoluble benzyl isothiocyanate, which is widely distributed as the aglucone, glucotropaeolin. The addition of myrosinase was found to be necessary since glucocheirolin in tests was found to be unchanged after incubation with rumen liquor at 37°C and pH 6·8 for 5 hr. Ascorbic acid (10^{-6}M) was included with myrosinase in the incubation mixtures to ensure the maximum rate of hydrolysis (Nagashima and Uchiyama 1959). In addition to glucotropaeolin and glucocheirolin, sinigrin (aglucone = CH_2: $CHCH_2NCS$) and progoitrin were also incubated under these conditions, the last-named in case its aglucone, 2-hydroxy-3-butenyl isothiocyanate, might be converted to thiourea derivatives in preference to cyclization with the formation of goitrin (5-vinyl-2-oxazolidenethione). All incubations were carried out at 37°C and pH 6·8 for 5 hr and the contents prepared for chromatography in CW as above. The papers were sprayed with Grote's reagent. The results are given in Table 9. Apart from glucocheirolin only glucotropaeolin (to a lesser extent) was converted to the corresponding disubstituted thiourea. A synthetic sample of dibenzylthiourea, m.p. 148–9°C (Salkowski (1891) recorded m.p. 148°C) had the same R_F, 0·96, as the unknown Grote-positive spot arising from glucotropaeolin. The negative result with sinigrin confirmed that obtained when the free aglucone was used (Table 8, compound 1).

IV. Discussion

The transformation of cheirolin to dicheirolin thiourea has been shown to be facilitated by a non-enzymic and heat-stable catalyst present in bovine rumen liquor. None of the compounds tested which could conceivably be present in rumen liquor, in particular reducing agents, was found to favour the conversion. The reaction, which takes place under both aerobic and anaerobic conditions, is almost certainly catalysed by a metal ion, since a sulphate ash of rumen liquor dialysate was active, while the dialysate was inactive in the presence of a chelating agent (EDTA). This aspect was, however, not thoroughly explored.

The restricted conversion of isothiocyanates to disubstituted thioureas (only two of a representative selection) is surprising, since three of these are closely related structurally to cheirolin (Tables 8 and 9). However, since the pH of incubation was the same in all cases, this did not allow for possible different pK values of the amines assumed to be formed as intermediates. By testing at varying pH values a greater number of positive results might have been obtained. According to Barnett and Reid (1961), the pH of rumen contents, depending on the nature of the diet and elapse of time from feeding, can vary from 5·0 to 7·5.

Thioglucosidases have been reported to be present in mammals (Goodman *et al.* 1959; Greer and Deeney 1959; Greer 1962) and it is thus of interest that in the

present work the thioglucosides tested were not hydrolysed by rumen liquor within 5 hr *in vitro* without the addition of myrosinase. The methods of collection and treatment of the liquor (collection from freshly slaughtered animals, filtration through gauze at 3°C, and storage in the frozen state) are unlikely to have inactivated any endogenous thioglucosidase which could be expected to be relatively stable (Neuberg and Wagner 1926; Reese, Clapp, and Mandels 1958). It therefore appears that an enzyme capable of splitting thioglucosides does not regularly occur in the rumen *in vivo*, although thioglucosidases must be consumed by a grazing animal, particularly where the pastures are contaminated by cruciferous weeds.

The relevance of the findings to the problem of endemic goitre will be discussed in Part III of this series of publications where the goitrogenic properties of cheirolin and other isothiocyanates and their derivatives are described.

V. Acknowledgment

One of us (H.S.B.) held a personal grant from the National Health and Medical Research Council who also contributed towards the costs of the investigation.

VI. References

Anschütz, R. (1910).—*Liebig's Ann.* **371**: 216.

Bachelard, H. S., McQuillan, Mary T., and Trikojus, V. M. (1963).—*Aust. J. Biol. Sci.* **16**: 177.

Bachelard, H. S., and Trikojus, V. M. (1960).—*Nature* **185**: 80.

Bachelard, H. S., and Trikojus, V. M. (1963).—*Aust. J. Biol. Sci.* **16**: 147.

Barnett, A. J. G., and Reid, R. L. (1961).—"Reactions in the Rumen." pp. 12–13. (Edward Arnold (Publishers) Ltd.: London.)

Connell, G. E., Dixon, G H., and Hanes, C. S. (1955).—*Canad. J. Biochem. Physiol.* **33**: 416.

Goodman, I., Fouts, J. R., Bresnick, E., Menegas, R., and Hitchings, G. H. (1959).—*Science* **130**: 450.

Greer, M. A. (1962).—"Recent Progress in Hormone Research." Vol. **18**. p. 187.

Greer, M. A., and Deeney, J. M. (1959).—*J. Clin. Invest.* **38**: 1465.

Grote, I. W. (1931).—*J. Biol. Chem.* **93**: 25.

Karrer, P., Scheitlin, E., and Siegrist, H. (1950).—*Helv. Chim. Acta* **33**: 1237.

Kjaer, A., Gmelin, R., and Larsen, I. (1955).—*Acta Chem. Scand.* **9**: 1143.

Nagashima, Z., and Uchiyama, M. (1959).—*Bull. Agric. Chem. Soc., Japan* **23**: 555.

Neuberg, C., and Wagner, J. (1926).—*Biochem. Z.* **174**: 457.

Reese, E. T., Clapp, R. C., and Mandels, M. (1958).—*Arch. Biochem.* **75**: 228.

Salkowski, H. (1891).—*Ber. dtsch. Chem. Ges.* **24**: 2724.

Sandberg, M., and Holly, O. M. (1932).—*J. Biol. Chem.* **96**: 443.

Schneider, W. (1910).—*Liebig's Ann.* **375**: 207.

Sell, E., and Proskauer, B. (1876).—*Ber. dtsch. Chem. Ges.* **9**: 1266.

Wagner-Jauregg, T., Hackley, B. E., Lies, T. A., Owens, O. O., and Proper, R. (1955).—*J. Amer. Chem. Soc.* **77**: 932.