ASCARIDOLE STUDIES

PART III. THE PURIFICATION AND CHARACTERISATION OF ASCARIDOLE

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In a previous communication[1] a polarographic method for the determination of ascaridole in oil of chenopodium and in solutions of oil of chenopodium in castor oil was described. A sample of pure ascaridole was required for the above and other investigations[2]. Numerous communications have appeared dealing with the purification of this substance[1,3–11] but the physical constants reported for ascaridole vary considerably (see Table I).

TABLE I

REPORTED PHYSICAL DATA FOR ASCARIDOLE

Reference	b.pt., °C.	m.pt., °C.	$n_D^{20°C.}$*	Density	$[\alpha]_D$	Assay (per cent. w/w ascaridole, iodimetric method)
1	84/3 mm.	—	1·4733	—	—	–
3	96 to 97/8 mm.	—	1·4769	$d_{20°C.}^{20°C.}$ 0·9985	$[\alpha]_D + 0·7°$	—
4	80 to 81/4 mm.	—	—	d 1·008	$[\alpha]_D^{20°C.} + 0·6°$	—
5	85 to 95/2–5 mm.	—	—	$d_{20°C.}$ 0·998	—	99·8
6	113 to 114/20 mm.	2	—	$d_{18°C.}^{18°C.}$ 1·0114	$[\alpha]_D - 0·03°$	106·4
7	115/15 mm.	—	1·4736	$d_{25°C.}^{25°C.}$ 1·0050	$[\alpha]_D^{25°C.} - 2·14°$	—
8	—	—	1·4732	$d^{20°C.}$ 1·0134	$[\alpha]_D^{22°C.} + 1·36°$	98
9	75/1·5 mm.	5	1·4738	$d_{25°C.}^{25°C.}$ 1·0061	—	—
10	83 to 84/3 mm.	3·2	1·4740	$d_{4°C.}^{20°C.}$ 1·0105	—	111,113
11	83/4–5 mm.	—	1·4743	$d_{15°C.}$ 1·0079	$[\alpha]_D - 4° 14'$	—
12	115/15 mm., 84/5 mm.	—	1·4743	$d_{15°C.}^{13°C.}$ 1·011	$[\alpha]_D - 4°$	—
This Paper	39–40/0·2 mm.	3·3	1·4731	$d_{20°C.}^{20°C.}$ 1·0113 $d_{4°C.}^{20°C.}$ 1·0103	$[\alpha]_D^{20} \pm 0·00°$	110·8

* Authors' values of n_D^t- recalculated by applying a correction of − 0·0004 per degree rise of temperature (see later) to enable comparison at 20° C. to be made.

Most workers have relied upon fractional distillation under reduced pressure[1,3–9,11] for purification, whilst a few have applied fractional recrystallisation at low temperatures[6,10]. In the former process, there is a possibility of the ascaridole rearranging during the distillation[13] thereby contaminating the product. This may explain the widely differing values quoted in Table I.

There is lack of agreement even in recently published work. Bitter[8] obtained a figure of 98 per cent. of ascaridole for his sample by the iodimetric method, but stated that this value was disputable since there was no control sample. Böhme and van Emster[10] obtained a sample

which gave results equivalent to 111 and 113 per cent. ascaridole by the same method. A carefully purified sample obtained by Szmant and Halpern[9] was presumed, from a consideration of its physical data, to be of higher purity than had hitherto been reported. However, the ultraviolet absorption curve for this sample exhibited a maximum at 240 mμ which is anomalous for a molecule possessing only one simple carbon-carbon double bond as its major absorbing group.

The problem of deciding when pure ascaridole has been obtained is complicated by the fact that the available methods of assay are either comparative, or else apply arbitrary factors originally obtained by the

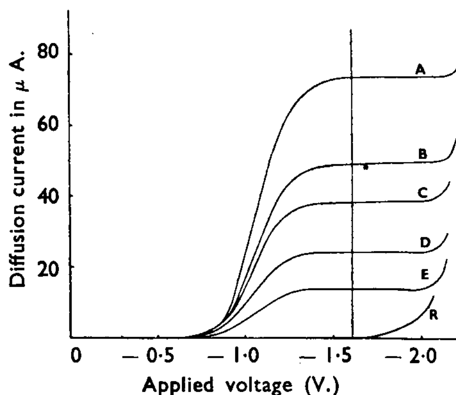

FIG. 1. Polarograms of ascaridole (in 97 per cent. ethanol and lithium acetate buffer).
Concentrations (g./l.):
A = 1·62 D = 0·54
B = 1·08 E = 0·27
C = 0·81 R = 0·00 (Residual current).

analysis of reputedly pure samples of ascaridole. The results obtained by the use of these factors are dependent upon the conditions used in the assay[2,10] and the correctness of these arbitrary factors has been questioned[2,10].

Because of the above discrepancies and difficulties, a detailed investigation of the problem of obtaining pure ascaridole has been made. Purification of several samples of oil of chenopodium, by various methods, has been carried out in an attempt to obtain identical products. Identity of the polarographic and other physical characteristics of these samples would constitute reasonable proof that pure ascaridole had been obtained.

METHODS AND RESULTS

3 methods of purification were attempted, namely, fractional recrystallisation at low temperature, chromatography and fractional distillation. A sample of ascaridole obtained by recrystallisation (crystallisation D1, Tables II and III) was taken as our basic standard of "100 per cent." ascaridole for this investigation.

Since ascaridole is reported to deteriorate upon storage, a secondary polarographic standard seemed necessary and *m*-dinitrobenzene, an easily purified substance, was found to be suitable. Polarographic assays were carried out at an applied voltage of 1·6 volts instead of 1·7 volts as previously recommended[1] since the former yields a flat portion of the plateau for the polarograms of both ascaridole and *m*-dinitrobenzene over the concentrations which have been used in this investigation (see Figs. 1 and 2).

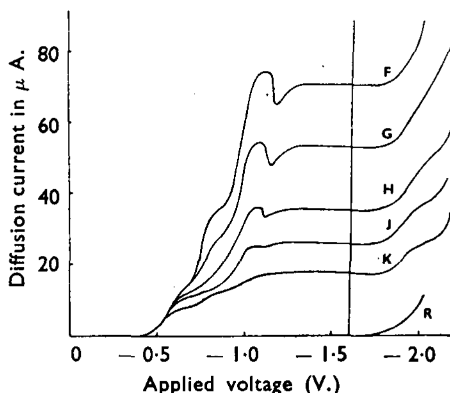

FIG. 2. Polarograms of *m*-dinitrobenzene (in 97 per cent. ethanol and lithium acetate buffer).
Concentrations (g./l.):

F = 0·331	J = 0·124
G = 0·248	K = 0·083
H = 0·165	R = 0·000 (Residual current).

Any possible variation in the results due to deterioration of the standard ascaridole or to changes in the capillary characteristics was eliminated in the following manner. Calibration curves of maximum diffusion current against concentration were plotted for "100 per cent." ascaridole and for recently recrystallised *m*-dinitrobenzene. These two curves were combined mathematically over the useful range of concentration to show the relationship between the diffusion currents of ascaridole and *m*-dinitrobenzene for identical concentrations of sample. From the equations for the straight line portions of the calibration curves, the following expression can be derived:

$$I_{asc} = \frac{m_1}{m_2}(I_{dnb} - c) + b$$

where I_{asc} = diffusion current for various weights of ascaridole,
I_{dnb} = diffusion current for the same weights of *m*-dinitrobenzene,
m_1 = slope of calibration curve for ascaridole (I_{asc} *v.* concentration),
m_2 = slope of calibration curve for *m*-dinitrobenzene (I_{dnb} *v.* concentration),
b = intercept on I_{asc} -axis,
c = intercept on I_{dnb} -axis.

A calibration curve can therefore be plotted for ascaridole by measuring I_{dnb} for known concentrations of *m*-dinitrobenzene and calculating I_{asc} for the same concentrations of ascaridole using the above relationship. By using *m*-dinitrobenzene to recalibrate the capillaries, allowance was made for slight changes in capillary characteristics and doubt was removed as to the stability of the stored "100 per cent." ascaridole sample. When the diffusion currents for ascaridole and *m*-dinitrobenzene were measured at various reservoir heights, the mathematically combined curves were identical, but the latter varied slightly when capillaries of widely differing characteristics were employed.

Experimentally determined calibration curves for "100 per cent." ascaridole stored for 3 months in the dark at room temperature and for ascaridole freshly purified by fractional recrystallisation, coincided with the calculated curves obtained upon recalibration of the capillaries with *m*-dinitrobenzene. Although stable under the above conditions, the ascaridole sample, after 2 months exposure to daylight, had deteriorated by about 5 per cent.

It has been stated [14] that certain difficulties have been encountered in the polarographic determination of ascaridole and that the results are not reproducible, using the method already published[1]. We have not experienced any difficulty using normal technique for polarographic analysis in organic media. Table II gives a routine set of results to indicate the reproducibility of the method.

In the present investigation, however, we have taken precautions additional to those required for routine analysis to enable detection of any anomalous results to be made immediately. These precautions are described in the Appendix along with a more detailed account of technique than was included in our earlier communication[1].

TABLE II

REPRODUCIBILITY OF THE POLAROGRAPHIC METHOD OF ASSAY OF ASCARIDOLE

Sample	Ascaridole Content (per cent.)								Mean
D1	100·1 99·8	100·4 100·3	99·8 99·8	99·9 99·6	100·5 100·7	99·3 100·5	99·5 100·6	99·3 100·0	100·0
D2	100·5	100·3	100·6	99·8	100·0	99·6	100·7	100·7	100·2

Purification Procedures

Fractional Recrystallisation. Of the many solvents investigated, a solution of 30 per cent. v/v of toluene in *n*-pentane was found to be the most suitable for recrystallisation. This mixed solvent gave smaller yields but purer products than *n*-pentane alone[6,10]. Before recrystallisation, the samples were partially purified by freezing out the ascaridole from oils of chenopodium or by fractional distillation under reduced pressure. They were then mixed with between 1 and 2 parts by volume of solvent and allowed to stand for a few hours in a stoppered flask surrounded by dry ice, with occasional shaking. The crystals were filtered, using reduced pressure, through a

sintered glass funnel surrounded by dry ice, pressed well, washed several times with *n*-pentane at − 50° C. to remove toluene, transferred to a container and allowed to melt at room temperature. The product was filtered through filter paper to free from traces of condensed moisture, and the remaining *n*-pentane was removed by passing dry nitrogen through the liquid for some hours. This method gives good yields of purified ascaridole and has the advantage that no heating is required at any stage. After 3 recrystallisations, the diffusion current per unit weight of sample showed no significant increase upon further recrystallisation. Some typical results are presented in Table III.

TABLE III

PURIFICATION OF ASCARIDOLE BY SUCCESSIVE FRACTIONAL RECRYSTALLISATION

Sample	Crystallisations	Weight taken, g.	Volume of Solvent,[a] ml	Yield, g.	$n_D^{20°\,C.}$	Ascaridole[b] content, per cent.
"Ascaridole" (containing 84·5 per cent. of ascaridole)	A	200	—[c]	101	1·4735	97·0
	B	100	100	65	1·4732	98·1
	C	60	60	45	1·4731	99·0
	D 1	20	20[d]	17·5	1·4731	100·0[e]
	D 2	20	20	16·5	1·4731	100·2
	E 1	10	40[d]	7·6	1·4731	100·1
	E 2	1·5	2	0·2	1·4731	100·0
Oil of Chenopodium (containing 64·5 per cent. of ascaridole)	1	88	130	60	1·4735	97·4
	2	60	60	40	1·4732	98·4
	3	40	40	35	1·4732	99·1
	4[f]	35	70	20	1·4731	100·1
	5[f]	10	20	8	1·4731	100·2

[a] 30 per cent. v/v of toluene in *n*-pentane unless otherwise stated.
[b] All assay figures in this paper are expressed w/w and are determined polarographically (unless otherwise specified), the mean of at least 6 determinations being recorded.
[c] Simple freeze out of ascaridole (*viz*., no solvent added).
[d] *n*-pentane only.
[e] Reference sample taken as "100 per cent." ascaridole and used as the polarographic standard.
[f] Samples used for infra-red analysis. The absorption curves of each were identical.

Chromatography. Solvent extracted silica gel (100 to 200 mesh), dried at room temperature, was used to prepare a chromatographic column (2 cm. in diameter) by a technique similar to that described by Miller and Kirchner[15], but omitting their starch treatment. 50 g. of the prepared material was used for 1 g. of partially purified ascaridole (see previous paragraph) dissolved in 20 ml. of *n*-hexane. After elution with about 200 ml. of *n*-hexane, the chromatogram was developed with 15 per cent. v/v of ethyl acetate in *n*-hexane, the eluate collected in 20 ml. fractions and the solvent removed under reduced pressure. The substitution of light petroleum (b.pt. 40 to 60° C.) or *n*-pentane for *n*-hexane gave equally satisfactory results. Rechromatography of all products which contained more than 99 per cent. of ascaridole resulted in a sample containing 99·8 per cent. (see Table IV).

In our earlier investigations, activated alumina was used as adsorbent with light petroleum (b.pt. 40° to 60° C.) as solvent and 20 per cent. v/v of benzene in light petroleum as eluant. The degree of purification varied with the activity of the alumina used and samples containing approxi-

mately 95 per cent. of ascaridole yielded, after rechromatography, only very small amounts of product analysing above 99 per cent. Subsequent work indicated that these difficulties were due to rearrangement or decomposition of the ascaridole on the alumina column. Chromatographic treatment of 1 g. of "100 per cent." ascaridole, using alumina, yielded 0·4 g. assaying at 100 per cent., 0·3 g. below 98 per cent. and 0·05 g. below 80 per cent. of ascaridole. In contrast, silica gel gave little rearrangement since all fractions were found to contain between 99·5 and 100 per cent. of ascaridole.

TABLE IV

CHROMATOGRAPHY OF "ASCARIDOLE" ON SILICA GEL

Sample	Eluant	ml.	Yield g.	$n_D^{20° C.}$	Ascaridole content, per cent.
2·0 g. of "Ascaridole" containing 84·4 per cent. of ascaridole	*n*-hexane	270	—	—	—
	15 per cent. v/v of ethyl acetate in *n*-hexane	20	0·2	1·4729	99·1
		20	0·5	1·4730	99·3
		20	0·4	1·4730	99·2
		20	0·1	1·4736	95·8
		20	negative	1·4740	—
1·0 g. of sample from above chromatography containing more than 99 per cent. of ascaridole	*n*-hexane	200	—	—	—
	15 per cent. v/v of ethyl acetate in *n*-hexane	20	0·3	1·4731	99·8
		20	0·25	1·4731	99·1
		20	0·1	1·4735	98·7
		20	negative	1·4737	—

Distillation. Samples of oil of chenopodium were fractionated under reduced pressure (0·1 to 0·5 mm.) using a column 2 cm. wide, packed for a length of 25 cm. with 2·5 mm. Fenske rings. Szmant and Halpern[9] described a distillation of oil of chenopodium under similar conditions and, as our results are substantially in agreement with theirs, our results are not reproduced here. Physical data reported for their purest product are included in Table I, but unfortunately they quote no assay figures. Their ultra-violet and infra-red absorption curves are, however, completely different from those we have obtained (see later). The purest product from our distillation experiments ($n_D^{20° C.}$ 1·4733, b.pt. 37 to 38° C./0·15 mm) contained 99·1 per cent. of ascaridole (110 per cent. by the iodimetric method) after four successive distillations.

Henry and Paget[13] have shown that ascaridole rearranges to the glycol anhydride on heating. Rearrangement seemed unlikely under the mild conditions of our distillation (40° C.), and this was confirmed by distilling 8·0 g. of "100 per cent." ascaridole as outlined above, the distillation being completed in 25 minutes. From 5·2 g. of distillate collected, 4·7 g. assayed at 99·8 per cent. of ascaridole and the remainder at 99·4 per cent., thus indicating negligible rearrangement. It appears possible, therefore, that a very efficient fractionating system would yield a pure product.

PHYSICAL AND ANALYTICAL DATA FOR PURE ASCARIDOLE

Ultra-violet Absorption. Measurements were made with a Unicam S.P. 500 spectrophotometer. Ethanolic solutions of ascaridole exhibited

no absorption maxima in the region 230 to 320 mμ (cf. Szmant and Halpern[9]).

Infra-red Absorption. Measurements were made with a Perkin-Elmer twin beam spectrometer. The absorption curves of thin films of ascaridole are shown in Figure 3. In Table V are listed the positions of the main absorption bands. Identical absorption curves were obtained on 2 successive recrystallisations (see Table III).

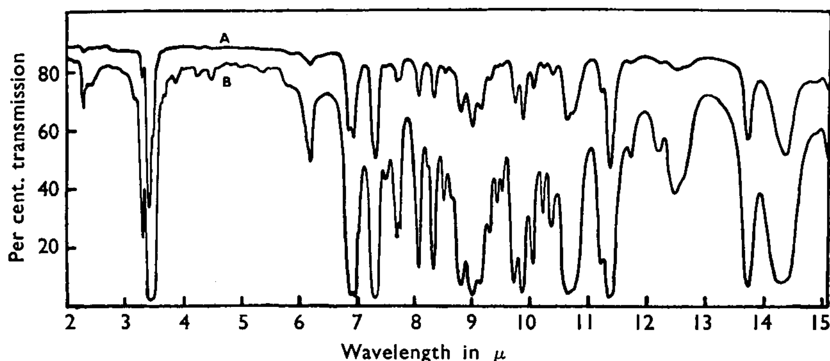

FIG. 3. Infra-red absorption of pure ascaridole.
A = Thin capillary film.
B = Path length 0·05 mm.

TABLE V

MAIN INFRA-RED ABSORPTION BANDS FOR ASCARIDOLE

Wavelength (μ)	Wavelength (μ)	Wavelength (μ)
3·30 (M)	7·70 } (M doublet)	9·87 (M)
3·40 } (VS doublet)	7·75 }	10·05 (M)
3·44 }	8·08 (M)	10·22 (W)
3·48 (shoulder)	8·24 (shoulder)	10·37 (M)
3·65 (shoulder)	8·34 (M)	10·63 } (M doublet)
3·70 (VW)	8·53 (W)	10·73 }
3·88 (VW)	8·66 (shoulder)	11·21 (M)
4·26 (VW)	8·82 (M)	11·37 (S)
4·50 (VW)	9·02 (M)	11·72 (W)
6·17 (M)	9·15 (M)	12·20 (W)
6·83 } (S doublet)	9·30 (M)	12·48 (W)
6·92 }	9·43 (W)	13·72 (S)
7·31 (S)	9·51 (W)	14·36 (S)
7·49 (VW)	9·73 (M)	—

Iodimetric Analysis. The samples gave results equivalent to 110·8 ± 0·5 per cent. of ascaridole (mean of several determinations) upon analysis by the iodimetric method of the British Pharmacopœia, 1953.

Miscellaneous Data. Elementary Analysis (by Mr. G. S. Crouch). Found: C. 71·2, H. 9·5 Calc. for $C_{10}H_{16}O_2$: C. 71·4, H. 9·6;

b.pt. 39° to 40° C./0·2 mm.
m.pt. + 3·3° C.
$n_D^{20° C.}$ 1·4731
$d_{20° C.}^{20° C.}$ 1·0113
$d_{4° C.}^{20° C.}$ 1·0103

$[\alpha]_D^{20° C.}$ ±0·00°.
$E_{1/2}$ − 1·01 volts *v.* S.C.E., lithium acetate buffer[1] in 97 per cent. v/v ethanol (cf. Bitter[8] − 0·89 volts in 0·2N lithium sulphate; Maruyama[16] − 0·786 volts in 0·1N ammonium chloride).

NOTES.—1. The melting point was determined by placing a 30 mm. immersion thermometer in the oil, cooling to $-30°$ C. and allowing to warm slowly, with stirring.

2. Values of refractive index were determined between 16° C. and 28° C. The temperature coefficient was $-0{\cdot}0004$ per degree rise in temperature and this value was used in Table I to adjust values of *n* obtained by other workers to 20° C.

DISCUSSION

Samples of ascaridole prepared by fractional recrystallisation to a maximum value of purity could not be further purified by the application of fractional distillation or chromatographic techniques. Purification by chromatography alone also gave samples identical with the recrystallised material, within the limits of experimental observation, whereas fractional distillation gave samples about 1 per cent. less pure. The production of identical samples by 2 of the above purification processes indicates that pure ascaridole has been obtained. Further confirmation of purity was obtained when samples from successive recrystallisations gave identical infra-red absorption curves.

Fractional recrystallisation is a much safer and more convenient method than fractional distillation for the preparation of large quantities of purified ascaridole. It is of interest to note that very good yields (75 per cent. of the ascaridole content) of almost pure material (97 per cent.) are obtained by freezing out the ascaridole from oil of chenopodium.

The physical data and analytical figures presented are in general agreement with those reported by Böhme and van Emster[10]. Although our infra-red absorption curve agrees with that reported by Maruyama[17] rather than the one by Szmant and Halpern[9] (see Fig. 3), we question the validity of the results obtained by the former worker because his infra-red analytical method gives results in fair agreement with the iodimetric method of the British Pharmacopœia. The iodimetric method has been shown to give results of 110 to 111 per cent. for pure ascaridole[2,10] and approximately 20 per cent. too high for oils containing 65 per cent. of ascaridole[2]. It is possible that other material with an infra-red absorption in the region of $10{\cdot}69\,\mu$ was present in the oils of chenopodium examined by Maruyama resulting in high values for ascaridole content by this method. None of our purified samples exhibited the anomalous absorption ($\epsilon_{max.}$ 3,700 at 240 mμ) reported by Szmant and Halpern[9] and we failed to find this absorption peak even in oils of chenopodium.

The use in polarography of an easily purified substance as a secondary standard if the compound being determined is unstable or difficult to purify has proved useful in the present investigation, although the method has limitations. It is analogous to the method described by Forche[18] for inorganic polarography, but there is a difference which may be due to the irreversibility of most organic oxidation-reduction systems, *viz.*, slight variations occur in the slope of the mathematically combined curve of diffusion currents of sample analysed *v.* those of the pure reference compound if capillaries of widely differing characteristics are used. Although

the method was proved to be applicable in the present investigation, further study is required before it can be generally applied.

However, such an investigation appears to be warranted because polarography has been introduced as a method of assay for certain organic compounds in official publications. Before the assay of a sample can be carried out, it is necessary to use pure material to prepare a calibration curve. Even if samples complying with certain purity tests and analytical procedures are available commercially, it does not follow that such substances are reliable as reference standards for polarographic work. An added complication is the difficulty of preparing identical reference samples in different laboratories if the material deteriorates or is not easily purified. It seems desirable to set up a number of easily purified, stable, reference compounds if polarography is to be used as a routine method for the quantitative analysis of organic susbstances.

APPENDIX

Polarographic Procedure

Apparatus

(1) Tinsley recording polarograph, Mark 12.

(2) Potentiometric manual polarograph, magic-eye model[19].

(3) Two dropping mercury electrodes connected to the same mercury reservoir.

(4) Thermostatic water supply adjusted to 20·0° ±0·1° C.

(5) Semi-micro polarographic cells (volume 2 to 3 ml.).

(6) Wash bottles containing 97 per cent. v/v ethanol.

Method. The two polarographs and electrodes were connected as shown in the simplified circuit diagram (Fig. 4), so that either instrument or capillary could be used at will.

Operation. With the switches S_1, S_2 and S_3 in the positions shown, potentiometers P_1 and P_2 are adjusted to give the required polarising voltage, V, for the recording and manual polarographs respectively. The diffusion current I'_{Dmax} is measured on the recording polarograph in the usual way. After operation of S_1,S_3, the diffusion current I''_{Dmax} is measured on the manual polarograph by determining the potential drop (V_1) produced across the standard resistance R (100KΩ ± 0·1 per cent.). This is achieved by opposing V_1 by an equal and opposite potential, V_2, applied by means of a low resistance potentiometer, P_3 (about 15Ω), to produce a potential difference, V_2, which is equal and opposite to V_1. At the balance point, observed by use of a magic eye null point indicator[19], there is no difference of potential between A and B. The above procedure is repeated after operation of S_2, thus replacing C_1 by the other polarographic cell C_2.

The use of two capillaries in polarographic work is strongly recommended since a capillary which loses its calibration is quickly detected and the necessary steps to remedy this can be taken. The capillaries must be stored in ethanol, since storage in distilled water may cause precipitation

of small droplets of the oil in the mouth of the capillary and thus alter its characteristics.

Method of analysis

10 to 20 mg. of the ascaridole sample was accurately weighed into a 10-ml. flask which had been allowed to reach equilibrium inside the balance case. 4 ml. of lithium acetate buffer[1] was added and the solution adjusted to 10 ml. with 97 per cent. v/v ethanol at 20·0° C. The polarographic cells were filled, after rinsing with solution, and deoxygenation was carried out

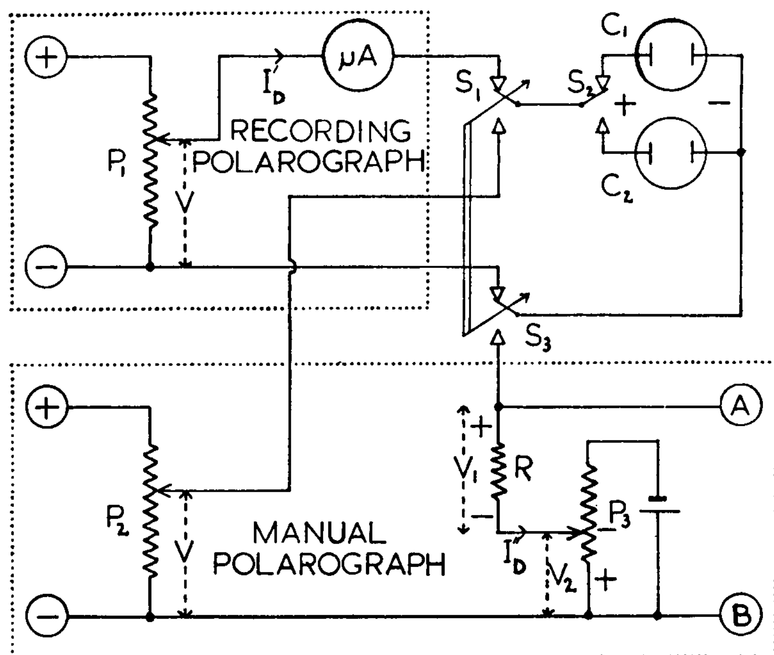

FIG. 4. Schematic circuit diagram of apparatus.

using nitrogen which had been passed through two wash bottles of 97 per cent. v/v ethanol at 20·0° C. Appreciable errors can arise when the polarographic cells and the wash bottles are at different temperatures. The diffusion current was determined as described above applying 1·6 volts between the dropping mercury cathode and the large pool anode. No damping was used and the diffusion current was measured at maximum drop size.

Calibration curves (I_D *v.* concentration) were plotted using suitable weights of pure ascaridole, or dilutions of standard solutions of pure ascaridole in ethanol.

In early experiments when recrystallised *m*-dinitrobenzene was used as a secondary standard, the same method was adopted, but the ascaridole was replaced by 2 to 4 mg. of *m*-dinitrobenzene per 10 ml. of solution.

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SUMMARY

1. Pure ascaridole has been obtained by fractional recrystallisation and by chromatography; it assayed at 110·8 ±0·5 per cent. w/w ascaridole by the B.P. iodimetric method. Fractional distillation gave a less pure product.

2. The physical constants of pure ascaridole are $d_{20^\circ C.}^{20^\circ C.}$ 1·0113, $n_D^{20^\circ C.}$, 1·4731, $[\alpha]_D^{20^\circ C.}$ ±0·00°, b.pt. 39° to 40° C./0·2 mm., m.pt. + 3·3° C., $E_{1/2}$ − 1·01 volts (*v.* S.C.E., lithium acetate buffer[1], 97 per cent. v/v ethanol).

3. Ultra-violet and infra-red absorption data are also reported.

4. A polarographic method is described. The application of two capillaries for routine analysis is recommended and the use of a secondary polarographic standard, *m*-dinitrobenzene, is described.

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