AN INVESTIGATION INTO CHEMICAL & BIOLOGICAL ASSAYS OF NEW COMPOUNDS FROM ALOES.

A Thesis presented for the degree

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by

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FOREWORD

Sincere appreciation is tendered to the following persons for their assistance.

a) for plant principles.
   MacFarlan, Smith LTD, Edinburgh. (aloin)

b) for a biological assay of aloes.
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c) for technical assistance with animals.
   Mr J. Nicholson. Dip. Agric. (McGill University).

d) to my director Dr. T.J.McCarthy, with whose help the accomplishment of this work was made possible.

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SPRAY REAGENTS.

Spray reagents have been used in this work, and have aided in the identification of glycanthrones, anthraquinones, glycosides and resins, and have been used for the identification of these on chromatograms. The following spray reagents have been used in this work.

ammonia vapours.

Fast Blue B. 0.5% aqueous.

sodium metaperiodate 5% aqueous.

magnesium acetate 0.5% methanolic solution.

sodium nitrate 5% aqueous, followed by glacial acetic acid.

potassium hydroxide 5% alcoholic.

INTRODUCTION

The drug aloes has been known since earliest times and is mentioned in the Ebers papyrus of circa 1,500 B.C. Alexander the great is reported to have sent a commission to Socotra to investigate the aloes grown there.(83). The chemical composition of aloes is complex, and being of plant origin, subject to variation. Both the complexity of the chemical constituents and their biological variation has resulted in a very large volume of conflicting material being published on this drug export.

Since aloes is used as a purgative for both human and veterinary use, it is obviously important that the dosage and consequently the active constituents, should comply to an accurate means of standardisation. To date, despite extensive world wide research into this drug, such standardisation has not been achieved. Even the methods used for the assay of the principal constituent, aloin, vary considerably in their results, and to complicate matters new chemical principles have been isolated from aloes in recent years.

Consequently the purpose of this work has been to investigate the main chemical assay methods currently in use, and to determine which was the most accurate, and why discrepancies occurred in the selected assay methods. Furthermore the results obtained by chemical assay have been compared with those obtained by biological assay in an attempt to correlate aloin content with purgative activity. Newly isolated compounds have been investigated biologically for the first time, and the biological assays of the resinous, glycosidal and other compounds of aloes have been performed.

The activity of aloin and aloes has been investigated in humans to determine whether there is any relationship between chemical assay, biological assay and activity in humans.

CHAPTER 1.

1.1 THE CHEMICAL ASSAYS OF ALOES.

The object of analysing and standardising crude drugs and their preparations is to produce a figure which will be directly related to therapeutic activity. To achieve this object it is necessary

a) to know precisely which chemicals present are the active ones,

b) to be certain that the final analytical figure obtained represents those chemicals quantitatively and exclusively; and

c) that the analytical procedure is sufficiently defined so that all workers using it will get the same figure for the same sample.

Neither the B.P.1968 (9)nor the U.S.P.XVII (85) have an assay for the aloin content of aloes, both using a limiting figure for the water-soluble extractive as the criterion of whether a sample is official or not.

There are numerous assays for the determination of aloin. The majority of these involve an initial separation of the aloin from the other constituents, or conversion of the aloin to its aglycone which is then separated from the other constituents, the final step usually being a spectrophotometric determination.

A summary of some of the methods appears in Table 1, while for more comprehensive reviews reference may be made to VAN OUDTSHOORN (86), HÖRHAMMER, WAGNER and BITTNER (50), KRAUS (56), FAIRBAIRN (29) or JANIAK & BÖHMERT (52).

Table 1.

Method	Reference.
Gravimetric.	SCHNEITER (ex 86) LISTER & PRIDE (60) SCHAEFFER. (44).
Spectrophotometric	PEYER. (ex 86). MÖHRLE. (70). MÖHRLE modified by HORHAMMER et alia. (50). MÖHRLE modified by BOHME & KREUTZIG. (70). FAIRBAIRN & SIMIC (39). JOINT COMMITTEE OF PHARMACY & ANALYTICAL CHEMISTRY. (53). GIBSON & SCHWARTING (43).

Method	Reference.
Spectrophotometric	AUTERHOFF & BALL (2). GSTIRNER. (45). HARDERS. (46). AWE & WACKSMUTHMELM. (3). OSTERREICHSES ARZNEIBUCH. (71) FORSDIKE. (40).
Thin Layer Chromatography.	GERRITSMA & VAN RHEEDE VAN OUDTSHOORN. (42). TEICHERT, MUTSCHLER & ROCHELMEYER (84). BÖHME & KREUTZIG. (6) HÖRHAMMER, WAGNER, and BITTNER (50) McCARTHY. (65).
Column Chromatography.	HÖRHAMMER, WAGNER & FÖCKING (51) JANIAK & BÖHMERT (52). D A B 7 (19). BRODY, VOIGT & MAHER. (12).
Paper Chromatography	BÖHME & KREUTZIG. (6). AWE & WACHSMUTHMELM (4).

Method.	Reference.
Paper Chromatography.	KRAUS. (55). BORKOWSKI, HENNEBERG & URSZULAK. (10). MARY, CHRISTENSEN & BEAL (63).
Paper Electrophoresis.	CORE & KIRCH. (18). PARIS & DURANT. (72).
Polarography.	STONE & FURMAN. (80). STONE. (81).
Potentiometric Titration.	RUGGIERI. (75).
Solvent extraction after clearing with lead acetate.	SMITH, JORDAN & DE KAY (78).
Chlorination.	LEGER. (46).
Pentose determination.	GOLDNER (46).
Persulphate method.	SEEL. (46).

In Table 2 are the results obtained by some of the methods stated in Table 1. Some of the earlier methods and in particular the calcium precipitation method, gave extremely low figures for aloin content.

Table 2.

Aloin % W/W		Method	Reference
Cape Aloes	Curaçao Aloes.		
4.04-5.32	16.88-17.24	TILDEN.	44
9%	25	CALCIUM ALOINATE.	48
10.5-14	2-31.	PARIS & DURAND	86
12-15.	-	-	
15-17	25-29.	SCHAEFFER.	44
18.5-19.2	35.9-36.6	GOLDNER (Pentose)	46
20.9-21.9	39.1-39.2	SEEL (Persulphate)	46
20.9-22.2	40.2-41.4	LEGER(Chlorination)	46
-	nearly 40	HARDERS.	46
up to 20	16-18	-	
ca 23	-	SMITH et alia.	78
15.16-24.6	-	KRAUS.	55
19.73-27.99	32.61	VAN OUDTSHOORN.	86
30.9	52.2	AUTERHOFF & BALL.	2
(Total anthraquinones.)			
	15-30.	SCHAEFFER .	23

This brief review shows quite clearly both the diversity of methods and the large variation in results obtained by the methods. The following methods have been selected for examination as they appear to satisfy some or all of the criteria of speed combined with accuracy.

1.2 THE METHOD OF FAIRBAIRN.

The chemical assay of aloes prepared by the Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry on recommended methods for the evaluation of drugs (53), will be referred to in this work as the assay of FAIRBAIRN, since it was based principally on his work.

Experimental procedure for the chemical assay of aloes.

1) Weigh accurately about 0.2 gm. of powdered Curaçao aloes or about 0.4 gm of powdered Cape aloes and place the sample in a 200 ml conical flask. Moisten with 2 ml of methanol, add 5 ml of water previously warmed to about 60°C, mix, add a further 75 ml of water at about 60°C and shake the mixture for 30 minutes. Cool the mixture, filter through a

Whatman No41 filter paper into a 100 ml calibrated flask, wash the conical flask with 20 ml of water, and make up to 100 ml with water.

ii) Transfer 10 ml of this solution into a round-bottomed 100 ml flask, add 1 ml of hydrochloric acid B.P., attach a water-cooled, double-surface condenser to the flask, place the flask in a bath of continuously boiling water (so that the water level is above that of the liquid level in the flask) and heat for 15 minutes.

Cool the solution, transfer it into a separating funnel and rinse out the round-bottomed flask with 5 to 10 ml of water, adding these washings to the contents of the separating funnel.

iii) Extract the contents of the separating funnel with two 20 ml portions of carbon tetrachloride. Wash the combined carbon tetrachloride layers with two 10 ml portions of water. Reject the carbon tetrachloride layer and return the washings to the aqueous acid layer in the first separating funnel. Transfer the contents of this funnel into a 100 ml calibrated flask, rinse the funnel with water, and make up to 100 ml with water.

iv) Place 10 ml of this solution in a round-bottomed 100ml flask containing 1 ml of 60 per cent. iron (iii) chloride solution and 6 ml of hydrochloric acid B.P. Attach a water-cooled, double-surface condenser to the flask, place the flask in a bath of continuously boiling water (so that the water level is above that of the liquid level in the flask) and heat for 4 hours.

Cool the solution, transfer it into a separating funnel, and rinse out the round-bottomed flask successively with 3 to 4 ml of water, 3 to 4 ml of N sodium hydroxide and 3 to 4 ml of water, adding these rinsings to the contents of the separating funnel.

v) Extract the contents of the separating funnel with three 20 ml portions of carbon tetrachloride. Wash the combined carbon tetrachloride layers with two 10 ml portions of water. Reject the washings. Extract the carbon tetrachloride layer with one 15 ml portion and with successive 5 ml portions of N sodium hydroxide until the final sodium hydroxide extract is colourless. Combine the alkaline extracts, heat them in a shallow dish on a bath of boiling water for 5 minutes, with constant stirring to remove carbon tetrachloride, cool and adjust to 50 ml with N sodium hydroxide. Determine the extinction of this solution in a 1 cm cell at 440 nm and at the maximum, at 500 nm, against N sodium hydroxide in a similar cell. The readings should be taken within 1 hour of beginning the alkaline extraction with one 15 ml portion of N sodium hydroxide (step v, line 5) and particular care should be taken to carry out these stages of the estimation in subdued light.

vi) Calculate the percentage of anhydrous barbaloin present, on the assumption that the E1%/1cm value at 500 nm of the red solution obtained from anhydrous barbaloin is 209. Also calculate the ratio value E 500/ E 440. If this ratio is less than 1.9 reject the result.

<u>Experimental procedure for the assay of anhydrous Barbaloin.</u>

i) Weigh accurately about 200 mg of barbaloin and dry it to constant weight at 105° to 110°C in vacuo, taking due care to dry the air readmitted to the vacuum oven. Calculate the moisture content of the sample of barbaloin. Discard the dry material.

At the same time weigh accurately about 50 mg of the sample of barbaloin, dissolve it in water and adjust the volume to 500 ml.

ii) and iii) Proceed with the method as for aloes, sections iv) and v), beginning at...Place 10 ml of this solution in a round-bottomed 100 ml flask... and continuing to the end of section v).

iv) Calculate the E 1%/ 1cm value for anhydrous barbaloin at 500 nm, as examined by this method. Also calculate the ratio values E 500 /E 440. If this ratio value is less than 1.9 reject the result.

The basis of the assay is the BORNTRÄGER reaction, whereby an anthraquinone in alkali produces a red colour.

The assay as laid down by the Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry on recommended methods for the evaluation of drugs (53), is based on the estimation of barbaloin in aloes by the method of FAIRBAIRN and SIMIC (39), with only minor modifications, which include the precise defining of the conditions used in the estimation. The purpose of the various steps is explained below.

Step ii) hydrolysis with hydrochloric acid destroys any O-glycosides which may be present (i.e. aloinosides A and B).

Step iii) removes any free aloe-emodin which is selectively soluble in carbon tetrachloride.

Step iv) here the barbaloin, which is unaffected by the mild acid hydrolysis of stage ii) is converted into aloe-emodin by the more drastic conditions of step iv) in which iron (iii) chloride and hydrochloric acid are used.

The aloe-emodin produced by the hydrolysis of the barbaloin is then extracted by carbon tetrachloride and finally determined spectrophotometrically after extraction with N sodium hydroxide.

The ratio value E 500/ E 440 was chosen to give a ratio of about 2.0. This being merely a measure of the cleanliness of the separations in the assay (20).

It is seen that if aloinosides A and B (rhamnose-

O-glycosides of aloin) are present, that they are converted to aloin by the removal of the rhamnose in step ii) and that they are included in the total aloin content, this not being a very serious error in view of the fact that work by HÖRHAMMER et alia (50) indicates that they have a similar purgative activity to aloin.

1.3 THIN LAYER CHROMATOGRAPHIC ESTIMATION OF ALOIN IN ALOES.

Thin layer chromatography was used to separate the aloin from the other constituents, a modification of VAN OUDTSHOORN'S (86) method being employed for analysis.

Preparation of Plates.

Plate glass chromatographic plates 13 cm x 9 cm x 0.4 cm were coated with a slurry of Silica Gel G (Merck), 8 parts of silica gel to 20 parts of distilled water. The plates were than allowed to dry in air.

Activation of Plates.

Immediately before use the prepared plates were placed in an oven at 105 °C for thirty minutes (STAHL (79)), to activate them. These plates were then cooled by standing on glass.

Application of Solutions.

In all the assays performed methanol was used as the solvent for both aloin and aloes. A micropipette (as per GERRITSMA and FREDERICKS (41) of volume circa 0.006 ml was calibrated accurately.

0.2 to 0.4 gm of aloe lump, finely powdered in a mortar and accurately weighed (containing thirty to eighty ug of aloin), was dissolved and made up to six ml with methanol.

The micropipette was then used to spot the plate one cm from the bottom of the plate applying three spots to each plate. This was done by placing the micropipette filled with the solution, on the surface of the silica gel plate, and allowing it to drain. Both before and after use, and in between applications of the same or different solutions, the micropipette was washed three times with methanol and allowed to drain onto filter paper.

Development.

The methanol from the aloe solution was allowed to evaporate and the plate was placed in a solvent tank closed with a glass top. The plates were placed back to back in the small tank to facilitate removal and to ensure no loss of silica during removal. They were then allowed to run until the solvent front had travelled a distance of ten to twelve cm. The solvent used was chloroform: ethanol (3:1 v/v).

Detection.

Many of the principles of aloe fluoresce brightly under ultra-violet light. Aloin fluoresces orange, in contrast to the blues, greys and browns of the other components, and the red of aloe-emodin. It is thus easily identifiable,and once identified the use of aloin as control is unnecessary.

ANALYTICAL PROCEDURE.

After development the plate was removed from the tank and dried in air. The plate was then placed under a long wave ultra-violet lamp and the silica gel above and below the aloin was carefully scraped away, leaving only the three aloin dots joined by silica gel.

Each aloin dot was then carefully scraped onto a clean glossy sheet of paper and transferred completely to a numbered centrifuge tube. Methanol, 5 ml, was then pipetted into each centrifuge tube and stirred with a glass rod. The solution was then centrifuged and the supernatant then filtered through Whatman 542 filter paper, directly into spectrophotometric cells. Triplicate readings were then made in a double beam spectrophotometer at a wavelength of 360 mm, using methanol as the blank. These readings were then averaged and the percentage aloin present determined by reference to a standard calibration curve.

1.5 THE METHOD OF MÖHRLE FOR THE DETERMINATION OF ALOIN IN ALOES AS MODIFIED BY BÖHME AND KREUTZIG (7).

Experimental procedure.

50 mg of aloes was finely powdered (sieve No.5) and accurately weighed to the fourth decimal place. This was then transferred to a 100 ml measuring flask with five 10 ml portions of water at 60 °C. This was then shaken well for five minutes. The solution was then cooled to 20 °C and made up to 100 ml with distilled water.

10 ml portions of this solution were then pipetted into two 50 ml measuring flasks. To the one flask 5 ml of 25% ammonia was added. To the other flask 1.0 ml of

5% sodium metaperiodate solution and 5 ml of 25% ammonia solution. Both flasks were then gently shaken and placed in the dark for two hours. After two hours the solutions were then made up to 100 ml and shaken well and allowed to stand in the dark for a further 15 minutes.

The red colour produced was then measured in a 1 cm cell, at a wavelength of 510 nm in a double beam spectrophotometer, using the aloe plus ammonia solution as a blank. An extinction of 0.250 represents 8.32 mg of anhydrous barbaloin.

It is seen that the above assay makes allowances for the aloe-emodin already present in the aloe and for the yellow colour that is produced by the resins and ammonia by using an ammoniacal solution of aloe as a blank.

1.4 THE METHOD OF MÖHRLE FOR THE DETERMINATION OF ALOIN IN ALOES AS MODIFIED BY HÖRHAMMER, WAGNER & BITTNER (1963) (50).

Experimental procedure.

35 mg of finely powdered aloe lump was accurately weighed off and transferred to a 100 ml measuring flask with five 10 ml portions of hot water. After shaking for

five minutes, the solution was cooled to 20 °C and made up to 100 ml with distilled water ; 10 ml of this solution was then pipetted into a 50 ml measuring flask. Then 1 ml of a 5% solution of sodium metaperiodate and 5 ml of 25% ammonia were added. The flask was well closed, shaken lightly and placed in the dark for two hours. After two hours the solution was made up to volume (i.e. 50 ml) with distilled water, shaken well and again placed in the dark for a further 15 minutes. The red colour produced was then measured at a wavelength of 505 nm, in 1 cm cells in a double beam spectrophotometer using water as the blank.

The aloin content was determined from the following formula.

$$\%\ \text{Aloin} = \frac{(E_{505} - 0.0325)\ 100}{0.31875 \times 3.5}$$

In the above assay the aloin in the aloe is oxidised by the sodium metaperiodate to aloe-emodin which then gives a red colour with the ammonia. No consideration is given to the aloe-emodin which is already present in the aloe, nor to the yellow colour which is produced by the resins with ammonia, which also reads at 505 nm.

1.6 DETERMINATION OF THE WATER-SOLUBLE EXTRACTIVE (B.P. 1968).

The B.P. Monograph for aloes lays down as the Official Standards: Solubility in alcohol, an Ash value, Loss on drying and a Water-soluble extractive.

Water-soluble extractive.

The B.P. states that this shall not be less than 75%, calculated with reference to the air dried drug, when determined by the following method.

Shake 1 gm, finely powdered aloes with 50 ml of water at 80°C in a stoppered flask until dissolved as completely as possible. Cool, dilute to 100 ml with water, and again shake. Set aside at room temperature overnight. Add 1 gm of kieselguhr, shake well and filter. Transfer 25 ml of the filtrate to a tared flat-bottomed dish, evaporate to dryness, dry the residue at 105°C for three hours, and weigh.

The U.S.P.XVII includes a very similar water soluble extractive method to that of the B.P., but the extractive required is only 50%.

CHAPTER 2.

COMPARISON OF THE ALOIN CONTENT OF DIFFERENT COMMERCIAL SAMPLES OF CAPE ALOES AS DETERMINED BY THE DIFFERENT METHODS OF ASSAY.

A series of commercial aloe samples was assayed by the assay methods already outlined. The aloin content of these samples varied between 11% and 30%. In addition the chemical constituents varied and the samples used contained all the chemical compounds known in aloes.

2.1. COMPARISON OF THE ALOIN CONTENT AS DETERMINED BY THE METHODS OF FAIRBAIRN AND BY THIN LAYER CHROMATOGRAPHY.

When various samples of aloes were assayed by the methods of FAIRBAIRN and thin layer chromatography the results obtained were not concordant. It can be seen from Table 3 that the thin layer method gave consistently higher results.

Table 3.

Sample	FAIRBAIRN	T.L.C.
1	13.3%	14.3%
2	15.9%	17.2%
3	19.9%	23.1%
4	12.9%	13.5%
5	19.0%	20.8%
6	14.9%	16.5%
7	12.8%	15.0%

2.1.1. INVESTIGATION INTO VARIANCE BETWEEN THE TWO ASSAY METHODS.

Two factors could give rise to a higher result with the T.L.C. method, these being:

i) Interference from other compounds.

ii) Interference from the silica gel.

i) Interference due to other compounds.

A light blue halo spreads above the aloin and merges with it at the top, (blue resin mentioned by

AUTERHOFF (1), BÖHME & KREUTZIG (5), HÖRHAMMER et alia (50) and others) and it is not possible to remove this completely from the aloin spot. Variations were tried in the solvent, thinly spread plates and thickly spread plates, different lengths of activation of the plates, but complete separation could not be obtained. The amount of overlap could be determined by spraying the resin spot with fast blue B, the resin spot turning orange both in daylight and under ultra-violet light.

Fortunately this resin has its peaks at 296, 253 and 245 nm and the reading it gives at 360 nm is negligible. This may hence be disregarded as a possible source of error.

ii) <u>Interference due to silica gel dissolving in the methanol.</u>

KORTE and SIEPER (54) in their work with Cannabis reported that the silica gel interfered with their readings below 500 nm. So it was decided to determine whether it interfered at 360 nm, in view of the fact that both BÖHME and KREUTZIG (6), and McCARTHY (68) had previously observed no interference with readings taken at this wavelength.

100 mg of silica gel was scraped off a plate which had been developed in chloroform:ethanol (3:1 v/v). This was transferred to a centrifuge tube, 5 ml of methanol pipetted in and well stirred. This was then centrifuged and the supernatant filtered through Whatman 542 filter paper directly into a 1 cm cuvette. The spectrum of this solution was then scanned, the readings obtained in the long wave region of the spectrum were very low but these increased as the ultra-violet region was approached. At 360 nm the readings obtained were quite appreciable.

This error can be obviated either:

i) By using a blank of silica gel in methanol or

ii) By introducing a correction factor.

2.1.2. DETERMINATION OF A CORRECTION FACTOR FOR SILICA GEL.

Graded weights of silica gel ranging from 25 to 100 mg were removed from a plate which had been run in chloroform:ethanol (3:1). These were transferred to centrifuge tubes 5 ml of methanol pipetted in and stirred well. The tubes were centrifuged and the supernatant liquid filtered through Whatman 542 filter paper directly into a 1 cm cuvette. This was then read at 360 nm against methanol.

Table 4.

weight of silica gel	reading 1	2	3	4
25mg	0.025	0.022	0.024	0.021
50mg	0.022	0.020	0.022	0.020
75mg	0.022	0.022	0.021	0.022
100mg	0.021	0.024	0.022	0.023

From Table 4 it is seen that a constant figure of 0.02(2) was obtained, irrespective of the weight of silica gel taken. This was then subtracted from all spectrophotometric readings of aloin, eluted from silica gel, and read at 360 nm.

It is highly probable that the interference due to silica gel varies from batch to batch, in view of the fact that BÖHME and KREUTZIG (6) and McCARTHY (68) found no interference.

2.1.3. COMPARISON OF THE ALOIN CONTENT OF ALOES AS DETERMINED BY THE METHODS OF FAIRBAIRN AND BY THIN LAYER CHROMATOGRAPHY CORRECTED.

Table 5 shows the results obtained by the method of FAIRBAIRN and corrected T.L.C. method.

Table 5.

Sample	FAIRBAIRN	T.L.C. corrected.
I	13.3%	12.5%
2	15.9%	15.3%
3	19.9%	20.5%
4	12.9%	12.2%
5	19.0%	19.1%
6	14.9%	14.4%
7	12.8%	13.2%
8	11.0%	10.5%
9	12.4%	11.7%
10	12.5%	11.5%
11	29.7%	29.8%
12	30.5%	29.9%
13	30.5%	29.8%

From this it is seen that there is close agreement between the results obtained by the two different assay methods. Especially when it is considered that the limits of error of the FAIRBAIRN method are ± 1% and that of the T.L.C. method are ± 2%

2.2 ALOIN CONTENT OF ALOES AS DETERMINED BY THE METHOD OF MÖHRLE (AS MODIFIED BY HÖRHAMMER et alia (50)) COMPARED WITH THAT OBTAINED BY THE METHODS OF FAIRBAIRN AND T.L.C.

HÖRHAMMER et alia (50) found excellent agreement between their modified method and thin layer chromatography. However in this work it was found that the results obtained by their method were generally # ca 20% too high, this being due in part to the yellow colour produced by reaction with the ammonia, this colour producing a reading at 505 nm and consequently giving a false high reading. A similar high reading is due to the presence of aloe-emodin in the sample.

Table 6 shows the values obtained for the different samples assayed by the three different methods.

Table 6.

Sample	FAIRBAIRN A	T.L.C.	MÖHRLE modified by HÖRHAMMER C	$\frac{C}{A}$ x 100
1	13.3%	12.5%	15.7%	118%
2	15.9%	15.3%	19.3%	121%
3	19.9%	20.5%	23.9%	120%
4	12.9%	12.2%	16.0%	123%
5	19.0%	19.1%	22.8%	120%
6	14.9%	14.4%	19.4%	124%
7	12.8%	13.2%	15.7%	122%
8	11.0%	10.5%	15.0%	136%
9	12.4%	11.7%	15.0%	121%
10	12.5%	11.5%	16.3%	130%
11	29.7%	29.8%	34.6%	117%
12	30.5%	29.9%	34.0%	112%

From this it is seen that the method of MÖHRLE as modified by HÖRHAMMER et alia (50) is far too inaccurate to be used as a quantitative assay for aloin in aloes.

This method can, however, be used for the assay of pure aloin for which it shows excellent agreement with the results obtained from the T.L.C. method. A sample of aloin containing 92.8% aloin and 7.2% moisture gave a result of 92.5% and 92.7% aloin by the T.L.C. method. When assayed by the method of MÖHRLE modified by HÖRHAMMER results of 91.35% were obtained.

2.3. ALOIN CONTENT OF ALOES AS DETERMINED BY THE METHOD OF MÖHRLE (AS MODIFIED BY BÖHME AND KREUTZIG (7)), COMPARED WITH THAT OBTAINED BY THE METHODS OF FAIRBAIRN AND T.L.C.

From Table 7 it is seen that very close agreement between aloin content is obtained by these three methods of assay.

The modified MÖHRLE method does, however, have two serious drawbacks, the first being that its reproducibility is temperature dependent. The second is that it cannot be used in its present form for the assay of pure aloin or samples of aloin containing a high percentage of aloin, but merely for aloes.

This is due to the fact that a solution of aloin and ammonia absorbs at 510 nm, hence if this is used as a blank, a low reading will be obtained for the aloe-emodin and ammonia in the other cell, which has maximum absorption at 510 nm.

Table 7.

Sample	FAIRBAIRN	T.L.C.	MÖHRLE as modifed by BÖHME & KREUTZIG.
1	13.3%	12.5%	13.3%
2	15.9%	15.3%	14.3%
3	19.9%	20.5%	19.0%
4	12.9%	12.2%	11.5%
5	19.0%	19.1%	19.0%
6	14.9%	14.4%	14.8%
7	12.8%	13.2%	8.5%
8	11.0%	10.5%	9.7%
9	12.4%	11.7%	12.0%
10	12.5%	11.5%	12.0%
11	29.7%	29.8%	29.0%
12	30.5%	29.9%	27.4%

This is demonstrated by the following assay of a sample of pure aloin. When, on arrival, the sample was unsealed and assayed by thin layer chromatography, it chromatographed pure and assayed as 100% aloin. The sample absorbed moisture on standing. This moisture content was determined by drying at 60°C. The sample was then assayed by two different workers, both by the T.L.C. method and by the method of MÖHRLE as modified by BÖHME and KREUTZIG.

Moist aloin	T.L.C. (two workers)	BÖHME & KREUTZIG	$\frac{\%\ \text{BÖHME \& KREUTZIG}}{\%\ \text{T.L.C.}}$
92.8%	92.5% :92.7%	83-84%	0.90 (i.e.90%)

The sample of aloin was allowed to stand for a further two days and again assayed by T.L.C. Furthermore the percentage purity was determined from the B.P.C. 1968 (8) and by the method of MÖHRLE as modified by BÖHME & KREUTZIG.

B.P.C.	T.L.C.(two workers)	BÖHME & KREUTZIG	$\frac{\%\text{BÖHME \& KREUTZIG}}{\%\text{T.L.C.\&B.P.C}}$
90.7%	90.3%	81.3%	0.896 (i.e. 89.6%

From this it is seen that the ratio of the method of MÖHRLE (as modified by BÖHME & KREUTZIG) to T.L.C. is the same in both cases, showing that the former method when used for aloin gives a consistently lower figure than the correct one. Hence it is seen that this method of assay cannot be applied to aloin of a high percentage purity.

2.4. COMPARISON OF THE ALOIN CONTENT OF ALOES AS DETERMINED BY THE METHOD OF MÖHRLE (AS MODIFIED BY HÖRHAMMER) AND THE METHOD OF MÖHRLE (AS MODIFIED BY BÖHME AND KREUTZIG)

Table 8 shows the percentages of aloin for the different samples of aloes as estimated by the two modification of the method of MÖHRLE.

Table 8

Sample	MÖHRLE modified HORHAMMER.	MÖHRLE modified BÖHME & KREUTZIG.
1	15.7%	13.3%
2	19.3%	14.3%
3	23.9%	19.0%
4	16.0%	11.5%
5	22.9%	19.0%
6	19.4%	14.8%
7	15.7%	8.5%
8	15.0%	9.7%
9	15.0%	12.0%
10	16.3%	12.0%
11	34.6%	29.0%
12	34.0%	27.4%

It is seen that the assay as modified by HÖRHAMMER et alia (50) gives a consistently higher result than the assay as modified by BÖHME and KREUTZIG, this being similar to the results obtained when BÖHME and KREUTZIG compared their assay with the original assay of MÖHRLE (70).

2.5. COMPARISON OF THE WATER-SOLUBLE EXTRACTIVE B.P. 1968 WITH THE ALOIN CONTENT.

Table 9 compares the water-soluble extractive of various aloe samples with their aloin content, determined by the method of FAIRBAIRN.

Table 9

Sample	water-soluble extractive (W.S.E.)	$\frac{\text{W.S.E.}}{5}$	Aloin %
1	76%	15.2%	13.4%
2	75%	15.0%	15.9%
3	83%	16.5%	19.9%
4	63.2%	12.6%	12.9%
5	78.4%	16.0%	19.0%
6	77%	15.0%	14.9%
7	59.6%	12.0%	12.8%
8	56.6%	11.3%	11.0%
9	72.5%	14.5%	12.4%
10	64.6%	12.9%	12.5%

The B.P. states that an aloe sample must have a water-soluble extractive of at least 75%. From this it is seen that many samples of Cape aloes would fail to meet this requirement. It is also seen that if a sample has a water-soluble extractive of less than 75% then the aloin content is generally below 14%. Also that if the water-soluble extractive is divided by five, the figure obtained is within 3% of the aloin content.

2.6. THE ASSAY OF ALOES CONTAINING DIFFERENT CONSTITUENTS.

The assay methods which have already been discussed were then used to assay samples of aloes containing different constituents from the normal commercial sample of Cape aloes.

2.6.1. ALOES CONTAINING ISOBARBALOIN.

Curaçao aloes contains isobarbaloin which is believed by most workers to be an optical isomer of aloin. This on hydrolysis yields the same aglycone as aloin, namely aloe-emodin (GARDNER & JOSEPH (ex 66)). Hence the methods of FAIRBAIRN, and MÖHRLE, for assaying aloin content estimate not only aloin but also the isobarbaloin which is also converted to aloe-emodin, whereas the thin layer chromatography method of assay should assay aloin alone.

On assaying a sample of Curaçao aloes by means of the methods of FAIRBAIRN, MÖHRLE (modified) and thin layer chromatography the following results were obtained

Table 10.

Assay method	FAIRBAIRN	T.L.C.	MÖHRLE (modified by BÖHME & KREUTZIG.
Aloin %	29.66%	28.25%	29.03%

From Table 10 it is seen that there is no great difference in the results obtained. This was found to be due to the fact that the solvent normally used i.e. chloroform:ethanol (3:1) does not separate aloin and isobarbaolin. This was demonstrated by spraying the plate with nitrous acid upon which the purple colour which developed with the isobarbaloin coincided with the aloin spot. In order to determine the aloin and isobarbaloin independently the plate must be developed in ethyl acetate:glacial acetic acid:water (5:1:4 v/v). With this solvent system the aloin and the isobarbaloin separate into two discrete spots which can be easily removed from the plate. Since aloin and isobarbaloin have the same spectrum with peaks at 350,294,268 and 222 nm it is possible to take the readings for both compounds at a wavelength of 360 nm. The results which were obtained are seen in Table 11.

<u>Table 11.</u>

isobarb plus aloin.(with solvent chloroform: ethanol (3:1))	28.20%
isobarbaloin. (separated with ethyl acetate:	12.5 %
aloin. Glacial acetic acid and water (5:1:4))	15.75%
	28.25%

2.6.2. ALOES CONTAINING THE ALOINSIDES.

The aloinsides were discovered by HÖRHAMMER et alia (50) in certain samples of Cape aloes and are found in *A. africana*. These are O-glycosides of aloin and have been shown to have a similar activity to aloin in mice. With both the assay of FAIRBAIRN and of MÖHRLE the aloinsides are first hydrolised to aloin and finally oxidised to aloe-emodin so that they are estimated in terms of aloin.

However when estimated by thin layer chromatography two spots appear if the solvent, chloroform:ethanol (3:1) is used to develop the plate, the upper spot being aloin and the lower spot combined aloinsides A and B. The spectrum of the aloinsides and aloin are very similar and the aloinsides may be determined in terms of aloin (VAN OUDTSHOORN (87)).

A sample of Coega aloes which contained the aloinsides was assayed by the three assay methods, Table 10 showing the results obtained.

Table 12.

FAIRBAIRN method.	MÖHRLE (modified by BÖHME & KREUTZIG)	T.L.C.
30.5%	27.4%	14.25% aloin 15.64% aloinsides. 29.89% total

From this it is seen that there is close agreement on total aloin content.

2.6.3. ALOES CONTAINING THE "PERIODATE POSITIVE" COMPOUND DESCRIBED BY BÖHME and KREUTZIG (5).

BÖHME and KREUTZIG (5) were the first to report a compound in Cape aloes which was periodate positive but which rapidly faded on standing. This spot was found in a sample of commercial aloes from Alicedale in the Eastern Cape. Although it was not possible to separate it from aloin using thin layer chromatography and chloroform:ethanol (3:1) as the developer it did not appear to interfere to any extent with the assay, as close agreement was obtained as to the aloin content of the sample by the three different methods of assay.

Table 13.

FAIRBAIRN method	MÖHRLE (modified by BÖHME & KREUTZIG	T.L.C.
19.05%	19.04%	19.08%

When assayed by the method of MÖHRLE (as modified by

BÖHME & KREUTZIG) on the addition of the periodate solution, the solution turned purple, but with the addition of ammonia it became yellow. The purple colour produced by the periodate does not interfere with the assay due to the rapidity with which it disappears. This "periodate positive" compound is of interest since it partially resembles isobarbaloin in its reaction to periodate, although for the latter compound the purple colour formed by periodate fades only after a considerable time. However it seems probable that this compound has purgative activity, since it is only this sample of aloes that has a purgative action greater than expected from the amount of aloin present. This has been confirmed in tests on rats and humans, and will be referred to in greater detail under section 4.6 .

2.6.4. ALOES CONTAINING HOMONATALOIN.

1) Assay of Homonataloin by Thin Layer Chromatography.

This was performed after the method of McCARTHY (67) utilising thin layer chromatography. The assay is performed as for the thin layer chromatographic assay of aloin, except that in this case the reading is made against a blank of methanolic silica gel extract at a wavelength of 294 nm. The brown homonataloin spot is isolated after location under ultraviolet light and then treated as described for aloin in section 1.3. Concentration of homonataloin is obtained from a standard graph.

2) Investigation to see whether the assay of FAIRBAIRN would detect Homonataloin.

Due to the fact that homonataloin is found in a large number of South African aloe species (over 20 to date) and that aloe juice is collected by unskilled labour, there is always the possibility that a commercial sample may contain homonataloin instead of aloin (e.g. the old Natal aloes). Thus it was decided to see if a laboratory worker performing an assay involving no visualization as in T.L.C. could detect the difference.

Homonataloin was taken and assayed as for aloin by means of the FAIRBAIRN method of assay. Everything proceeded normally until step 2 section 1.2. which involves the alkali extraction of the carbon tetrachloride containing the aloe-emodin. On the first alkali extraction the resulting solution gave a red brown colour.

On the second extraction with N. NaOH a mauve coloured solution was obtained. On combination of these two alkali extracts, removal of any carbon tetrachloride present by warming, making up to volume and reading in a spectrophotometer against N. NaOH, it was found that the main peak was at 460 nm instead of 505 nm as for aloe-emodin in NaOH. This fact automatically makes it impossible for the desired value of 2 for the ratio of reading at 500 nm to reading at 440 nm to be obtained by virtue of the fact that the reading at 440 nm will nearly always be higher than that at 500 nm. This will immediately indicate the presence of components other than aloin, e.g. homonataloin, and this indicates adulteration.

Investigation to see whether the assay of MÖHRLE would detect Homonataloin.

The assay of homonataloin by the method of MÖHRLE was performed as for aloin. On the addition of the periodate solution to the solution of homonataloin a purple colour immediately developed, which changed to a yellow colour on the addition of ammonia solution.

After standing in the dark for two hours an orange colour was found to have developed. Although this orange colour gave a fairly high reading at 510 nm it was found that the maximum reading occurred at a wavelength of 460 nm. Hence it is seen that a laboratory worker could possibly assay a sample of aloe containing homonataloin and, not realising this, express his results as aloin.

From this it is seen that a ratio of the spectrophotometric reading at 510 nm and that at 460 nm should be introduced. For the assay to be a valid estimation of the aloin content of a sample of aloes this ratio should be greater than one. In this manner the danger of assaying homonataloin as aloin could be removed completely even if the laboratory worker failed to see the purple colour produced initially by the addition of the periodate solution, or the fact that the colour at the end of the assay is orange and not red.

2.7 Breakdown products of Aloin.

2.7.1. Beta-barbaloin.

β-barbaloin is mentioned in the B.P.C.1968(8) as one of the constituents of aloes. "β-barbaloin is amorphous, water-soluble, isomeric with barbaloin and formed from it upon heating at high temperature due to prolonged heating of the juice: Cape aloes contains most (about 8%), due to prolonged boiling of the juice and the high temperature attained towards the end of boiling". DENSTON (22).

Formation of β-barbaloin.

Aloin was placed in a glass ampule and heated in an oven at 170°C for three hours. On removal, the yellow aloin had changed to give a dark brown-black, metallic-appearing substance. On adding methanol this did not all go into solution.

The methanolic solution was then chromatographed using chloroform:ethanol (3:1 v/v). On observation of the plate under an ultraviolet lamp it was seen that two new spots had appeared, the one being just above the aloin and the other (a smaller spot) lying just above this. They were a similar colour to aloin, except being slightly darker. These spots were then sprayed with the following reagents:

1) KIO_4 - no colouration was obtained, hence it cannot be isobarbaloin.
2) Ammonia - Yellow under U.V. light (same as aloin).

3) Mag. acetate. - Yellow under U.V. light (same as aloin)

4) Fast Blue B. - no reaction, hence it is not resinous.

The spots were removed from a thin layer plate, eluted with methanol, centrifuged and filtered through Whatman 542 filter paper into a 1 cm cell and the U.V. spectrum determined. There was no peak at 359 nm as there is with aloin. The main peak occurred at 295 nm with a small peak at 252 nm. Both the spots gave the same spectra. The peak at 295 nm is typical of anthraquinone compounds.

A chromatogram was then developed using ethyl acetate: glacial acetic acid:water (5:1:4) as a solvent system. Here only one spot was found, this occurring immediately above the aloin and merging slightly with it. On standing for a few days this spot became visible in daylight as a light brown streak.

2.7.2. ALOIN CONTENT AFTER HEATING AT 150°C FOR TWO HOURS.

Pure aloin was heated at 150°C for two hours and then assayed for aloin content by thin layer chromatography and by the method of FAIRBAIRN.

Method	Thin layer chromatography	The FAIRBAIRN Method.
% aloin	84.0%	84.5%

From this it is seen that there has been a loss of 16% aloin. From the thin layer plate it was seen that only a small amount of aloe-emodin had been formed. Hence the 16% of aloin must have been converted to β-barbaloin, aloe-emodin and the methanol-insoluble residue.

Determination of whether the spots which arise from heating aloin might exist in aloe.

The spots which arose from heating the aloin have not been observed in aloes when chromatographed, but the possibility exists that they might be masked by some other spots. In order to determine whether this was so a chromatographic plate was spotted with a methanolic solution of aloin which had been heated at 150°C for three hours. This spot was then overspotted with a methanolic solution of aloes, the plate chromatographed (using chloroform:ethanol 3:1) and examined under a U.V. lamp. The new spots above aloin could not be seen as they were obscured by the resin spots of aloes which lie immediately above the aloin spot. Hence β-barbaloin would not normally be observable in aloes.

2.7.3. The breakdown of Aloin in aloes.

In the preparation of aloe lump, which involves the boiling of aloe juice for several hours, there is a considerable loss in the aloin content. This has been demonstrated by VAN OUDTSHOORN (86) who found that the loss after two hours of heating was only 3%, but at the end of the process was ca. 10% aloin. McCARTHY

(65) dried aloe sap at 150°C in an electric oven and demonstrated that the aloin loss was dependent on time of heating and the thickness of the layer of aloe sap. A thick layer (1-2 cm) gave a relative aloin loss of 11.4 - 13.0%

A thin layer (1 - 2 mm) gave a relative aloin loss of 4.1 - 7.7%

Thus it is seen that there is a considerable loss of aloin on heating. However on assaying the aloe lump for aloe-emodin it is found that there is only a very low percentage of aloe-emodin present.

FAIRBAIRN (53) states that the aloe-emodin percentage seldom exceeds 0.5% when expressed in terms of aloin. To investigate this further, four samples of aloe lump were assayed for aloe-emodin by the following method:

1 g of aloe lump was shaken with 80 ml of hot water for 5 minutes, allowed to cool to 20°C and then made up to 100 ml with distilled water, 10 ml of this solution was then pipetted into a separating funnel, where it was extracted with 3 x 15 ml portions of carbon

tetrachloride. The aqueous layer was then rejected and the combined carbon tetrachloride portions were washed with 2 x 10 ml portions of water. The aqueous washings were then rejected. The carbon tetrachloride layer (containing the aloe-emodin) was then extracted with one 10 ml portion of N NaOH and one 5 ml portion. The combined alkali layers were then warmed in a small porcelain vessel over a water bath for 5 minutes with constant stirring in order to remove any carbon tetrachloride which might still be present. The solution was then made up to 25 ml and the red colour measured at a wavelength of 500 nm against a blank of N NaOH.

The results obtained for the four different samples expressed as aloin appear below.

Sample	Curaçao	Coega	Kirkwood	Cape aloes (lot 39)
	0.29%	0.125%	0.162%	0.23%

These results are in agreement with those obtained by FAIRBAIRN (53). From this it is seen that although there is approximately a 10% loss of aloin in the preparation of aloe lump, there is a final concentration of aloe-emodin of less than 0.5%. Consequently the total%

aloin altered by heating is not converted to aloe-emodin.

By virtue of the fact that the nature of the work performed was divided into two different techniques, namely chemical assays and biological assays, this work has been divided into two main sections, Chapters 1 and 2 dealing with the chemical assays and Chapters 3,4 and 5 dealing with the biological assays. There will be a short resumé at the end of each section on the work performed in that section, with a final summary and discussion at the end.

Resumé of Chemical Assays.

In Chapter 1 the different chemical methods of assaying aloes are outlined in a brief scheme. The chemical methods of assay which were used in this work are then described in detail.

In Chapter 2 the results obtained for a series of commercial samples of aloes when assayed by the different chemical assays investigated are compared. Here it was found necessary to introduce a correction factor for the thin layer chromatographic method of assay due to the interference caused by silica gel.

The assay methods were also used for the assay of pure aloin and here it was demonstrated that the method of MÖHRLE as modified by BÖHME & KREUTZIG cannot be used in its present form for the assay of aloin of high purity.

The assays were also performed on aloe samples known to have chemical constituents different to the normal commercial samples of Cape aloes to determine whether these interfere in any way with the assay.

The relationship between aloin and the water soluble extractive (B.P. 1968) was also investigated.

CHAPTER 3

3.1 THE USE OF BIOLOGICAL ASSAY METHODS FOR INVESTIGATING PURGATIVE ACTIVITY.

Biological methods of assay are used to measure the potency of drugs for which there are no satisfactory physical, chemical or physico-chemical methods by observing their pharmacological effects on living animals or tissues.

They are particularly useful for substances which have not been obtained in a pure form and cannot be assayed in the impure state by chemical methods, particularly if the nature of the active substance is not known.

Drugs which are assayed biologically are compared for potency against a standard preparation of the same substance wherever possible, or at second best against a substance possessing similar activity. Before stable standard preparations were introduced it was customary to express potency in animal units, i.e. the weight of digitalis lethal to one gram of frog was called a frog unit. This was however unsatisfactory due to the weight variance, state of nutrition, sex variance and variation between different strains of laboratory animals. Even though the use of standard preparations eliminates these errors it does not compensate for variations in the animals used in a particular laboratory. These errors, however, can be estimated by statistical methods and can be reduced by using a sufficiently large group of animals.

Much of the progress in biological standardisation has been due to the realisation of the need for a strict statistical analysis of the results.

EMMENS (24) summarised the general principles to which any biological standardisation must conform as follows:

1) A standard reference preparation must be used simultaneously with the preparation under test.

2) The assay must prove a valid, unbiased estimate of the potency of the preparation under test and the limits of error of this estimate at any required probability.

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3) The assay must provide evidence that the actions of the preparation under test and of the standard preparation do not differ.

4) The most accurate method will be that for which the quantity s/b is minimal, where s is the standard deviation of an individual result and b is the slope of the dose response line.

5) The living material receiving each dose of the standard and unknown must be as uniformly distributed among such dosage groups as is possible.

Potential sources of variation such as difference in response between litter mates, sexes or strains of animals must be minimised as far as possible by allocating animals to dosage groups so that their influence can be isolated and examined in the subsequent statistical analysis.

Biological assays may be divided into two classes, viz.

1) <u>Graded Response Assays</u>.

These depend upon the measurement of graded response i.e. a relationship is obtained between a dose and the response to that dose. Here the experiment is repeated at different dose levels and a plot of the dose against the response, or of the log dose against response obtained. The log dose is generally related linearly to the response, and if this is plotted for both the standard and the unknown, a set of parallel lines should be obtained, the separation between them giving a measure of the potency difference, accurate results only being obtainable if the lines are linear and parallel.

2) Quantal Assays.

These are commonly referred to as the all-or-none assay, meaning that either a positive result is obtained or a negative result . There is no gradation and the curve obtained is always S shaped i.e. at a low dose a negative result is obtained and at high dose positive results are obtained with all the test animals. This is used for toxicity tests and for poisons and at low dose levels all the test animals live (negative result) while at high dose levels all the animals die.

In Biological assays one of the most important factors is the Laboratory animals used, their accommodation and treatment.

3.2 Procurement of Animals.

In laboratories it is generally best to breed the small laboratory animals from a good strain of animals. This inbreeding of animals is said to have the following faults:

1) Differences are not great enough for practical interest.
2) They are said to be difficult to breed.
3) The technique of inbreeding is laborious.
4) If colonies are established in different places genetic divergence will arise between them.

W. LANE-PETTER (57) maintains that 1) and 2) are incorrect but does agree with 3) and 4) and he advocates "Traffic Light sub-cultivation", where primary colonies of various strains are kept in one place and constantly controlled, genetically, health-wise and specific response-wise. Foundation stock from such primary colonies may be cultivated elsewhere for up to three generations. This would obviate 3) and 4).

BROWN and HUGHES (14) maintain that insufficient consideration is given to the differences that occur between animals to disease susceptibility, responses etc. They found the response variation in the writhing response to mild analgesics (used in the screening of analgesics) was such that certain strains of mice gave non-reproducible results, whereas others gave reproducible results.

ANNIE M. BROWN (13) working with insulin on mice obtained similar variation in results, so that it can be a great advantage to obtain a strain of animal which gives a definite response in the test work for which it will be used, to one which gives only an intermediate response.

Wherever the animals are procured from it should be from a stock which is known to be healthy and disease free. This is particularly important if it is not possible to breed animals, and all have to be obtained from a commercial breeder.

Handling and Psychology of Laboratory Animals.

A good worker will be able to handle the animals sympathetically and win their confidence, while if he is afraid that they may bite him or if they are afraid of him, the methodology is at fault. The animals should be tame and hence should be regularly handled, as this facilitates manipulation during actual experiments and gives better results.

3.3. THE ANIMAL HOUSE.

Too often finances dictate the planning of this, and not sufficient stress is laid on its importance.

Siting.

The animals should be kept near to the laboratory but preferably not in it. The breeding stock should also be kept separate from the normal stock, and in no circumstances should the animals be returned to the normal stock from the experimental side.

The animal house should therefore be a separate building, wing or floor of the laboratory building. In any event it must be physically separate.

Construction.

The animal house should be so constructed as to permit maintenance of a constant temperature, and constructed of such materials as to permit frequent washing down of the walls and the floors, which must have a smooth hard surface.

Heating, Ventilation and Lighting.

Animal rooms should be maintained at a constant temperature which should not vary by more than 5°F all year round. The best method is the delivery of heated air through ducts with some form of forced exhaust.

Any form of artificial lighting can be used, as all the animals except monkeys are colour-blind and hence only respond to the intensity of the light.

3.4 THE CAGES.

These must fulfil four functions:

1) It must confine the animal and be constructed of material that the animal cannot break, distend or destroy.

2) The cages must allow the animal to live in health and comfort. Here the size is the main consideration and this is dependant upon the length of time the animal is to be confined in the cage.

3) A cage must be economical in first cost, accommodation and maintenance. Simplicity of design should be the first aim. As well as being the most hygienic these are also usually the best. Inconvenient catches, badly placed doors, ill-conceived trays etc. are all some of the inconveniences that can be caused by a badly designed cage.

4) A cage must satisfy experimental requirements. For certain work a normal cage is not suitable and a specially designed cage is necessary e.g. in the metabolism experiments, LOU'S mice cages etc.

3.5 THE BIOLOGICAL ASSAY OF PURGATIVES.

Most of the work that has been done in this field has been done on the anthraquinone purgatives namely cascara, aloes, rhubarb and senna.

Purgatives evoke various responses in laboratory animals and hence there are various responses which can be used as criteria for the biological assay of purgative activity. These include peristaltic stimulation as used by MACHT (ex 17) and by LISH and DUNGAN (59). Weight of faeces passed was used by LIDDEL, KING and BEAL (ex 17). Visual appearance of the faeces was originally used by LOEWE (ex 17) and GEIGER (ex 17), then later by COLLIER, FIELLER, and PARIS (17), by LOU (61) and by FAIRBAIRN (25 to 38) and others. Faecal consistency was used by means of the principle of "wire adhesion" by LATVEN, MUNCH and SLOANE (58), working with rats.

Apart from the various responses to purgatives that can serve as biological criteria, mention must be made of variation due to animal species themselves. Thus MUNCH (65) commenting on the purgative activity of aloes in several animals, stated"that mice are unreliable, that fish are of no use, that rabbits are too sensitive, that cats are good, but variations in sensitivity occur, while dogs are more difficult to standardise than cats". VIEHOEVER (65) has even used the water flea. RAMSTAD (73) states that a biological assay is more reliable than chemical evaluations, using the mice faeces technique of FÜHNER. He stated that man is about 15 times more sensitive than the mouse to senna 50 times more sensitive to rhubarb root, 130 times to cascara sagrada and 800 times to aloes.

From this it is seen that a wide variety of animals have been used in the bioassay of purgatives. Mice are the animals which have been most frequently used for bioassays, and with some success as well, with certain of the purgatives. Below is some of the work which has been performed using mice.

In 1925 KIEFFER (ex 88) used mice in a bioassay of aloes, and he came to the conclusion that the resinous fraction contributed chiefly to the purgative effect, with the aloin and the aloe-emodin having little effect. By 1939 LOEWE (ex 17) had adapted the peristalsis method of MACHT (ex 17) to the mouse. STRAUB and GEBHARDT (ex 61) in 1936 used white mice to determine the minimum effective dose of senna infusions; the potency was expressed as the number of such doses per ml of senna infusion. In 1940 GEIGER (ex 17) used mice when he compared the potency of the test preparation with the standard (a 5% infusion of senna leaf) by comparing the percentage of mice which produced a positive response. In 1944 GROTE & WOODS (ex 61) modified this by using powdered senna as their standard. HAZELTON et alia (ex 61)(1942-1945) working with mice introduced the Threshold Cathartic Dose i.e. the dose producing catharsis in approximately 50% of the test animals. In 1948 COLLIER, FIELLER and PARIS (17) used mice for their evaluation of senna extracts, which they compared against a reference standard of Alexandrian senna in the form of an extract. The mice were placed in gas jars fitted with wire mesh bottoms, the faeces being allowed to fall through onto filter paper, and were later classified as formed faeces and unformed faeces (shapeless, large and capable of staining the filter paper). They used the ratio of unformed faeces to total faeces as the criterion of the individual animal response, and demonstrated that the log dose-response line was reasonably linear. They also made the interesting discovery that while senna given orally was active, senna given intravenously had no effect at all, this being in conflict with the theory of STRAUB and TRIENDL (ex 31) namely that the anthraquinone glycoside is first absorbed into the blood stream and then later being secreted in the colon.

In 1949 LOU (61) decided that the method of GEIGY as modified by COLLIER et alia was the most convenient especially as mice are so relatively easy to handle. He modified it further by introducing the following improvements: he designed special cages of suitable dimensions fitted with detachable wire mesh bottoms to facilitate inspection of faeces. He added a definite proportion of water to the food (most workers had witheld water during testing even though water plays an important part in purgation) and used counts of wet faeces only as the criteria of activity. Powdered senna fruit was used as the standard of purgative activity. The response was expressed as the number of wet faeces per Kg. of mouse. He showed that while the plot of dose against response was not linear, the plot of log dose against response was linear. This assay is still the bioassay which is most commonly used today for some of the anthraquinone purgatives. BRITAIN, D'ARCY and GRIMSHAW (11) investigated the mouse assay method for investigating purgative activity. They used the purified sennosides as a standard and maintained that it was a valid standard for the bioassay of senna preparations. They showed the assay to be suitable for the assay of senna, its extracts and its preparations but that it was not so well suited for cascara and rhubarb and that it did not demonstrate the purgative action of aloin. (D'ARCY, GRIMSHAW and FAIRBAIRN (21).)

AUTERHOFF and BALL (2) in 1954 used mice on various aloe extracts and came to the conclusion that the mouse gave a false impression of the activity when related to humans. In 1958 LISH & DUNGAN (59) used the LOU (61) mouse assay to investigate the peristaltic stimulating and faecal hydrating properties of dioctyl sodium sulfosuccinate, Danthron and various cascara extracts. However, they expressed their results as an $E.D_{50}$. FAIRBAIRN has in recent years used the assay of LOU (61) with great success on his work on senna, cascara, rhubarb and various pure anthracene derivatives (25 to 38).

In 1963 HÖRHAMMER et alia (50) separated the aloinosides from aloes, and work with mice indicated that these have the same activity as aloin. The rat has also been used in the bioassay of purgatives with some success.

In 1931 MACHT & BARBA-GOSE (ex 17) found that a meal dyed with charcoal was propelled from the pyloric sphincter to the anal sphincter of the rat more rapidly than usual after the administration of a purgative and they used this response for the biological estimation of purgative activity.

In 1958 LISH & DUNGAN (59) used the method of MACHT & BARBA-GOSE (ex 17) to determine propulsive rates of a charcoal mix. In their investigations using dioctyl sodium sulfosuccinate, Danthron and various cascara extracts, the charcoal mix was administered 3¼ hours after the drug via a stomach tube. The rats were sacrificed forty five minutes later : control animals received the vehicle alone. A self-contained four point assay was used and Peristim (methanol extract of cascara bark) was arbitrarily chosen as the standard. The mean % of intestinal travel was then determined and this was found to give an approximately linear log dose/response relationship when the furthest charcoal travel lay between 65% and 85% . The caecum at the 90% level was found to act as a block to charcoal travel, GREEN, KING & BEAL (ex 17) 1963 and LIDDLE, KING and BEAL (ex 17) 1942 used the weight of faeces passed, by rats, in a given time as an index for purgative activity.

LATVEN, SLOANE & MUNCH (58) developed a bioassay for aloes, using rats as the test animals. Throughout their initial investigations they used a single sample of aloes known to be effective in people. They first determined the average alimentary passage time. This was done by first determining the normal alimentary passage time (by administering a solution of carmine to the rat by oral intubation), and the time taken between dosage and its appearance in the stools was taken as the "carmine passage time". This was found to be 408 minutes in rats. Aloes was then administered at the same time as the carmine and this was found to have no significant effect on the passage time. LATVEN then determined the time taken for laxative action by administering doses of aloes ranging from 10 to 400 mg/Kg to a group of rats. Faecal samples were then manually expelled at two hour intervals. Normal samples were found to be relatively hard, dry and of a blackish colour. There was no change in appearance for seven hours after administering aloes. On the eighth hour, however, specimens were found to be of a brown to a tan colour, and on the tenth hour the sample was also found to be soft, and at the higher dosage levels the samples were semi-liquid. The intensity of these effects were found to be maximal at ten to twelve hours, decreasing slowly and progressively at later periods, while normalcy returned within 24 hours at the lower dosage levels but only after 48 to 72 hours at the higher dosage levels. Unfortunately LATVEN et alia (58) had no reference standard against which to express potencies, so they expressed their results as a Median Defaecatory dose, which of course is only valid for the rats which he used.

LISTER & PRIDE (60) used this Median Defaecatory dose method on barbaloin, aloin B.P. 1953, and amorphous aloin, and found a relative potency for each of 100, 82 and 27 respectively. This latter figure agrees with the range of 20.4% to 32.2%(from four samples of amorphous aloin prepared directly from aloe juice)obtained by McCARTHY (65).

FAIRBAIRN (30) has reported that he has had no success with the method of LATVEN et alia (58) but that recent work with a special strain of the Wistar rat was proving encouraging. He also reports (31) that in 1963, 32 out of 36 pure strain Wistar rats were sensitive to barbaloin, but that of a batch bought from the same source in 1964, only 5 out of 24 were sensitive although all were found to be reactors when subjected to the dextran test (47). SCHMIDT in 1959 (77) successfully used rats in an investigation of the laxative effect of various synthetic laxatives. In this work he included aloin, cascara sagrada, 1:8-Dioxy-anthraquinone and senna glycosides.

, Reckitt and Sons LTD of Hull (74) are at present performing a bioassay for aloes using rats in which the number of wet faeces produced is used as the criterion for purgative activity. Aloin is used as the standard.

Besides the mouse and rat some of the other animals used for purgative assays are mentioned below. STRAUB & TRENDL (ex 61) used cats in 1937 to determine the route of absorption of senna extracts. GREEN, KING & BEAL (ex 17) 1936 and LIDDLE, KING & BEAL (ex 17) working with guinea pigs, found that the weight of faeces passed in a given time was a reliable index of their response to purgatives.

LOEWE (ex 61) used Rhesus monkeys in his assay method.

From the above assays it is seen that mice are unsuitable for the assay of aloes, it was for this reason that rats were employed as the test animals in this work.

The Accuracy of These Assays.

Unfortunately many workers have not given a figure for the degree of accuracy which they obtained. Bearing in mind that b is the slope of the dose/response line and s is the standard deviation, COLLIER (17) stated that his standard deviation was usually about 20% with a ratio of $b^2/s^2 = 16$. His limits of error would have been 63% and 160%. However with a ratio of $b^2/s^2 = 30$ his limits of error would have been 71% and 141%.

LOU (61) determined his standard deviation as being 15.714% and his limits of error (P;0. 99) was 100 ± 40.5%

which was a higher accuracy than that of COLLIER.

LATVEN et alia (58) unfortunately gave no indication of the accuracy of his assay.

3.6 FACTORS EFFECTING THE BIOASSAY OF PURGATIVES.

BRITTAIN, D'ARCY and GRIMSHAW (11) examined the method of LOU to improve its accuracy and precision.

They used sennoside A as a reference standard, and maintained this was a valid basis for assay. They investigated some of the following points for mice.

The Effect of Dose Volume.

Different groups of mice were given doses in volumes of 0.25, 0.5 and 1.0 ml. In all three dose volume groups the responses were linear : however the "within dose error" for the 0.5 ml dose volume group was least.

Adjustment of Dosage for Bodyweight.

It was found that there was no gain in precision by dosing on a bodyweight basis.

The Effect of the Bodyweight of the Mice on the Accuracy

Here they used groups of mice within narrow bodyweight ranges. It was found that the accuracy improved to some extent by using heavier mice and that satisfactory results were obtained where the bodyweights lay between 27 and 31 g.

The Effect of Training Mice.

Conditioning of the mice before routine testing greatly improved the precision and accuracy of the assay. This conditioning involved subjecting the mice at weekly intervals to the assay procedure in the testing laboratory for the normal duration of the assay, for 2 to 5 weeks after which the mice were used for routine assays. As the conditioning progressed the fiducial limits narrowed and the index of precision became narrower.

The Effect of Sex Variation.

COLLIER (17) reported that with Tuck No 1 white strain mice that he could detect no difference in response between male and female mice.

LOU (61) however reported that HAZELTON had detected a sex difference, so he used only male mice.

INVESTIGATIONS USING RATS.

The Effect of Using Different Strains of Rats.

LATVEN et alia (58) performed assays using Charles River rats and Blaine and Wistar rats, the results that he obtained indicated a difference of the ED_{50} between the different strains of rats, but the response within a single strain appeared to be reasonably uniform. LATVEN et alia (58) working with Charles River rats discovered that the female rat was nearly twice as resistant to aloe as was the male.

3.7 MEDICINAL APPLICATION OF THE BIOASSAY OF PURGATIVES

The main application is in the standardisation of purgatives, an example of this being the placing of Sennokot, a standardised senna preparation , on the market.

Another very important application is the determination of the active constituents of vegetable purgatives, FAIRBAIRN in particular doing much work in this field (16).

CHEMISTRY OF ANTHRACENE PURGATIVES.

The anthracene derivatives occur either in free form or in the form of glycosides, usually with glucose, or occasionally rhamnose as the sugar. The following aglycones have been reported:

1) Anthraquinone compounds: e.g. emodin, aloe-emodin, rhein. These occur in cascara, aloes, rhubarb and senna, although only in very small amounts in aloes and senna.

2) Anthranol compounds: anthranol and its tautomeric isomer, anthrone, are reduced forms of anthraquinone. Hydroxy derivatives of anthranol and anthrone, corresponding to those of anthraquinone occur frequently e.g. aloe-emodin anthranol in aloes.

3) Oxanthrone compounds, these being intermediate between anthraquinones and anthranols.

4) Dianthranol compounds: STOLL suggested that the

aglycones of senna might be dianthranol or dianthrone compounds which can be oxidised to rhein-like compounds.

For the purposes of his investigation FAIRBAIRN divided these into the following three groups;

a) Glycosides.
b) Free anthranol compounds.
c) Free anthraquinone compounds.

Working with senna, cascara and rhubarb he then determined which of a, b or c contained the active constituents. This he did in the following manner:
i) He took senna leaf and subjected it to varying degrees of hydrolysis and then compared the purgative activity of these fractions with the unhydrolised drug. He found that mild hydrolysis had little effect, but that vigorous hydrolysis and possibly oxidation led to a marked loss of activity. He then took a solution of pure Sennoside A and divided it into three portions, one of which was used as the standard, another was hydrolised and the liberated aglycone extracted and purified. The third portion was hydrolised and oxidised. These three portions were then dosed to mice

and the results showed that the aglycone possessed 1/3 of the activity of the glycoside and that the oxidised aglycone possessed no activity at all. These results were confirmed using the senna pod.

ii) In rhubarb there is present a large proportion of free aglycones. FAIRBAIRN (26) extracted the free emodins with ether and acetone, and the dried exhausted drug was then administered to mice, and the results obtained showed that there was no loss of activity, indicating that the main activity lay in the ether-insoluble glycosides. The free aglycones consisted almost entirely of anthraquinone compounds, which would account for the entire absence of activity in this fraction.

iii) Working with a potent extract of cascara which also contains a large proportion of free aglycone as well as the glycosides, again similar results as were obtained with rhubarb resulted.

This work indicated that the main purgative activity of the anthracene derivatives is shown when they are in the glycoside form, and that the free anthranol form though less active than the glycosides, is more active than the anthraquinone form.

It was hence suggested that the sugar moiety acts as a transporter and protects the orally active anthranol from oxidation to the inactive anthraquinone.

From the above it can be seen that chemical assays which are based on the determination of the total content of anthracene derivatives-irrespective of their form-will not give a true indication of the purgative activity and will not give the same results as a bioassay.

FAIRBAIRN and SALEH (38) used the assay of LOU in the detection of a third active glycoside of senna. They discovered that although samples of senna leaf contained a smaller proportion of sennosides than the leaf, they had a greater biological activity. On investigation they found that the mucilage had no activity, nor was there any synergism between sennosides A and B. They then discovered a non-rhein glycoside (10-15% of the total glycosides of the leaf), but only 2-5% of the total in the pod. This glycoside was shown to be as active as the sennosides and also when mixed with sennosides A and B so that it represented 14% of this mixture, this third glycoside exerted a marked synergistic effect, increasing the total activity by about 1.7 times.

Furthermore they found that the glycosidal content only accounted for 60% to 70% of the activity of the crude drug, indicating that there is still some other factor responsible for the remaining 30% to 40% of the activity.

From the preceding review it is seen that although a considerable amount of work has been performed on several of the anthraquinone containing purgatives (in particular senna) it is seen that the constituents of aloes have not been separated and their individual purgative activities determined by the method of LATVEN.

In consequence in this work several principles of aloes have been separated, including principles as yet not used to ascertain purgative activity, and these findings appear in Chapter 4.

3.8 THE PHARMACOLOGY OF ANTHRACENE PURGATIVES.

The site of action of the anthracene purgatives is the large intestine. STRAUB AND TRIENDL (ex 31), and OKADA (ex 31) showed that when the free aglycone was applied directly to the large intestine, marked peristalsis was produced, but that the glycosides had no effect until they were hydrolised. GEBHARDT, STRAUB and TRIENDL (ex 31) and OKADA (ex 31) concluded that the glycosides of senna are absorbed in the small intestine and transported by the bloodstream to the large intestine where they are hydrolised to the aglycone by the enzymes of Escherichia coli. OKADA demonstrated that hydrolysis was effected by the contents of the large intestine but not those of the small intestine. The hydrolytic ability was destroyed by heating the contents of the large intestine at 60 °C, indicating that the micro-organisms (especially E. coli) are responsible for the hydrolytic effect. FAIRBAIRN (31) demonstrated the necessity of the presence of live E. coli for the activity of the anthracene glycosides by sterilising the gut of white mice with succinyl sulphathiazole before administering senna, after which there was a considerable decrease in the activity of the senna.

SCHMIDT (76), however, concluded that the glycoside passed directly from the small intestine into the large intestine. This supports the fact that COLLIER et alia (17) found that the intravenous injection of senna had no purgative effect. FAIRBAIRN (31) confirmed this using partially purified glycosides of senna and pure sennosides.

The importance of the glycoside is its ability to withstand the detoxication mechanisms of the body. FAIRBAIRN (31) suggests that this protective function of the sugar may be performed in two ways:

1) Increased solubility in water prevents the drug from passing into the bloodstream and being detoxified by the liver.

2) The sugar may prevent enzymes from attacking the aglycone.

Originally it was thought that the hydrolysis produced hydroxyanthraquinones and that the quinone form was the active substance. However FAIRBAIRN et alia (26) demonstrated that the anthrones are more active than the quinones and anthrone glycosides. SCHMID (76) showed that the anthrones were many times more active than the corresponding anthraquinones when applied directly to the large intestines. He also showed that E. coli reduces anthraquinones to anthrones.

3.9 THE CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTIVITY OF PURGATIVES.

Since all the vegetable purgatives contain anthracene derivatives, it is natural to assume that these are the active principles. Attempts have been made to associate the activity of these to phenolphthalein, another powerful purgative.

KAUFMAN (ex 25) in his attempts to correlate the chemical structure and pharmacological activity of these phenolic substances stated "that the laxative group in a number of aromatic compounds was a substituted 4,4'dihydroxydiphenyl methylene group".

HUBACHER, DOERNEBERG and HORNER (ex 25) prepared 36 compounds, mostly phthaleins and anthraquinones, and tested their laxative potencies on Rhesus monkeys. They showed that none of the previous generalizations, including those of KAUFMAN, satisfactorily explained the results obtained. They themselves were unable to come to any reliable generalization, except that all compounds showing laxative activity possessed at least two phenolic hydroxyl groups and at least three rings in their molecules. VIEHOEVER (ex 16) in 1935, from data based on the research of earlier workers who studied the effect of various oxyanthraquinones - in cats, stated that 1:2:6 and 1:2:7 trioxy derivatives were very active in doses of 300 and 500 mg respectively, but the 1:2:3 derivatives required 1,000 mg to achieve the same effect. No catharsis resulted from doses of 500 - 1,000 mg of 1:2,1:4, penta - and hexaoxy derivatives, although 1:2:3:4-tetraoxyanthraquinone (Alizarin Bordeaux) showed activity at 1,000 mg but none at 500 mg. Emodin (1:6:8:-trioxy-3-methyl anthraquinone gave only a slight action at 500 mg dosage.

FAIRBAIRN (25) used white mice to estimate the purgative activity of aloe-emodin, chrysophanol-emodin, and of the fungal anthraquinones catenarin and helminthosporin, and he noted that:

a) those with one hydroxy group were inactive.

b) those with two hydroxy groups were active.

c) those with three hydroxy groups were active provided that all three were not α groups (viz catenarin and helminthosporin.)

FERGUSON (ex 25) made the interesting observation that those anthraquinone compounds having two -OH groups in the 1:8 position and none in the 2:3:6 or 7 positions produced "senna type" stools i.e. watery in mice, whereas those with hydroxy groups in positions 2:3:6 or 7 produced "aloe type" stools (soft). It should be noted, however, that aloin is a 1:8-dihydroxy compound. He also confirmed that mice were extremely resistant to aloin.

The importance of the phenolic groups is shown by the fact that removal of one or all phenolic groupings from known cathartics results in reduced or no cathartic activity.

CHAPTER 4.

BIOASSAY METHOD.

For the bioassays the method of LATVEN, SLOANE and MUNCH was used (58).

4.1. TEST ANIMALS.

Albino, inbred male rats acquired from the S.A.I.M.R. were used as test animals. The weights of these were in the range of 185 g to 240 g.

4.1.1. HOUSING OF ANIMALS.

The rats were individually housed in wire cages 9" x 11" x 9" high, fitted with a sliding metal tray to facilitate cleaning. Each cage was fitted with side-glasses 3" high to minimise any possible drafts. On each cage, positioned so that the rats could not destroy it, was a card with the number and weight of every rat. On this was entered the date, dosage, drug and the response obtained from every experiment.

Each cage was fitted with a plastic food bowl of standard dimensions. Water was provided by means of water bottles to prevent the rats from fouling their water as they would in an open dish. These were 4 oz medicine flats fitted with a cork, through which passed a glass tube of $\frac{1}{4}$ inch diameter. To prevent airlocks forming in the tube, plastic coated wire was passed down the centre of the glass tube. The bottles were inverted and placed in metal containers which were wired to the tops of the cages, the glass tubing passing through a hole in the bottom of the container and ending 6" above the floor of the cage. The rats obtained water by standing on their hind legs and sucking on the glass tube. Metal containers were used when it was found that the rats gnawed the corks of the water bottles if these were placed directly on the tops of the cages.

Bedding.

Sawdust, which had been sterilised by heating for three hours at $150^{\circ}C$ to prevent the danger of any infection from wild rats, was used as bedding, this being replaced every three to four days with fresh bedding.

Food.

For food, chinchilla pellets (Epol) were supplied once a day (20 g. per rat). These proved to be an excellent food for the rats.

4.1.2. THE ANIMAL ROOM.

This was a room of 24' x 15' x 15' high, fitted with windows 11' above the floor. The cages were placed 2½' above the floor. This room was situated next to the experimental laboratory.

Temperature.

The animal room was maintained at a temperature of 18° - 24°C (65° - 75°F) by means of a hot-air heater which circulated the air throughout the room.

Light.

Normal daylight was supplemented with neon lights.

Humidity.

Good ventilation was maintained by means of the windows and fan, this being essential as dry air can cause ringtail, while excessive dampness shortens the life of adult rats.

4.1.3. HANDLING OF THE RATS.

Entrance to the animal room was restricted to those handling and caring for the animals. Sudden and loud noises were avoided at all times, but especially while handling the rats, which are easily startled. They were picked up by placing one hand across the back and closing the fingers lightly but firmly under the chin and body, Gloves were never worn, thus preventing clumsiness during handling.

4.1.4. TRAINING OF THE RATS.

BRITTAIN, D'ARCY and GRIMSHAW (11) showed that the training of the mice in the mouse assay of LOU (61) increased the accuracy of the assay. Consequently for three weeks before being used in assays the rats were allowed to settle in their cages and were frequently handled to accustom them to the procedure employed.

Initially they were dosed with plain water, they were later dosed with a coloured solution of tartrazine to ensure the full dose was being delivered into the stomach. If it was not, the fact would immediately be obvious by the yellow colouration around the mouth.

4.1.5. DOSING OF THE RATS:

Due to the bitter taste of aloes and many of its constituents the only way the drug can be administered orally is by means of a stomach tube. The apparatus used for dosing consisted of a graduated 2 ml syringe fitted with a large gauge hypodermic needle. To this needle was attached a rubber catheter 5" long ("BRESCO" 3.E.G.) which passed through a thin glass tube, supported for most of its length by a thicker polythene tube (Total length 7½")

The syringe was filled with the required dose. The catheter was then dipped in glycerine to act as a lubricant and withdrawn into its protective glass tubing. The rat was held by an assistant who immobilised its feet. The thin glass tube was placed in the rat's mouth and held in place by the index finger and thumb of the left hand. The syringe was then pushed down into the protective tubing, thus passing the catheter through the oesophagus and into the stomach. The plunger was then depressed to administer the dose and the catheter was then withdrawn into its protective sheathing to prevent it being bitten by the rat upon removal from the rat's mouth.

4.1.6. EXPULSION OF FAECES.

The rectum of the rat invariably contains one or more faecal pellets, which can be manually expelled or extruded for examination, this being done in the following manner. The rat was held in the left hand with the fingers across the back and the thumb on its chest: with the forefinger and thumb of the right hand, the skin over the rectum was compressed as far forward as possible and the contained faecal mass was then forced out. Normal faeces are dark brown to black in colour and very hard and dry. After receiving an effective dose of aloes the faeces became light brown or tan coloured, soft and moist.

4.1.7. OBSERVATIONAL CRITERION.

The criterion used was the same as that used by LATVEN et alia (58) namely that of "wire adhesion". The faecal sample was expelled manually from the rat and was allowed to fall upon a clean, oil-free glass plate. Within five minutes a 17G hypodermic needle was inserted vertically downward into the pellet. The wire was then lifted. Specimens of normal consistency adhered to the wire and were lifted off the glass plate: however "soft stools" adhered to the glass by virtue of the greater quantity of surface mucous, as well as the greater contact area of adhesion.

In this way a positive or negative response was obtained. A slight modification was used to obtain a 75% response, in that if a pellet just failed the test the same pellet was resubmitted to the test and if it adhered to the glass plate the second time it was taken as 75% positive.

4.1.8. BIOASSAY METHOD.

The rats to be used in the assay were starved overnight. A suspension of the drug was prepared by triturating the drug, in a small glass mortar, with sufficient acacia to give 2% of the final suspension. Water was then added and the suspension transferred to a 10 ml measure. The mortar was washed and the washings were added to the measure which was finally made up to volume. LATVEN et alia (58) used a dispersing agent but as surfactants have been shown to effect the permeability of membranes and hence the rate of absorption no dispersing agent was used here. Evenly spaced dosage levels encompassing the entire dose-response curve were used, with four rats at each level. Faeces were first examined for normality before the rats were dosed.

The rats were dosed with a stomach tube and immediately fed, water being supplied ad lib. After ten to twelve hours, faecal samples were taken and the "wire adhesion" test applied. Twenty to thirty minutes later a second faecal sample was taken and similarly examined: the maximum effect demonstrated by either of the samples was taken as the characteristic response. Simultaneously the same procedure was performed with the standard aloin.

After being used in a bioassay the rats were rested for at least a week before being used again.

4.1.9. THE REFERENCE STANDARD.

It was decided to use aloin as a reference standard in the biological assays, since the work of AUTERHOFF & BALL (2) with humans indicates that this is the active principle. In addition Reckitt laboratories are using aloin as a reference standard in their biological assay of aloes. In order for a reference standard to be a valid standard for biological assays the slope of the plot of log dose/ % response must be parallel with that of the unknown. This was shown to be the case in section 4.4

4.2 PREPARATION OF STANDARDS.

4.2.1 PREPARATION OF ALOIN.

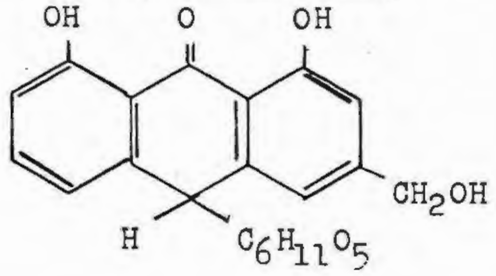
Method of HARDERS 1940 (46).

10 g of Cape aloes was dissolved in 50 ml. of water with heating. To this solution 0.4 ml of 25% HCl and 4 g of calcium chloride was then added. After twenty four hours the clear supernatant liquid was decanted.

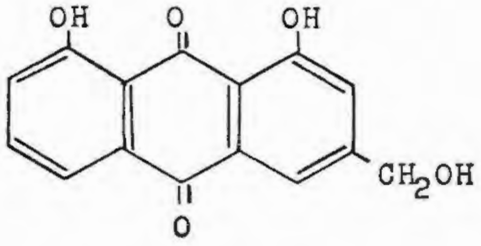
To this was added 10 ml of 20% ammonia solution and the solution was well stirred. After ten minutes the precipitate was filtered off with a Buchner funnel and then placed in a beaker. Hydrochloric acid was then added until all had gone into solution. The slightly turbid solution was then stored under ether in a cool place until all the aloin had crystallized out. This took approximately 24 hours. The crystals were then filtered off on a sintered glass filter and dried in a vacuum dessicator. These were then recrystallized twice from methanol and dried as before. Long, pale yellow needle-like crystals were obtained which gave a melting point of 147 °C – 148 °C. When assayed by the

FIGURE I.

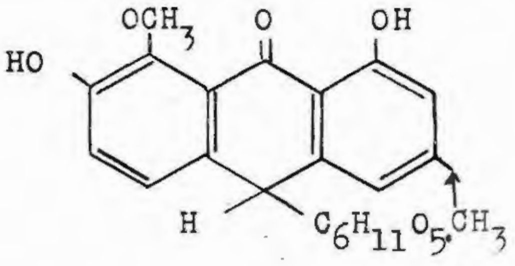
STRUCTURAL FORMULAE



ALOIN



ALOE - EMODIN.



HOMONATALOIN

thin layer chromatographic method they assayed as 99.9% pure.

4.2.2. THE EXTRACTION OF HOMONATALOIN.

In 1917 LEGER (52) isolated homonataloin from Natal aloes. HAYNES, HENDERSON and TYLER (49) in 1960 elucidated its structure and showed it to be 10-D-glucopyranosyl 2-8-dihydroxy-1-methoxy-6-methylanthrone, a C-glycoside. McCARTHY (66) showed that homonataloin is present in a large number of species of aloe, numbering over twenty to date. One of the specimens in which it is most abundant is A. speciosa, which grows in a grove some sixteen miles from Grahamstown, at Hellspoort, and thus it was decided to use this as a source for homonataloin. The method of HAYNES et alia (48) for the isolation of homonataloin from Natal aloes was employed.

A large number of the leaves were cut, longitudinal slashes were made on their surface and they were allowed to drain for fifteen minutes in a glass tank. This was then repeated with fresh batches of leaves and the sap was then allowed to stand overnight. The following morning the yellow solid which had precipitated from the sap was filtered off and sucked dry on a Buchner funnel. A yield of 41 g was obtained.

This was then shaken with 80 ml of acetone for 18 hours. The acetone-soluble fraction was then filtered off. The acetone-insoluble fraction was then refluxed with 200 ml of 2:3 aqueous ethanol for thirty minutes and the resulting solution filtered. This filtrate was set aside overnight to crystallize. Pale yellow crystals were deposited, and on filtering off, a yield of 8.7 g was obtained. The crystalline homonataloin chromatographed pure, but was recrystallized once from methanol.

Ultfaviolet spectrum in methanol showed the following maxima: 347, 294, 273 (inflection) and 220 nm.

4.2.3. THE EXTRACTION OF ALOE-EMODIN.

A very old sample of Coega aloe juice (6 years) previously containing both aloin and aloinsides, was chromatographed and it was noticed that there was no aloin or aloinosides present, but that they had all been converted to aloe-emodin with a resulting high concentration of this. Thus it was decided to use this sample for the extraction of aloe-emodin. 75 g of the aloe juice was refluxed over a waterbath for thirty minutes with 100 ml of ethyl acetate, This was then filtered off and the residue was refluxed with a further 100 ml of ethyl acetate for a further thirty minutes. This was filtered off and the two orange coloured filtrates were combined, washed well with water and evaporated under reduced pressure until the solid just commenced to precipitate. Then acetone was added to redissolve and the solution was spread on a large glass plate and the solvent allowed to evaporate off, leaving an orange coloured solid on the plate (yield 5.5 g.). The latter step was done to prevent loss by sublimation under reduced pressure.

The aloe-emodin was then crystallized out of methanol.

The ultraviolet spectrum of aloe-emodin gave peaks at 225, 254, 287 and 430 nm.

When chromatographed, only aloe-emodin was present. The fact that it was aloe-emodin was confirmed by its Rf value and by spraying the plate:

1) With ammonia, upon which a bright red colour was produced.

ii) With magnesium acetate reagent followed by heating at 105 °C for ten minutes, upon which a bright red colour was also produced.

4.2.4. THE EXTRACTION OF ALOESIN.

Aloesin is the name given to a new C-glycosyl chromone from aloes, the structure of which has been recently elucidated by HAYNES and co-workers (to be published). Aloesin has been shown to be present in several species of South African aloes.

Normally the extraction of aloesin would be complicated by the presence of aloin due to their similar solubilities in the various solvents. However aloin is not as stable as aloesin, and the aloin in aloe juice on standing breaks down to aloe-emodin. Thus the sample of Coega aloe juice in which all the aloin and aloinsides had broken down was used. The aloe-emodin present was first extracted with ethyl acetate, and the residue was then refluxed with acidified water (dil HCl to pH 3) on a water bath. The insoluble resins which precipitated were then centrifuged off after standing overnight. The aqueous supernatant was then filtered and evaporated to dryness under reduced pressure, yielding a pale yellow solid.

A methanolic solution of this when spotted on filter paper and allowed to dry gave the following reactions when treated with the following reagents.

Table 15

Reagent	Daylight.	Ultraviolet light
KOH 5% alcoholic	yellow	bright blue
Ammonia vapour	yellow	bright blue.
Fast Blue B	orange	orange red.

The reactions obtained were the same as those observed by McCARTHY (66) when working with a sample of aloesin provided by HAYNES.

The ultraviolet spectrum was the same as that of the sample from HAYNES, this being a broad clearly-defined peak at 296 nm, with a valley rising sharply to give two small, sharp peaks at 252 and 244 nm.

On chromatographing the sample using chloroform:

ethanol (3:1 v/v) the aloesin was evident at its normal Rf while at the base was seen a small fluorescent white spot which is normally never seen in aloes as it is masked by a brown spot. Above the aloesin were seen traces of the light blue resin spot which normally occurs just above the aloin spot in aloes.

On spraying the plate with Fast Blue B the aloesin spot turned orange while the small fluorescent white spot at the base turned a pale purple. Both of these are resin reactions.

4.2.5. THE PREPARATION OF RESIN.

Resin was prepared from commercial samples of Cape aloes by preparing an aqueous solution of this and then precipitating the resins by acidification. The gummy residue that precipitated was then washed several times with hot water to remove as much aloin as possible, and the residue was finally spread on a glass plate and sun-dried. When assayed for aloin this gave 5% by the thin layer chromatography method, showing the protective effect of resin on the aloin.

Resin was also prepared from that which was precipitated in the extraction of aloesin. This was washed with water, sun-dried and powdered. This was then extracted with successive portions of methanol until the methanol no longer became coloured. The methanolic solution was then filtered off and evaporated to dryness under reduced pressure to yield a dark brown solid. When this was chromatographed in chloroform:ethanol (3:1 v/v) traces of aloe-emodin were observed, a dark brown spot immediately below the aloe-emodin, faint traces of aloesin and a faint trace of aloin.

4.3. DETERMINATION OF THE SENSITIVITY OF THE RATS.

Due to the variation in response by different strains of rats to purgatives (58), no idea was had of what dosage levels would be effective. The rats were first dosed with a sample of aloes at the same dosage levels as used by LATVEN, SLOANE and MUNCH (58). Table 16.

Table 16.

Dose of aloes.	Positive	Negative	% Respons
4 rats 10mg/Kg rat	2	2	50
4 rats 15mg/Kg rat	–	4	0%
4 rats 20mg/Kg rat	–	4	0%
4 rats 30mg/Kg rat	–	4	0%

With the exception of the first dose level all results obtained were negative. The positive doses obtained from the two rats was presumably due to other causes as both of them were later dosed at a far higher dosage level, where they both yielded negative responses

Simultaneously another group of rats were dosed with amorphous aloin (containing 30% crystalline aloin), according to the dose levels as used by LISTER and PRIDE (60) Table 17 tabulates the results.

Table 17.

Dose amorphous aloin (30% aloin)	Positive	Negative	% Response
4 rats 25mg/KG	–	4	0%
4 rats 35mg/kg	–	4	0%
4 rats 50mg/Kg	–	4	0%
4 rats 70mg/Kg	–	4	0%

The negative results obtained in the above two experiments demonstrated that the rats being used were less sensitive than those used by the authors mentioned.

By increasing the dose levels positive responses were obtained in the region of 200 – 300 mg aloe/200g rat. Table 18 tabulates the results obtained.

Table 18.

Dose of aloes	Positive	Negative	% Response
4 rats 300mg/200g rat	4	–	100%
4 rats 150mg/200g rat	1	3	25%
4 rats 75mg/200g rat	–	4	0%

It is seen from this that 100% response is obtained at the upper dose level, while 100% negative response is obtained at the lower dose level indicating that the rats were giving a uniform response.

4.3.1. DETERMINATION OF THE DOSAGE LEVEL OF ALOES.

Because of the varying effect of the different samples of aloes on the rats in the bioassay, it was found that it was necessary to determine in which range the E.D.50 lay before a quantitative assay could be performed (see section 4.3.2). To do this four rats were taken the day before the assay was due to be performed and each was dosed at a different dosage level, these being normally 300, 250, 230 and 200 mg/200 g rat. From the response obtained the dosage levels for the quantitative assay could be determined. In this assay the different dose levels differed by 15 mg.

4.3.2. DETERMINATION OF THE E.D.50 OF ALOIN.

The E.D. 50 of aloin is that dose of aloin which will produce a positive response in 50% of the rats used. As mentioned earlier, the dose levels required for aloin and aloe were found to be considerably higher with the rats being used than those used by LISTER and PRIDE (60), LATVEN, SLOANE and MUNCH (58) and by Reckitt Laboratories (74).

From a series of experiments using single rats at various dosage levels it was found that the E.D. 50 was in the region of 170mg/200g rat. An assay was then performed using groups of four rats at four different dosage levels, viz 200mg, 185mg, 170mg and 150mg, to obtain a plot of log dose against response. The following day to the assay was again repeated using another sixteen rats at the same dosage levels as before.

From Table 19 it is seen that the responses obtained were very consistent and that the slope of the plot of the log dose against percentage response was the same in both assays (graph 1)

Table 19.

Aloin dose levels	Positive	Negative	% Response.
200mg/200g rat	4	–	100%
185mg/200g rat	3	1	75%
170mg/200g rat	2	2	50%
150mg/200g rat	2	2	50%

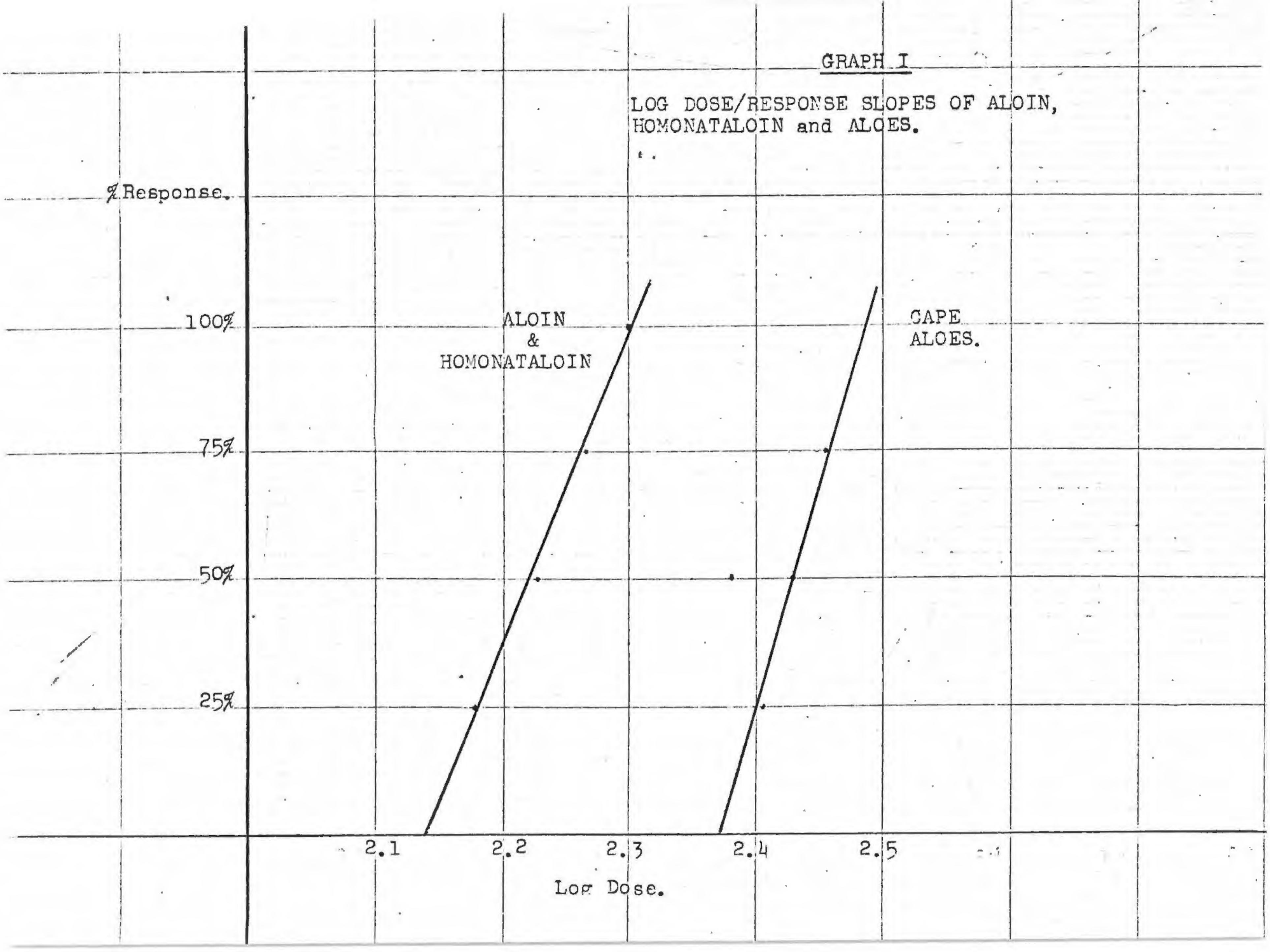
Table 19.

Aloin dose levels.	Positive	Negative	% Response
200mg/200g rat	3¾	¼	94%
185mg/200g rat	3	1	75%
170mg/200g rat	2	2	50%
150mg/200g rat	1	3	25%

From the plot of log dose against % response it is seen that the E. D. 50 of aloin for the rats being used is 177 mg/200g rat.

4.4. DETERMINATION OF WHETHER THE SLOPE OBTAINED WITH THE LOG/DOSE RESPONSE PLOT IS THE SAME FOR ALOES AND FOR ALOIN.

In order for aloin to be a valid standard, for aloes as defined for biological assays, the slope of the log dose/response plot must be parallel to that of aloes. To ascertain whether this was the case, a sample of Cape aloes was used in a bioassay and the response obtained is tabulated in the next Table.



GRAPH I

LOG DOSE/RESPONSE SLOPES OF ALOIN, HOMONATALOIN and ALOES.

Table 20.

Aloes dose levels.	Positive	Negative	%Response.
285mg/200g rat	3	1	75%
270mg/200g rat	2	2	50%
255mg/200g rat	1	3	25%
240mg/200g rat	2	2	50%
Aloin dose levels.	**Positive**	**Negative**	**%Response.**
200mg/200g rat	4	–	100%
185mg/200g rat	3	1	75%
170mg/200g rat	2	2	50%
155mg/200g rat	1	3	25%

On comparing the plot of log dose/%response of this sample with that of Aloin B.P.C. it is seen that the two slopes are parallel (graph 1). In subsequent bioassays it was found that the slopes for aloin and aloes in all the assays performed were, for all practical purposes, parallel (graph 2).

4.5 DETERMINATION OF WHETHER THE PURGATIVE ACTIVITY OF ALOES IN RATS IS DEPENDENT UPON THE ALOIN CONTENT.

LISTER and PRIDE (60) using the bioassay method of LATVEN et alia (58), and working with crystalline aloin, Aloin B.P. and amorphous aloin demonstrated that their activity decreased in order of their descending aloin content. However they did not work on aloes. If the other constituents have no purgative activity then an equivalent dose of aloe and aloin should give the same response. To determine whether this was true for aloes the following experiments were performed. Three groups of rats, consisting of four rats to a group, were dosed with aloes (containing 11% aloin) at a dosage level of 300mg, 150mg and 75mg/200g rat respectively. At the same time three groups of rats, four rats to a group, were dosed with equivalent doses of aloin, viz 30 mg, 15mg, and 7.5mg/200g rat. The responses obtained are tabulated in Table 21.

Table 21.

Dose of Aloes 11% Aloin. Sample A	Positive	Negative	% Response
300mg/200g rat	4	–	100%
150mg/200g rat	1	3	25%
75mg/200g rat	–	4	0%
Dose of Aloin	**Positive**	**Negative**	**% Response**
30mg/200g rat	–	4	0%
15mg/200g rat	–	4	0%
7.5mg/200g rat	–	4	0%

From this it is seen that aloin alone cannot be responsible for the activity of aloe in the rat.

In the second series of assays the rats were dosed with amorphous aloin (sample B containing 30% crystalline aloin) and a sample of aloes (sample C containing 20% of aloin), both of which possessed approximately double the quantity of aloin which sample A contained (i.e. 11%) to see if the product of percentage aloin times dose was constant. If the aloin is the only constituent responsible for purgative action, then the dose of 100mg of samples B and 150 mg C should give the same response as 300mg of sample A. The response obtained is tabulated in Table 22.

Table 22.

Dose of aloes sample C	Positive	Negative	% Response
200mg/200g rat	–	4	0%
150mg/200g rat	–	4	0%
100mg/200g rat	–	4	0%
Dose of aloin sample B.	**Positive**	**Negative**	**% Response**
200mg/200g rat	–	4	0%
150mg/200g rat	–	4	0%
100mg/200g rat	–	4	0%

From this it is seen that the product of percentage aloin times dose is not constant i.e. samples of aloes containing equivalent quantities of aloin do not give equivalent responses in the rat (i.e. 300 mg of sample A should have given the same response as 150 mg of samples B and C.).

A series of bioassays were performed on a number of aloe samples of known aloin content to determine what relationship existed between purgative activity and aloin content. The results obtained are tabulated in Table 23.

Table 23.

Dose of aloin	Positive	Negative	% Response
200mg/200g rat	4	-	100%
185mg/200g rat	3	1	75%
170mg/200g rat	2	2	50%
150mg/200g rat	1	3	25%
Dose of aloes Mossel Bay 20% aloin.	Positive	Negative	% Response
285mg/200g rat	3	1	75%
270mg/200g rat	2	2	50%
255mg/200g rat	1	3	25%
240mg/200g rat	2	2	50%
Dose of aloes Alicedale 19% aloin.	Positive	Negative	% Response
200mg/200g rat	$3\frac{3}{4}$	$\frac{1}{4}$	94%
185mg/200g rat	$2\frac{3}{4}$	$1\frac{1}{4}$	69%
170mg/200g rat	2	2	50%
150mg/200g rat	1	3	25%
Dose of aloes Coega 30%us aloin	Positive	Negative	% Response
285mg/200g rat	2	2	50%
270mg/200g rat	$2\frac{3}{4}$	$1\frac{1}{4}$	69%
255mg/200g rat	2	2	50%
240mg/200g rat	1	3	25%

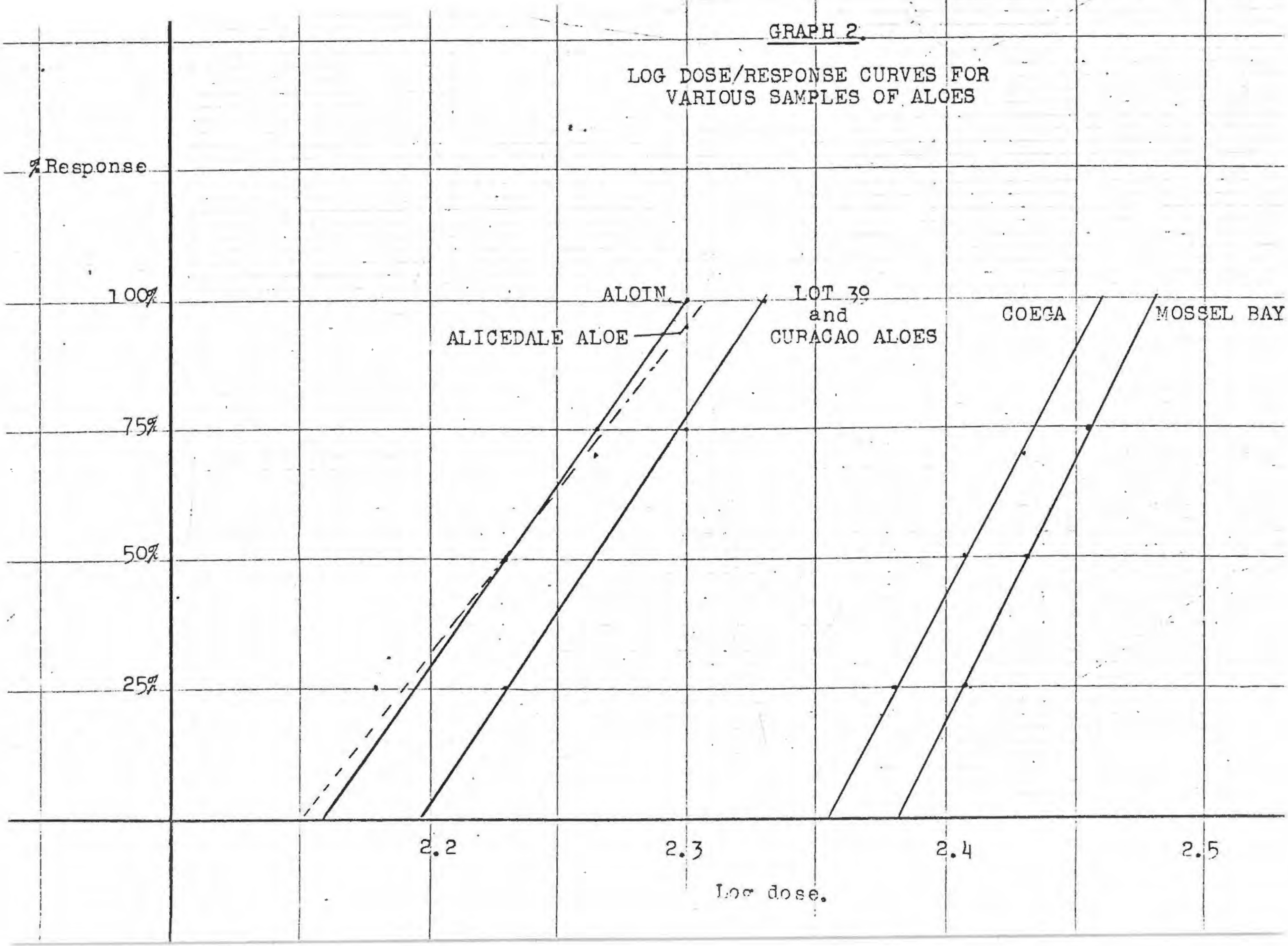
Dose of aloes Lot 39 12% aloin	Positive	Negative	%Response
215mg/200g rat	4	-	100%
200mg/200g rat	3	1	75%
185mg/200g rat	3	1	75%
170mg/200g rat	1	3	25%

Dose of aloes Curaçao 30% aloin.	Positive	Negative	%Response
215mg/200g rat	4	-	100%
200mg/200g rat	3	1	75%
185mg/200g rat	3	1	75%
170mg/200g rat	1	3	25%

Table 24 shows the activity of the different samples in terms of the standard aloin, their aloin content and their water-soluble extractive.

Table 24.

Sample	Aloin	Alice-dale	Lot 39	Coega	Curaçao	Mossel Bay
activity.	100%	100%	85%	66%	85%	63%
aloin content.	100%	19.0%	11.5%	29.89%	29.6%	20.5%
water-soluble extractive	100%	78.4%	64.6%	83.6%	82.0%	83%


GRAPH 2.
LOG DOSE/RESPONSE CURVES FOR VARIOUS SAMPLES OF ALOES
% Response
100%
75%
50%
25%
ALOIN
ALICEDALE ALOE
LOT 39 and CURACAO ALOES
COEGA
MOSSEL BAY
2.2
2.3
2.4
2.5
Log dose.

From Table 23 and graph 2 it is seen that there is no apparent connection between purgative activity in rats and aloin content of aloes. Coega aloes (30% aloin) has the highest aloin content of the samples used, yet it is only slightly more active than Mossel Bay aloes. Alicedale aloes (19%) aloin) although having approximately the same aloin content as the Mossel Bay sample has far greater activity, in fact this activity being the same as for pure aloin. Lot 39 aloes (11-12% aloin) although having by far the lowest aloin content is, however, more active than Mossel Bay and Coega aloes which have two and three times the aloin content respectively.

4.6 INVESTIGATION OF THE HIGH ACTIVITY OF THE ALICEDALE SAMPLE OF ALOES.

From the bioassays performed above it was obvious that the activity of the sample of aloe lump from the Alicedale region was far superior to the activity of any other sample assayed, even though its aloin content was the same as some other samples assayed. Thus it was decided to subject this sample to further chromatographic investigation. Both the Alicedale and Mossel

Bay samples were chromatographed in two different solvent systems (chloroform:ethanol 3:1, and ethyl acetate:water:glacial acetic acid 5:4:1.) On examination under ultraviolet light the two samples appeared to be identical. However when they were sprayed with nitrous acid a purple spot appeared below the aloin spot of the Alicedale aloe. This spot rapidly faded and disappeared within five minutes. When sprayed with sodium metaperiodate reagent the same purple spot appeared and also faded and disappeared as it had done with nitrous acid. When unsprayed plates were allowed to stand for a few days the spot became visible as a black spot in daylight.

This spot would appear to be the same "periodate positive" spot which was mentioned by BÖHME & KREUTZIG (5). It would appear that the greater biological activity of the Alicedale sample is due to this compound since all the other factors were identical for the two aloe samples.

4.7 COMPARISON OF RESULTS OBTAINED WITH THOSE OF RECKITT & SONS LTD (PHARMACOLOGY LABORATORY.)

It was decided to see if there was any agreement between the bioassay being used for this work and that being used by Reckitt Laboratories. To this effect two samples of aloes were sent to them to be assayed (per kind assistance of Dr Daglish). The samples sent were the Alicedale sample and a Mossel Bay sample.

The samples were tested on Reckitt's "dirty rats" weighing 135-190 g. A purified extract of (barb)aloin B.P. was used as the standard purgative. It was found that the material of unknown potency produced greasy, immiscible drops on contact with water, so they were first dissolved in 95% ethanol and subsequently precipitated with cold distilled water to provide a fine suspension in 5% ethanol. The standard barbaloin was suspended in a similar manner, each rat receiving 10 ml /Kg of the suspension.

Groups of 10 rats, starved overnight were dosed orally with barbaloin (75,150 or 300mg/Kg) or single doses of the samples of Alicedale or Mossel Bay aloes. The number of wet faeces was recorded for 28 hours after dosing. Water was available throughout the test and food was available immediately after administration of the purgatives.

RESULTS:

Table 25.

Sample	% Effect.
Barbaloin (150mg/Kg)	100%
Alicedale aloe	88%
Mossel Bay	51%

The purgative potency of the Alicedale aloe is not significantly different from the potency of the standard barbaloin, but the Mossel Bay sample is significantly less potent as a purgative agent.

Unfortunately there was insufficient material of each of the test samples to allow a parallel line assay, which would permit accurate determination of relative potencies.

From this and Table 23 it is seen that there is close agreement between the two bioassay methods, despite the fact that the rats being used here require five times the dose of Reckitt's "dirty rats".

4.8 BIOLOGICAL ACTIVITY OF ALOESIN.

i) Determination of a dose level.

Six rats were taken and dosed with aloesin at each of the following dose levels.

Table 26.

Rat 1	2	3	4	5	6
300 mg	300 mg	255 mg	255 mg	240 mg	200 mg
positive	negative	positive	negative	negative	negative

From this it is seen that the E.D.50 must lie in the range of 255-300mg/200g rat, which indicated that aloesin has a considerably lower activity in rats than has aloin.

ii) Determination of Time of onset of action of Aloesin.

Since aloesin is not an anthraquinone glycoside but a benzpyrone derivative the length of time which it takes to exert its action is not necessarily the same as aloin. To determine this, two rats were dosed with 300 mg of aloesin; every two hours a faecal pellet was expelled and examined by the "wire adhesion" test. It was found that after eight hours a positive result was obtained whereas LATVEN et alia (58) found that aloe exerted its action after ten hours. However, the presence of aloesin in the faeces was detected within four hours of dosing the rat . The method of detection was as follows:

Detection of Aloesin in faecal pellets.

It was decided to see if aloesin was hydrolised or whether it was voided in the form of unchanged aloesin. The first step was to determine if it could be easily detected in the faeces. A rat was dosed and after ten hours a faecal pellet was expelled and shaken with ammonia. The resulting solution was filtered and placed under an ultraviolet lamp. A white fluorescence was observed. At the same time a standard was run using the pellet of a rat which had not been dosed with aloesin, to obviate the possibility of benzpyrones in the food. This also gave similar fluorescence. However when spotted onto filter paper and allowed to dry, the aloesin plus ammonia solution fluoresced a bright white blue, and this when sprayed with Fast Blue B solution produced an orange colour, while the standard did neither.

When a thin layer chromatographic plate was run in chloroform:ethanol (3:1), using an ammoniacal extract of a pellet the aloesin was observed at the same Rf as a control of aloesin run at the same time. The presence of aloesin was further established by taking an ammoniacal extract of a faecal pellet, filtering through Whatman 542 filter paper and diluting this with water. A spectroscopic examination was then made of this solution. This was then compared with that of aloesin plus ammonia and the peaks obtained were identical in both cases. This indicates that a large proportion of the aloesin passes through the gut of the rat unchanged.

4.9 BIOLOGICAL ACTIVITY OF HOMONATALOIN.

Homonataloin is a C-glycoside, the structure of which was elucidated by HAY and HAYNES (48). This has two hydroxy groups and one methoxy group so it should, according to the number of hydroxy groups present, (by classification of FAIRBAIRN), be active. As yet it has never been used in a bioassay, although at one stage Natal aloes were marketed and these contained homonataloin as their main constituent. VAN OUDTSHOORN (87) however mentioned that the reason these fell into disuse was due to the fact that they had a lesser purgative activity than Cape aloes (which is aloin containing).

Determination of dosage levels for Homonataloin.

Since homonataloin is a 1,8 dihydroxy compound determination of its dosage level was performed to see whether the presence of hydroxyl groups in these positions is required for a purgative activity equal to that of aloin.

Four rats were taken and dosed as follows two with 75 mg/200g rat of homonataloin, one with 100mg/200g rat and one with 150mg/200 g rat of homonataloin.

Table 27.

Rat 1	Rat 2	Rat 3	Rat 4
75 mg Negative	75 mg Negative	100 mg Negative	150 mg Positive

From this it was decided to dose the homonataloin on the same dosage schedule as for aloin. Table 28 shows a comparison of the results obtained for homonataloin and aloin.

Table 28.

Dose of homonataloin	Positive	Negative	% Response
200mg/200 g rat	4	-	100%
185mg/200 g rat	3	1	75%
170mg/200 g rat	2	2	50%
150mg/200 g rat	1	3	25%
Dose of aloin	**Positive**	**Negative**	**% Response**
200mg/200 g rat	4	-	100%
185mg/200 g rat	3	1	75%
170mg/200 g rat	2	2	50%
150mg/200 g rat	1	3	25%

From the plot of log dose/ % response graph (graph 1) it is seen that the slopes of the two plots are parallel and the potency of the homonataloin is the same as that for aloin.

From this it would appear that homonataloin could be used as a purgative for humans in the same dosage as for aloin. In addition it is far simpler to extract than aloin. However, <u>A. speciosa</u>, the main source of homonataloin is not nearly as abundant as <u>A. ferox</u>, and homonataloin occurs mainly in relatively small <u>Aloe</u> species which contain little sap.

4.10 BIOLOGICAL ACTIVITY OF RESIN.

The work of AUTERHOFF & BALL (2) with mice indicated with these animals that the resin was more active than the aloin. The evidence with regard to the pharmacologic action of the resins is conflicting. KIEFER (88) isolated three active resins from Cape aloes and thinks that the purgative action is due to the three resins, and that the aloin and aloe-emodin play a minor part in producing this effect.

In contrast, TSCHIRCH & HOFFBAUER (65) came to the conclusion that only the resin in aloe is totally inactive or without purging properties, while aloin, aloe-emodin and anthraglucosides have a purging effect. According to RAMSTAD (73) the resins of aloes require the presence of bile acids in order to act. Aloe enemas are stated to have no stronger effect than water, but the addition of a small amount of bile produces an instantaneous, drastic action. In this connection it is noteworthy that the South African Bantu merely use a teaspoonful of fresh aloe juice in warm water as an enema, apparently with complete effect.

Resin containing 5% of aloin was dosed to the rats. The resin was suspended and dosed to the rats as for aloes. The results obtained are tabulated in Table 29

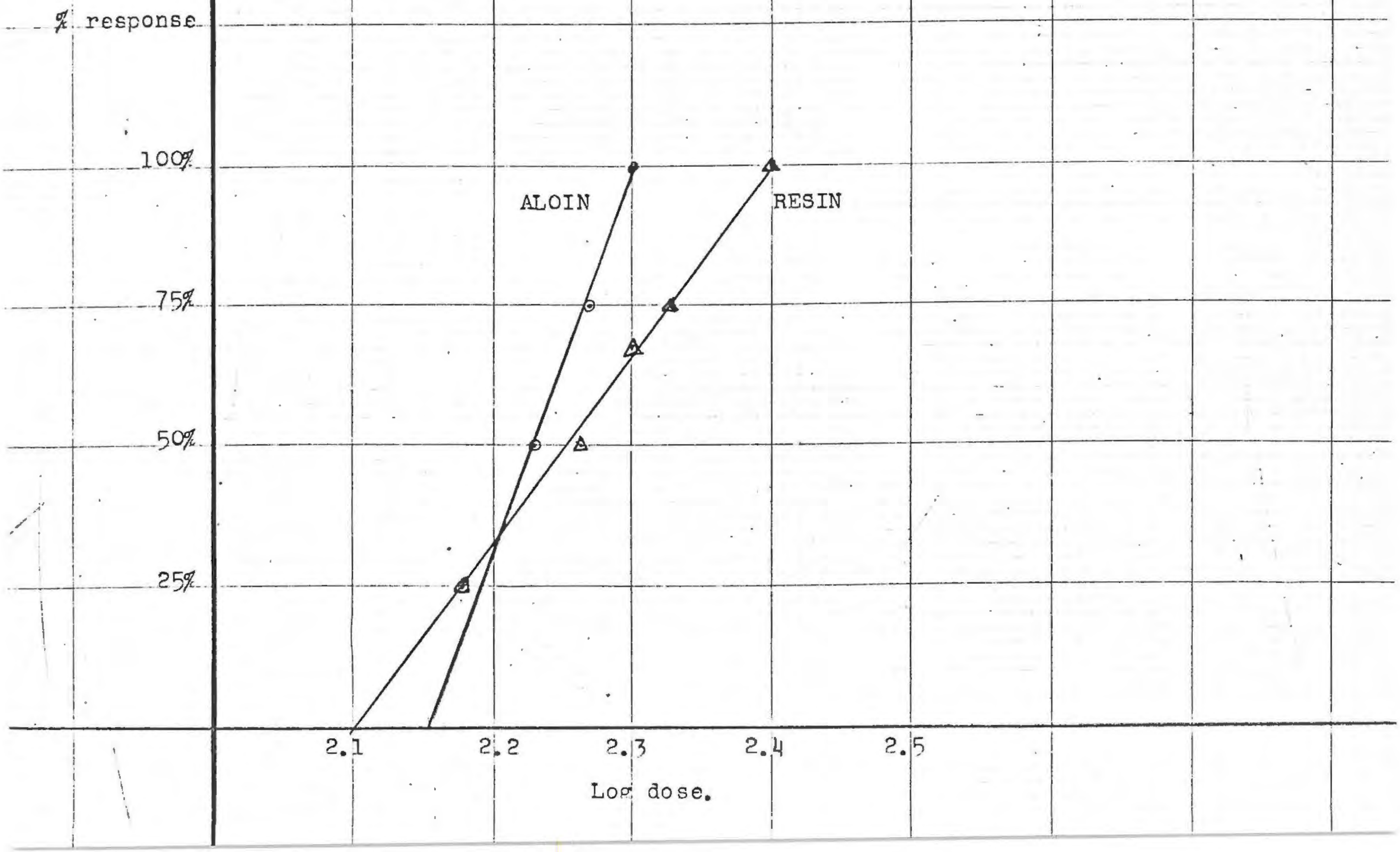
Table 29.

Dose of Resin	Positive	Negative	% Response
250mg/200g rat	4	-	100%
215mg/200g rat	3	-	75%
200mg/200g rat	2¾	1¼	68%
185mg/200g rat	2	2	50%
150mg/200g rat	1	3	25%

From the plot of log dose/ %response it is seen that the resin is only slightly less active in the rat than the aloin and that the slopes of resin and aloin diverge. This is however possibly due to the wider separation

GRAPH 3.

LOG DOSE/RESPONSE SLOPES OF ALOIN and RESIN.



between the dose levels than with those used for aloin. (graph 4)

Although the resin has a definite purgative effect in rats it will be shown that with humans, resin appears to have minimal action.

CHAPTER 5.

5.1. TO DETERMINE WHETHER ALOIN IS THE ACTIVE CONSTITUENT IN ALOES WITH RESPECT TO HUMANS.

AUTERHOFF and BALL (2) performed an interesting series of experiments with human volunteers in which they separated aloes into the following fractions.

Table 30.

	Fraction	% aloin	Biological activity
I.	Cape aloes	30.9%	24 out of 45 people regard II as more active than I & III
II.	Water extractive	36.8%	
III.	Acetone extractive.	34.4%	
IV.	Residue of water extractive.	23.6%	
V.	Methyl Acetate extractive.	37.6%	10 out of 11 people consider VI to be worth less.
VI.	Residue of Methyl Acetate extractive.	10%	5 out of 6 people consider VII to be more active than V.
VII.	Ammonia soluble drug.	32%	
VIII.	Crystalline Aloin.	99.7%	13 people consider VIII IX and I in equivalent doses (93mg, 220mg and 300mg) to have the same activity.
IX.	Aloin amorphous (Aloetin Merck)	42.6%	

Table 30 shows the results which AUTERHOFF & BALL (2) obtained and from which they concluded that aloin is the active constituent of aloes and that the activity of aloes is dependent upon its aloin content. A closer look at these figures shows the remarkable tie-up between aloin content and dosage. If one considers fractions I, VIII and IX, then the following interesting figures relating dosage to percentage aloin arise:

Ratios	aloin (inverse)	dosage
$\frac{\text{IX}}{\text{VIII}}$	$\frac{99.7}{42.6} = 2.34$	$\frac{220}{93} = 2.37$
$\frac{\text{VIII}}{\text{I}}$	$\frac{99.7}{42.6} = 3.22$	$\frac{300}{93} = 3.22$
$\frac{\text{I}}{\text{IX}}$	$\frac{42.6}{30.9} = 1.38$	$\frac{300}{220} = 1.36$

It was decided to investigate this due to the fact that the biological assays using rats showed no direct relationship between aloin content of the aloes and the biological activity.

Student volunteers were used. They were dosed according to bodyweight and did not know which drug (aloin or aloes), nor which dose level each was receiving. In addition they were informed that there were placebos among the capsules which they received. The major difficulty was to select a criterion of activity. BROWNE, EDMUNDS & REID (15) performing clinical trials on senna preparations used a criterion of the total number of bowel movements occurring within a set period of time. McNICOL (69) working with standardised senna preparation used the number of bowel movements in the 23 hours after medication as well as the consistency of the stools. In this trial a great deal of reliance was placed on individual assessment of response and the following system was used.

i) Nil response	–	
ii) Mild response	M.	motion is easily suppressed.
iii) Moderate	Mod.	motion is compulsive but transient.
iv) Severe	S.	motion is compulsive and repeated.

Each student was provided with a card on which was noted his/her name, the response, side-effects, time of action and comments.

5.2. DETERMINATION OF WHETHER THERE IS A SEX VARIATION IN HUMANS.

The first step was to determine whether there was any sex variation in humans as there were in rats and mice. McNICOL (69) working with senna had not found any with this drug. Male and female students of the same weight (120 lb. corrected) were dosed with the equivalent aloes and aloin at different dose levels, the results obtained being tabulated below in Table 31.

Table 31.

Aloes 200 mg.		Aloes 400 mg.	
males	females	males	females
MILD	MILD	MOD	MILD
–	MILD	MILD	MOD
–	–	MOD	MOD
	–		MILD
Aloin 20 mg		**Aloin 40 mg.**	
males	females	males	females
–	MILD	MILD	MILD
–	MILD	MILD	MILD
MILD	MILD	–	MOD
MILD	MOD	MOD	MOD
	–		MILD

From this it is seen that there is no great difference in response between the two sexes and thereafter no differentiation was made between the two in tests.

5.1.2. ATTEMPT TO PLOT A LOG DOSE/RESPONSE SLOPE FOR ALOIN.

The B.P.C. gives a dosage range for aloin of 10 to 60 mg, so this was used as a guide for the dose levels used. The students were divided into four broad groups according to weight. This was to facilitate dosage. Each group (10 to a group) was then administered Aloin B.P.C. in the form of a capsule (Aloin B.P.C. containing 90% crystalline aloin)

Group 1 received 20mg, equivalent to 18mg crystalline aloin
2 received 40mg, equivalent to 36mg crystalline aloin
3 received 45mg, equivalent to 41mg crystalline aloin
4 received 65mg, equivalent to 59mg crystalline aloin

The dose in mg per lb body weight was then determined for each result and the results regrouped on this basis. The percentage response was then determined for each group.

A mild response was arbitrarily taken as being half a response.

A moderate response was taken as being a full response.

The results obtained are tabulated below, in Table 32.

Table 32.

Dose mg/lb body-weight.	Log dose	% response
0.15	$\bar{1}.1761$	25
0.25	$\bar{1}.3979$	50
0.30	$\bar{1}.4771$	60
0.36	$\bar{1}.5563$	78.5

The percentage response was then plotted against the log dose in mg per Lb body weight. The plot obtained for this was found to be linear (graph 4).

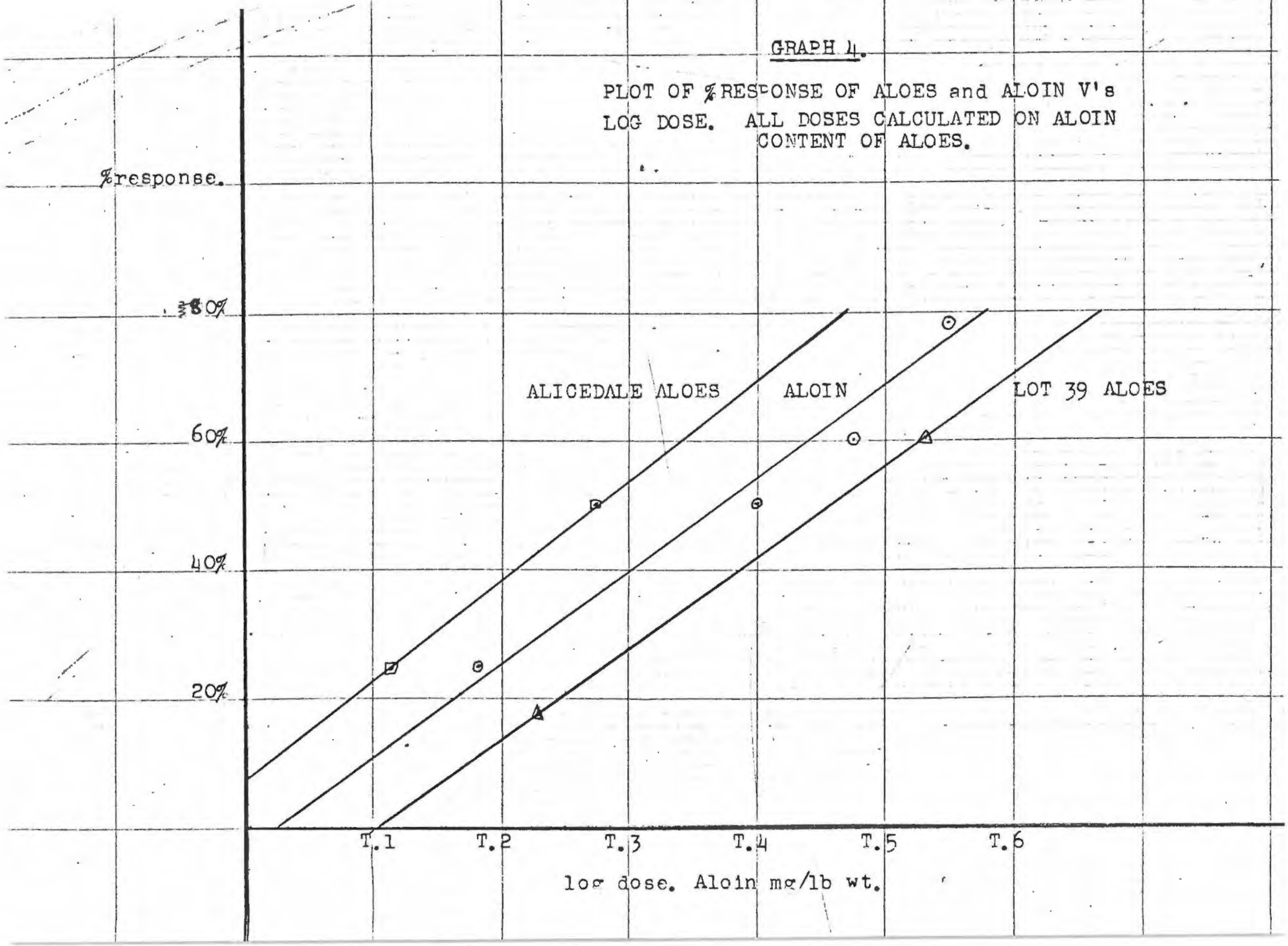
5.1.3. Comparison of the slope obtained for aloes with that of aloin.

For an assay to be valid, evidence must be provided that the action of the preparation under test and the standard preparation do not differ. This is normally indicated by the slopes of the log dose/response curve for the unknown and the standard being parallel. To do this it was decided to use a two dose level assay of the aloes with ten volunteers at each dose level. Two different samples of aloes were used and these gave the following results.

Table 33.

Sample	Dose mg/lb based on aloin.	Log dose	% response
Alicedale	0.1296	$\bar{1}.1121$	25%
Alicedale	0.187	$\bar{1}.2718$	50%
Cape aloe Lot 39	0.1715	$\bar{1}.2304$	18%
Cape aloes Lot 39	0.34	$\bar{1}.5315$	60%

On plotting the log dose/%response curve for the two samples of aloe and comparing this with that obtained for aloin (graph 4) it is seen that the aloin and Lot 39 are parallel and that the slope of the Alicedale aloes diverged very slightly from the other two, the divergence being so small as to be negligible for all



GRAPH 4.

PLOT OF % RESPONSE OF ALOES and ALOIN V's LOG DOSE. ALL DOSES CALCULATED ON ALOIN CONTENT OF ALOES.

practical purposes. Hence the potency of the aloes may be calculated in terms of the standard aloin.

5.1.4. COMPARISON OF THE POTENCY OF ALOIN WITH THAT OF ALOES.

The potency of two different commercial samples of aloes were compared with that of aloin by means of the log dose/response curves obtained above. Due to the interesting results obtained with the rats in which the Alicedale aloe showed a greater potency than would be expected from its aloin content, it was decided to use this as one of the test substances to see if it would show similarly increased action in humans. The other samples of aloe chosen was a commercial sample containing only 11% aloin. Both samples were given in doses according to their aloin content.

From the graph obtained (graph 4) plotted on the aloin content it is seen that the Alicedale sample, which showed the "periodate positive" spot of BÖHME & KREUTZIG (5) shows the greatest activity, followed by the aloin, with the sample containing 11% aloin showing the lowest activity. However the activities are fairly close as is shown by the E.D.50 of each when expressed in terms of mg of aloin a 150 lb person would receive.

Alicedale	Aloin	Lot 39
30mg	37.5mg	45 mg

From this it would appear that the activity is not always entirely dependent upon the aloin content of the aloes as is shown by the greater activity of the Alicedale sample.

It was thought that the lower activity of sample 39 might possibly be due to its high resin content. This resin would be precipitated at the acid pH of the stomach and might possibly trap some of the aloin which would not be free to exert its action. The resins are, however soluble in alkaline solution and since the pH of the small intestine is normally 8, the resin might possibly redissolve, thus it was decided to see whether aloe was soluble in simulated alkali pancreatic juice.

5.1.5. INVESTIGATION OF THE SOLUBILITY OF ALOES IN SIMULATED PANCREATIC JUICE.

Alkaline pancreatin solution as used by the B.P.

1968 (9) for the testing of enteric coated capsules was used. This consisted of

Pancreatin		3 g
$NaHCO_3$		15g
Na taurochlorate		5 g
Aqua	ad	1,000 ml.

A fixed weight of aloes (lot 39) was placed in 100 ml of this solution, shaken and then placed in an incubator at 35°C for 14 hours, the solution being shaken occasionally. After 14 hours all the aloes had dissolved to yield a red coloured solution. From this it would appear that the resin passes into solution in the small intestine and must hence release any trapped aloin. However the possibility still remains that when the resin gets precipitated in the stomach it traps some of the aloin and remains here for a longer period of time than the water-soluble fractions which will pass through more rapidly and exert their action.

The solution which had resulted from incubating aloes with alkaline pancreatin for 14 hours was evaporated to dryness with hot air, dissolved in methanol and chromatographed in chloroform:ethanol (3:1), and was then examined under an ultraviolet light whereupon no aloin could be detected, although aloesin and aloe-emodin were present. To determine whether the aloin had been destroyed by the pancreatin or merely by the alkaline solution the following experiment was performed:

15 mg of aloin was dissolved in 50 ml of alkaline pancreatin solution B.P. 1968 and 15 mg of aloin was dissolved in 50 ml of a solution containing 1.5% $NaHCO_3$. Both solutions were then incubated at 35°C for 14 hours; on removal both solutions had turned red. Both solutions were then centrifuged for the same length of time and 5 ml of the supernatant diluted to 100 ml with water. These two solutions were then read against water in a double beam spectrophotometer at 360 nm. The readings which were obtained were identical in both cases indicating that any aloin which had been broken down in the solutions had been as a result of the pH of the aqueous solutions. (14% was broken down in both cases).

5.1.6. THE FATE OF ALOIN IN THE RAT.

It was decided to see what percentage of aloin is voided unchanged in the rat. To do this a rat was dosed with 200 mg of aloin, and all the faeces passed for the next 24 hours were collected. These were then repeatedly extracted with acetone until no colouration of fresh acetone occurred. The acetone extract was then filtered and the volume reduced by evaporation under reduced pressure. This was then made up to a convenient volume and chromatographed. The aloin spot was then scraped off and assayed using the thin layer chromatographic method. When this was related to the amount of aloin with which the rat had been dosed it was found that 15% of the original aloin remained unchanged.

5.2. IN VITRO ATTEMPT TO LYSE ALOIN BY THE USE OF E. coli.

As already mentioned earlier it is believed that the sugar molecule is split off the anthracene nucleus by the action of E. coli thus resulting in the production of the anthrone. No reference could be found as to this being done 'in vitro' with a C-glycoside, all previously being done with O-glycosides.

The major difficulty in detecting the anthrone of aloe-emodin spectrophotometrically was due to the similarity of the peaks of aloe-emodin and its anthrone. So it was decided that the simplest method would be to follow the decrease in aloin content.

Tubes of Difco broth were prepared (0.2g per 250 ml), each tube being filled with 10 ml of broth and autoclaved for 30 minutes at 15lb/sq inch. Six of the tubes were inoculated with a 14 hour culture of E. coli and allowed to grow for 8 hours at 35°C. The other six tubes were used as a control. 5 mg of aloes was aseptically dissolved in 10 ml of sterile water and 0.01 ml of this was added to each tube, flaming before and after addition. The tubes were then once more incubated and removed at set time intervals. Then a tube containing bacteria and a control tube were centrifuged to remove the bacteria from the suspension. Since the medium also precipitates, the control tube was required. The supernatant liquid of both the experimental tube and the control tube were then read in a Beckman spectrophotometer at 360 nm, using water as a blank. It was seen that there was no appreciable difference between the reading for the control and the experimental tube. Hence it was apparent that in vitro E. coli does not split a C-glycoside within 40 hours. This would possibly explain the reason why aloin and other C-glycosides are so inactive in the rat and mouse since all previous work in these animals was performed using O-glycosides which are easily split by E. coli.

RESUMÉ

In Chapter 3 biological methods of assaying purgatives are reviewed with reference to the animals used, their housing, and to various factors which can affect biological assays.

In Chapter 4 the experimental methods which were employed are discussed, these including - handling, training, dosing of rats, criterion of purgative activity and the reference standard employed. The methods of isolation of the various constituents which were used in the biological assays are then detailed. Dosage levels for aloin and different aloe samples were determined and the E.D.50 for the different samples established. The fact was established that the slopes of the aloin and aloes were parallel.

An attempt was made to correlate the biological activity of the different samples with their aloin content. The activity of aloesin, homonataloin and resin were investigated in the rat.

The results obtained for two samples of commercial aloes were compared with those obtained by an overseas pharmacology laboratory.

In Chapter 5 the activity of aloes and aloin in humans was investigated. It was demonstrated that there is no sex variation in the response to both aloes and aloin. The slopes of the log dose/response curve of aloin and two different samples of aloes were shown to be parallel, and it was shown that the activity of a sample was closely related to the aloin content.

SUMMARY and DISCUSSION.

The object of the work performed was to determine whether there is any relationship between the chemical assay, the biological assay and the activity of aloes in humans. If a relationship could be found between any of these then the drug, aloes, could be standardised as a purgative.

To this effect it was first necessary to determine the best method for the chemical assay of aloes, since there are numerous different chemical methods of assay which give varying results. Some of the current methods which were investigated include:

1) The method of FAIRBAIRN (53) which involves ferric chloride oxidation of aloin.

2) The method of MÖHRLE as modified by HÖRHAMMER et alia (50) which involves a periodate oxidation of aloin.

3) The assay of MÖHRLE as modified by BÖHME & KREUTZIG (7).

4) The thin layer chromatographic method.

5) The water-soluble extractive of aloes. (B.P. 1968)

The object was to find an assay which is both rapid and simple to perform and is nevertheless accurate.

The method of FAIRBAIRN was investigated by five British analytical laboratories (53) and excellent concordance was obtained by them. In consequence, and considering their analytical error of only 1% on aloe samples containing circa 20% aloin, it was decided to equate this method to 100% accuracy, and to determine the effectiveness of the other methods relative to this. In addition, the FAIRBAIRN method eliminates false positives by a wavelength ratio as reported in the text.

The thin layer chromatographic method of assay was found to give close agreement with the FAIRBAIRN method. once interference from silica gel had been corrected for. However, this method does not give a total aloin value for samples containing aloinosides. Thus a technician not familiar with these compounds, could by this method omit them completely, and thus omit anthracene derivatives of known purgative activity. Samples of Cape aloes and Zanzibar aloes occasionally contain aloinsides and consequently this factor must be borne in mind. It also has the advantage that a hybrid of aloin and homonataloin, with resultant enhanced purgative activity, could be determined by this method.

It was found that the method of MÖHRLE as modified by HÖRHAMMER et alia gives far too high a reading for the aloin content of aloes, due to the fact that the resins and ammonia form a yellow colour which reads at the wavelength at which the readings are taken, the error being in the region of 20% too high. This method can, however, be used for the assay of samples of aloin of a high purity.

The method of MÖHRLE as modified by BÖHME & KREUTZIG was found to give an accurate result, provided that the temperature is kept constant at 20°C. It cannot, as is shown here, be used for the assay of pure aloin or aloin of a high percentage of purity. Moreover this assay, as it stands, has the disadvantage that it cannot differentiate between aloin and homonataloin, hence it is recommended that a ratio similar to that as employed by the FAIRBAIRN method be introduced. The most effective manner in which this could be done would be to calculate the ratio value E510nm/E 460nm, since the peak for nataloe-emodin and alkali occur at 460 nm. If the ratio value is greater than 1 then the assay will be valid for aloin.

It is thus seen that comparable results are obtained by three of the assay methods investigated. However the time taken to perform the different assays varies considerably. In the majority of analytical laboratories the time taken to perform an assay is a very important factor. Below is a table of the total time taken to perform each assay and of the actual working time for each of the above assays.

Table 34.

	FAIRBAIRN	MÖHRLE	T.L.C.
Total time	6.5 hours	2.75 hours	1.75 hours.
Working time.	2.25 hours	45 mins.	45 mins.

From this it is seen that the assay of FAIRBAIRN, although being the most accurate, is far more timeconsuming than the other methods, and it is felt that the majority of analytical laboratories would be prepared to sacrifice a small degree of accuracy for the substantial saving in time (tables 5 to 7 refer), particularly as the drugs concerned are given in comparitively large doses for purgative effect. From the work performed it is felt that the method of thin layer chromatographic analysis should be adopted due to its speed and relative accuracy.

For the biological assay of aloes, the method of LATVEN et alia (58) was used. In this work several different samples of Cape aloes and a sample of Curacao aloes with varying aloin content were used. No relationship could be found between the aloin content of the samples used and their biological activity in the rats, which were used in these bioassays. The picture is considerably confused by the fact that the resins, which appear to have no purgative activity in humans, have an activity nearly as great as that of aloin in the rats used.

Furthermore no relationship could be found between the activity of aloe samples in humans and the same aloe samples when administered to the rats. Although the activity of the two samples used was found to be similar when used in the rat, in humans a considerable difference in activity was observed. In accordance with the work of AUTERHOFF & BALL the activity of aloes in humans is closely related to the aloin content of the aloes. In agreement with their work it is found here that people may usually be dosed in accordance with the aloin content of aloes, but that occasionally as with the Alicedale sample, an unknown principle can enhance the purgative activity of aloes.

From this it is seen that the biological assay of aloes using rats gives no idea of the potency of aloes when related to humans, but that the activity in humans can be determined by chemical assay of aloes. In addition, no difference in response to aloes or aloin could be found between the two sexes.

Homonataloin was isolated and its purgative activity in rats was investigated, where it was found to have the same activity as aloin. A search of the literature has revealed no similar work having been performed on homonataloin. In similar manner the benzpyrone, aloesin was isolated and its purgative activity in rats investigated, this being found to be considerably less than that of aloin, as was anticipated from its chemical nature.

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