REVIEW ARTICLE

PHYSIOLOGY OF ALKALOIDS*

By Kurt Mothes

Professor of Botany, Institut für Biochemie der Pflanzen, Deutsche Akademie der Wissenschaften zu Berlin, Halle/Saale

We have little idea of the total number of alkaloids or their distribution in the plant kingdom. Quite apart from the well-known sources of alkaloids found within systematic natural orders, traces of the selfsame alkaloids may be found widely scattered throughout unrelated generat Their presence and amount is largely governed by the ability of the plan. to effect biosynthesis and also its ability to tolerate these products.

We know today that nicotine occurs not only in the genus *Nicotiana* but that it is found, in addition, in Equisetaceae, Lycopodiaceae, Crassulaceae, Papilionaceae, Cannabinaceae, Chenopodiaceae, Asclepiadaceae and Compositae. In the family Solanaceae we find it also in the genus *Duboisia*, *Solanum* and *Atropa*, but mostly only in traces. If nicotine is introduced into *Atropa* or *Lycopersicum*, for instance, by suitable grafting on a *Nicotiana* root stock, the leaves of the scion when exposed to strong light show bleaching of the chloroplasts. Such leaves may become completely white, and live as parasites on the stock, which nourishes the scion with its own leaves. *Atropa sp.* can form nicotine, but cannot withstand high concentrations of this substance[1].

Although most scions of different species upon *Nicotiana* stock are damaged by the nicotine formed in the root and ascending to the shoot, there are remarkable exceptions. Thus the composite *Zinnia* grows well. This we explained by the finding that *Zinnia* itself synthesizes nicotine, and that its nicotine-content is not less than that of low-alkaloid tobaccos[2]. This means that during evolution a resistance must have developed parallel with the development of other new characteristics. This resistance is likely to depend upon a complex of factors, and to effect a marked increase in the content of alkaloid by an artificial mutation, caused say by X-rays, is not easy if the factor of resistance is not already developed.[3]

One of the main causes for the limitation of the occurrence of alkaloids could be due to incompatibility. Extremely high alkaloid concentrations are very rare in living tissue. Careful and extensive selection work has shown that aberrant types with remarkably high alkaloid content do occur occasionally but are localised. Obviously high alkaloidal content is associated with a decreased vitality[3–5].

An instructive example is provided by ergot of rye. We know of strains that contain more than 1 per cent of alkaloid in the sclerotium, but for

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artificial culture on rye-fields only strains which contain 0·3 to 0·5 per cent of alkaloid are used. Probably the high-alkaloid strains have less vitality, which means a much reduced yield. This suggests that the optimum alkaloidal content is genetically fixed and shows only limited variation. This is also true for the alkaloid composition of ergot. It has been possible to select strains that contain a preponderance of alkaloid, which is valuable for industrial production[5,7]. We have found such a strain to keep its exclusive character, no matter whether cultivated on rye or on wild grasses[8]. Although the number of ergot strains in nature is enormous, we ourselves have isolated thousands, it is interesting that some are geographically localised.

I think that the selection of stable races, which are quantitatively and qualitatively valuable, is the necessary presupposition for the permanent use of medicinal plants in therapeutics and for the production of distinct plant substances. If by quality-breeding the step from the wild to the cultivated plant could be speeded, it would take only a short time for the drug market to have sufficient materials of reliable source and at low cost.

Alkaloid Synthesis

Site of Formation

The development of the alkaloid character is not only a genetic problem but also a problem of physiology. Unfortunately the number of well-investigated alkaloidal plants is very small, and limited to plants with alkaloids which are predominantly or exclusively formed in the root, and accumulation of which in the organs of the shoot is the result of a translocation. This is true for the synthesis of nicotine in some tobacco species, and of hyoscyamine-hyoscine in *Datura*, *Atropa* and *Hyoscyamus*.

Several institutes have assisted in this discovery. The most important proofs are those that follow. Excised roots in sterile culture are able to form alkaloids and can excrete large quantities into the surrounding liquid medium. It may be that this cannot be altered, as the prevention of the flow of alkaloids from the root to the shoot would effect a high accumulation of alkaloids in the root, and probably this would mean a self-toxication of the root-tissue. Isolated leaves, however, form no alkaloid or only a little. But when they are rooted, alkaloid appears. If a scion of an alkaloid-free plant is grafted on the root of *Nicotiana*, *Atropa*, *Datura* and others, the alkaloids characteristic of the stock appear in a short time. Investigation of the bleeding-sap of such graftings showed that the alkaloids migrated from the root to the shoot. If, on the other hand, a scion of *Nicotiana*, *Atropa* or other alkaloidal plant is grafted to an alkaloid-free root (e.g., *Cyphomandra*), the scion remains low in or devoid of alkaloid. Summaries of the history of these investigations have been published by Dawson[9], Iljin[10], James [11] and Mothes[12].

There are other alkaloids which are formed predominantly in the shoot (solanidines)[13–15], and alkaloids which cannot be formed in the isolated root (lupine-alkaloids)[16–18]. Probably the number of alkaloids synthesised in aerial parts is much greater than those in the roots. This is indicated by the investigations of Cromwell on *Conium*[19], of Shibata on *Ephedra*[20]

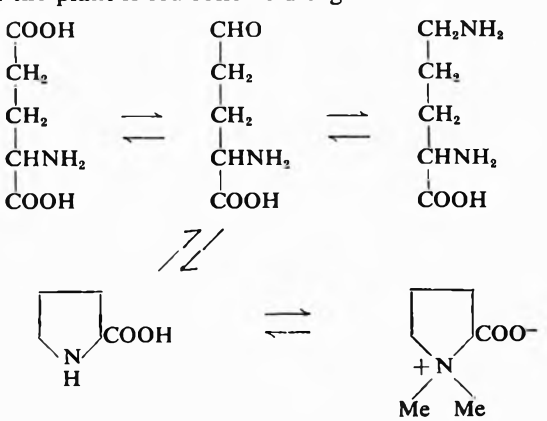
and of Leete on *Catha*[26]. The arbitrary division of the alkaloids according to whether they are formed in root or shoot is not possible, as, for example, when the synthesis of nicotine had been discovered to take place in the tobacco root we had already called the root the "Hauptbildungsstätte" (chief place of formation) with some reservation[21], because we had found that young leaves could also synthesize nicotine[22]. The formation in the shoot of alkaloids normally synthesised in the root has since been confirmed by various investigators[22–25].

In plants where the root is the "Hauptbildungsstätte" of the alkaloids the ability of the shoot to form alkaloid seems to be associated with the influx of certain precursors from the root. We found[24,27], that the hyoscyamine-synthesis in *Atropa* scions on various tomato strains was not always the same. This might indicate that a leaf or a shoot during its development loses its capacity to perform by itself all the reactions necessary for the complete synthesis of an alkaloid. A fully developed leaf behaves like a mutant, a gene of which has become ineffective for the alkaloid synthesis.

Influence of Development and Ageing

The influences of development and ageing on the metabolism have been rarely investigated. I am sure that some negative results found in the investigation of the synthesis of alkaloids using isotopes were due to insufficient attention being directed towards these points of the physiology of development. There are numerous publications reporting experiments in which a probable precursor in its labelled state was fed to plants, and where the lack of success led to the conclusion that the substance investigated could not be the precursor. In these experiments, however, whether any synthesis occurred during the experimental time was not observed.

Thus it was found that seedlings of *Medicago sativa* were incapable of forming stachydrine from [2-^{14}C]-ornithine[28,29], though the synthesis of proline from ornithine has been proved using several organisms. Recently it was shown that these seedlings can convert ornithine to glutamic acid, and that, in the presence of pyridoxal, both ornithine and glutamic acid are converted to proline. The methylation of proline to stachydrine is possible when the plant is fed folic acid together with methionine[30].



Obviously the young plants require the precursors in an amount sufficient for the deamination and activation of amino acids as well as for the methylation of the N-atom.

In the growing plant a similar differentiation of the chemical performance takes place in the root. For instance, nicotine is produced only in any quantity in the growing tip of the tobacco root. In the older parts there is little or no synthesis at all, although they are still growing in thickness, and cell division takes place. All the factors promoting cell elongation and branching of the root also promote alkaloid synthesis. But it is not yet known to which cells of the root-tips and to which processes the alkaloid synthesis is bound. A remarkable effect is observed when the number of growing root-tips is experimentally increased, which can easily be done by repeatedly cutting the roots. Thus a threefold increase in the absolute nicotine production can be produced over 3 months[31].

TABLE I

LEAF-CUTTINGS OF *Nicotiana rustica*. 136 DAYS OLD. CONTROL: ROOTS NOT CUT. EXPERIMENT: ROOTS CUT THREE TIMES AFTER 45, 84 and 105 DAYS. ALL THE RESULTS FOR THE CUT ROOTS ARE INCLUDED IN THE TOTAL ANALYSIS. RESULTS RELATED TO 10 LEAF-CUTTINGS

	Analysis after days	Fresh weight g.	Dry weight g.	Total N mg.	Protein N mg.	Soluble N mg.	Nicotine N mg.
Control							
Lamina	136	118	13·2	753	256	497	115
Midribs and stalks	136	60	6·9	320	138	182	41
Roots	136	237	14·2	638	312	326	32
Total		415	34·3	1711	706	1005	188
Experiment							
Lamina	136	110	11·7	750	200	550	245
Midribs and stalks	136	54	6·6	318	138	180	63
Roots 1st cut ..	45	34	1·9	89	43	46	2
" 2nd cut ..	84	53	3·7	155	70	85	9
" 3rd cut ..	105	45	3·0	130	55	75	10
End of experiment	136	76	6·5	307	137	170	30
Total		372	33·4	1749	643	1106	359

Nitrogen Uptake

It should be noticed that the excess production of alkaloid is not related to an excess absorption of nitrogen: the control plants with intact root systems take up just as much nitrogen from the medium, but they form much larger quantities of amides, especially glutamine, and accumulate them in the leaves as the plants with cut roots accumulate the nicotine. Up to date it has not been possible to effect a synthesis of nicotine or hyoscyamine in excised roots which have been deprived of their tips. Moreover, the roots must be growing, as roots without tip-growth cannot perform this synthesis.

Low concentration of auxin promotes the root growth in terms of length and dry weight. Such roots form much nicotine. Higher concentrations inhibit the growth in length and the formation of dry weight; they also inhibit the nicotine synthesis, but not to the same extent as the protein

synthesis. On the contrary, these low-alkaloidal roots are high in protein, and they respire intensively[32,33]. (Tables II and III.) Thus it is possible that the elongation of the cells of the root-tips, but not their division, is closely related to the alkaloid synthesis.

TABLE II

NICOTINE PRODUCTION, GROWTH AND RESPIRATION ACTIVITY OF EXCISED ROOT SEGMENTS OF *Nicotiana tabacum* VAR. TURKISH, DURING CULTURE FOR THE PERIODS INDICATED (SOLT)[32]

Segment type and treatment	Initial			1 week			2 weeks	
	Nicotine µg.	Dryweight mg.	QO_2	Nicotine µg.	Dryweight mg.	QO_2	Nicotine µg.	Dryweight mg.
Mature segments ..	286	18·8	4·7	284	17·8	1·3		
Tip segments	65	3·0	12·4	773	30·0		4437	130·0
Tip segments with 10^{-6} M indole acetic acid ..				134	15·4	5·9	147	29·0

TABLE III

Nicotiana rustica. DEVELOPMENT OF ROOTED LEAF-CUTTINGS IN A MEDIUM OF ZINZADZE CONTAINING VARIOUS CONCENTRATIONS OF INDOLE ACETIC ACID (IAA). DURATION: 37 DAYS[33]

No.	IAA concentration mg./l	Organ	In 10 leaf-cuttings			In per cent dry weight	
			Dry weight g.	Protein N mg.	Nicotine N mg.	Protein N	Nicotine N
0	Control	Leaves	2·3	84	6	3·65	0·026
1	0	Leaves	19·3	439	126	2·27	0·652
		Roots	16·6	270	49	1·62	0·295
		Total	35·9	709	175		
2	1	Leaves	20·7	500	142	2·41	0·689
		Roots	20·6	406	48	1·97	0·230
		Total	41·3	906	190		
3	3	Leaves	15·4	389	60	2·53	0·390
		Roots	14·7	332	21	2·25	0·144
		Total	30·1	721	81		
4	10	Leaves	12·7	355	32	2·80	0·249
		Roots	9·2	322	3	3·50	0·030
		Total	21·9	677	35		

Some years ago we studied this problem of the relation between nitrogen absorption and alkaloid synthesis, because nitrogen assimilation in many plants can be closely related to the formation of very specific root-substances, which represent primary nitrogen assimilates and translocation forms of nitrogen. Thus we found that in the roots of Boraginaceae, Platanaceae, Aceraceae and many Papilionaceae, allantoin and allantoic acid[34–36] are formed, and probably citrulline in the roots of Betulaceae[37] and acetylornithine in the roots of Fumariaceae[38]. These four substances occur in the roots and the bleeding-sap of the various species of these families in large amounts.

To study experimentally the relations between nitrogen absorption and nitrogen assimilation on the one hand and alkaloid synthesis on the

other we grafted *Atropa* upon *Lycopersicum*. In the root of *Lycopersicum* no hyoscyamine was formed. We did not feed these graftings as usual

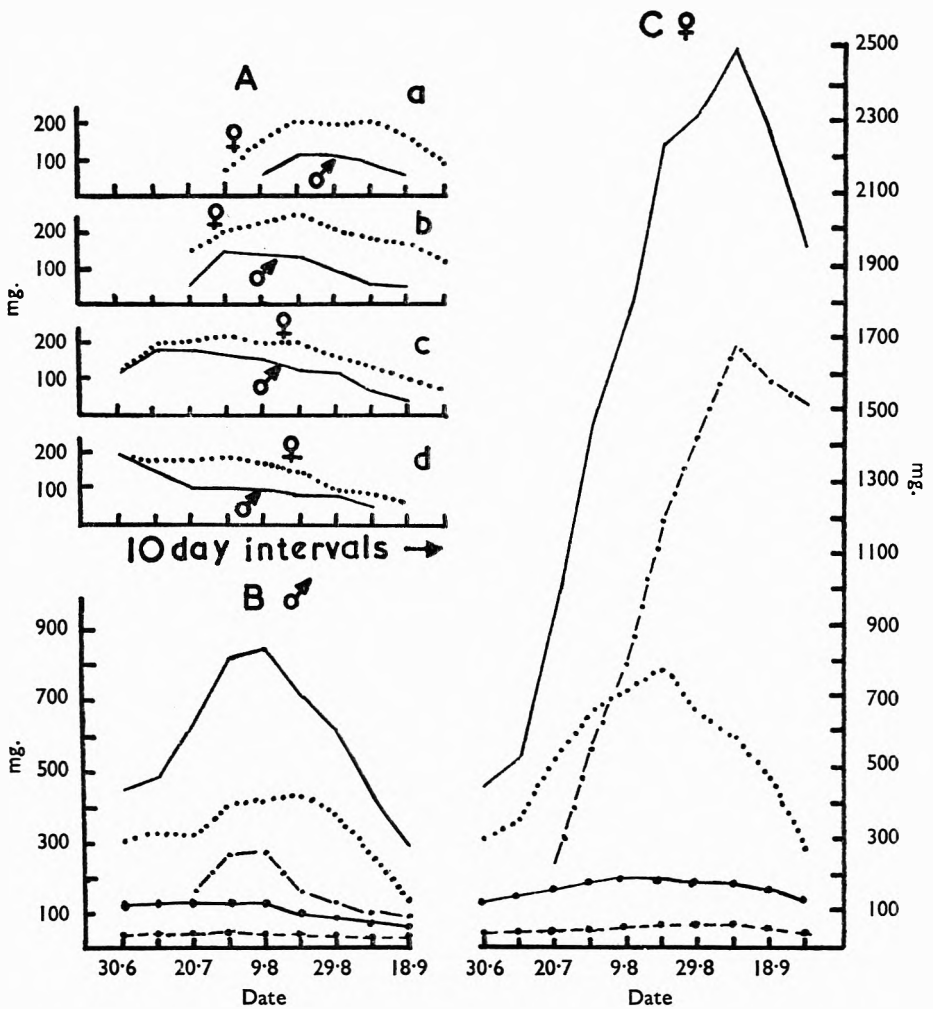


FIG. 1. *Cannabis sativa*. The development of total nitrogen in male (B) and female (C) individuals, mg. total nitrogen in one plant.

A. Leaves at different insertions, (*a*) 150–210 cm. high. (*b*) 100–150 cm. high. (*c*) 50–100 cm. high. (*d*) 0–50 cm. high.
B. Male plant. ——— Whole plant. . . . . Leaves ●—●. Flowers and side shoots ●—●. Stalk and stem ●—● Root.
C. Female plant, same key as male plant.

by the roots but only by spraying the leaves with solutions of ammonium nitrate, thus shifting the primary nitrogen assimilation to the leaves. We did not observe a noticeable alkaloid synthesis[39,40].

But if we fed intact plants of *Atropa*, *Datura* and *Nicotiana* by spraying the leaves, without adding nitrogen to the soil, alkaloid was produced in large quantities. *Atropa*, *Datura* and *Nicotiana* roots are, therefore, able to synthesize alkaloid, receiving the necessary nitrogen from the leaves. The decisive factor for the alkaloid production was not the process of nitrogen assimilation, but that of root growth.

Influence of the Shoot and Flowering

The growth of roots and therefore their activity in alkaloid synthesis is decisively influenced by the shoot. This is particularly so with the hapaxanthic plants, which have a terminal inflorescence, when the development of flowers often effects a permanent suppression of the roots. This suppression not only results in a check of growth but also of nitrogen-absorption. This phenomenon can be observed particularly well with male individuals of hemp (*Cannabis indica*) (Fig. 1). When the male flowers develop, the absolute nitrogen content of the plants begins to fall. The roots excrete nitrogen into the medium. This inhibition of the roots may be effected partly by an acute carbohydrate deficiency because the flowers absorb the stream of assimilates. Obviously there are still other causes involved, probably those of a hormonal character[41].

The conditions are similar, though less extreme, with *Nicotiana* species, especially with those strains which during and after flowering do not branch.

All the factors inhibiting the formation of flowers promote alkaloid synthesis. For a long time agriculturalists have used this fact when topping and pruning the plants[42,43]. Hofstra[44] and Reuter[45] have clearly shown that the excess of alkaloid in such plants is not caused by concentration of the normal content of alkaloid into a smaller shoot-volume, but that there is excess production. We have noted that the leaves of an *Atropa* scion on tobacco root are bleached by the nicotine from the tobacco root. If the *Atropa* scion flowers it does not bleach. We found that under the influence of the flowering *Atropa* the nicotine content of the tobacco root decreases[1]. This effect of flowering can be demonstrated under more physiological conditions with plants photoperiodically sensitive. Among others we used *Nicotiana sylvestris*, a long-day plant. Under short-day conditions it remains in the rosette-stage and continuously produces new leaves, and its root system grows without inhibition. Under long-day conditions the plant shoots, forms flowers and ceases to produce leaves. Its root growth is inhibited from this stage. Such a plant contains only about one-fourth of the amount of alkaloid compared to the plant under short-day conditions[31]. (Table IV.)

There are still other differences in the alkaloidal content. The flowering plant under long-day conditions contains predominantly nornicotine, the plant of the same age under short-day conditions contains predominantly nicotine. (Table V.)

This process of demethylation usually does not take place in the root. There we find approximately the same ratio of nicotine to nornicotine independent of the day length. Possibly the nornicotine of the root is not formed from nicotine, but has a primary character. It is also found

in young roots, in the culture of excised roots[46], and in the bleeding-sap[44,45,47] of various *Nicotiana* species. The nornicotine of the shoot, however, is undoubtedly formed in the greatest amount secondarily from nicotine[48,49]. In certain tobacco strains there is practically no demethylation, in others it occurs only during the drying of the leaves or during fermentation. These strains represent an important group of low-nicotine tobaccos. They are, however, not originally low in nicotine. Other

TABLE IV

PHOTOPERIODICAL INFLUENCE UPON ALKALOID SYNTHESIS IN *Nicotiana sylvestris*. (Mothes and others[31])

Date	Experiment	State of development	In one plant						Nicotine in per cent dry weight		
			g. dry weight			mg. nicotine					
			root	shoot	total	root	shoot	total	root	shoot	total
29.2.56	beginning	rosette	0·21	1·00	1·21	1·8	6·2	8·0	0·86	0·67	0·66
13.3.56	long day	leaves expanded	1·46	6·15	7·61	13·1	33·6	46·2	0·90	0·55	0·61
	short day	rosette	1·72	4·11	5·83	14·8	21·5	36·3	0·85	0·52	0·60
22.3.56	long day	elongation of shoot	5·0	14·5	19·5	31·3	36·2	67·5	0·62	0·25	0·35
	short day	rosette	5·8	10·0	15·8	80·5	45·6	126·1	1·39	0·46	0·80
3.4.56	long day	first flowers	9·5	24·1	33·6	63·5	44·7	108·2	0·67	0·19	0·32
	short day	rosette	13·1	10·8	23·9	141·3	58·7	200·0	1·07	0·54	0·84
16.4.56	long day	full blossom	7·8	27·0	34·8	62·4	26·2	88·6	0·80	0·10	0·25
	short day	rosette	17·8	12·3	30·1	264·3	94·4	358·7	1·48	0·37	1·20

TABLE V

PHOTOPERIODICAL INFLUENCE UPON SYNTHESIS OF NORNICOTINE IN *Nicotiana sylvestris*. (Mothes and others[31]). (Nicotine = 100.)

Date	Experiment	Root			Shoot		
		Nicotine	Nornicotine	Anabasine	Nicotine	Nornicotine	Anabasine
3·4	long day	100	1·5	1	100	1	trace
	short day	100	1·5	1	100	1	trace
16·4	long day	100	2·5	2	100	10–15	trace
	short day	100	2	1·5	100	4	trace
24·5	long day	100	4	3			
	green leaves				100	25	trace
	yellow leaves					nearly all nornicotine	
	total				100	500	trace
	short day	100	3	2			
	green leaves				100	3	trace
	yellow leaves					nearly all nornicotine	
	total				100	30	trace

strains and species demethylate in ageing leaves, for example, our *Nicotiana sylvestris*, or they immediately demethylate in the shoot the nicotine ascending from the root, e.g., most strains of *Nicotiana glutinosa*[31,50]. Thus the difference between long-day and short-day plants of *Nicotiana sylvestris* is only indirectly caused by flowering. Since flowering plants have old leaves only, they demethylate relatively more strongly than plants which do not flower and which continuously form new leaves. In both cases the old yellow leaves contain only nornicotine.

The process of demethylation is not reversible, even if CH_3-donators are given to the leaves[51]. Probably the demethylation is achieved by an oxidative degradation of the methyl group and not by transmethylation[52,53]. Thus old and young, growing and full-grown organs, differ in their capacity

to add methyl groups at the N-atom. Probably there is a lack of sufficient activity in the corresponding enzyme systems, in which folic acid plays a special rôle. These changes in an ageing organ are of a general importance. I refer to the investigations of Cromwell and Rennie[54], who found that leaves of *Beta vulgaris* are able to oxidize choline and betaine aldehyde to betaine, but that only to a small extent can they methylate glycine, even if methionine is fed.

Since this process of methylation also plays a decisive rôle in the synthesis of the more important substances, for example, the purines, lack of activity in synthesizing alkaloids and betaines in full-grown or ageing organs reflects a state of general impotence.

TABLE VI

Nicotiana sylvestris, MG. OF SCOPOLETINE IN PHOTOPERIODICALLY TREATED PLANTS. (Kala[57].)

Experiment	Stage	Root		Stem		Leaves		Flowers	
		per plant	per cent dry weight	per plant	per cent dry weight	per plant	per cent dry weight	per plant	per cent dry weight
Short day	rosette	0·36	1·3	—	—	0·05	1·2	—	—
Long day	flowering	1·2	29·0	0·08	2·0	0·40	6·0	0·6	4·0

But it would be wrong to draw general conclusions from the few cases investigated up till now. Thus we have studied the biosynthesis of cumarines, which can also be synthesised in the root. Reciprocal graftings of legumes and the culture of excised roots have shown this distinctly[55,56]. But to what extent they can be formed in the shoot is not yet certain. Their synthesis is not restrained by flowering but, on the contrary, it is promoted[57]. (Table VI.)

Influence of State of Development

Thus, besides the inherited characteristics, the chemical activity of the plant plays an important rôle which changes quantitatively or even qualitatively during the course of development. It is also the cause of some apparent contradictions in the publications of several authors.

I refer to the investigations of our laboratory concerning the formation of hyoscine in *Datura*. Usually in the roots of *Atropa*, *Datura* and *Hyoscyamus*, hyoscyamine and hyoscine are formed side by side. It is not yet certain whether the epoxide is formed primarily or from the hyoscyamine. Young plants (few weeks old) contain usually more hyoscine than hyoscyamine[58–60], but in the shoot there are great differences: some species contain almost exclusively hyoscyamine, others hyoscine. Romeike[61] found that in *Datura ferox* the hyoscyamine on ascending into the leaves is oxidised to hyoscine. Leete, Marion and Spenser[62] fed *Datura* with [α-^{14}C]-ornithine and obtained radioactive hyoscyamine; hyoscine, however, was not radioactive. They concluded that the two alkaloids had different precursors. It seems to be more probable that

during the time of experiment no hyoscine has been formed from hyoscyamine. In principle it is a similar problem to that encountered in the synthesis of stachydrine.

There are other examples. Trautner[63] reports that in *Duboisia myoporoides* of a Northern Australian origin hyoscine prevails throughout life, whereas in those of a Southern Australian origin the formation of hyoscyamine begins after 4 to 6 months and almost completely disappears in older plants. This is of commercial importance.

The influence of the ageing of organs and of the state of development of the plant is frequently dismissed but the relationship is not always clear. In barley the methylated derivatives of tyramine arise from tyramine and tyrosine in the roots of young plants only[64,65]. According to the investigations of my collaborator Rabitzsch, in seedlings, tyramine, methyltyramine, hordenine and candicine are successively formed. After about 30 days these substances disappear.

Areschkina[66] reports that in the growing parts of *Senecio platyphyllis*, platyphylline-*N*-oxide primarily occurs. After flowering it disappears from the aerial parts and platyphylline accumulates in the rhizome.

Smirnova turkestana, in May, contains only smirnovine; in August, only sphaerophysine[67].

The results of Sander[14] are very interesting. He showed that the steroid alkaloids are not exclusively, but primarily, formed in the shoot, young organs being especially active. After flowering, the alkaloid content decreases, but *Nicotiana* is in contrast to this because the developing fruits are a preferred site of tomatine degradation.

As the degradation or loss of alkaloids in older organs has often been described (*Nicotiana*, *Atropa*, *Datura*, *Lupinus*, *Papaver*, etc.), I shall not discuss it. In many instances the time of flowering is the time of peak of the alkaloid content.

Besides its genetical constitution, the state of development of a plant or an organ can have great influence on its alkaloid content. This becomes more evident when we study the effect of external factors on the qualitative and quantitative development of the alkaloidal character. There is an immense literature on this subject, but a literature full of contradictions.

To help eliminate the effects of development and especially of flowering, we recently worked with isolated leaves of various alkaloid plants, which were rooted by a short auxin treatment. Such leaf-cuttings do not form shoots and flowers, and show a completely uniform growth of roots and lamina for several months. In the growth of a leaf, cell division plays a minor part. The intercostal parts grow only by cell enlargement. The growing leaves provide excellent conditions for the growth of roots, since no buds, no flowers or fruits compete with them. Carbohydrates, auxins and other leaf-substances are at the disposal of the roots in an optimal way, and a richly branched active root system is formed.

The chemical performance of this root system can be elucidated by the analysis of the lamina. We can distinguish between two groups of substances: proteins, amino acids, purines, and nucleic acids, which follow

the growth in an almost stoicheiometric way, and alkaloids, amides and nitrates, the content of which is related not to the activity of the lamina but to the root system. These substances are secondarily accumulated in the lamina, and can be found there in enormous quantities. A leaf which under normal conditions contains 1 per cent nicotine may increase its

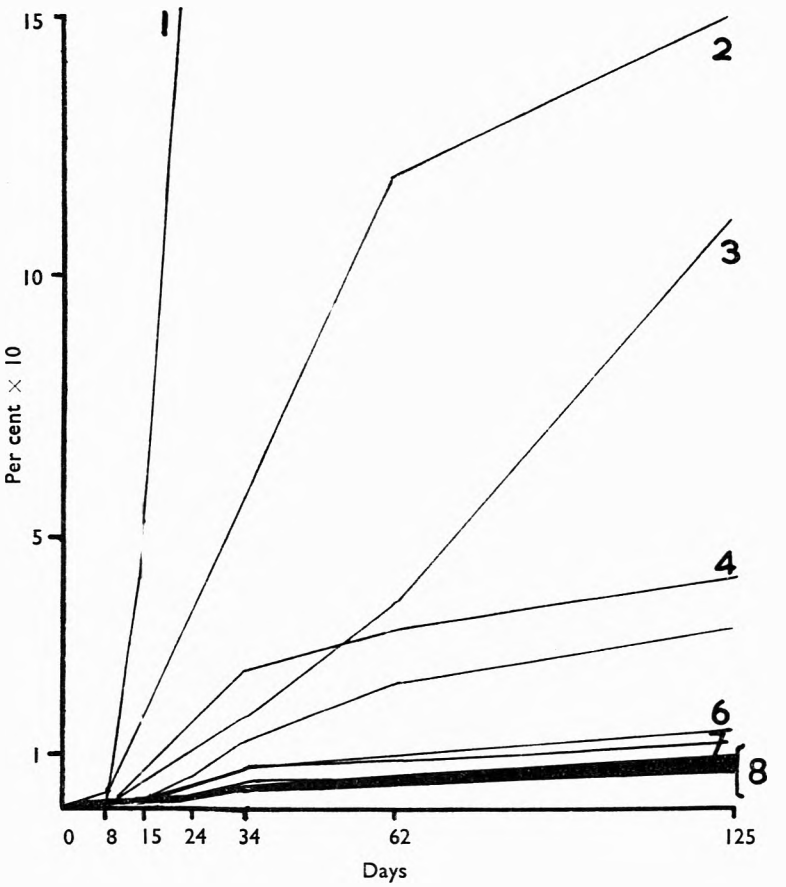


FIG. 2. *Nicotiana rustica.* Rooted leaf-cuttings. Half of the lamina was used as a control and the amount of total nitrogen was arbitrarily taken as 100. The amounts of the other half after different periods of cultivation is related to this control. (See ref. 68).

1. Nitrate-N. 2. Amide-N. 3. Nicotinic-N. 4. Ammoniacal-N. 5. N in solution. 6. Total-N. 7. Amino-N. 8. Fresh weight, dry weight, protein-N, purine-N.

content up to 20 per cent. Three or four months after the beginning of the experiment, the protein- and purine-nitrogen increases with the dry weight some 10 times, the nicotine-nitrogen 100 times, the amide-nitrogen 150 times, and the nitrate-nitrogen 1500 times (Fig. 2). The accumulation proceeds very uniformly for the first 3 to 4 months[68], enabling a close study to be made on the effect of the external conditions.

External Conditions

Temperature

An external condition which is most difficult to regulate when alkaloid plants are cultivated in the field is the temperature of the soil. Its effect on the growth and the activity of roots has been little studied. We have made some preliminary experiments in which roots were exposed to different temperatures (11–12°, 20–21°, 30–31°) while keeping the shoots under uniform conditions. We worked with plants that had been deprived of all their leaves and buds except one leaf. As compared with leaf-cuttings, such plants have a stem, and this allows the leaf to be isolated thermally from the soil. But deductions must make allowances for the fact that variation of temperature may also effect a change in the oxygen supply.

These experiments were made with *Nicotiana rustica*, and they showed that the most intensive protein synthesis in the root took place at low temperatures, but that the most nicotine was formed at medium temperatures. A soil temperature of 30° inhibited growth, nitrogen-uptake, protein and nicotine synthesis[33]. This may not be the same for other species, but that protein and nicotine synthesis have different optimum temperatures seems to be of importance.

Light

The effect of light is much better studied than the effect of temperature, but unfortunately the two are mostly not differentiated from one another. In regions with a high insolation, the alkaloid content of the plants does not usually exceed that of the northern regions, and is not only related to modifications but also to genetical fixations. There are in Bulgaria *Atropa* strains with an unusually high content of alkaloid, which is conditioned genetically[69]. The same is true of ergot strains in warm dry Southern Europe. How such strains arise, and why they can survive in these climates, is as yet unanswered.

Examples such as these are often described, but whether they are a matter of genetical fixation or of a temporary modification by the surroundings has not been sufficiently investigated.

We made some experiments, again with non-flowering leaf cuttings, where only the light factor was varied. In an earlier publication[68] we showed with *Phaseolus* and *Symphytum* that with high light-intensity the root nitrogen uptake and nitrogen assimilation is limited by the nitrogen concentration. However, if the leaves are shaded, light is the limiting factor.

The confining effect of the light factor is well elucidated by the synthesis of nicotine. This influence of light can manifest itself very differently. A presupposition for the growth of the roots and therefore for the nicotine formation is that the roots are well supplied with carbohydrates; that means that the light intensity is sufficiently high. A very high intensity promotes the whole metabolism strongly for some days or weeks but soon causes remarkable damage, which becomes apparent as a strong depression of photosynthesis, a decrease of soluble sugars in the root, a complete

check of the nitrogen-absorption, a stoppage of root growth, and therefore a complete check of nicotine synthesis[70]. This light effect needs a more detailed analysis. Probably the lack of efflux of carbohydrates effects an inhibition of photosynthesis and the absorbed light, conducted to a "wrong substrate", damages the chloroplast apparatus photodynamically. Our collaborator Parthier showed in unpublished experiments that even low light intensities have a detrimental effect if they are offered continuously. Enormous quantities of coarse-grained starch accumulate in the chloroplasts and gradually burst the plastids, which are apparently injured mechanically in an irreversible way. But one might as well take into consideration a photo-oxidative destruction of auxin. We can suppose that such processes also play an important part in an intact ageing plant.

It is possible that the detrimental effects of the light factor become manifest only in isolated leaves. Generally the alkaloid content follows the light intensity. The efficiency of the different spectral regions has not been studied sufficiently. It has frequently been reported, however, that the synthesis of the steroid alkaloids is promoted by ultra-violet light[71–72]. It may be that the same is true for the alkaloids of the sclerotia of *Claviceps purpurea*. If the rye plants, which nourish the sclerotia, are grown under glass, or if the ears are enveloped with Cellophane, the alkaloid content is much lower than that obtained with normal light[73]. Whether decreasing the light necessary for assimilation brings about a parallel decrease in alkaloid content must be investigated, for we know that the awn and glume of the ears play an important rôle in nourishing the grains[74–76]. The physiology of the ears of cereals is in need of thorough investigation.

Effect of Nutrients

In some feeding experiments with ergot we injected into the pith of rye plants amino acids which were likely to take part in the synthesis of ergot alkaloids. We did not succeed in changing the mixture of alkaloids, nor were we able to influence the total quantity of alkaloids—perhaps with one exception: tryptophane seemed to increase the alkaloid content. These results agree with the fact that in the mature ergot all the amino acids involved in the alkaloid formation are already present, and therefore they cannot be a limiting factor in the alkaloid synthesis, except the tryptophane, which mostly seems to be missing[77]. The honey-dew, a product of the activity of the young mycelium, also contains all the essential amino acids[78]. It has not yet been proved how far these amino acids are assimilated from the rye plant or are synthesised in the mycelium itself[79,80].

At any rate the alkaloid composition cannot be changed by the character of the host[8], or by nutrition[72], or in saprophytic culture[81]. Therefore it is remarkable that injection of labelled tryptophane into the rye stem produces alkaloids labelled at the lysergic acid ring[82].

Concerning the root alkaloids, we studied the effect of some nutrients on the formation of nicotine in rooted leaf cuttings of *Nicotiana rustica*. In agreement with experiments with whole tobacco plants described in the literature we found that with increasing nitrogen supply the nicotine production increases both absolutely and relatively (dry weight basis).

A high nitrogen supply, however, can only be fully utilised if the plant is also sufficiently supplied with carbohydrates. If carbohydrates are deficient a high nitrogen supply inhibits both the root growth and the alkaloid formation[68,33]. A decrease in the concentration of general minerals increases the effectiveness of the N supply. This may essentially be conditioned by the promotion of the nitrogen uptake and the greater accumulation of soluble nitrogen containing compounds.

These results are interesting because, in contrast to intact plants, in leaf cuttings there is no formation of flowers to be inhibited, and no immature vegetative tissues to be developed by a high nitrogen supply. Alkaloid formation in ergot is also promoted by nitrogen-containing fertiliser, but we do not know why. Perhaps a good supply of nitrogen preserves the metabolic acitivity of the photosynthesising parts of the ear for a longer time.

There is a voluminous literature about the effect of potassium. We are especially interested in the statements that potassium deficiency brings about an increase in the acidity of the cell-sap, an increased nitrogen uptake and an accumulation of ammonia. We have made the same observations with leaf-cuttings. As potassium deficiency was found[83,84] to cause an accumulation of putrescine in barley and of arginine in flax, nicotine was investigated to see if it behaved similarly. This, however, is not the case. Potassium deficiency inhibits the nicotine synthesis, but an excess of K also effects a lower nicotine content. A "normal" potassium supply (0·215 g. K_2SO_4/1. nutrient solution) appears to be optimal[33].

An interesting problem is whether a nutrient-factor exists which in a specific way promotes alkaloid synthesis. One such factor seemed to be boron[85,86]. Boron deficiency was thought to bring about a high nicotine content in tobacco plants. The investigations of our laboratory with leaf-cuttings did not confirm this[87]. Boron deficiency inhibits the development of the root, and therefore the alkaloid synthesis. Possibly in the experiments of Steinberg the inhibition of flower formation by a slight boron deficiency gave rise to a promotion of root activity[88]. Excised roots of *Atropa* cultivated under conditions of boron deficiency also showed a very bad growth and a low alkaloid production[89].

All our investigations with leaf-cuttings resulted in our establishing that nicotine production is closely related to root growth. Growth itself, however, is a complex phenomenon. We must distinguish between growth of plasma, division of the nucleus and the cell, and enlargement of cells. We are not yet quite sure to which sort of growth the nicotine synthesis is associated. The branching of the root is not the decisive factor, as boron deficiency may effect a rich branching, but the nicotine synthesis is low under these conditions. To effect a full nicotine synthesis the growth must be vigorous and the roots must look white and healthy. Perhaps the experiments of Solt[32] and those of our own[33] with auxin indicate a closer relation of the nicotine synthesis to cell-elongation.

Relation between Alkaloid and Protein Synthesis

In most cases growth is linked to an increase of cell protein. Therefore it was supposed that nicotine synthesis was bound to protein synthesis[90],

and an almost stoicheiometric ratio of the two substances was found in the plant: half a molecule of nicotine was supposed to arise for every one molecule of amino acid bound in the protein of the root. But we could not confirm this. The ratio total nicotine-N: root protein-N fluctuates considerably, from 0·1 to 1·5[33,39]. The occasional validity of this "rule" only seems to be an expression for the observation that growth is mostly combined with protein synthesis. But we have no explanation why alkaloid synthesis primarily takes place in the growing tissue; this is apparently a widespread phenomenon and not limited to root alkaloids. For example, it is true for the solanidines[13,14]. The relation between the alkaloid synthesis and the metabolism of growing organs leads to the question: what is the function of alkaloids in the life of plants? This problem has long been treated in a teleological way only.

Function of Alkaloids

Doubtless for many animals alkaloids are only poisons preventing plants from being eaten. By no means must we overlook the interesting special cases, where apparently alkaloids play a rôle in defence, for example, against the Colorado beetle (*Leptinotarsa decemlineata*) by *Solanum* species, the fungus *Phymatotrichum* by *Mahonia* and *Fusarium* by *Lupinus* or *Lycopersicum*. There are a number of such examples, but they should not be generalised. Certainly even alkaloidal plants have their animal and vegetable enemies. The general value for the selection of the character "alkaloidal" is therefore dubious. If the alkaloids had a great value in selection, the alkaloidal plants would constitute a higher percentage of the individuals living on the earth. But only rarely do they predominate in the vegetation, as for example on the alpine pastures of our mountains, where around the cottages of the shepherds the cattle crowd in large numbers, there are great amounts of *Veratrum*, *Delphinium* and *Aconitum*. Or in the more humid parts of the asiatic deserts, where the camel caravans make a halt and great herds of antelopes gather, *Anabasis* and other alkaloid-containing Chenopodiaceae grow. We can take it for granted that in these two examples the character "alkaloid" has a positive value for selection, since vegetation is threatened almost exclusively by mammals. But these are rare cases. They correspond to the extensive spread of thistles on our pastures. Perhaps it is the same problem that in free nature "sweet lupines" are rare and the alkaloid-containing strains predominate. On the other hand I stress the fact that a high content of alkaloid seems to decrease vitality. This is even probable for lupines and seems to be one more cause for the fact that these "poisonous plants" are not very frequent.

This prompts the examination of plant tissue to see whether alkaloids can accumulate to such an extent during the development of an individual that they gradually produce detrimental effects. But this examination is not easy. A substance applied externally to plant tissue may have a different mode of action from one that is enclosed in the vacuole and separated from the protoplasm by the tonoplast.

If we cultivate excised tobacco roots under aseptic conditions we find that nicotine in the concentration of 10^{-4} g./l. inhibits growth. In the

plant, however, the concentrations can be much higher. An excised tobacco root continuously excretes nicotine into the medium. Possibly the inhibition of growth in the excised root is related to the accumulation of root-substances, which would normally pass into the shoot, producing a self-poisoning.

Another important question is whether alkaloids are essential and indispensable substances with important functions in metabolism and growth. Such functions have as yet not been proved, but we are not inclined to deny their existence. The fact that we did not succeed in producing artificial X-ray-mutants devoid of alkaloid might possibly indicate that such mutants come into existence but are not able to survive. Perhaps the low alkaloic races have a decreased vitality[91,92].

Fisher and Loomis describe a flower-promoting effect of nicotine sprays with soya-bean[93,94]. Our collaborator Dr. Ramshorn could not confirm this.

If *Cyphomandra* is grafted on *Nicotiana rustica* the scion flowers very soon, but the flower-promoting factor is not nicotine. If *Hyoscyamus muticus* is grafted on *Nicotiana rustica* the vegetative growth is remarkably promoted, but again the promoting factor is not nicotine.

Thus there remain but a few facts, which might indicate a possible physiological function of the alkaloids. Ramshorn[95,96], Solotnizkaja[97] and Izard[98] found that nicotine in a specific way affects the auxin-complex; in low concentrations it promotes growth of the *Avena* coleoptile, in concentrations more than 10^{-5} M it inhibits. Müller and Ramshorn found that nicotine influences the permeability for water during deplasmolysis in a specific way[99]. But those are effects on plants which do not contain nicotine, where nicotine is a drug and not a physiological substance. Bachmann ascribes an effect similar to auxin to all the pyridines[100]. It is very difficult to make similar experiments with tobacco, since the presupposition of such experiments is a comparable control plant devoid of nicotine, while all the tissues of tobacco contain this alkaloid.

There are many reports of pharmacological investigations on alkaloids, investigations concerning the effect upon the nervous system, and more detailed work on the effect upon enzyme systems and particularly the respiratory enzymes[101–108].

It is possible that we may erroneously conclude a physiological function from the pharmacological effect.

As nature excites us by a continuous play with colours and odours, as she stimulates the morphologist by an abundance of forms, which cannot be understood merely teleologically, thus as an artist she creates for the chemist an enormous number of the simplest and most complicated substances. Certainly each of these in the course of evolution may receive a function. But we should not look upon nature as so limited that she had to exhaust her creative genius purposefully.

Only a small part of the recent literature could be considered in this article. According to the subject given to him the author has put in the foreground the work of his own laboratory, for which he asks to be excused.

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References

1. Mothes and Romeike, *Flora*, 1954, **142**, 109.
2. Schröter, *Arch. Pharm.*, 1955, **288**, 141.
3. Mothes, Romeike and Schröter, *Naturwiss*, 1955, **42**, 214.
4. Manske, in Manske-Holmes: *The Alkaloids*, **1**, Academic Press, New York, 1950, p. 1.
5. Gröger, *Abh. dtsch. Akad. Wiss. Kl. Chem.*, 1957, (1956), (7), 243.
6. Mothes, 1957, in Gröger 1957, ref. 5, p. 250.
7. Gröger, *Die Kulturpflanze*, 1956, Supplement **1**, 226.
8. Meinicke. *Flora*, 1956, **143**, 395.
9. Dawson, *Adv. Enzymol.*, 1948, **8**, 203.
10. Iljin, *Biochimija* (*russ.*), 1949, **14**, 554.
11. James, 1950 (*see* ref. 4, **1**, 15).
12. Mothes, *Hdb. Pflanz. physiol.*, 1958, **8**, 989.
13. Prokoschew, Petrotschenko, Iljin, Baranowa and Lebedewa, *Dokl. Akad. Nauk* (*Moskau*), 1952, 83, 881.
14. Sander, *Planta*, 1956, **47**, 374.
15. Gusewa and Paseshnichenko, *Biochimija*, 1958, **23**, 412.
16. Smirnowa and Moschkow. *Sowj. Bot.*, 1941, **1941**, 32.
17. Peters, Schwanitz and von Sengbusch, *Die Kulturpflanze*, 1956; *Supplement*, **1**, 247.
18. Mothes and Engelbrecht. *ibid.*, 1956, **1**, 258.
19. Cromwell, *Biochem. J.*, 1956, **64**, 259.
20. Shibata, *Planta medica.*, 1956, **4**, 74.
21. Mothes and Hieke, *Naturwiss.*, 1943, **31**, 17.
22. Hegnauer, *Pharm. Weekbl.*, 1951, **86**, 935.
23. Mashkootsev and Sirotenko, *Dokl. Akad. Nauk* (*Moskau*), 1951, **79**, 487.
24. Mothes and Romeike, *Flora*, 1952, **139**, 181.
25. Solt, *Plant Physiol.*, 1957, **32**, 484.
26. Leete, *Chem. Ind.*, 1958, **1**, 1088.
27. Romeike, *Pharmazie*, 1953, **8**, 668.
28. Leete, Marion and Spenser, *J. biol. Chem.*, 1955, **214**, 71.
29. Morgan and Marion, *Canad. J. Chem.*, 1956, **34**, 1704.
30. Wichler and Marion, *J. biol. Chem.*, 1958, **231**, 799.
31. Mothes, Engelbrecht, Tschöpe and Hutschenreuter-Trefftz, *Flora*, 1957, **144**, 518.
32. Solt, *Plant Physiol.*, 1957, **32**, 480.
33. Mothes and Engelbrecht, *Planta medica*, 1958, **6**, 317.
34. Mothes and Engelbrecht, *Flora*, 1952, **139**, 586.
35. Mothes and Engelbrecht, *ibid.*, 1954, **141**, 356.
36. Engelbrecht, *ibid.*, 1954, **141**, 501; **142**, 25.
37. Reuter, *ibid.*, 1957, **144**, 420; **145**, 326.
38. Reuter, *ibid.*, 1957, **145**, 326.
39. Mothes, Trefftz, Reuter and Romeike, *Naturwiss*, 1954, **41**, 530.
40. Trefftz, *Kulturpflanze*, 1956, **4**, 325.
41. Mothes and Engelbrecht, *Flora*, 1952, **139**, 1.
42. Mothes, *Planta*, 1928, **5**, 563.
43. Hofstra and Keuls, *Meded. Inst. Vered. Tuinbouwgewassen Wageningen*, 1952, **36**, 1.
44. Hofstra, Proefschrift Groningen, 1952.
45. Reuter, *Kulturpflanze*, 1957, **5**, 139.
46. Schröter and Engelbrecht, *Arch. Pharm.*, 1957, **290**, 204.
47. Tso and Jeffrey, *Plant Physiol.*, 1957, **32**, 86.
48. Dawson, *Amer. J. Bot.*, 1945, **32**, 416.
49. Iljin, *Dokl. Akad. Nauk* (*Moskau*), 1948, **62**, 247.
50. Tso and Jeffrey, *Plant Physiol.*, 1956, **31**, 433.
51. Dawson, *Amer. J. Bot.*, 1952, **39**, 250.
52. Egri, *Fachl. Mitt. Österr. Tab. Regie*, 1957, p. 19.
53. Iljin and Serebrowskaja, *Dokl. Akad. Nauk* (*Moskau*), 1958, **118**, 139.
54. Cromwell and Rennie, *Biochem. J.*, 1954, **58**, 318.
55. Mothes and Kala, *Naturwiss*, 1955, **42**, 159.
56. Reppel and Wagenbreth, *Flora*, 1958, **146**, 212.
57. Kala, *Planta medica*, 1958, **6**, 186.
58. Hegnauer, *Pharm. Weekbl.*, 1951, **86**, 805.
59. Romeike, *Pharmazie, Berl.*, 1953, **7**, 496.

60. Evans and Partridge, *J. Pharm. Pharmacol.*, 1953, **5**, 293, 772.
61. Romeike, *Flora*, 1956, **143**, 67.
62. Leete, Marion and Spenser, *Canad. J. Chem.*, 1954, **32**, 1116.
63. Trautner, *Austr. Chem. Inst. J. Proc.*, 1947, **14**, 411.
64. Leete, Kirkwood and Marion, *Canad. J. Chem.*, 1952, **30**, 749.
65. James and Butt, *Abh. dtsch. Akad. Wiss. Kl. Chem.*, 1957, No. 7, 182.
66. Areschkina, *ibid.*, 1957, No. 7, 196.
67. Rjabinin and Iljina, *Dokl. Akad. Nauk SSSR*, 1951, **76**, 851.
68. Mothes and Engelbrecht, *Flora*, 1956, **143**, 428.
69. Deltscheff, *Pharmazie, Berl.*, 1957, **12**, 686.
70. Engelbrecht and Unverricht, *Flora*, 1957, **145**, 236.
71. Conner, *Plant Physiol.*, 1937, **12**, 79.
72. Schreiber, *Abh. dtsch. Akad. Wiss. Kl. Chem.*, 1956 (7), 143.
73. Mothes and Silber, *Forsch. Fortschritte*, 1954, **28**, 101.
74. Archbold, *Ann. Bot.*, 1942, **6**, 488.
75. Sagromsky, *Z. Pflanzenzüchtg*, 1954, **33**, 267.
76. Watson, Thorne and French, *Ann. Bot.*, 1958, **22**, 321.
77. Gröger and Mothes, *Pharmazie, Berl.*, 1956, **11**, 323.
78. Gröger. *Arch. Pharm.*, 1958, **291**, 106.
79. Rothstein and Miller, *J. biol. Chem.*, 1954, **211**, 851.
80. Holtz and Miller, *Naunyn-Schmiedeberger Arch.*, 1925, **105**, 25.
81. Rochelmeyer, *Abh. dtsch. Akad. Wiss. Kl. Chem.*, 1957, 1956 (7), 250.
82. Mothes, Weygand, Gröger and Grisebach, *Z. Naturforsch.*, 1958, **13***b*, 41.
83. Richards and Coleman, *Nature Lond.*, 1952, **170**, 460.
84. Coleman and Richards, *Ann. Bot.*, 1956, **20**, 393.
85. Steinberg, *Plant Physiol.*, 1955, **30**, 84.
86. Steinberg and Jeffrey, *Plant Physiol.*, 1956, **31**, 377.
87. Scholz, *Z. Pflanzenernährg*, 1958, **80**, 149.
88. Steinberg and Jeffrey, *Plant Soil*, 1957, **9**, 64.
99. Barceló, Manrique and Morēno, *Bull. Soc. Chim. biol., Paris*, 1952, **34**, 1106.
90. Schmid and Serrano, *Experientia*, 1948, 6, 311.
91. Troll, *Abh. dtsch. Akad. Wiss. Kl. Chem.*, 1957, 1956 (7), 65.
92. Von Velsen, *Z. Pflanzenzüchtg*, 1950, **28**, 356.
93. Fisher and Loomis, *Science*, 1954, **119**, 71.
94. Fisher, *Bot. Gaz.*, 1955, **117**, 156.
95. Ramshorn, *Flora*, 1955, **142**, 601.
96. Ramshorn, *Kulturpflanze*, 1956, Beiheft **1**, 264.
97. Solotnizkaja, *Bull. Bot. Gart. Erevan*, 1948, (6) 29.
98. Izard, *Ann. Inst. Tabac Bergerac*, 1957, **2**, 11.
109. Müller and Ramshorn, *Flora*, 1957, **145**, 264.
100. Bachmann, *Phytopathol. Z.*, 1957, **29**, 117.
101. McIlwain, *Biochem. J.*, 1950, **46**, 612.
102. Frenyo, *Ann. Biol. Univ. Budap*, 1950, **1**, 88.
103. Case and McIlwain, *Biochem. J.*, 1951, **48**, 1.
104. Fahmy and Walsh, *ibid.*, 1954, **58**, 231.
105. Adams and Quastel, *Proc. Roy. Soc. Biol. Sci. B.*, 1956, **145**, 472.
106. Beránková, and Sorm, *Chem. Listy*, 1956, **50**, 2018.
107. Burton, Kaplan, Goldin, Leitenberg, Humphreys and Sodd, *Science*, 1958, **127**, 30.
108. Izard and Cuzin, *Ann. Inst. Tabac. Bergerac*, 1956, **2** (3), 11.

RESEARCH PAPERS

THE STABILITY OF RESCINNAMINE IN SOLUTION

From The Joint Committee of The Pharmaceutical Society of Great Britain and The Society for Analytical Chemistry on Methods of Assay of Crude Drugs

Analytical Methods Committee, Society for Analytical Chemistry,
14 Belgrave Square, London, S.W.1

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The ultra-violet absorption characteristics of solutions of rescinnamine in various solvents change when the solutions are exposed to daylight. The change, which is thought to be due to a *cis-trans* isomerism, is not accompanied by any detectable alteration in pharmacological properties.

IN 1956 the Pharmaceutical Society of Great Britain and the Society for Analytical Chemistry formed a Joint Committee to investigate methods of assay of crude drugs. A Working Panel* was set up, under the direction of the Committee, to examine methods of assay for rauwolfia.

While examining a method of assay for rauwolfia described by Carol, Banes, Wolff, and Fallscheer[1] variable results were obtained in some cases. The assay method, which was claimed to give a measure of both the reserpine and rescinnamine contents of rauwolfia, depends upon separation of these two weak bases from others present, followed by their hydrolysis to trimethoxybenzoic and trimethoxycinnamic acid respectively. The two acids are then determined in solution in chloroform by an ultra-violet absorption method making use of a two-point correction technique.

TABLE I

THE STABILITY OF TRIMETHOXYCINNAMIC ACID IN BORATE BUFFER SOLUTION (pH 10) WHEN STORED EXPOSED TO DAYLIGHT

Time after preparation of solution	*E* (1 per cent, 1 cm.) values at 290 mμ			
	Laboratory			
	A	B	C	D
Immediately	776	810	784	798
10 minutes	772	788	762	436
20 minutes	772	774	756	292
30 minutes	772	756	731	288
2 hours	718	560	589	288
24 hours	384	286	265	290
24 hours (in dark) ..	796	808	788	802

Samples of pure trimethoxybenzoic and trimethoxycinnamic acids were prepared and their light absorption characteristics in various solvents determined. Although good agreement could be obtained between four laboratories for the trimethoxybenzoic acid there was a wide discrepancy

*Mr. C. A. Johnson (*Chairman*) (Boots Pure Drug Co. Ltd.) and Messrs. T. Davies (CIBA Laboratories Limited), F. G. Farrell (The Pharmaceutical Society of Great Britain). J. J. Lewis (Department of Materia Medica, University of Glasgow) and A. W. Peacock (Riker Laboratories Ltd.) with Miss A. M. Parry as Secretary.

in results for the trimethoxycinnamic acid. Further tests showed that although solutions of the latter acid are stable for several hours when protected from light the ultra-violet absorption characteristics of solutions exposed to daylight change rapidly. Solutions were prepared in various solvents and exposed to daylight, and the extinction of 1-cm. layers were measured at intervals during 24 hours. The figures recorded in Table I show that although the rate of change was not the same in the various laboratories, the trend was similar in each case. In solvents such as borate buffer solution (pH 10) and methanol the wavelength of maximum absorption changed from about 290 mμ to about 270 mμ and the extinction of a 1-cm. layer of an 0·0005 per cent w/v solution at 290 mμ fell from about 0·400 to 0·145 within 24 hours or a little more. Further exposure to daylight brought about no further change. In chloroform, the solvent used by Carol and others, the maximum absorption of a fresh solution occurred at about 310 mμ and on exposure to light the extinction value decreased markedly although the wavelength of maximum absorption did not change very much. Figure 1 shows the absorption curves of a 0·0005 per cent solution of trimethoxycinnamic acid in chloroform before and after exposure to daylight.

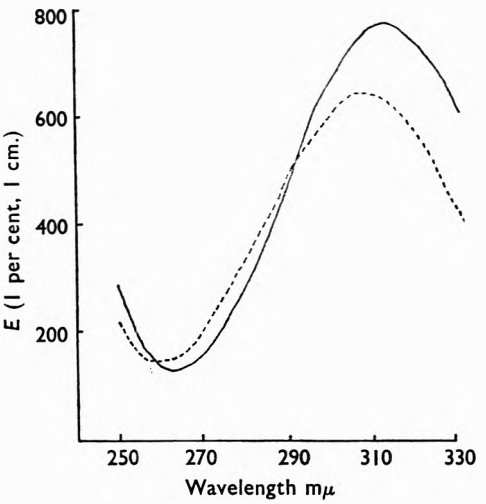

FIG. 1. Absorption curves of trimethoxycinnamic acid (0·0005 per cent) in chloroform

——— Before exposure to light.
- - - - - After 24 hours' exposure to light.

Because of these findings the light absorption characteristics of reserpine and rescinnamine were examined in a similar manner. Those of reserpine

TABLE II

THE STABILITY OF RESCINNAMINE IN ACETIC ACID SOLUTION WHEN STORED EXPOSED TO DAYLIGHT

Time after preparation of solution	*E* (1 per cent, 1 cm.) values at 300 mμ			
	Laboratory			
	A	B	C	D
Immediately	411	400	411	435
10 minutes	411	357	363	358
20 minutes	405	332	339	340
30 minutes	397	325	333	336
2 hours	356	315	322	332
24 hours	322	315	322	335
24 hours (in dark) ..	411	400	411	435

solutions showed little alteration when exposed to light for several hours, but those of rescinnamine solutions showed a marked and rapid change. Tables II,III and IV show the values of the extinction coefficients recorded for approximately 0·002 per cent w/v solutions of rescinnamine in 10 per

TABLE III

THE STABILITY OF RESCINNAMINE IN CHLOROFORM WHEN STORED EXPOSED TO DAYLIGHT

Time after preparation of solution	E (1 per cent, 1 cm.) values at 306 mμ			
	Laboratory			
	A	B	C	D
Immediately	393	385	—	394
10 minutes	375	355	—	320
30 minutes	351	332	—	312
2 hours	317	305	—	300
24 hours	295	293	—	296
24 hours (in dark) ..	394	385	—	407

cent acetic acid, chloroform and methanol respectively, while Figure 2 shows light absorption curves of a solution in acetic acid before and after exposure to daylight for 24 hours. The discrepancy in the initial extinction values obtained in the different laboratories might be due to changes already taking place during preparation of the solutions. About 24 hours exposure to daylight brings about the changes after which further exposure has no effect.

Two possible reasons for the changes have been considered and these are, (i) splitting of the double bond, and (ii) *cis-trans* isomerism.

The light absorption curve of a solution of trimethoxycinnamic acid in borate buffer solution is much more like that of a solution of trimethoxybenzoic acid after irradiation than it was originally. The relationship between the three curves is shown in Figure 3. This seems to support the first of the two possibilities but on the whole the second seems the more likely explanation. Changes in configuration can occur in substances capable of existing in geometrically isomeric forms when their solutions are exposed to light. For example,

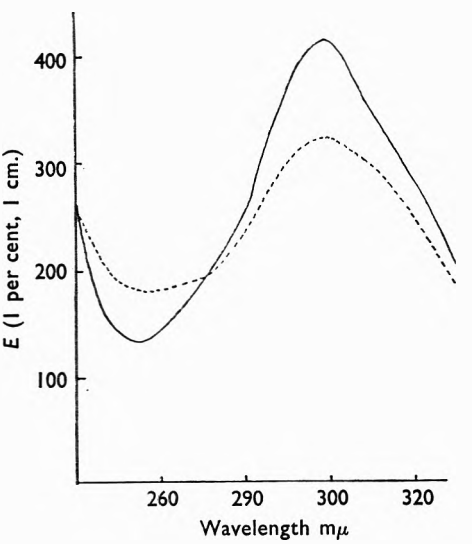

FIG. 2. Absorption curves of rescinnamine (0·002 per cent) in acetic acid

——— Before exposure to light.
- - - - - After 24 hours' exposure to light.

on exposure to ultra-violet light either maleic or fumaric acid is converted into an equilibrium mixture of the two acids. It is also well known that the light absorption characteristics of geometrical isomers may differ considerably.

TABLE IV

THE STABILITY OF RESCINNAMINE IN ABSOLUTE METHANOL WHEN STORED EXPOSED TO DAYLIGHT

Time after preparation of solution	E (1 per cent, 1 cm.) values at 302 mμ			
	Laboratory			
	A	B	C	D
Immediately	426	472	455	—
10 minutes	401	400	455	—
30 minutes	365	372	425	—
2 hours	343	370	412	—
24 hours	342	370	392	—
24 hours (in dark) ..	419	472	455	—

It was of importance to establish whether or not this change affected the pharmacological action of rescinnamine. An aqueous solution with the minimum quantity of dilute acetic acid was prepared to contain 2 mg. of the alkaloid in each millilitre and was divided into two equal portions, one of which was exposed to daylight for several hours while the other was protected from light. Suitable dilutions were examined spectrophotometrically to ensure that the change had occurred; the solutions were also tested by a modification of the colorimetric method of assay proposed by Banes, Wolff, Fallscheer and Carol[2]. This latter method is based upon the indole moiety of the rescinnamine molecule and thus, if the change were solely due to changes in the trimethoxycinnamoyl moiety of the molecule, it would be expected to give a similar result with solutions both protected and exposed to light. This was found to be the case. The main bulk of each of the two solutions was used for pharmacological tests.

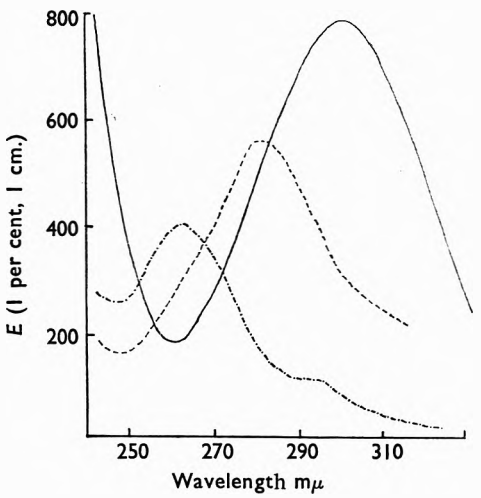

FIG. 3. Absorption curves of trimethoxybenzoic and trimethoxycinnamic acids in borate buffer solution (pH 10)

— · — Trimethoxybenzoic acid.
——— Trimethoxycinnamic acid before exposure to light.
- - - - - Trimethoxycinnamic acid after 24 hours' exposure to light.

The pharmacological tests were carried out by methods similar to those described by Zoha, Kirpekar and Lewis[3]. Briefly, the test solutions were

compared with a control solution on the blood pressure of anaesthetised cats and also upon certain vasopressor reflexes, namely those elicited by stimulation of the central end of the cut vagus, and by compression of the abdominal aorta and of the common carotid arteries. The effect upon the response of blood pressure to stimulation of the splanchnic nerve was also ascertained. No differences could be observed between the control and test solutions.

These qualitative investigations thus indicate that certain pharmacological properties of rescinnamine are not altered by exposing solutions to daylight and the problem therefore resolves itself into a purely analytical one. It is apparent that, without modification, the method of assay of rauwolfia proposed by Carol and others[1] is untrustworthy. It is also clear that the ultra-violet absorption characteristics of rescinnamine cannot be used as a basis for the determination of the alkaloid. It may be that the amount of trimethoxycinnamic acid or of rescinnamine in a solution might be obtained by subjecting the solution to irradiation with ultra-violet light until the light absorption characteristics became constant. Such a procedure could not readily be applied to solutions containing mixtures of reserpine and rescinnamine or of the acids resulting from their hydrolysis, however, since the effect of irradiation is to impair the favourable conditions necessary for the application of the two-point correction procedure.

References

1. Carol, Banes, Wolff and Fallscheer, *J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 200.
2. Banes, Wolff, Fallscheer and Carol, *ibid.*, 1956, **45**, 708.
3. Zoha, Kirpekar and Lewis, *J. Pharm. Pharmacol.*, 1958, **10**, *231T.*

THE STABILITY OF THIOGLYCOLLATE SOLUTIONS

PART I. EFFECTS OF METHOD OF PREPARATION OF SOLUTIONS, pH AND TEMPERATURE UPON THE OXIDATION OF THIOGLYCOLLATE

BY A. M. COOK AND K. J. STEEL

From the Department of Pharmaceutics, School of Pharmacy, University of London, Brunswick Square, London, W.C.1

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The pH of, and amount of oxidation occurring in, thioglycollate solutions is influenced by the method of preparation of the solutions from thioglycollic acid. When heat is employed to effect solution, the oxidation of the resultant thioglycollate increases with increase in pH and temperature of storage. Solutions prepared without heat may exhibit a more alkaline reaction than expected. Storage of such solutions causes a fall in their pH, the extent of which is determined by the temperature and time of storage. The effect of storage at different temperatures upon the oxidation of thioglycollate is not as great with unheated solutions as in heated solutions. Oxidation of thioglycollate is increased by dilution. The dithiodiglycollate, produced on oxidation of thioglycollate, itself undergoes decomposition in alkaline conditions.

AMONGST the many diverse uses of thioglycollic acid is its use in bacteriology, both in the preparation of media for supporting the growth of anaerobic organisms[1] and as an inactivating agent for mercurial bacteriostats when testing preparations for sterility[2–5].

The use of sodium thioglycollate is common in the United States for these purposes, whilst it is more usual in this country to prepare the sodium salt *in situ* by neutralisation of the acid with sodium hydroxide. In his original paper, Brewer[1] considered sodium thioglycollate to be relatively stable and preferable to the acid which is a syrupy liquid and requires pH adjustment when incorporated into media.

Doubts on the stability of sodium thioglycollate have been expressed[3] and it has the disadvantages of being hygroscopic and tending to discolour on exposure to air, an aqueous solution of the discoloured material being yellowish.

During an investigation of the inactivation of mercurial bacteriostats by thioglycollate media, it was observed that peptone water containing 0·01 per cent of thioglycollic acid (neutraised to pH 7 with sodium hydroxide) after standing at room temperature for six hours, gave no red colouration with ammoniacal sodium nitroprusside whereas a freshly prepared sample produced an immediate red colour, indicating the presence of sulphydryl groups. Heating the older sample at 98–100° for up to 20 minutes and cooling before testing did not affect the result. Failure to detect sulphydryl in the stored medium implies that oxidation of the thioglycollate has occurred. As the value of thioglycollate media, both for the cultivation of anaerobes and the inactivation of mercurial

bacteriostats, is dependent upon the availability of sulphydryl groups, their oxidation will render the media useless for these purposes. It was decided therefore to investigate the oxidation of thioglycollic acid and factors associated with it.

Experimental and Results

The thioglycollate medium has been prepared by the addition of a sterile solution of thioglycollic acid, neutralised to pH 7 with sodium hydroxide, to the peptone water immediately before use. The final medium had a pH of 7·4–7·5.

The peptone water and the thioglycollate solution were each tested for the presence of metallic ions which could catalyse the oxidation of thioglycollate. Peptone is known to contain traces of metals but these could not be detected in a 1 per cent solution. No positive reactions for cupric ions were obtained with α-benzoin-oxime ("cupron"), dithio-oxamide ("rubeanic acid") or sodium diethyldithiocarbamate; for ferric ions with ammonium thiocyanate or 7-iodo-8-hydroxyquinoline-5-sulphonic acid ("ferron"); or for ferrous ions with dimethylglyoxime or 2:2′-dipyridyl. As no gross contamination with metallic ions, which could catalyse the oxidation, was demonstrable, it was assumed that the loss of thioglycollate was a result of atmospheric oxidation, which might be expected to be accelerated at this low concentration and slightly alkaline reaction of the medium.

Effect of Heat on pH

One per cent solutions of thioglycollic acid were prepared with the addition of 10·7, 10·85 and 11 ml. of N sodium hydroxide solution per 100 ml. of final solution. Theoretically, 1 g. of thioglycollic acid requires 10·85 ml. of N NaOH for neutralisation.

Determined at ten minute intervals up to three hours after preparation, the range of pH values (glass electrode) were 8·25 to 8·35, 8·45 to 8·6 and 8·95 to 9·15 respectively. Portions of the solutions autoclaved at 115–116° for 15 minutes and cooled had pH values of 5·4 ,7·2 and 8·8 respectively.

Effect of Storage on pH

One per cent thioglycollic acid solutions containing varying amounts of sodium hydroxide solution were again prepared, half of each solution being autoclaved and the remainder unheated. The solutions were stored at 4, 20 and 37° in glass-stoppered bottles in the dark and their pH was determined initially and after 3, 8, 14, 24 and 40 days storage. As the dissociation constant Ka varies with temperature, samples were allowed to reach 20° before measuring their pH. The solutions prepared with heat maintained their reactions within the following limits, under the three storage conditions, "acid" solution, pH 5·35 to 5·45; "neutral" solution, pH 6·95 to 7·10; "alkaline" solution, pH 7·80 to 8·00.

The results for the solutions prepared without heat mostly showed a considerable fall, see Table I.

Storage of the autoclaved thioglycollate solutions produced no appreciable change in their pH. Solutions prepared without heat and stored at 37° showed a fall in pH, which after 14 days reached a value similar to that of the corresponding heated solutions. The unheated "acid" solution stored at 20° showed a gradual fall in pH until after 40 days storage its pH was only slightly above that of the heated "acid" solution.

TABLE I

EFFECT OF STORAGE UPON THE pH OF THIOGLYCOLLATE SOLUTIONS PREPARED WITHOUT HEAT

Days of storage	0	3	8	14	24	40
"Acid" solution at 4°	8·30	8·45	8·60	8·80	8·20	7·05
20°	8·30	8·25	8·15	7·55	6·75	5·55
37°	8·30	7·50	5·75	5·50	5·50	5·40
"Neutral" solution at 4° ..	8·40	8·55	8·80	8·90	8·50	8·10
20° ..	8·40	8·45	8·45	8·30	7·75	6·95
37° ..	8·40	8·10	7·20	7·00	7·00	7·00
"Alkaline" solution at 4° ..	8·50	8·65	8·90	9·00	8·80	8·60
20° ..	8·50	8·55	8·70	8·90	8·60	8·20
37° ..	8·50	8·30	8·00	7·60	7·60	7·60

The reactions of the remaining unheated solutions increased up to 8 to 14 days, after which their pH began to fall. All the unheated solutions stored at 4° were more alkaline after 40 days storage than the corresponding heated solutions, whilst the unheated solutions after 40 days storage at 20° had a pH similar to those of the heated solutions.

Effect of Preparation upon the Oxidation of Thioglycollate

One per cent thioglycollic acid solutions containing the theoretical quantity of sodium hydroxide solution for neutralisation were prepared. Half of the solution was autoclaved at 115–116° for 30 minutes whilst the remainder was sterilised by passing through a 5/3 sintered glass filters. After sterilisation, the solutions were stored in 100 ml. glass-stoppered bottles at 20° in the dark. Samples were removed for assay initially and after 24, 90 and 120 hours storage. Their thioglycollate content was determined by titration with potassium iodate solution in acid conditions[6], with the results shown in Table II.

Effect of pH and Method of Preparation on the Oxidation of Thioglycollate during Storage

On the results of the above preliminary experiments, the following investigation was designed. Solutions containing 1 per cent of thioglycollic acid and varying quantities of sodium hydroxide solution were prepared, and sterilised by heating in an autoclave or by filtration. The alkali content of each solution and its initial pH is shown in Table III. Portions of each solution were stored in glass-stoppered bottles in the dark at 4, 20 and 37°. After varying intervals, samples were withdrawn and their thioglycollate content determined by the potassium iodate method. As temperature is reported[7] to markedly affect the iodine consumption of thioglycollic acid, samples for assay were rapidly cooled to about

10° before being titrated. Some typical results are shown in Table IV which relates the percentage of the thioglycollate which has oxidised to the storage temperature and initial reaction of the solutions. Repetition of the experiments with 0·1 per cent thioglycollate solutions showed a much more rapid oxidation rate, up to 25 per cent of the thioglycollate being oxidised in 24 hours.

TABLE II

THIOGLYCOLLATE CONTENT (per cent w/v) OF NOMINAL 1 PER CENT SOLUTIONS STERILISED BY AUTOCLAVING OR FILTRATION, AFTER STORAGE AT 20°

	Time of storage in hours			
	0	24	90	120
Autoclaved	1·0	0·964	0·609	0·233
Filtered	0·907	0·871	0·677	0·534

TABLE III

ALKALI CONTENT AND INITIAL pH OF 1 PER CENT THIOGLYCOLLIC ACID SOLUTIONS, STERILISED BY AUTOCLAVING OR FILTRATION

Solution	Amount of N NaOH per 100 ml. of solution	Initial pH	
		Autoclaved	Filtered
"Acid"	10·7 ml.	5·4	8·3
"Neutral" ..	10·85 ml.	7·4	8·4
"Alkaline" ..	11·0 ml.	7·8	8·6

TABLE IV

PERCENTAGE OXIDATION OF THIOGLYCOLLATE OCCURRING IN AUTOCLAVED AND FILTERED 1 PER CENT THIOGLYCOLLATE SOLUTIONS, AFTER STORAGE AT DIFFERENT TEMPERATURES FOR 24 HOURS

Storage temperature	Autoclaved solution, oxidation per cent			Filtered solution, oxidation per cent		
	Acid	Neutral	Alkaline	"Acid"	"Neutral"	"Alkaline"
4°	2·8	5·4	8·0	5·0	6·2	6·5
20°	4·3	7·1	9·7	5·3	8·1	8·5
37°	8·0	9·9	11·8	5·3	6·3	6·3

Presence of Dithiodiglycollate in Thioglycollate Solutions after Storage

Thioglycollate solutions containing 1·0 or 0·1 per cent of the acid when first prepared and adjusted to an acid, neutral or alkaline reaction, were tested for the presence of sulphydryl groups, with ammoniacal sodium nitroprusside solution, after two months storage at 4, 20 or 37°. Samples in which a positive reaction was obtained were diluted with purified water until no red colouration was produced on addition of the reagent; dilution more than ten times was never required. The solutions were then tested for the presence of disulphide by the method of Walker[8], which reduces disulphides to sulphydryl compounds with potassium cyanide. Of the solutions tested, only those having an initial alkaline reaction and stored at 37° failed to give a positive reaction for the presence of disulphide.

Discussion

Thioglycollic acid may be prepared by the action of sodium chloracetate on sodium hydrosulphide or by the reduction of dithiodiglycollic acid, prepared from sodium chloracetate and sodium disulphide. Besides thioglycollic acid, the reaction mixture before extraction and purification contains both dithiodiglycollic and thiodiglycollic acids with dithioglycollide and other thioglycollides[9]. After extraction, purification is by vacuum distillation.

Thioglycollic acid is commercially available in two forms, anhydrous and an aqueous preparation, containing about 97–98 and 75 per cent of thioglycollic acid respectively. Each contains a small quantity of dithiodiglycollic acid (about 0·5 per cent) and the remaining 2 per cent or so of unaccounted material, in the anhydrous product, probably consists of dithioglycollide.

By heating thioglycollic acid in a stream of nitrogen, esters of varying molecular weight have been isolated[10] which are considered to be straight chain polythioglycollic esters of structure

$$HSCH_2CO(SCH_2CO)_nSCH_2COOH$$

where n can be any integer from 7 to 21.

$$(n+2)\ HSCH_2COOH \longrightarrow HSCH_2CO(SCH_2CO)_nSCH_2COOH + (n+1)H_2O$$

It is believed that on heating, these esters rearrange to form the cyclic dithioglycollide which can be separated by vacuum distillation. Dithioglycollide (2:5-dioxo-1:4-dithiane) may also be derived by the condensation of two molecules of thioglycollic acid with the elimination of the elements of two molecules of water[11], the reaction being analogous to the formation of lactide from lactic acid. Mulvaney[9] considers that such a dehydration occurs readily at the temperature required for distillation and expresses the reaction thus:

$$n\,HS{\cdot}CH_2{\cdot}COOH \longrightarrow (S{\cdot}CH_2CO)_n + nH_2O$$

It should be noted however that a monomeric cyclic thio-ester where $n = 1$ does not exist[11].

Dithioglycollide also forms at normal room temperatures, up to 4 per cent of the thioglycollic acid in a freshly distilled sample being dehydrated in a month[9]. The reaction resulting in its formation is reversible[9] but the rate of hydration is slow at ordinary temperatures; it may be accelerated by dilution, temperature rise or the use of mineral acid as catalyst.

Schöberl and Krumey[11] report that prolonged heating of dithioglycollide with water causes its hydrolysis, whilst alkalies rapidly hydrolyse it, the resultant thioglycollic acid being detectable both acidimetrically and iodimetrically. Their results showed that one of the CO–S bonds is rapidly broken by alkaline hydrolysis even at room temperatures forming *S*-thioglycollylthioglycollic acid. Other properties of dithioglycollide have been reported by Schöberl and Wiehler[10,12].

The heated thioglycollic acid solutions had the expected pH, whereas solutions prepared without heat remained alkaline (Table III). In the unheated solutions, it appears that all the thioglycollic acid has not reacted with the sodium hydroxide and it may be inferred that this unreacted acid is in some form other than thioglycollic acid. It could be present as dithiodiglycollic acid or as dithioglycollide. The first of these two compounds being a dicarboxylic acid would be expected to react with alkali in a manner similar to thioglycollic acid, and it is therefore more probable that dithioglycollide accounts for the unreactable material. The titration of thioglycollic acid with alkali does not show a drifting end point and presumably the dithioglycollide is stable towards alkali in the cold. In this respect it differs from the lactide of lactic acid, but this might be expected since sulphur compounds usually have a greater stability than the corresponding oxygen compounds.

In the heated thioglycollate solutions, more sodium hydroxide was utilised, causing a fall in pH, and this infers that hydrolysis of the dithioglycollide occurs on heating with alkali. This hydrolysis may be partial to produce *S*-thioglycollylthioglycollic acid, or complete to produce thioglycollic acid; in either case the production of carboxylic acids will decrease the amount of free alkali in the system.

Assay of samples of thioglycollic acid will not determine any dithioglycollide present, although some of it may be hydrolysed during the Pharmacopoeial assay process and its hydrolysis products will then be determined[6]. In a fresh sample of thioglycollic acid, the dithioglycollide content may be expected to be small (less than 2 per cent) but in older samples this value may be increased. If a sample contains an appreciable amount of dithioglycollide, the addition of sufficient alkali to neutralise the thioglycollic acid (based on the assay results) will produce a solution the pH of which will vary with the heat used in its preparation.

In bacteriological work, sterile thioglycollate solutions are usually required, and it may be questioned whether these solutions should be sterilised by heat or by filtration. Solutions sterilised by heat have the desired pH immediately; sterilised without heat, the use of sintered glass filters is essential to avoid contamination with metals. This method of preparation has the disadvantage that the final solution may have a more alkaline reaction than expected, especially if the thioglycollic acid had a high dithioglycollide content.

From the results in Table I of the effects of storage upon the pH of thioglycollate solutions, it is evident that some change is occurring in the unheated solutions which does not occur in heated solutions. The most probable explanation is that hydrolysis of dithioglycollide results from prolonged contact with alkali, since all the unheated solutions were initially alkaline. Schöberl and Krumey[11] have shown the hydrolysis of dithioglycollide by alkali to be accelerated by increase in temperature. This would explain the more rapid decrease in pH observed with those solutions stored at 37° compared with those stored at 20°, and the slight fall in pH of solutions stored at 4°.

The Oxidation of Thioglycollic Acid

The main loss of thioglycollic acid on storage is by oxidation to dithiodiglycollic acid. The oxidation is catalysed by copper[9,13–16], iron [9,13,14,17] and cobalt[18] but not by zinc[9]. The rate of oxidation varies with the pH[19], presence or absence of buffer[14] and the concentration of metallic catalyst. The disulphide formed on oxidation also acts as a catalyst in the auto-oxidation of thioglycollic acid[14,15,19,20]. Other reported oxidation products include hydrogen peroxide[16,21] and sulphuric acid[13].

Two main problems, apart from pH, appear to be associated with the question of sterilisation of thioglycollate solutions; first, does heat treatment accelerate the oxidation and, secondly, does the increased surface area of the solution during filtration cause increased oxidation? From the results in Table II it is seen that the unheated solution had a lower thioglycollate content than the heated solution when first prepared. As both solutions were made with the same quantity of thioglycollic acid, it is inferred that the different thioglycollate content is due to the presence of unreactable dithioglycollide in the unheated solution. On storage at 20°, the heated solution appeared to undergo oxidation more rapidly than the unheated solution. Fuller conclusions can be drawn from the later investigation, of which the results shown in Table IV are an example. From this more extensive investigation, the following conclusions were drawn.

Solutions which have been heated have a higher thioglycollate content initially than unheated solutions. The rate of oxidation of thioglycollate in heated solutions increases with increasing pH and storage temperature. The effects of the reaction of the solutions prepared without heat were not as obvious as those of the heated solutions. Storage of unheated solutions at 20° produced more oxidation than storage at 4° or 37°. It is believed that storage at 4° minimises oxidation, as in the case of heated solutions, whereas storage at 37° allows some hydrolysis of dithioglycollide which increases the sulphydryl content and reduces the alkalinity. "Acid" solutions appeared to undergo less oxidation than the "neutral" or "alkaline" solutions, but the difference between these two was much less well marked than in the heated solutions. Solutions containing 0·1 per cent of thioglycollate underwent much more rapid oxidation than the corresponding 1 per cent solutions.

The positive results obtained with the test for disulphide in old samples of thioglycollate solutions may be assumed to be caused by dithiodiglycollic acid, or its sodium salt, which is the oxidation product of thioglycollic acid. From the results reported it is seen that oxidation of thioglycollate occurred most rapidly in solutions adjusted to an alkaline reaction and stored at an elevated temperature; by the absence of disulphide in such solutions is it inferred that the disulphide has itself undergone further decomposition. The decomposition of sodium dithiodiglycollate has been reported[15,19,22,23], although the pH at which this decomposition occurs (above about 9 to 9·5) is higher than that in the solutions tested (initial pH about 8·5). Amongst the reported decomposition products are sodium glyoxalate[19] and sodium oxalate[22].

From this investigation, the following recommendations are proposed. For maximum stability, thioglycollate solutions should be adjusted to maintain an acid reaction; in the case of solutions which are to be mixed with bacteriological media, the buffering capacity of the medium should be normally sufficient to maintain the final mixture at the required pH. Similarly, thioglycollate solutions should be stored at as low a temperature as possible, preferably in a refrigerator. These solutions should be stored in well filled, well closed containers to reduce the volume of air in contact with the solution. Sterile solutions of thioglycollate may be prepared by autoclaving.

References

1. Brewer, *J. Amer. med. Ass.*, 1940, **115**, 598.
2. Marshall, Gunnison and Luxen, *Proc. Soc. exp. Biol. N.Y.*, 1940, **43**, 672.
3. Daily and Blubaugh, *J. Bact.*, 1941, **42**, 147.
4. Graydon and Biggs, *Med. J. Aust.*, 1942, **2**, 513.
5. Nungester, Hood and Warren, *Proc. Soc. exp. Biol. N.Y.*, 1943, **52**, 287.
6. Steel, *J. Pharm. Pharmacol.*, 1958, **10**, 574.
7. Lucas and King, *Biochem. J.*, 1932, **26**, 2076.
8. Walker, *ibid.*, 1925, **19**, 1082.
9. Mulvaney, *Proc. Sci. Sect. Toilet Goods Assoc.*, 1946, **5**, 33.
10. Schöberl and Wiehler, *Ann.*, 1955, **595**, 101.
11. Schöberl and Krumey, *Ber.*, 1944, **77B**, 371.
12. Schöberl and Wiehler, *Angew. Chem.*, 1954, **66**, 273.
13. Wieland and Franke, *Ann.*, 1928, **464**, 101.
14. Elliott, *Biochem. J.*, 1930, **24**, 310.
15. Kharasch, Legault, Wilder and Gerard, *J. biol. Chem.*, 1936, **113**, 537.
16. Holtz and Triem, *Z. physiol. Chem.*, 1937, **248**, 1.
17. Schubert, *J. Amer. chem. Soc.*, 1932, **54**, 4077.
18. Michaelis and Schubert, *ibid.*, 1930, **52**, 4418.
19. Dixon and Tunnicliffe, *Proc. Roy. Soc. Lond. B.*, 1923, **94**, 266.
20. Harrison, *Biochem. J.*, 1927, **21**, 1404.
21. Schales, *Ber.*, 1938, **71B**, 447.
22. Schöberl, *Ann.*, 1933, **507**, 111.
23. Shinohara and Kilpatrick, *J. biol. Chem.*, 1934, **105**, 241.

EFFECTS OF CHLORPROMAZINE, PROMAZINE, DIETHAZINE, RESERPINE, HYDROXYZINE, AND MORPHINE UPON SOME MONO- AND POLYSYNAPTIC MOTOR REFLEXES*

BY B. SILVESTRINI AND G. MAFFII

From the Department of Pharmacology, Lepetit S.p.A., Milan, Italy

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The effects of chlorpromazine, promazine, diethazine, reserpine, hydroxyzine and morphine on some mono- and polysynaptic motor reflexes have been investigated in intact, spinal and decerebrate cats and rabbits. Chlorpromazine, promazine, reserpine and hydroxyzine selectively depress the monosynaptic spinal reflexes (knee jerk) of the intact animals. Polysynaptic reflexes (linguo-mandibular and crossed extensor) are slightly affected. In both cats and rabbits the brain of which was disconnected from the spinal cord by surgical sections at different levels, the inhibitory action of these drugs disappears. Morphine and diethazine, on the contrary, selectively depress the polysynaptic reflexes (linguo-mandibular) and in a minor degree the monosynaptic ones (knee jerk): this action is retained in spinal animals. These findings are discussed and related to other pharmacological properties of the compounds.

How chlorpromazine and reserpine affect the spinal reflex activity appears to depend upon experimental conditions. With reserpine, Schneider and colleagues[1] reported a facilitation of the monosynaptic responses in spinal and decerebrate cats, while Krivoy[2] observed a predominant inhibition of the monosynaptic transmission in the spinal cord of intact and decerebrate animals. Bein[3] reported that no inhibition of either mono- or polysynaptic reflexes occurred after reserpine. With chlorpromazine, Dasgupta and Werner[4] described an inhibition of the crossed extensor reflex in decerebrate cats: in the spinal animal this reflex was less affected. Preston[5], working on spinal cats, did not observe chlorpromazine to have any effect even in very high doses, on spinal reflex discharges recorded from the ventral roots. Krivoy[2] found a predominant inhibition of the monosynaptic transmission in intact and decerebrate animals. Bein[3] showed that the inhibition of mono- and polysynaptic spinal reflexes produced by chlorpromazine is suppressed after a transection of the cervical spinal cord.

In view of these contrasting results it seemed of interest to make a systematic reinvestigation of the actions of reserpine and chlorpromazine at the level of motor reflex activity in intact, spinal and decerebrate animals and a comparison of their effects with those of other related compounds.

EXPERIMENTAL

Methods

Twenty-two cats and 41 rabbits, of both sexes, weighing respectively 2–3 and 1·9–2·8 kg. were used.

* A preliminary report of this work was presented at the X Congresso della Società Italiana di Farmacologia in Naples (Italy), October 7–8, 1958.

The knee jerk was elicited by tapping with a mechanical hammer the patellar tendon every 1 or 2 seconds. The linguo-mandibular reflex was obtained by stimulating the tongue through silver electrodes (square wave pulses; 100 cycles/sec.; 1 msec. duration) at the rate of one every 2 to 6 seconds. The crossed extensor reflex was elicited every 2 or 4 seconds by means of the stimulation (square wave pulse; 100 cycles/sec.; 0·5 msec. duration) of the central end of the contralateral sciatic nerve. The voltage was just above threshold. A Grass Model S4D stimulator with isolation unit was used. Reflex responses were kymographically recorded. Aqueous solutions of different concentrations were used.

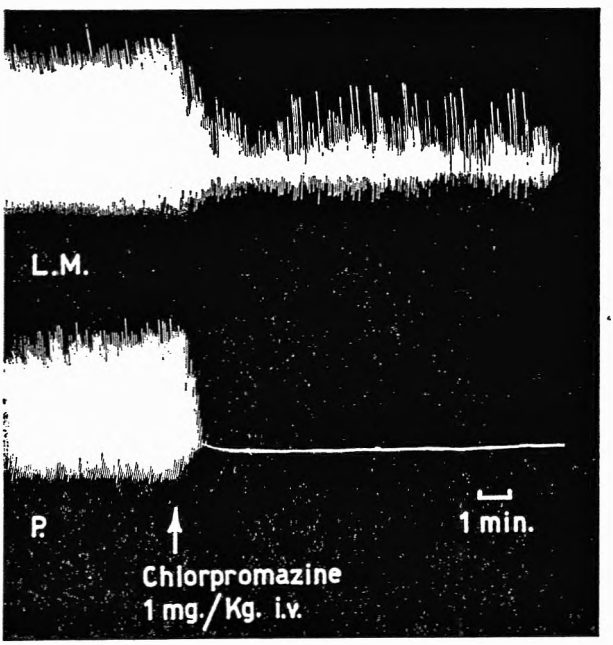

FIG. 1. Effects of chlorpromazine on linguo-mandibular (upper tracing) and patellar (lower tracing) reflexes. After 1 mg./kg. of chlorpromazine the knee jerk is suppressed and the linguo-mandibular reflex is decreased. Cat weighing 2·9 kg., anaesthetized with chloralose.

Solution of reserpine was prepared thus: reserpine 0·25 g., benzyl alcohol 2 ml., citric acid 0·25 g., propylene glycol 10 ml., distilled water to 100 ml.

In some experiments the blood pressure was recorded from the right common carotid artery.

In a first set of experiments intact animals were anaesthetized either with chloralose (70 mg./kg.) i.p. or urethane (1 g./kg. s.c.) or chloralose and urethane together (50 and 500 mg./kg.). By this procedure, eight of ten animals gave successful experiments. An additional group of animals was deeply anaesthetized with ether for section of the spinal cord (at cervical or lumbar level) or of the mesencephalon. Records were made

when the animals had recovered. In some experiments the spinal sections were made on animals previously anaesthetized with chloralose or urethane, to ascertain the effects of anaesthetics on spinal preparations. Here, six of ten experiments were successful.

Results

From Table I, which presents our results, it may be seen that in the intact rabbit and cat, anaesthetized with chloralose and urethane or both, the patellar reflex is markedly inhibited by *chlorpromazine* in doses of 0·5–1·5 mg./kg. The onset of this action was quick and its duration very prolonged (Fig. 1: lower tracing). The linguo-mandibular reflex was markedly suppressed in 3 of 11 experiments (Fig. 1: upper tracing), in the others being unaffected or slightly inhibited. Figure 2 shows that mephenesin acts in an opposite manner. In spinal cats and rabbits, chlorpromazine may still inhibit the linguo-mandibular reflex, but it does not affect the knee jerk. After the chlorpromazine, a fall in the blood pressure (10–30 mm. Hg) was observed in both intact and spinal animals, but no clear relation exists between the hypotensive effect and degree of inhibition of the reflexes. With decerebrate rabbits, a slight inhibition of the patellar reflex was caused by the drug when the section was made at the intercollicular level, while after a pontine or prepontine section the effects disappeared.

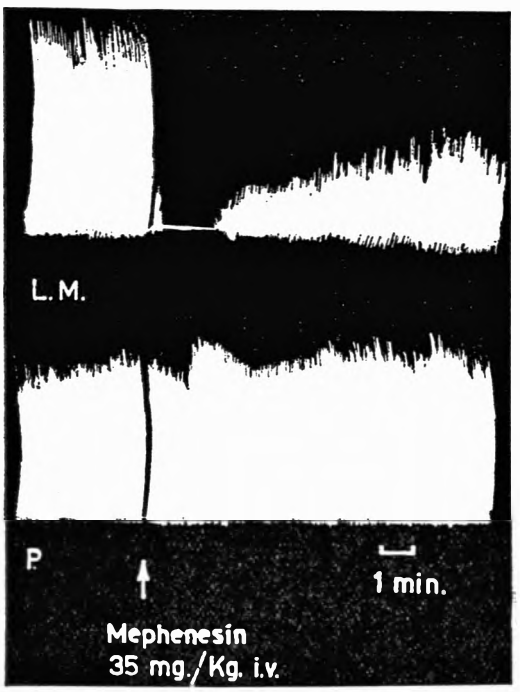

FIG. 2. Effects of mephenesin on linguo-mandibular (upper tracing) and patellar reflexes (lower tracing). After 35 mg./kg. of mephenesin the linguo-mandibular reflex is inhibited, while the patellar one is almost unaffected. Cat weighing 3 kg., anaesthetized with chloralose.

The effects of *reserpine* were studied on the linguo-mandibular, patellar and crossed extensor reflex and were different in rabbits and cats. After 0·4–1 mg./kg. the patellar reflex is slowly depressed in the intact rabbit, the decrease being usually complete in 15 minutes and lasting over two hours. In the intact cat the effects were less constant. In one experiment

no effect was observed after 2 mg./kg. In the others the inhibition sometimes lasted about one hour and often appeared after an initial facilitation of the knee jerk response (Figs. 3 and 4). The effects of reserpine on the polysynaptic reflexes were similar in both species, these reflexes being less

FIG. 3. Effect of reserpine on the patellar reflex of intact cat. 1 mg./kg. of reserpine induces an increase and then a disappearance of the knee jerk. Cat weighing 2·9 kg., anaesthetized with chloralose and urethane.

affected than the monosynaptic one (Fig. 4), being clearly inhibited only in 3 of 12 experiments. After the sectioning the spinal cord the knee jerk was not inhibited and in the cat usually became irregular and was even enhanced. In one rabbit and one cat the inhibition of the patellar reflex produced by reserpine in the intact animals was suppressed after a spinal section. However, this procedure produced in other animals a fall in the blood pressure such to induce us to disregard the results. Reserpine in decerebrate rabbits, in doses of 1–1·5 mg./kg., slightly inhibited the knee jerk reflex in one of 3 experiments. In this instance the section was intercollicular, while in the other two experiments the upper portion of the pons was involved. Blood pressure of intact animals is regularly and slowly lowered by reserpine. In spinal animals no effect was normally observed, though in some instances sudden falls of the blood pressure appeared. Concurrently with these pressure changes, both the patellar and linguo-mandibular reflexes may be temporarily depressed.

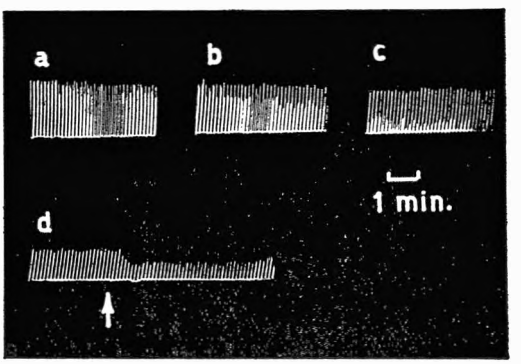

FIG. 4. Effects of reserpine on the patellar and the crossed extensor reflexes of intact animal. (*a*) Simultaneous recording of both patellar and crossed extensor reflexes. (*b*) 20 minutes after 0·5 mg./kg. of reserpine. The knee jerk appears to be inhibited, while the crossed extensor reflex is almost unaffected. (*c*) 30 minutes after reserpine. The knee jerk is markedly inhibited; the crossed extensor reflex is almost unaffected. (*d*) 60 minutes after reserpine. Mephenesin, 20 mg./kg. (at the arrow) produces an inhibition of the crossed extensor reflex. Cat weighing 2·2 kg., anaethetized with chloralose.

TABLE I

INDIVIDUAL RESULTS

Animal	Preparation	Drug mg./kg.	Linguo-mandibular reflex	Patellar reflex	Crossed extensor reflex	Depression of blood pressure in mm. Hg
Rabbit ..	Intact (chloralose)	Chlorpromazine 1	Inhibited	Inhibited	—	− 30
Rabbit ..	Intact (chloralose)	Chlorpromazine 0·5	Inhibited	Inhibited	—	—
Rabbit ..	Intact (chloralose)	Chlorpromazine 1	Slightly inhibited	Inhibited	—	—
Rabbit ..	Intact (chloralose)	Chlorpromazine 1	Unaffected	Inhibited	—	− 20
Rabbit ..	Intact (chloralose)	Chlorpromazine 1·5	Slightly inhibited	Inhibited	—	− 15
Rabbit ..	Intact (chloralose + urethane)	Chlorpromazine 1·5	—	Inhibited	—	—
Rabbit ..	Intact (chloralose + urethane)	Chlorpromazine 0·5	—	Inhibited	—	—
Rabbit ..	Intact (chloralose + urethane)	Chlorpromazine 1	Slightly affected	Inhibited	—	—
Cat ..	Intact (chloralose)	Chlorpromazine 0·5	Inhibited	Inhibited	—	—
Cat ..	Intact (chloralose)	Chlorpromazine 0·5	Slightly affected	Inhibited	—	—
Cat ..	Intact (chloralose + urethane)	Chlorpromazine 1	Unaffected	Inhibited	—	− 30
Rabbit ..	Spinal (C1)	Chlorpromazine 1	Inhibited	Unaffected	—	—
Rabbit ..	Spinal (L1)	Chlorpromazine 1	Slightly inhibited	Unaffected	—	—
Rabbit ..	Spinal (L1) (chloralose)	Chlorpromazine 1	—	Unaffected	—	—
Rabbit ..	Spinal section (L1) chloralose + urethane)	Chlorpromazine 1·5	—	Unaffected	—	− 10
Rabbit ..	Intercollicular section	Chlorpromazine 1	—	Slightly inhibited	—	—
Rabbit ..	Prepontine section	Chlorpromazine 1	—	Unaffected	—	—
Rabbit ..	Pontine section	Chlorpromazine 1	—	Unaffected	—	—
Cat ..	Spinal (C1)	Chlorpromazine 1 + 1	Unaffected Inhibited	Unaffected Unaffected	—	—
Cat ..	Spinal section	Chlorpromazine 1·5	Slightly affected	Unaffected	—	—
Rabbit ..	Intact (chloralose)	Reserpine 0·3	Almost unaffected (irregular)	Slightly inhibited (irregular)	—	—
Rabbit ..	Intact (chloralose)	Reserpine 1 Mephenesin 20	Unaffected Inhibited	Inhibited Unaffected	—	—
Rabbit ..	Intact (chloralose)	Reserpine 0·4	—	Inhibited	—	—
Rabbit ..	Intact (chloralose)	Reserpine 1	Unaffected	Enhanced	—	− 10
Rabbit ..	Intact (chloralose) Spinal section (L1)	Reserpine 0·4	— —	Inhibited Return	—	—
Rabbit ..	Intact (urethane)	Reserpine 0·5	Slightly inhibited	Inhibited	—	—
Rabbit ..	Intact (chloralose + urethane)	Reserpine 0·4	No effect	Inhibited	—	− 10
Cat ..	Intact (chloralose)	Reserpine 1 + Reserpine 1	Almost unaffected	Almost unaffected	—	—
Cat ..	Intact (chloralose)	Reserpine 1	Inhibited	Enhanced and then inhibited	—	—
Cat ..	Intact (chloralose)	Reserpine 1·5	—	Inhibited 1 hour later	—	− 30
Cat ..	Intact (chloralose)	Reserpine 1	Slightly inhibited	Enhanced, inhibited 1 hour later	—	− 20

TABLE I—*continued*

Animal	Preparation	Drug mg./kg.	Linguo-mandibular reflex	Patellar reflex	Crossed extensor reflex	Depression of blood pressure in mm. Hg
Cat ..	Intact (chloralose)	Reserpine 1·2	Slightly inhibited	Inhibited 1 hour later	Slightly inhibited	—
Cat ..	Intact (chloralose)	Reserpine 1	—	Enhanced and then inhibited	Unaffected	—
Cat ..	Intact (chloralose)	Reserpine 2	Slightly inhibited	Inhibited	—	−20
	Section in L1			Return		
		Chlorpromazine 1		No effect		
Rabbit ..	Intercollicular section	Serpentine 1	—	Slightly inhibited	—	—
Rabbit ..	Pontine section	Serpentine 1·5	—	Unaffected	—	—
Rabbit ..	Pontine section	Serpentine 1	—	Unaffected	—	—
Cat ..	Spinal (C1)	Reserpine 2·5	Slightly affected	Unaffected	—	—
		Morphine 8	Inhibited	Inhibited	—	—
Cat ..	Spinal (C1)	Reserpine 1	Unaffected	Enhanced	—	−10
Rabbit ..	Intact (chloralose)	Promazine 2	Inhibited	Inhibited	—	—
Rabbit ..	Intact (chloralose + urethane)	Promazine 2	Unaffected	Inhibited	—	—
Rabbit ..	Intact (chloralose)	Promazine 1·5	Slightly inhibited	Inhibited	—	−20
Cat ..	Intact	Promazine 1	Unaffected	Inhibited	—	—
Rabbit ..	Spinal section (L1)	Promazine 2	Slightly inhibited	Unaffected	—	—
Rabbit ..	Prepontine section	Promazine 2	—	Almost unaffected	—	—
Rabbit ..	Pontine section	Promazine 2	—	Unaffected	—	—
Cat ..	Spinal section (C1)	Promazine 3	—	Unaffected	—	—
Rabbit ..	Intact (chloralose)	Hydroxyzine 10	—	Inhibited	—	—
Rabbit ..	Intact (chloralose)	Hydroxyzine 10	Slightly inhibited	Inhibited	—	—
Rabbit ..	Intact	Hydroxyzine 5+	Unaffected	Unaffected	—	—
	(chloralose + urethane)	Hydroxyzine 5	Unaffected	Inhibited	—	—
Cat ..	Intact (chloralose)	Hydroxyzine 10	Slightly inhibited	—	—	—
Rabbit ..	Prepontine section	Hydroxyzine 10	—	Slightly inhibited	—	—
Rabbit ..	Pontine section	Hydroxyzine 10	—	Unaffected	—	—
Cat ..	Spinal section (C1)	Hydroxyzine 10	—	Unaffected	—	—
Rabbit ..	Intact (chloralose + urethane)	Morphine 10	Slightly inhibited	Inhibited	—	—
Cat ..	Intact (chloralose + urethane)	Morphine 10	Inhibited	Inhibited	—	−20
Rabbit ..	Prepontine section	Morphine 10	—	Inhibited	—	—
Rabbit ..	Pontine section	Morphine 10	—	Inhibited	—	—
Cat ..	Spinal (C1)	Morphine 8	Slightly inhibited	Inhibited	—	−30
Rabbit ..	Intact (chloralose)	Diethazine 2	Inhibited	Slightly inhibited	—	—
Rabbit ..	Intact (chloralose urethane)	Diethazine 5	Inhibited	Inhibited	—	—
Rabbit ..	Pontine section	Diethazine 5	—	Slightly inhibited	—	—
Cat ..	Spinal section	Diethazine 10	Inhibited	Inhibited	—	—
Cat ..	Spinal (C1)	Diethazine 10	Inhibited	Inhibited	—	−15

Promazine acts on spinal reflexes as does chlorpromazine. Amounts of 1·5–2 mg./kg. decrease the knee jerk in the intact animals, while the linguo-mandibular reflex is inconsistently affected. After sectioning the

spino-cerebral axis at C1, L1, prepontine or pontine levels, effects on the knee jerk reflex disappear.

Hydroxyzine, in doses of 5–10 mg./kg., promptly suppresses the patellar reflex of the intact animals (Fig. 5), but only inconsistently depresses the linguo-mandibular reflex. In the spinal and decerebrate animals the patellar reflex is unaffected after the same dosage of hydroxyzine.

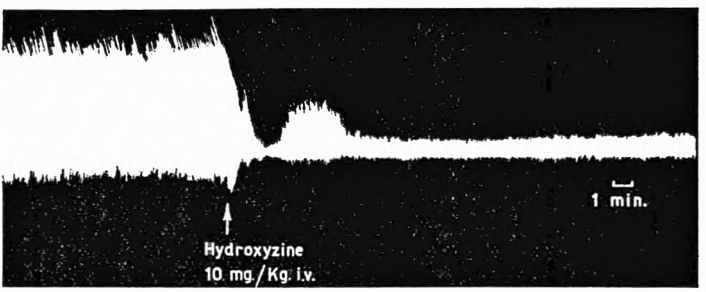

FIG. 5. Effects of hydroxyzine on the patellar reflex of intact animal. 10 mg./kg. of hydroxyzine results in an inhibition of the patellar reflex. Rabbit weighing 2·8 kg., anaesthetized with chloralose.

Morphine, in doses of 8 to 10 mg./kg., inhibits in intact, spinal and decerebrate animals, the polysynaptic reflex and to a minor extent the monosynaptic one (Fig. 6B and C).

Diethazine in doses of 2–5 mg./kg. was found to depress reflexes both in intact and in spinal animals, though in the latter case higher doses were required. The linguo-mandibular reflex is usually affected after 1–2 mg./kg.

DISCUSSION

Our results together with those obtained by other authors are summarised in Table II. These show that an intact connection between spinal cord and brain is essential for chlorpromazine, promazine, reserpine and hydroxyzine to produce inhibitory effects on spinal reflexes. The influence of the traumatic shock produced by the spinal sections on some patterns of reflex activity must not be disregarded, however. We did not observe any consistent change in the activity of morphine, diethazine and mephenesin on spinal reflex arcs before and after the sections of the spinal cord. The effects of anaesthesia cannot explain the peculiar behaviour of these drugs, as the experiments made on spinal animals previously anaesthetised with chloralose or urethane gave the same results. Haemodynamic depression may also be excluded, because it is known that polysynaptic arcs show a great sensitivity to changes in blood pressure and the effects on linguo-mandibular reflex were the same before and after the spinal sections.

Some findings indicate a possible role of the mesencephalon in these effects. Our experiments show that mesencephalic sections are critical, as small differences in the level of this section produce substantial changes in the action of these drugs. Schneider and others[1] did not find reserpine

to have any inhibitory effect in decerebrate animals, while Krivoy[2] has observed that the inhibitory action of reserpine and chlorpromazine is retained in this preparation. This may be explained by differences in the sections. On the other hand, many reported data demonstrate the presence of inhibitory and facilitatory centres in the reticular formation[10–13].

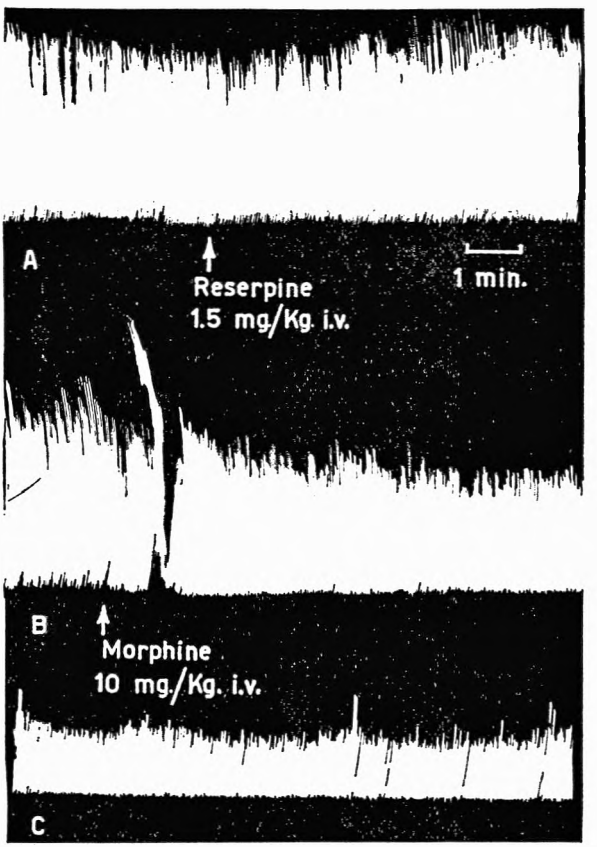

FIG. 6. Effects of reserpine and morphine on the knee jerk of a spinal animal.
(A) The patellar reflex is unaffected after 1·5 mg./kg. of reserpine. (B) 60 minutes after reserpine, the injection of 10 mg./kg. of morphine provokes an inhibition of the reflex response. (C) The inhibitory effect of morphine is evident 15 minutes after the injection. Cat weighing 2·5 kg., sectioned at C1 anaesthetized with ether.

Chlorpromazine, promazine, reserpine and hydroxyzine could act on these centres and two possible mechanisms may be considered. Firstly, the drugs might directly stimulate cerebral centres which exert an inhibitory action on motor reflexes. Secondly, the drugs might prevent some inhibitory cerebral centres from influencing opposing mechanisms.

Other drugs need the integrity of brain–spinal cord connections to inhibit spinal reflexes. Scopolamine was found by Teuchmann[14] to decrease the ipsilateral flexor reflex in the thalamic cat while no action in decerebrate and decapitate animals was found. De Maar[9], experimenting with cats the brain of which was sectioned at different levels, showed that an intact connection between diencephalon and spinal cord is necessary to demonstrate the actions of scopolamine and atropine on spinal reflexes

TABLE II

SUMMARY OF RESULTS

	Reflexes	Chlor-promazine	Promazine	Reserpine	Hydroxyzine	Morphine	Diethazine
Intact Animal	Linguo-mandibular	Incon-sistently inhibited	Incon-sistently inhibited	Incon-sistently inhibited	Slightly inhibited	Inhibited	Inhibited
	Monosynaptic	Inhibited (1 and 2)	Inhibited	Inhibited (1)	Inhibited (3)	Slightly inhibited (4)	Slightly inhibited
	Other polysynaptic	Inhibited (1 and 2)	—	Slightly inhibited (1)	—	Inhibited (4)	Inhibited (5)
Spinal Animal	Mono-synaptic	Unaffected (6 and 2)	Unaffected	Unaffected or en-hanced (7)	Unaffected (3)	Slightly inhibited (4)	Slightly inhibited
	Poly-synaptic	Unaffected (2, 6 and 8)	—	Unaffected (2)	—	Inhibited (4)	—

(1) See also Krivoy[7].
(2) ,, ,, Bein[3].
(3) ,, ,, Hutcheon and others[6].
(4) ,, ,, Wikler[7] and Takagi and others[8].
(5) ,, ,, De Maar[9].
(6) ,, ,, Preston[5].
(7) ,, ,, Schneider and others[1].
(8) Dasgupta and Werner[4] reported that the crossed extensor reflex was less inhibited in the spinal than in the decerebrated cats.

According to Bijlsma[15], orphenadrine (Disipal) inhibits the spinal reflexes through a cerebral mechanism, while other drugs used in paralysis agitans, such as diethazine, caramiphen and benzhexol, show a more widespread inhibitory action, similar to that displayed by mephenesin and morphine.

A second peculiar feature in the effects of chlorpromazine, promazine, reserpine and hydroxyzine appears to be the predominant inhibition of monosynaptic reflex activity. From a physiological point of view this finding is not completely surprising. In fact, King and others[12] have observed that the stimulation of certain centres of the bulbar reticular formation results in a facilitation of the linguo-mandibular reflex and in a simultaneous inhibition of the knee jerk. Alternatively, the stimulation of more rostral regions of the reticular formation, from the pons to the diencephalon, produces opposite effects. On the basis of these results one might assume the mono- and polysynaptic reflexes to be under the control of different cerebral structures, some of which would be affected by chlorpromazine, promazine, reserpine and hydroxyzine. We have been unable to find any mention of other drugs which predominantly inhibit monosynaptic reflexes. On the contrary, a selective inhibitory

action of polysynaptic reflexes has been considered to be a property common to many C.N.S. depressants[16]. Also, morphine and diethazine behave like other central depressant agents, and selectively affect polysynaptic arcs. This fact may be of some interest as morphine has been found to block the conditioned avoidance response of rats[17] as well as the secondary conditioned response[18], and in this resembles chlorpromazine and reserpine. However, morphine potentiates the stereotyped response induced by mescaline in mice, while promazine and chlorpromazine clearly abolish it[19]. The present study confirms that morphine has different and in some mechanisms opposite effects upon the regulation of spinal cord reflex activity to the tranquillising drugs examined.

On the other hand, diethazine, the chemical structure of which is strictly related to promazine, has been found to differ from the two other phenothiazines in its action on spinal reflexes. Diethazine is a drug of value in counteracting the symptoms of Parkinson's disease and it is supposed to act upon the reticular formation of the brain stem[20]. However, according to Balestrieri and Fadiga[21], chlorpromazine blocks the EEG arousal reaction which remains unchanged under diethazine treatment. This finding could be related to the differences of action on reflex activity, found by us, between chlorpromazine and diethazine, and perhaps may have some connection with the differences in therapeutic activity of the two drugs. In fact, it is known that diethazine does not possess the "tranquillising" activity of chlorpromazine and promazine.

The true significance of these findings is not clear, and it has not been possible to demonstrate any direct relation between the actions on reflex responses and the behavioural effects of the drugs investigated. We hope that from the present study a general picture of the actions of some psychotropic drugs on the spinal reflex activity may result which perhaps will have a practical importance for searching and investigating new drugs.

References

1. Schneider, Plummer, Earl and Gaunt, *Ann. N.Y. Acad. Sci.*, 1955, **61**, 17.
2. Krivoy, *Proc. Soc. exp. Biol. N.Y.*, 1957, **96**, 18.
3. Bein, Proceedings of the Int. Symposium on Psychotropic Drugs, Milan, May 9–11, 1957, Elsevier Publishing Co., 1957, p. 325.
4. Dasgupta and Werner, *Arch. int. Pharmacodyn.*, 1955, **100**, 409.
5. Preston, *J. Pharmacol.*, 1956, **118**, 100.
6. Hutcheon, Scriabine and Schrogie, *ibid.*, 1957, **119**, 154.
7. Wikler, *Proc. Soc. exp. Biol. N.Y.*, 1945, **58**, 193.
8. Takagi, Matsumura, Yanai and Ogiu, *Jap. J. Pharmacol.*, 1955, **4**, 176.
9. De Maar, *Arch. int. Pharmacodyn.*, 1956, **105**, 349.
10. Magoun, *Science*, 1944, **100**, 544.
11. Magoun and Rhines, *J. Neurophysiol.*, 1946, **9**, 165.
12. King, Minz and Unna, *J. Compar. Neurol.*, 1955, **102**, 565.
13. Hankinson and Namin, *Amer. J. Physiol.*, 1954, **179**, 643.
14. Teuchmann, *Arch. int. Pharmacodyn.*, 1949, **79**, 257.
15. Bijlsma, *C. R. du 51ème Congr. Int. de Therapie*, Utrecht, 4–6, VI, 1957, p. 31.
16. Domino, *Ann. N.Y. Acad. Sci.*, 1956, **64**, 705.
17. Cook and Weidley, *ibid.*, 1957, **66**, 740.
18. Maffii, *J. Pharm. Pharmacol.*, 1959, **11**, 129.
19. Maffii and Soncin, *ibid.*, 1958, **10**, 541.
20. Bovet and Longo, XXth International Physiological Congress, Brussels, July 30–August 4, 1956.
21. Balestrieri and Fadiga, *Arch. Sci. Biol.*, 1954, **38**, 620.

THE DETERMINATION OF THE ORIGIN OF OPIUM

PART II. SIMULTANEOUS ASSAY OF NARCOTINE, THEBAINE AND PAPAVERINE IN OPIUM BY INFRA-RED SPECTROSCOPY*

BY V. J. BAKRE†, ZISAN KARAATA‡, J. C. BARTLET AND C. G. FARMILO

From the Food and Drug Directorate, Department of National Health and Welfare, Tunney's Pasture, Ottawa

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A rapid and reliable method for the simultaneous determination of the percentage of narcotine, thebaine and papaverine in opium by an infra-red method has been developed. Examination of the infra-red spectrum of the carbon tetrachloride solution of the dried chloroform residue from an acetic acid-water opium solution revealed a quantitative relationship between the absorbances at 1767, 1602 and 1160 cm.$^{-1}$ and the concentration of narcotine, thebaine and papaverine.

A RECENT international conference of a committee of experts on opium origin determinations at the United Nations[1] recommended that research on opium be continued in order to obtain rapid, reliable and reproducible quantitative analytical values for the major alkaloids contained in opium. They pointed out that the main disadvantage of present unified methods of analysis of opium[2–7,12] is the long time required in the separation and purification of each alkaloid before assay. The simultaneous quantitative determination of papaverine and oxycodone (which contains a C = O group) by infra-red spectroscopy of chloroform solutions was made in 1955[8]. The possibility of simultaneous determination of narcotine, thebaine and papaverine became evident in this laboratory, when it was found that their infra-red spectra in chloroform[9], and carbon tetrachloride showed quantitative relationships between concentration and absorbances at 1767, 1602 and 1160 cm.$^{-1}$ respectively. Further evidence of the possibility of using infra-red analysis for the quantitative estimation of mixtures of papaverine and narcotine was shown and later the determination of narcotine in the presence of papaverine in mixtures of pure drugs was reported[10,11]. It is the object of this paper to describe an infra-red method for the simultaneous assay of narcotine, thebaine and papaverine in opium.

EXPERIMENTAL

Reagents

Reference Standards: Narcotine (100 mg.), (B.P.C. grade) thebaine (70 mg.) and papaverine (50 mg.) (B.P.C. grade) were dissolved in anhydrous carbon tetrachloride and made up to 25 ml. in individual volumetric flasks and stored in the dark.

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† Colombo Plan Fellow, Opium Origin Research Programme.

‡ United Nations Fellow, Opium Origin Research Programme.

Phosphoric acid: 85 per cent.

Persulphate test solution: potassium persulphate (0·1–0·12 g.; reagent grade) was dissolved in concentrated sulphuric acid (5 ml., 95·5 per cent).

Froehde's (fuming) test solution: Dissolve sodium molybdate (0·5 g.) in sulphuric acid (5 ml., 95·5 per cent and dilute with fuming sulphuric acid in the proportion of 1 to 15.

Preparation of Opium Extract[12]

Opium (4·5 g.) powdered and sieved to 20:40 mesh size particles was triturated with glacial acetic acid (20 ml.) to a smooth paste (10–20 minutes rubbing) in a mortar, to which was added water (25 ml.) from a burette, slowly and with continuous stirring. The aqueous acetic acid-opium mixture was filtered through a Whatman 42H filter paper into a narrow graduated cylinder to prevent excessive evaporation. An aliquot (10 ml.) of the opium solution equivalent to 1 g. of opium was taken for analysis and extracted with chloroform according to the scheme given in Table I.

Group separation of Narcotine, Thebaine and Papaverine for Infra-red Analysis

Five clean, dry separatory funnels (125 ml.) were taken and their stop-cocks greased with Apiezon M, or Lubriseal, then washed with chloroform to remove excess grease and assembled on a rotating table. An aliquot (10 ml.) of opium extract was placed in funnel (1). Water, sodium hydroxide, etc. were added to funnels (2) to (5) as shown in Table I.

TABLE I

ARRANGEMENT OF SEPARATORY FUNNELS FOR EXTRACTION OF NARCOTINE, THEBAINE AND PAPAVERINE

Number of funnel	Contents of funnel	Quantity used
(1)	Acetic acid-water-opium solution ..	10 ml.
(2)	Water	10 ml.
(3)	Sodium hydroxide water (1 : 1) ..	15 ml.
	Water	10 ml.
	Sodium bisulphite	50 mg.
(4)	Water	10 ml.
(5)	Water	10 ml.

The contents of funnel (1) were shaken with chloroform (10 ml.), the layers separated, the chloroform transferred to funnel (2) and the shake-out repeated. At the same time another portion of fresh chloroform (10 ml.) was added to funnel (1). The shake-outs were continued until 4 portions of chloroform had been successively transferred into funnel (1) to (5) in turn. At funnel (2) a test for thebaine was made, using two drops of the chloroform layer, with syrupy phosphoric acid with which it gives a golden yellow colour. Further chloroform extractions were made until the thebaine test was negative. Tests for narcotine and papaverine using persulphate in concentrated sulphuric acid (red orange colour) and fuming Froehde's reagents (intense violet colour) were then made.

The chloroform solution from funnel (5) was filtered through a cotton wool pad, and after evaporation of the chloroform, the residue was dried in a desiccator and triturated with anhydrous carbon tetrachloride. The resulting solution was filtered and washed through a sintered glass funnel under reduced pressure and made up to volume in a volumetric flask (25 ml.) with anhydrous CCl_4. The infra-red spectrum of a portion of this solution was obtained using a 1 mm. NaCl cell by the usual techniques in a Perkin Elmer Model 21 recording spectrophotometer. Chemical determinations of opium alkaloids were made by the modified K/34 method[11,12].

Results and Discussion

Group Separation of Narcotine, Thebaine and Papaverine and Minor Phenolic Alkaloids for the Infra-red Analysis

The filtered solution of the acetic acid extract of opium alkaloids plus other extractives (i.e., coloured materials, etc.) was shaken with chloroform, which divides the alkaloids into two main groups, those readily extracted by chloroform from unbuffered acetic acid solution, and those not readily extractable. The former group consists of narcotine, papaverine, thebaine and about one third of the total amount of minor phenolic alkaloids including nearly all of the porphyroxine-meconidine. The other group remaining in the aqueous solution consists of morphine, codeine, cryptopine, an unknown base, narceine, and about two thirds of the minor phenolic alkaloids.

The chloroform extracts are washed with unbuffered acetic acid solution and then with alkali solution. The chief purpose of which is to remove the acetate ions. The minor phenolic alkaloids that are readily extracted from unbuffered acetic acid are not held by alkali against chloroform.

The complete separation of the group of alkaloids, thebaine, papaverine and narcotine and minor phenolic substances, is indicated by the completion of the thebaine extraction, since narcotine and papaverine are removed from the aqueous phases more readily. The unknown base, which may be extracted slightly, yields a thebaine-like colour rection with sulphuric acid (2 + 1), a slight reaction with syrupy phosphoric acid, and scarcely any colour with concentrated hydrochloric acid. However the latter reagent does not react well with minute amounts of thebaine. A little experience indicates the completion of the thebaine extraction using syrupy phosphoric acid as the main test solution.

The minor phenolic alkaloids are insoluble in carbon tetrachloride and remain in the filter bed after trituration of the chloroform residue of the groups of alkaloids.

Calculation of Concentrations of Narcotine, Thebaine and Papaverine from Infra-red Spectra

The infra-red spectra of pure narcotine, thebaine and papaverine individually and in a mixture are shown in Figure 1. The absorbance

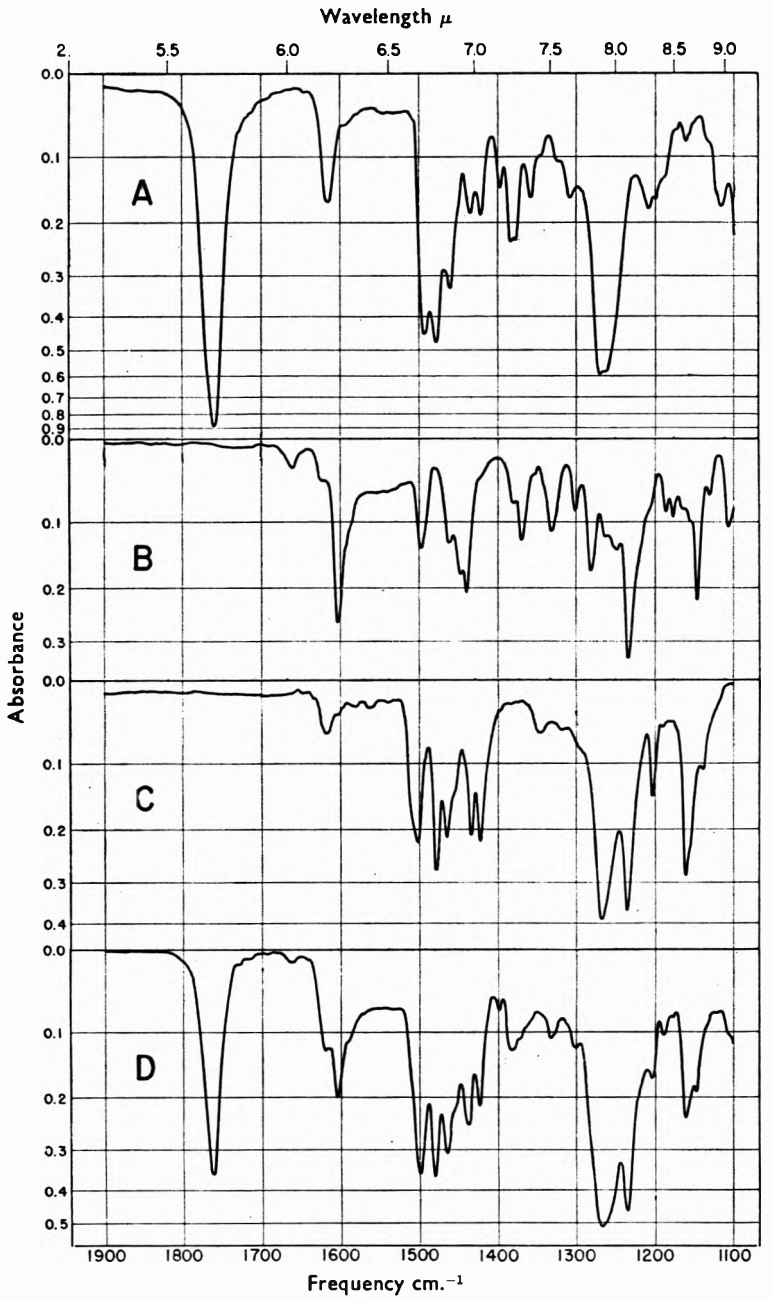

FIG 1. Infra-red absorption spectrum of narcotine (A) (100 mg.); thebaine (B) (70 mg.), papaverine (C) (50 mg.); mixture (D) of narcotine (38·2 mg.); thebaine (38·2 mg.); papaverine (29·3 m.g.) in CCl_4 (25 ml.)

TABLE II

ABSORBANCES AT CHARACTERISTIC FREQUENCIES OF STANDARD PURE BASES

Alkaloid	Concentration mg./25 ml. CCl_4	Absorbances at frequencies, $cm.^{-1}$								
		A_{1767}	A_{1850}	$A_{1767}-A_{1850}$	A_{1602}	A_{1645}	$A_{1602}-A_{1645}$	A_{1160}	A_{1172}	$A_{1160}-A_{1172}$
Narcotine	100	0·900	0·020	0·880	0·065	0·000	0·065	0·078	0·058	0·020
Thebaine ..	70	0·010	0·000	0·010	0·265	0·012	0·253	0·083	0·070	0·013
Papaverine	50	0·015	0·000	0·015	0·040	0·000	0·040	0·288	0·048	0·240

data of Table II were derived from the spectra of these pure alkaloids. From the absorbance data, the following equations were derived for the calculation of percentages of narcotine, thebaine and papaverine:

Per cent narcotine =

$$\frac{(A_{1767} - A_{1850}) - \frac{0{\cdot}01}{0{\cdot}0253}(A_{1602} - A_{1645}) - \frac{0{\cdot}015}{0{\cdot}240}(A_{1160} - A_{1172})}{0{\cdot}00880} \times \frac{100}{1000}$$

Per cent thebaine =

$$\frac{(A_{1602} - A_{1645}) - \frac{0{\cdot}065}{0{\cdot}880}(A_{1767} - A_{1850}) - \frac{0{\cdot}040}{0{\cdot}240}(A_{1160} - A_{1172})}{0{\cdot}00361} \times \frac{100}{1000}$$

Per cent papaverine =

$$\frac{(A_{1160} - A_{1172}) - \frac{0{\cdot}020}{0{\cdot}880}(A_{1767} - A_{1850}) - \frac{0{\cdot}013}{0{\cdot}253}(A_{1602} - A_{1645})}{0{\cdot}00480} \times \frac{100}{1000}$$

The first term of each equation is the net absorbance of the "peak" for each alkaloid measured above a base line. The next two terms are minor corrections for the other two alkaloids. The denominator is based on the net absorbances per milligram obtained from the standard solutions of pure alkaloids, while the final factor converts the calculated concentration from milligrams to per cent.

Figures 2 and 3 show the characteristic features of the infra-red spectra of carbon tetrachloride solutions of chloroform residues from opium samples of two different countries of origin. Figure 2 is the spectrum obtained from an Indian export opium having very low papaverine, and average thebaine and narcotine content, while Figure 3 shows the spectrum of an Iranian type opium with high papaverine and thebaine, and average narcotine content. Table III lists the infra-red data obtained from Figure 2 along with the absorbance correction and sample calculations of alkaloid content.

Comparison of the K/34 *and Infra-red Methods*

Quantitative results. Table IV lists results of assay by the infra-red method and the comparative assay. The sample of Indian opium

Ex 3398, which has been adopted as a comparison sample in all this work was analysed by the K/34 unified chemical method of analysis described by Fulton in the United Nations document[12]. Samples F112, F113,

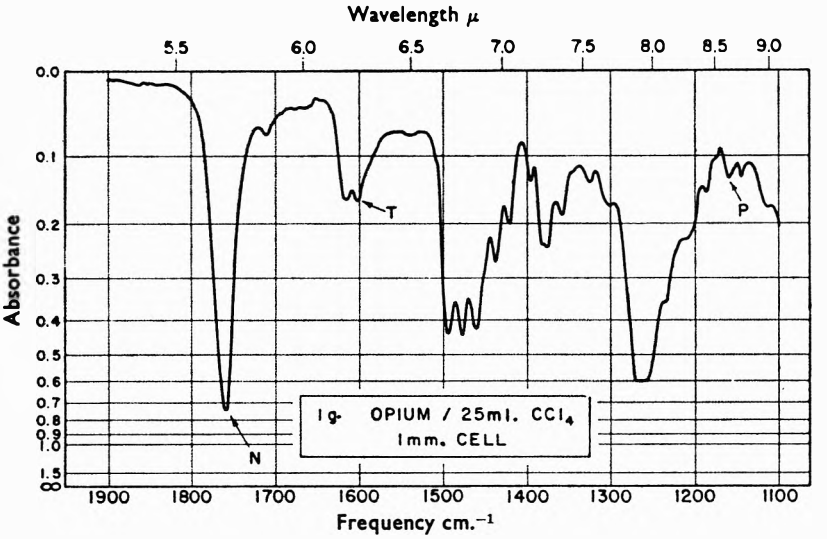

FIG. 2. Infra-red absorption spectrum of extract of Indian export opium reference standard.

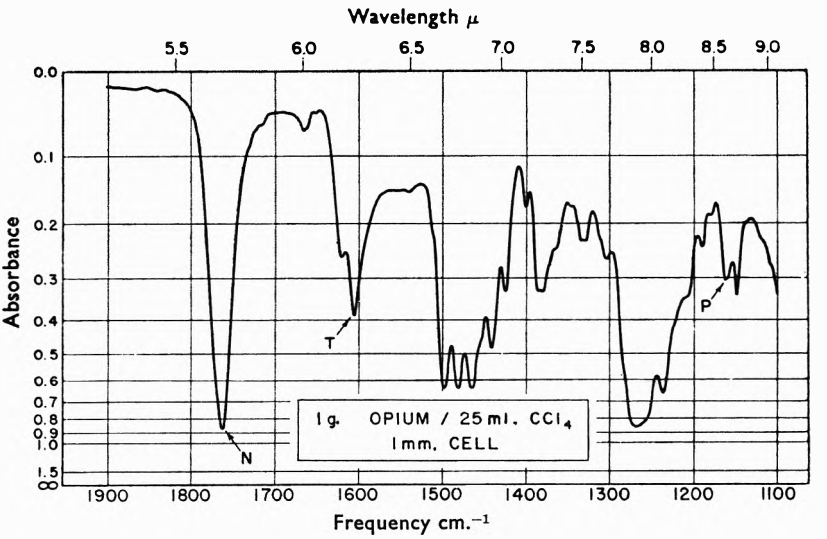

FIG. 3. Infra-red absorption spectrum of extract of Iranian opium (F112).

F119 and F120 which were supplied to us for origin determinations were assayed by a modification of the K/34 method[11]. In addition, a study of the variance of the K/34 method using the Indian export sample Ex 3398, on the results from 8 determinations by 4 different analysts on 4 replicate

samples analysed in duplicate by each analyst was made. The analysts worked at the same time under the same conditions to obtain these data: The modification of the K/34 method[11] was then made and by comparison, yields similar results to the K/34 method[12]. Seven samples were analysed

TABLE III

EXAMPLE OF CALCULATIONS OF ALKALOID PERCENTAGES FROM FIGURE 2

Alkaloid	Peak		Base line		Correction for other alkaloids	Net absorbance	Factor	Per cent
	Frequency cm.$^{-1}$	Absorbance	Frequency cm.$^{-1}$	Absorbance				
Narcotine ..	1767	0·726	1850	0·015	−(0·005+0·003)	0·703	$\frac{100}{0·0088 \times 1000}$	7·99
Thebaine ..	1602	0·167	1645	0·031	−(0·053+0·006)	0·077	$\frac{100}{0·00361 \times 1000}$	2·13
Papaverine ..	1160	0·129	1172	0·090	−(0·016+0·006)	0·017	$\frac{100}{0·00480 \times 1000}$	0·35

TABLE IV

COMPARATIVE RESULTS OF ANALYSIS OF OPIUM SAMPLES BY INFRA-RED AND CHEMICAL METHODS

Opium Sample No.	Content of alkaloid, per cent					
	Narcotine		Thebaine		Papaverine	
	IR[a]	Chem[b]	IR	Chem	IR	Chem
F112 .. .. (UN 197)	8·50 8·58	8·83	6·15 6·70	6·26	2·02 2·39	2·31
F113 .. .. (UN 198)	8·76 9·04	7·02	5·79 5·76	6·05	2·29 2·35	2·19
F119 .. .. (UN 226)	9·74 9·51	8·69	1·21 0·95	1·07	4·25 4·25	4·01
F120 .. .. (UN 227)	5·42 5·55	5·11	2·30 2·71	2·43	1·33 1·30	1·43
Indian .. Ex 3398	7·99 7·58	7·8	2·13 1·97	2·10	0·35 0·25	0·42

a IR = Infra-red method of assay.
b Chem = Chemical methods of assay cited in references 11 and 12.

TABLE V

COMPARISON OF 95 PER CENT CONFIDENCE LIMITS FOR NARCOTINE, THEBAINE, PAPAVERINE

Method	Confidence limit for		
	Narcotine	Thebaine	Papaverine
K/34 ..	± 1·27 (10)	± 0·56 (17)	± 0·37 (9)
IR	± 0·40 (7)	± 0·19 (7)	± 0·18 (7)

* Numbers in brackets are degrees of freedom.

in duplicate by the infra-red method beginning with the same Indian sample. All data were submitted to the Biometrician[13] for statistical analysis to establish the confidence limits shown in Table V.

Steps of the K/34 method. For purposes of discussion of the advantages of the infra-red method compared with the K/34 method and its modification, a brief description of the general steps is given.

1. Initial extraction of the raw opium to prepare a solution containing all of the opium alkaloids.

2. Separation of the alkaloids into three groups, (A) morphine and codeine, which remain in acid containing funnels; (B) thebaine which is retained in sulphuric acid containing separatory funnels; (C) narcotine and papaverine which are contained in the chloroform.

3. Countercurrent extraction and separation and purification of morphine, codeine, thebaine, papaverine, narcotine, narceine and an unknown base along with minor phenolic alkaloids.

4. Assay of the purified alkaloids by aqueous or non-aqueous titration.

The K/34 method requires fifty-six separatory funnel solutions and an average of six to eight solvent passes per funnel to remove the alkaloids. The modifications introduced reduce the number of separatory funnels to 20 and thereby the time of analysis appreciably.

Time required for analysis. The time required for the determination of morphine, codeine, thebaine, papaverine and narcotine by the K/34 method is five days per sample. For the modified K/34 method 3 days per sample is required. Determination of only thebaine, narcotine and papaverine by the modified K/34 method takes two days per sample compared with half a day per sample by the infra-red method. Since a large number of samples of opiums will be required to be analysed to meet the needs of the UN opium research programme the time saved is an important consideration.

Reproducibility of the K/34 and infra-red methods. A study of the causes of variance of the K/34 method has been made by Farmilo and others (in preparation). The chromatographic purity of isolated alkaloids, and the amounts in replicate samples in a specially prepared and accurately sampled Indian export opium (T. & H. Smith Ex 3398) were found. The 95 per cent confidence limits for each alkaloid value were determined by statistical analysis of the data[13]. In the infra-red study, confidence limits for the mean values were obtained using the same Indian export opium sample. These two sets of data are compared in Table V and the number of degrees of freedom are shown in brackets after the limit values. It can be readily seen that the infra-red method shows a distinct improvement in reproducibility of results.

Study of Substances with Possible Interfering Infra-red Absorbances

Stopcock grease. It was observed in preliminary experiments after the chemical extraction, when Celloseal* grease had been used on the stopcocks of the separatory funnels shown in Table I, that the narcotine lactone carbonyl band at 1765 cm.$^{-1}$ sometimes showed a shoulder at 1740 cm.$^{-1}$. This indicated additional C = O (not lactone) absorption. A spectrum of the Celloseal grease in carbon tetrachloride showed strong absorption at 1737 cm.$^{-1}$. The spectra of Apiezon-M† and Lubriseal‡

* Stopcock grease sold by Fisher Scientific Supply Co. Montreal, P.Q.
† Edwards High Vacuum, (Canada Ltd) P.O. Box 515, Burlington, Ontario.
‡ Arthur H. Thomas, Supply House, Philadelphia, U.S.A.

greases were free from carbonyl absorption and were satisfactory for this purpose.

Opium fat. The infra-red spectrum of the naturally occurring light petroleum solubles in opium was obtained in carbon tetrachloride solution. This spectrum resembled the spectra of glycerides in general and had a strong absorbance band at 1725 $cm.^{-1}$ which was absent in the spectra of the alkaloid extracts obtained in the usual way. Presumably the glyceride material is removed during the filtration from the initial aqueous acetic acid opium extract. The Indian export opium sample, before and after fat extraction with light petrol yielded identical infra-red spectra.

Secondary alkaloidal constituents. Minor phenolic alkaloid fractions obtained from the study of chemical methods[11] (also paper in preparation) were used to demonstrate the lack of interference of these secondary alkaloids with the narcotine, thebaine, and papaverine during infra-red analysis. A carbon tetrachloride extract of the combined minor phenolic alkaloid fractions was made. An infra-red spectrum of this solution showed little absorbance over the entire wavelength range.

Colouring matter in opium. The carbon tetrachloride solutions of the chloroform residues from the opium extractions are yellow to yellow brown in colour. This colour may be removed by passing the carbon tetrachloride solution through a bed of calcium hydroxide,with some loss of narcotine. An infra-red spectrum of the decolourised solution showed a lower narcotine absorbance, but otherwise was identical with a spectrum of the coloured solution. The colouring matter is not present in the carbon tetrachloride solution in concentrations which affect the infra-red spectrum.

Other major alkaloid constituents. It has been found that codeine is very slightly extracted by chloroform from aqueous acetic acid opium solutions during chemical separation of the thebaine, papaverine and narcotine from morphine and codeine. A study of the chromatograms of concentrated carbon tetrachloride solutions of chloroform residues showed negligible quantities of codeine. This quantity does not affect the characteristic infra-red absorbances of narcotine, thebaine and papaverine. No other alkaloids could be detected chromatographically in these residues.

Acknowledgements. The authors wish to acknowledge the technical assistance of Mrs. Ruth Lane in preparing opium extracts and carrying out the chromatography and of Miss C. Cox for statistical analysis of the data. The financial assistance provided by the Government of Canada through the Colombo Plan to V. J. Bakre and of the United Nations Technical Assistance Administration to Z. Karaata is acknowledged. The help and assistance of the Indian and Turkish Governments in supporting this endeavour is gratefully acknowledge. The help of the Division of Narcotic Drugs of United Nations and the Department of Health and Welfare, Ottawa, is appreciated. We wish to thank Drs. L. I. Pugsley, R. A. Chapman and L. Levi for valuable discussions and suggestions.

REFERENCES

1. Report of the Committee of Experts on the United Nations Programme of Opium Research, United Nations Document E/CN7/338, February 20, 1958. Report to Analysts, *Analyt. Chem.*, 1958, **30**, No. 8 19A.
2. Anneler, *Festschrift Barell*, 1936, 344–362.
3. Fulton, *Royal Canadian Mounted Police Crime Detection Laboratory Seminar Report*, 1954, II, 141.
4. Klyatschkina and Feldmann, League of Nations Document, O.C. 1946 (1)e, Geneva, April 17, 1937.
5. Klyatschkina, *Arch. Pharmaz. Berdtsch. pharmaz. Geo.* 1934, **272**, 558.
6. Pfeifer, *Pharmazie*, 1958, **13**, 100.
7. Holubek, Kudrnac and Novak, *ibid.*, 1958, **13**, 95.
8. Salvesen, Domange and Guy, *Ann. pharm. franc.*, 1955, **13**, 354.
9. Levi, Hubley and Hinge, *United Nations Bull. Narcotics*, 1955, VII, 42.
10. Lee Kum-Tatt, Rockerbie and Levi, *J. Pharm. Pharmacol.*, 1958, **10**, 621.
11. Lee Kum-Tatt and Farmilo, *ibid.*, 1958, **10**, 427.
12. Fulton, United Nations Document, ST/SOA/SER. K/34, 1954.
13. Cox, Communication from Biometrics Section, Food and Drug Directorate, Ottawa.

THE GLYCOSIDIC CONSTITUENTS OF *HYDROCOTYLE VULGARIS* L.

By Chr. J. K. Mink

From the Department of Pharmacognosy, State University, Utrecht

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From *Hydrocotyle vulgaris* L. the amorphous saponin earlier designated Hydrocotyle-saponin B has been isolated and its hydrolysis products studied.

A preliminary study of the saponins of *Hydrocotyle vulgaris* was reported in 1956[1]. The object was to ascertain whether the chemical components of this European plant were similar to those of a closely allied plant, *Centella asiatica* L., which occurs in Madagascar and Ceylon.

From the Madagascar variety the crystalline triterpene glycoside, asiaticoside, has been isolated[2]. Hydrolysis of this produced two molecules of glucose, two of rhamnose and one of a crystalline α-amyrin derivative asiatic acid ($C_{30}H_{48}O_5$)[3,4], which contains three hydroxyl groups and a carboxyl group.

The components of the Ceylonese variety were an amorphous triterpenoid glycoside, centelloside, as well as three amorphous polyhydroxy triterpenic acids, namely centic acid, centoic acid and centellic acid. On hydrolysis, centelloside split off glucose, fructose and centellic acid in the molecular ratio of 10 : 2 : 1[5,6]. None of these compounds appeared to be identical with asiatic acid or asiaticoside.

Previously[1] *H. vulgaris* in contrast to *C. asiatica*, was shown to possess haemolytic activity, equivalent to that of senega root. Using paper chromatography, four haemolytically active substances were detected which were named component S_4, hydrocotyle-saponin A_1 and A_2, and hydrocotyle-saponin B. Quantitatively this latter substance forms the most important haemolytically active component of the plant. Further work[7] was aimed at obtaining more information on the composition of hydrocotyle-saponin B isolated for this purpose from the dialysed methanol saponin prepared as described in earlier work[1].

Experimental

Purification of Saponin B

The dialysed methanol saponin[1] contained a small amount of coloured matter and saponin A_1, as impurity. This was removed by adsorption chromatography on slightly acid alumina and eluting saponin B with methanol containing 0·1–0·3 per cent w/v of formic acid. Saponin B was further purified by shaking a 1 per cent solution of the saponin in methanol (98 per cent v/v) with a little activated charcoal. After filtration and evaporation to dryness, the residue was taken up in 0·1N NaOH and electrodialysed to remove traces of $Al(OH)_3$ eluted from the column. The product was obtained as a white amorphous solid, free from ash, melting at 209–210° (decomp.). Hydrolysis, effected by heating in a

sealed tube at 100° for 17 hours a solution containing 2 per cent w/v of the saponin in equal volumes of methanol (98 per cent v/v) and 3 per cent sulphuric acid, gave products identified by paper chromatography as glucose, arabinose and glucuronic acid in about equal amounts.

From the products of hydrolysis the tentative formula $C_{47}H_{74}O_{20}$ or $C_{47}H_{76}O_{21}$ is suggested. After drying over P_2O_5 *in vacuo* for 72 hours (110° at 0·1 mm. Hg), when the moisture content was found to be 9·09 per cent, analysis gave the following results.

Found: C, 56·08; H, 7·98 per cent. $C_{47}H_{74}O_{20}$ requires C, 58·86; H, 7·78 per cent: $C_{47}H_{76}O_{21}$ requires C, 57·77; H, 7·84 per cent.

As saponin B retains solvent tenaciously, this may be responsible for the differences in the found and calculated percentages.

Hydrolysis Products

The aglycone obtained from hydrolysis was amorphous, and could be separated into three components by adsorption chromatography. The aglycone in solid state was first extracted with hot chloroform. This effected a partial separation of the components, which were named A-, I- and K-aglycone. Both the A- and I- and some K-aglycones were chloroform soluble. The chloroform insoluble fraction was entirely K-aglycone. The chloroform soluble fraction was evaporated to dryness and the residue taken up in a mixture of chloroform and ether (1 : 9 v/v). It was then chromatographed on slightly acid alumina, eluting the components with mixtures of chloroform-ether and chloroform-methanol in sequence of increasing polarity.

A-glycone. An amorphous haemolytically active substance of melting point 243–246° (decomp.). After drying over P_2O_5 *in vacuo* for 48 hours at 100°–0·1 mm. Hg, found: C, 72·0; H, 10·0 per cent. Calculated for $C_{30}H_{48}O_5$: C, 73·7; H, 9·9 per cent.

I-Aglycone. An amorphous haemolytically inactive substance of melting point 224–227° (decomp.). After drying over P_2O_5 *in vacuo* for 72 hours at 100° and 0·1 mm. Hg, found: C, 70·1; H, 9·6 per cent. Calculated for $C_{30}H_{50}O_6$: C, 71·1; H, 9·9 per cent. The differences between the found and calculated percentages are attributed to the tenacious manner in which the solvent is retained, an experience in common with Bhattacharrya[8], who was working with amorphous triterpenic acids from the Ceylonese variety of *C. asiatica.*

K-Aglycone. A crystalline haemolytically inactive substance of melting point 283° (decomp.). After drying over P_2O_5 *in vacuo* for 24 hours at 20° and 0·1 mm. Hg, found: C, 70·9; H, 10·1 per cent. Calculated for $C_{30}H_{50}O_6$: C, 71·1; H, 9·9 per cent.

Molecular weight estimated according to the method of Smit and others[9], found: 500 and 513, calculated: 506·7.

Acetates of K-aglycone. Penta-acetate (amorphous): after drying over P_2O_5 *in vacuo* for 48 hours at 60° and 0·1 mm. Hg, found: C, 67·0; H, 8·5; $COCH_3$, 29·0 per cent. Calculated for $C_{40}H_{60}O_{11}$: C, 67·0; H, 8·4; $COCH_3$, 30·0 per cent. Melting point: 223–226°.

Tri-acetate (crystalline): after drying over P_2O_5 *in vacuo* for 48 hours at 60° and 0·1 mm. Hg, found: C, 68·0; H, 8·8; $COCH_3$, 18·4 per cent. Calculated for $C_{36}H_{56}O_9$: C, 68·3; H, 8·9; $COCH_3$, 20·4 per cent. Melting point 242°.

The A- and K-aglycones are interrelated. Heating the A-aglycone for 16 hours at 100° with 50 per cent v/v methanol containing 10 per cent w/v sulphuric acid was shown by adsorption chromatography to yield 16 per cent of K-aglycone, and refluxing the A-aglycone with 1 per cent methanolic potassium hydroxide to yield 46 per cent of the K-aglycone. Insufficient I-aglycone was available for analogous tests.

Infra-red Data

The infra-red spectra of aglycones (NaCl prism, KBr phase, concentration 0·3 per cent) suggested that the A- and I-aglycone possess a carbonyl function (C = O stretching absorption at 5·90 μ), which was absent with the K-aglycone. As no carboxyl or ester function could be detected, the carbonyl function is interpreted as a ketone or aldehyde group.

The practically equal intensity of the O–H stretching absorption of I- and K-aglycone was found to be greater than that of A-aglycone. The presence of a cyclic-ether linkage in the K-aglycone is considered probable in connection with the presence of a C–O stretching absorption at 8·85 μ (1130 $cm.^{-1}$) which is lacking in the spectra of the other aglycones and is typical for a C–O–C group[10] in the region between 8·77 and 9·35 μ (1140 and 1070 $cm.^{-1}$). The absence of the intense = C–H bending absorption at 11·30–11·34 μ (882–885 $cm.^{-1}$) which is characteristic of the vinylidene group of the lupeol side chain, indicates that the aglycones are derivatives of the oleanane or ursane series[11–13].

Discussion

Production of isomeric aglycones or artifacts has been observed frequently with steroid saponins[14–16] during the hydrolysis of a single glycoside. But for triterpenoid saponins only aescin[17,18] and the triterpenoid glycoside from *Lemaireocereus stellatus*[19] have been reported as behaving in this manner. The infra-red spectrum of the components isolated from saponin B and the relation between the A- and K-aglycone suggest that they arise from the A-glycone as a result of hydrolysis, and the relation may be expressed thus:

$$\text{A-aglycone} \xrightarrow{+H_2O} \text{I-aglycone} \xrightarrow[\text{tautomerism}]{\text{ring-chain}} \text{K-aglycone.}$$

There were no indications that the K-aglycone is identical with any of the pentacyclic triterpenoids so far isolated.

References

1. Mink, *J. Pharm. Pharmacol.*, 1956, **8**, 1155.
2. Bontems, *Bull. Sci. pharm.*, 1942, **49**, 186.
3. Boiteau, Buzas, Lederer and Polonsky, *Nature, Lond.*, 1949, **163**, 258.
4. Polonsky, Thesis, Paris, 1953.

5. Bhattacharyya and Lythgoe, *Nature, Lond.*, 1949, **163**, 259.
6. Lythgoe and Trippett, *ibid.*, 1949, **163**, 259.
7. Mink, Thesis, Utrecht, 1957.
8. Bhattacharyya, *J. Ind. chem. Soc.*, 1956, **33**, 579; 630.
9. Smit, van Wijk and Ruiter, *Rec. trav. chim.*, 1959, **77**, in the press.
10. Bellamy, *The Infra-red Spectra of Complex Molecules*, Muethen & Co. Ltd., London, 1956, p. 100.
11. Cole and Thornton, *J. chem. Soc.*, 1957, 1332.
12. Arthur, Cole, Thieberg and White. *Chem. Ind.*, 1956, 926.
13. McMurry and Thornton, *Analyt. Chem.*, 1952, **24**, 318.
14. Marker and Rohrmann, *J. Amer. chem. Soc.*, 1940, **62**, 896.
15. Marker, Wagner, Ulshafer, Wittbecker, Goldsmith and Ruof, *ibid.*, 1943, **65**, 1248.
16. Marker and Lopez, *ibid.*, 1947, **69**, 2387.
17. Hofer, Thesis, Zürich, 1950, 64.
18. Ruzicka, Baumgartner and Prelog, *Helv. chim. Acta*, 1949, **32**, 2057; 2069.
19. Djerassi, Liu, Farkas, Lippman, Lemin, Geller, McDonald and Taylor, *J. Amer. chem. Soc.*, 1955, **77**, 1200.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Ergot Alkaloids, Separation and Determination of, by Paper Chromatography. M. Pöhm. (*Arch. Pharm.*, 1958, **9**, 468.) In this process the following ergot alkaloids can be identified and determined: ergometrine, ergotamine, ergosine, ergocristine, ergocornine and ergocryptine and their respective *dextro* isomers. The chromatography is carried out on a strip 15 cm. by 45 cm. of Whatman No. 1 filter paper which has been impregnated with formamide, with the mobile phase descending. This latter consists of a mixture of solvents of low polarity —carbon tetrachloride—chloroform—benzene in a ratio 7:2:1 or carbon tetrachloride-*n*-dibutyl ether, 8:2. After the solvent front has travelled about 35 cm., the chromatography is stopped and the positions of the spots of alkaloids localised in ultra-violet light. The limit of sensitivity of this observation by means of fluorescence is about 0·2 μg. of a single alkaloid. A method is described of identifying the alkaloids (if necessary) by comparison with the movement of authentic samples. The quantitative determination is carried out by cutting out the spots and shaking with a known amount of 1 per cent tartaric acid solution and the *p*-dimethylaminobenzaldehyde-sulphuric acid reagent of the British Pharmacopoeia until the paper has disintegrated, and measuring the extinction of the blue solution at 625 mμ. In this preliminary separation certain alkaloids which are not separated require further chromatography. The water-soluble alkaloids ergometrine and ergometrinine remain at the starting point. The spot is cut out and secured in a fold in a second formamide -impregnated strip and developed with a benzene-pyridine (6:1) mixture. The water-insoluble alkaloids ergocristine and ergocornine and ergocristinine and ergocorninine require further chromatography and are treated in the same way as the water-soluble alkaloids. A process for the hydrolysis of the ergot alkaloids and the separation and quantitative determination of the resulting amino acids valine, leucine and phenylalanine is described. D. B. C.

Phenothiazine Compounds, a New Method of Determination of. G. Dusinský. (*Die Pharmazie*, 1958, **8**, 478.) A titrimetric determination of the phenothiazine compounds promethazine, diethazine, chlorpromazine and prochlorperazine is reported based on selective oxidation using either ceric sulphate or potassium bromate. A red radical is first formed by the loss of one electron, the colour of which is discharged at the end point after the loss of a second electron. The progress of the titration can thus either be followed visually or electrochemically by means of a dead-stop method. With promethazine accurate results were only obtained by the dead-stop method. The method is more specific than other methods, for example, direct neutralisation or determination with silicotungstic acid, since it is unaffected by the presence of alkaloidal bases, etc., for example, caffeine, amphetamine, codeine, barbituric acid derivatives and tablet excipients. The accuracy of the method is $\pm$ 1·5 per cent for promethazine and $\pm$ 0·5 per cent for the other compounds. D. B. C.

Thujone, Colorimetric Estimation of, with 3:5-Dinitrobenzoic Acid. D. H. E. Tattje. (*Pharm. Weekbl.*, 1958, **93**, 689.) Data are presented showing the effect of varying the concentration of each reagent involved, and also the effect of temperature, and from these data the following optimum conditions have been worked out. To 4 ml. of a solution of thujone in ethanol containing about 0·5 mg. per ml. is added 5 ml. of a 4 per cent solution of 3:5-dinitro benzoic acid in ethanol and 3 ml. of 4N sodium hydroxide and the intensity of colour measured in a 0·5 cm. cell at 5375Å against a blank identical in all respects except for the omission of thujone. There should be a fresh blank for each estimation, and the temperature should be exactly 20°. α and β-Thujones take different times to attain the maximum extinction which is also different for the two optical isomers. The method requires much less sample and is much more rapid than the hydroxylamine method. Where only the thujones are present in an oil, the two methods are in good agreement, but where other ketones and/or aldehydes are present, low results are obtained due to the specificity of the colorimetric method for thujone. Wormwood oil, however, sometimes gives anomalously high results for β-thujone. D. B. C.

GLYCOSIDES

***Digitalis purpurea*, Influence of Fermentation on the Glycosidal Content of.** D. H. E. Tattje. (*Pharm. Weekbl.*, 1958, **93**, 819.) Twenty-four batches of leaves were taken and four batches were fermented at 35° during periods each of 1, 2, 3, 4, 5 and 6 days and finally dried at 70°. Two other control batches were immediately dried at 70° after harvesting. The digitoxigenin and gitoxigenin glycosides were determined colorimetrically in all the batches, and the glycosidal content calculated on crude fibre. The gitoxigenin content of the fermented leaves was 25 per cent higher than that of the non-fermented leaves, but there was no significant difference in the content of digitoxigenin glycosides for a time of fermentation not greater than 3 days. After a longer fermentation time, however, there was a significant fall in digitoxigenin content. D. B. C.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Allicin, Inhibitory Action on Degranulation of Mast Cells Produced by Compound 48/80, Histamine Liberator from Ascaris, Lecithinase A and Antigen. B. Högberg and B. Uvnäs. (*Acta physiol. scand.*, 1958, **44**, 157.) The authors have already produced evidence that 48/80 degranulates mast cells by activating an enzyme mechanism. It was suggested that this enzyme, possibly carrying essential amino groups, was attached to the mast cell membrane. It is now shown that very low concentrations of allicin, a potent enzyme inhibitor prepared from garlic, block the *in vitro* degranulation of rat mast cells produced by compound 48/80, by a histamine liberator prepared from *Ascaris lumbricoides*, by antigen-antibody reaction using horse serum, and by lecithinase. Excess glutathione reversed the blocked degranulating processes. These observations support the theory that the degranulation of mast cells is due to the activation of a lytic enzyme attached to the mast cell membrane. This enzyme is dependent on sulphhydryl groups for its activity. Whether it is also dependent upon NH_2 groups is now being investigated. M. B.

BIOCHEMICAL ANALYSIS

Bromides, Application of Semimicro Determination of, to Physiological Fluids. D. Kaplan and I. Schnerb. (*Analyt. Chem.*, 1958, **30**, 1703.) This method is suitable for quantities of 0·01 mg. of bromide ion with an error of ± 3 per cent and depends upon the oxidation of bromide to bromate with sodium hypochlorite, and determination of the bromate iodimetrically. In the case of urine, the total halides are precipitated from protein-free urine with excess silver nitrate. The washed silver halides are treated with hydrochloric acid and zinc to displace the silver which is filtered off, and the filtrate analysed for bromide as before. For blood or serum, protein is first removed with sodium tungstate, and the protein-free fluid analysed as for urine. Bromide can also be determined in sweat, saliva and cerebrospinal fluid. D. B. C.

Catechol Amines in Urine, Simple Biological Test for. M. A. Floyer. (*Lancet*, 1958, **2**, 1154.) A rapid, although approximate, estimation of urinary catechol amines can be made by measuring the rise in blood pressure, after intravenous administration of neat, neutralised urine, in the anaesthetised rat. This method is suitable for the screening of large numbers of patients with hypertension to exclude phaeochromocytoma. Up to 15 urine samples can be tested on the same rat. The assay results are expressed as noradrenaline. Added adrenaline does not increase the pressor effect of the noradrenaline when the two are injected together so this method probably measures noradrenaline only and may therefore give a low result for total catechol amines in patients excreting a high proportion of adrenaline. In all six patients with proven phaeochromocytoma the pressor effects of the urine confirmed the diagnosis, whereas in very few instances did the urine from hypertensive patients give a measurable pressor response. There may very occasionally be some doubt in differentiating the latter cases from those of phaeochromocytoma in whom the excretion is low, but in the great majority of cases the separation is easy and reliable. M. B.

Phenylmercury Compounds, Micro-estimation of, in Animal Tissues. V. L. Miller, D. Lillis and E. Csonka. (*Analyt. Chem.*, 1958, **30**, 1705.) By this method, 5 to 20 μg. amounts of phenylmercury acetate per g. of animal tissue or 5 ml. of urine may be determined. For urine, the sample is heated with N sodium hydroxide on a water bath under a reflux condenser. The cooled mixture is treated with potassium permanganate. Excess permanganate is destroyed by adding an ammoniacal solution of hydroxylamine sulphate. Ammonium sulphamate is then added to destroy the oxidising material formed during the alkaline reduction of the excess permanganate by hydroxylamine (probably an oxide of nitrogen). The mixture is acidified with strong hydrochloric acid and extracted with chloroform into which the mercury passes as phenylmercuric chloride. The chloroform is then washed with dilute hydrochloric acid and treated with excess dithizone reagent and the transmittance at 620 mμ determined in order to determine the excess (green) dithizone present rather than the yellow phenylmercury dithizonate. The procedure is similar for kidney, liver, muscle and spleen. For brain, it is necessary to heat under reflux on a water bath with the permanganate. The method was checked by adding quantities of 2 to 20 μg. of phenylmercury acetate to urine, liver, kidney and brain and assaying. Results were within 1 μg. of the expected value.
D. B. C.

PHARMACOLOGY AND THERAPEUTICS

Chlorothiazide Derivatives, Diuretic Activity of. W. Logemann, P. N. Giraldi and M. A. Parenti. (*Nature, Lond.*, 1958, **182**, 1510.) Chlorothiazide (6-chloro-7-sulphamyl-1 : 2 : 4-benzothiadiazine-1 : 1-dioxide) is a very active diuretic drug and it can also relieve hypertension in many patients. It has an inhibitory action on carbonic anhydrase and it also causes a diuresis like that observed with the organic mercurial compounds, resulting in an increase in the excretion of sodium salts as well as water. An analysis is made of the structural conditions necessary for this diuretic activity. It was found that the activity is not largely due to the sulphamyl group. Ring closure to thiadiazin-dioxide is not necessary for diuretic activity. Chlorine cannot be omitted in these compounds and it cannot be replaced by an amino group. A free sulphonamide group is not necessary. Activity decreases rapidly with increasing molecular weight of the alkyl group. The dimethyl compound still has activity and the morpholine compound slight activity, but the *cyclo*hexyl compound is inactive.

M. B.

Chlorpropamide in the Treatment of Diabetes. I. Murray, M. J. Riddell and I. Wang. (*Lancet*, 1958, **2**, 553.) Forty-three diabetic patients were treated with the oral hypoglycaemic agent chlorpropamide (*N*-propyl-*N*-(*p*-chlorbenzenesulphonyl)urea). The patients selected were less than 10 years diabetic, over 40 years old, and had taken insulin, if at all, for not more than two years. The dose of chlorpropamide was 1 g. daily at breakfast. Urine tests (Clinitest) were made before each of the three main meals of the day. A satisfactory response was obtained in 28 of the 43 patients, and was usually evident by the second or third day, and always within 7 days. In the successful group the mean blood sugar fell from 273·5 mg./100 ml. before treatment to 161·8 mg. at the end of two weeks, while in the unsuccessful cases the comparable values were 364·9 mg. and 304·5 mg. In the successful cases the mean daily output of urinary glucose was reduced from 35·5 g. to 3·4 g. daily, whereas the mean figures in the unsuccessful cases were 58·3 g. and 59·6 g. Among the failures with chlorpropamide, 6 had been treated with the other sulphonylureas; all had failed with tolbutamide but 3 had responded to carbutamide, giving the impression that chlorpropamide is somewhat less potent than carbutamide but definitely more potent than tolbutamide. There were no serious toxic effects, but 9 patients showed side-effects (in 4 cases after the dose had been increased to 2 g.); nausea occurred in 7 cases, an erythematous rash in 1, and drowsiness in 1. In most cases which respond a single daily dose of 1 g. is sufficient and this amount should not be exceeded. If after a few days' treatment a good response is obtained the dose should be reduced. S. L. W.

5-Hydroxytryptamine, Radioprotective Action of. H. A. S. van den Brenk and K. Elliott. (*Nature, Lond.*, 1958, **182**, 1506.) The effect of pretreating rats with antagonists and specific antimetabolites, preceding the administration of 5-hydroxytryptamine (5-HT) and of tryptamine, and its effect on the acute lethality from total body X-irradiation has been investigated. Acute lethality was assessed for a 30-day period following irradiation. The loss in body weight and incidence of diarrhoea was also recorded. The antagonist used was 1-benzyl-2 : 5-dimethylserotonin (BAS phenol) and the antimetabolites used were (+)-lysergic acid diethylamide (LSD) and its brominated derivative (BOL 148).

It was found that both antagonist and antimetabolites of 5-HT alone had no significant protective action, but inhibited that of 5-HT and tryptamine. Antihistamines failed to influence the protective action of 5-HT but the radioprotective action of histamine was inhibited. Atropine and dibenzyline, which respectively block the two types of tryptamine receptors, failed to influence the protective action of 5-HT. Tranquillisation of rats with reserpine did not influence the lethality significantly. However, administration of 5-HT 5 minutes before irradiation of reserpinised rats resulted in a radioprotective effect. The results obtained suggest a close correlation between the radioprotective effect of certain amines and their pharmacological actions. M. B.

5-Hydroxytryptamine, The Action of, on the Human Uterus. W. J. Garrett. (*Arch. int. Pharmacodyn.*, 1958, **117**, 435.) Strips of myometrium cut from the anterior wall of non-pregnant human uteri removed at hysterectomy were suspended in oxygenated Ringer-Locke solution and their movements recorded on a smoked paper by conventional methods. Spontaneous rhythm occurred after a latent period of 2–3 hours. 5-Hydroxytryptamine (5-HT) in concentrations up to 100 μg./ml. usually decreased the background tone of the muscle and the frequency of the spontaneous contractions. The amplitude of the spontaneous contractions was, however, increased. Larger concentrations of 5-HT (100–500 μg./ml.) caused a sustained contraction of the muscle upon which the spontaneous contractions were superimposed. Kymograph records of uterine activity in volunteer patients were taken in pregnancy and labour by means of a small hydrostatic balloon passed into the lumen of the uterus through the cervical canal. Intravenous infusions of 5-HT from 2·2 to 300 μg./min. had little effect on uterine contractility and no marked side effects were observed. In one case, 200μg. of 5-HT was given by rapid intravenous injection and in this case mild stimulation of the uterus was observed. The author concludes that 5-HT has little significance in the physiology of human myometrial contraction since the observed effects were produced only by concentrations considerably higher than those which exist under physiological conditions. W. C. B.

Iron Preparation, Oral, Gastrointestinal Tolerance to. D. N. S. Kerr and S. Davidson. (*Lancet*, 1958, **2**, 489.) Ferrous sulphate, ferrous gluconate, ferrous succinate, ferrous calcium citrate, and "known" and "unknown" control pills containing lactose, were administered to 93 healthy young women in a double-blind trial. The ferrous sulphate, gluconate, and succinate pills each contained 35 mg. of iron; of these, and of the control pills, the dose was one three times daily. The ferrous calcium citrate was prepared in pills containing 17·5 mg. of iron, and these were given in a dose of two pills three times daily. All the pills were identical in size, shape, colour and coating. The pills were taken from Mondays to Fridays during 6 successive weeks, and at the end of each week a questionnaire was completed giving details of any symptoms experienced during the preceding week. Virtually no toxic effects were reported from the "known" control pills, but the exactly similar "unknown" control pills, which were thought by the subjects to contain iron, produced as many side-effects as the pills which did contain iron. None of the four iron pills was found significantly more toxic than the inert pills. It was therefore concluded that intolerance to these iron preparations, in the dosage given, was mainly psychological in origin. S. L. W.

Reserpine, Researches on the Mechanism of Sedative Action. S. Garattini and L. Valzelli. (*Science*, 1958, **128**, 1278.) A series of experiments were carried out on female rats kept in constant temperature rooms at 0°, 22°, 29° or 37° to determine whether a relationship exists between the sedative action of reserpine, 5-hydroxytryptamine (5-HT) release and hypothermia. The sedative action of reserpine was evaluated by the potentiation of sleeping time after the intraperitoneal administration of pentobarbitone. Brain 5-HT was extracted and measured spectrophotometrically and rectal temperature was determined with a resistance thermometer. The rats were injected with 2·5 mg./kg. of reserpine 4 hours before the determinations were made. The results showed that increasing room temperature from 22° to 37° did not change body temperature, nor did it affect the sleeping time after pentobarbitone. It did, however, cause a small increase in the content of brain 5-HT. After reserpine was injected, barbiturate sleeping time was prolonged only when 5-HT was released. This occurred, together with a fall in body temperature, with the rats kept at 22° and 29°. With those kept at 37°, body temperature was unchanged and brain 5-HT increased after the administration of reserpine; under these conditions reserpine showed no evidence of sedative activity. Reserpine significantly decreased the body temperature of rats kept at 0° but there was no change in brain 5-HT and no sedation. The results supply further evidence in support of the hypothesis that the sedative action of reserpine takes place only when there is 5-HT release. The onset of hypothermia was not always associated with sedation and it was not correlated with 5-HT release. W. C. B.

Tessalon in Pulmonary Insufficiency and Irritative Cough. R. L. Wilson, S. M. Farber and W. Mandel. (*Antibiotic Med.*, 1958, **5**, 567.) The drug, ω-methoxypoly(ethyleneoxy)ethyl *p*-butylaminobenzoate (Tessalon), is a cough suppressant that appears to act by suppressing the afferent arm of the Hering-Breuer reflex. It is chemically related to the local anaesthetic, amethocaine, with the addition of a long chain glycol. The respiratory effects of intramuscular and oral preparations of the drug were studied subjectively and by tests of physical effort in 30 patients with pulmonary fibrosis or emphysema. The drug was given intramuscularly in a dose of 5 mg. in 17 of the patients, the standard physical tolerance test being evaluated one hour after injection. Some of the tests were repeated following a week on the oral preparation in a dose of 150–200 mg. daily. The most constant finding was an increase in the amount of physical effort that could be tolerated, which occurred in 12 out of the 17 patients. This was associated in those patients with an increase in the tidal volume as the exercise progressed, and a less rapid augmentation of the respiratory rate than occurred before the drug was administered. In some patients the recovery from maximal hyperventilation to normal respiration appeared accelerated. The recovery to normal respiration after a standard exercise was markedly improved in almost every case. In 20 patients who were given the drug orally a subjective evaluation only showed that 18 were improved or much improved, with less cough and less shortness of breath and increased effort tolerance. In a further group of 37 patients with pulmonary tuberculosis the daily administration of 150 mg. orally had a beneficial effect on the cough in a significant number. There is evidence that the effects of the drug may persist beyond the period of administration. The only toxic reactions noted were one case of mild erythema and one of transient skin itching.

S. L. W.

Triamcinolone Arthritis. R. Wells. (*Lancet*, 1958, **2**, 498.) Fourteen patients, all of whom had previously been receiving optimal doses of prednisolone for various conditions without any incidence of important side-effects, were transferred to triamcinolone. Of 9 with no previous history of joint symptoms, 3 developed arthritis; in 2 of these the condition resembled acute rheumatoid arthritis. It is suggested that arthritis may be a side-effect of triamcinolone therapy. S. L. W.

APPLIED BACTERIOLOGY

Blankets and Hospital Infection. H. Schwabacher, A. J. Salsbury and W. J. Fincham. (*Lancet*, 1958, **2**, 709.) Two experiments were made to assess the effect of blankets on bacterial infections in hospital. The first experiment showed that the use of freshly washed blankets, disinfected with quaternary ammonium compounds, for each patient admitted to a test ward, reduced the total bacterial count (and especially the *Staphylococcus aureus* phage-type 80, which was previously endemic), and also decreased cross-infection. The second experiment showed a moderate reduction of the bacterial count when terylene blankets were used instead of woollen blankets. But the total bacterial count was reduced more, and there was no cross-infection, when cotton blankets were used. After the first washing, cotton blankets did not produce the fluff which seems to a major factor in the spread of infection. It is suggested that the following measures would probably result in a great diminution in bacterial counts in hospitals: (1) woollen blankets should be replaced by cotton ones washed at 100° before use; (2) on admission, every patient should be given a bed covered with freshly washed sheets and cotton blankets; (3) all blankets should remain on the beds—ambulant patients may have separate blankets, which should be regularly washed, for use when not in bed. S. L. W.

Blankets and Hospital Infection: Fibre Composition of Hospital Dust. T. A. Pressley. (*Lancet*, 1958, **2**, 712.) All samples of airborne dust in three hospitals (in Melbourne) consisted essentially of cellulose fibres; very few wool fibres could be found. The cellulose fibres presumably came from sheets, towels, clothes, ward dressings, and bandages. Since dust from one hospital using all-wool blankets showed a fibre distribution similar to that from two other hospitals using wool-cotton union blankets it is unlikely that the cotton warp of the union blankets contributed materially to the air-borne cellulose. Most samples subjected to bacterial examination contained coagulase-positive *Staphylococcus aureus*. This suggests that the cross-infection with the *Staph. aureus* is primarily due to transfer of the bacteria by some agency other than the fluff from blankets, and therefore the replacement of woollen blankets with those made from other textile fibres, or the application of an oiling technique to blankets only, is unlikely to reduce cross-infection. S. L. W.

***Salmonella typhimurium* Infection, Dust-borne.** J. G. Bate and U. James (*Lancet*, 1958, **2**, 713.) In the infants' ward of a children's hospital seven outbreaks of gastro-enteritis over 11 months were caused by *Salmonella typhimurium*, phage-type 2. The infection was not being spread by human carriers nor from the central milk-kitchen. The source of infection was finally found in the dust-bag of a vacuum floor-polisher. Since suction-cleaning and polishing of the floors by machines fitted with disposable paper dust-bags has been substituted, there has been no case of *S. typhimurium* infections. S. L. W.

BOOK REVIEW

DISINFECTION AND STERILIZATION. Theory and Practice. By G. Sykes. Pp. xviii + 396 (including Index). E. and F. N. Spon, Ltd., London, 1958. 75s.

There are several established works on disinfection and sterilisation, but the rapid accretion of information on these subjects makes any new publication acceptable if it has been competently prepared. This book is welcome on this score as well as on others. The greater part of the text is so well written as to be understandable, and this is an achievement in a field in which the vast amount of experimental results obtained has not been matched by a corresponding advance in fundamental knowledge. Microbiologists derive from a variety of scientific disciplines; many experiments providing comparable results have been designed for quite different purposes and, too often, with an inadequate understanding of the essential principles of biological investigations. The writer of such a book must attempt the almost impossible task of correlating published information, and most microbiological books suffer in this attempt. The author of this book has not always been more successful in this than his predecessors; for example, parts of the chapter on phenols and related substances lack the precision of most of the text. Occasionally the evidence presented does not seem to support the author's conclusions. This however is far from being general throughout the book, and those parts of the subject where the experimental evidence is consistent and particularly those in which the author has had personal experience are presented with admirable clarity.

The general plan is not very different from that of similar publications, and this is perhaps unfortunate as the first part on the Theory of Disinfection and Methods of Testing is not the best in the book. Possibly because bacteria often behave similarly to chemical reagents microbiologists have attempted to explain the metabolism, reproduction and death of cells in terms of the equations and graphs of physical chemistry. Mr Sykes, although himself a chemist, clearly recognises this as an oversimplification, and commences his chapter on the theory of disinfection by a discussion on microbial enzymes. He continues, however, with a section on the "dynamics of disinfection" with the latest elaborations, which he does not marry too happily with the remainder of the chapter. The next two chapters on methods of testing disinfectants and antiseptics reflect the author's great practical experience although there is considerable variation in the amount of critical attention given to the different methods referred to. It hardly seems necessary to describe the phenol coefficient tests in full detail as this detail is readily available to those who need it, and the space so used could have been allocated to lengthen descriptions of other tests and to an assessment of their merits and applications. So many of these tests, and indeed of the experiments assessing sterilisation procedures, depend on the practicability of producing visible growth from small numbers of damaged cells, that a chapter might well have been allocated to this aspect.

Part II, which discusses methods of sterilisation, and Part III, on disinfection and sterilisation, are very good, and all pharmacists who are concerned with these subjects should read them. Nevertheless, anyone who expects to find a complete pharmaceutical treatise telling him how to sterilise any particular pharmaceutical preparation or material is likely to be disappointed. Information on the sterilisation of hypodermic syringes, plastic tubing and rubber gloves and dry powders where it occurs is incidental and sometimes is not even indexed. Chemical

disinfectants are discussed in five chapters following the usual pattern in such books, and there are references to most substances in current use. It is a commentary on the quality of much experimental work in this field that the author, after noting the limitations of the phenol coefficient earlier, has perforce to refer to such coefficients in providing quantitative information on a variety of substances for which this coefficient has little significance. There are no chapters on the antibiotics and sulphonamides because, as the author explains, they are already covered in other volumes. So are most other antibacterial substances, and it is doubtful if antibiotics can be denied a major role in a modern book of this sort; indeed there are frequent references to them in the text. The last part of the book is on preservatives and preservation and deals with a subject in which the author has himself worked. As with the rest of the book wherever this applies, the section is well done. It is a pity that it could not have been expanded as it is obvious that the author has much more to say on this subject. Sufficient is written, however, to show how much more work has to be done on the preservation of pharmaceutical preparations.

The book is a competent and useful work which does credit to the author and to the publishers. The first impression is likely to be rather more favourable than subsequent impressions because the subjects described are in a condition which makes any book on them unsatisfactory to its readers unless the author chooses to simplify by an autocratic selection of evidence which Mr. Sykes does not. It should not be regarded as a pharmaceutical textbook written primarily for the pharmacists and students of pharmacy; it is obviously not intended to be so circumscribed. However, the author's long experience in pharmaceutical microbiology produces this effect to some degree, and many pharmacists and pharmaceutical students will find the book useful and, until a more specialised work becomes available, even essential.

K. R. CAPPER.