C O R D Y C E P I N

A N E W A N T I B I O T I C

A T H E S I S

submitted to

THE UNIVERSITY OF GLASGOW

in fulfilment of the

requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

KENNETH G. CUNNINGHAM

October, 1951.

The Work Described in this Thesis Appears in the Following Publications;

1) Cordycepin, A Metabolic Product isolated from Cultures of Cordyceps militaris (Linn.) Link.
Nature, 1950, 166, 949.

2) Cordycepin, A Metabolic Product from Cultures of Cordyceps militaris (Linn.) Link. Part I. Isolation and Characterisation.
J. Chem. Soc.,(in print) received April 1951.

3) Cordycepin, A Metabolic Product from Cultures of Cordyceps militaris (Linn.) Link. Part II. The Structure of Cordycepin.
J. Chem. Soc.,(in print) received April 1951.

4) Micro-determination of the Molecular Weights of Picrates by a Spectrophotometric Method.
J. Chem. Soc.,(in print) received April 1951.

5) And in Part;

Hydroxamic Acids. Part II. The Synthesis and Structure of Cyclic Hydroxamic Acids from Pyridine and Quinoline.
J. Chem. Soc., 1949, 2091.

The Author wishes to express his sincere thanks to Professor F. S. Spring for his excellent supervision of this Research.

The thanks of the Author are also due to Dr. H. R. Bentley for valuable criticism and advice, to Dr. S. A. Hutchinson without whom this work would not have been undertaken, to Mrs. Dorothy Hodgkin and Dr. I. A. Brownlie for X-ray and infra-red examinations, and to William Dawson for collaboration in the work described in the Appendix to this Thesis.

The Author is indebted to the Governors of the Royal Technical College, Glasgow, for the award of the Nobel Research Scholarship.

C O N T E N T S

SUMMARY

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A strain of the mould Cordyceps militaris (Linn.) Link has been promoted on nitrogenous media to yield solutions which inhibit the growth of several micro-organisms. It has been shown that the total activity of the solutions can be absorbed upon activated charcoal from which the active principle, cordycepin, has been isolated. Dilute aqueous solutions of cordycepin reproduce the inhibitory effect of the culture solutions towards the test organism, and the activity is sufficiently great to term cordycepin an antibiotic.

The metabolic product contains the elements carbon, hydrogen, oxygen and nitrogen and is optically active. It forms salts with picric acid, picrolonic acid and flavianic acid, and elemental analyses of the metabolic product and its simple derivatives suggested the empirical formula $C_8H_{10}O_2N_4$ or $C_{10}H_{13}O_3N_5$. The solubility properties of cordycepin are such that a standard method of molecular weight determination could not readily be employed to distinguish between these alternatives; an accurate micro-method for the molecular weight determination of bases such as cordycepin was therefore devised and is discussed in the Appendix. The

molecular weight of cordycepin was found to be 254, in close agreement with that required by $C_{10}H_{13}O_3N_5$ (251).

The first indication of the structure of the metabolic product was obtained from a study of its ultraviolet light absorption which shows marked similarity to that of some pyrimidine and purine compounds. Cordycepin is resistant to reduction and hydrogenation, but benzoylation yielded a tetrabenzoate and from the acid hydrolysis of this ester, adenine hydrochloride was isolated, thus accounting for the total nitrogen content of the molecule. The metabolic product itself was found to be unstable in dilute hydrochloric acid, hydrolysis occurring to yield solutions from which adenine was isolated and characterised as its picrate. Solutions of hydrolysed cordycepin have been shown to react with 2:4-dinitrophenylhydrazine in hydrochloric acid, yielding the 2:4-dinitrophenylosazone of a molecule $C_5H_{10}O_4$ and this suggests that the linkage between adenine and the residual fragment is glycosidic rather than amide.

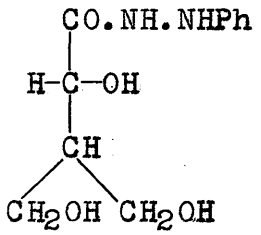
No carbon-methyl, methoxyl or nitrogen-methyl group was detected in cordycepin, and the absence of the grouping $CH_3.CHOH$ or $CH_3.CO$ in the side chain was confirmed by the negative result of the iodoform test,

but the presence of a terminal hydroxymethyl group was proven by the tosylation of cordycepin.

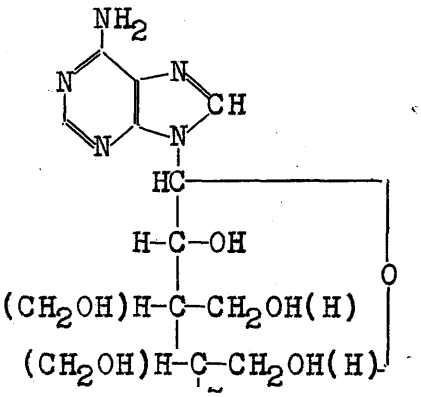
Deamination of cordycepin yielded hypoxanthine, proving the molecule to be unsubstituted on the 6-amino group of the adenine nucleus. The glycosidic bond therefore occurs at the 7- or 9-position and the formulation of cordycepin as an adenine-9-glycoside is much preferred on the basis of the ultraviolet absorption spectrum. Since an osazone of the glycosidic fragment has been isolated corresponding to a deoxypentose (cordycepose), cordycepin cannot be an adenine-9:2'-deoxypentoside; evidence that the molecule is an adenine-9:3'-deoxypentoside has been provided by the fact that cordycepin is resistant to periodate oxidation.

Careful hydrolysis of cordycepin followed by removal of adenine and hydrochloric acid yielded analytically pure cordycepose as a pale straw-coloured syrup which reduces Fehling's solution; bromine oxidation of the sugar gave the corresponding lactone, cordyceponolactone, $C_5H_8O_4$. The latter yields a crystalline phenylhydrazide and cordycepose must be a deoxyaldopentose. The four stereoisomeric phenylhydrazides corresponding to a straight-chain 3-deoxyaldopentonic acid have been described previously, and differ markedly in physical

properties from cordyceponic acid phenylhydrazide. Cordycepose is therefore a branch-chain sugar, and this theory is strongly supported by the fact that no optical activity could be detected in ethanolic solutions of cordycepose p-bromophenylosazone. Cordyceponic acid phenylhydrazide contains only one assymetric carbon atom, and being dextrorotatory, it is formulated (on the basis of the phenylhydrazide rule) as (i), whilst cordycepin is represented by one of the two stereoisomeric forms of (ii) of which the configuration about the glycosidic linkage has not been determined.



(i)



(ii)

A number of attempts to confirm the structure of cordycepose synthetically (principally through the compound 2-bromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal) failed, due principally to the fact

that mixed products were obtained which could not be separated by distillation, decomposition occurring even in high vacuum.

A method by which the molecular weight of cordycepin was determined (and which is applicable to any base or hydrocarbon yielding a pure picrate) has been devised and proven, against a wide range of compounds, to give results with an accuracy of ±2%. The method depends upon the fact that the ultraviolet light absorption of picric acid is intense in the near-visible range of the spectrum, (a range in which few colourless organic compounds exhibit appreciable absorption) and is unaltered in this region by the absorption of another molecule at lower wavelength. Thus, for all picrates the value of ε is a constant for any given wavelength in the near-visible range and M (molecular weight, the only unknown) is evaluated by calculation.

INTRODUCTION.

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The biochemical and chemical investigation of the life processes of bacteria and moulds has recently been stimulated by the discovery that among the metabolic products of these micro-organisms are compounds with considerable antibacterial properties, and hence of potential chemotherapeutic value. In fact, the present chemical interest in micro-organisms is largely conditioned by the concept that of the many bacteria, and more especially moulds, which have not yet been investigated, a number must exist which are capable of producing compounds of medicinal importance.

The classes of micro-organisms which lend themselves most readily to chemical investigation, because of their relative ease of cultivation on synthetic media, are the Schizomycetes (bacteria), the Eumycetes (moulds) and the Actinomycetes, the last-named being unicellular organisms closely resembling bacteria in their structure, but producing a mycelial "felt" similar to that of a mould. In general, the culture medium is an aqueous solution of compounds which supply the elements required by the

micro-organism for its propagation. Carbon is usually made available in the form of carbohydrate, although alcohol or carboxylic acid are also employed, and combined nitrogen is frequently supplied as amino-acids. The elements sulphur, phosphorus, magnesium and potassium, and traces of the heavy metals iron, copper and manganese may also be required.

The sterilized medium is inoculated with the micro-organism under investigation, and is then incubated at the optimum temperature until maximum growth has been obtained. If the micro-organism is one which produces in the culture medium a metabolic product with antibacterial properties, then the progress of the culture is observed by assaying the activity of the medium until maximum results are obtained.

The commonly employed methods of assaying the activity of culture filtrates, concentrates or solutions of pure compounds, are based upon the inhibition of growth of a control organism. The methods are arbitrary and correlation of results is very difficult. In the agar-streak method (1), varying dilutions of the active substance are mixed with specific volumes of nutrient agar medium, which

is then allowed to set and is streaked with the test organisms. The dilution at which the growth of a specific organism is inhibited under standardised conditions gives a measure of the potency against that organism. The serial dilution method (2), on the other hand, utilises a liquid medium made to a standard volume with different concentrations of the active compound. Inoculation with the test organism and incubation under controlled conditions evaluates the highest dilution at which growth is completely inhibited, as shown by the lack of development of turbidity in the medium. In the diffusion cup method (3), the nutrient agar is inoculated with the control organism and is allowed to set before discs of the medium are removed and are replaced by cylindrical porous cups. The active solution is placed in the cups and diffuses into the agar during incubation, thus creating an area of inhibition around each cup. The diameter of this area is taken as a measure of the activity of the compound against the test organism.

Although the term "antibiotic" was introduced by Waksman (4) to include all of those compounds produced by micro-organisms which are active against other micro-organisms, the definition was made more strict

a fundamental metabolic process of a mould, but may be determined by nutritional supply and culture conditions. Nor is antibiotic production necessarily a manifestation of microbial antagonism, since an organism may produce a compound active only against other organisms with which it does not normally compete for existence (5). Different strains, and even different genera, may produce the same antibiotic compound, thus patulin (from Penicillium patulum, 8), clavacin (from Aspergillus clavatus, 9), clavatin (from Aspergillus clavatus, 10), claviform (from Penicillium claviforme, 11), and expansin (from Penicillium expansum, 12) are all the same compound. On the other hand, the promotion of one specific strain under varied conditions may yield more than one antibiotic. Waksman (13) has reviewed numerous attempts dating from the late 19th Century, to apply metabolic products to therapeutic purposes, and of these the work of Emmerich and Löw (14) merits particular mention. From cultures of Pseudomonas pyocyanea they prepared an active heat-resistant preparation "pyocyanase" which was employed in particular against Bacillus anthracis infection. Although never definitely proven beneficial to man,

this preparation was commercially available in Germany over a period of 3 decades.

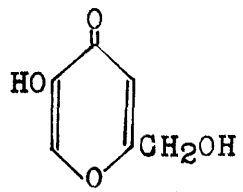
The first isolation of an antibiotic elaborated by a mould, and active against pathogenic bacteria, was carried out by Alsberg and Black (15) who obtained penicillic acid from cultures of Penicillium puberulum. The metabolic product was found to inhibit the growth of Bacillus coli. In 1929, Fleming (16) reported that culture filtrates of Penicillium notatum exhibited extreme antibacterial activity, particularly against Gram-positive cocci, and it was later shown (17) that penicillin could also be obtained in a synthetic medium, but could not be isolated from the acidified culture filtrates by ether extraction. It was not until 1940 that Florey and co-workers (18) obtained a stable penicillin preparation suitable for investigation and chemotherapeutic examination. The activity of penicillin is much greater, and more specific, than that of the sulphonamides, no toxic effects having been observed by the local administration of large amounts, and it has been employed against Gram-positive infections by intravenous and subcutaneous injection with remarkable success.

Many antibiotics isolated from mould and bacteria

cultures have been studied during the past ten years and those have led in turn to the synthetic investigation of compounds containing a specific functional group with the object of isolating compounds of therapeutic value. The result of this intensive research has been the isolation and characterisation of a large number of antibacterial compounds, of which a small percentage have found clinical application. The volume of this work is so great that no detailed discussion of it is possible; however, the subject has been frequently reviewed (19).

A second class of compound isolated from media on which micro-organisms have been promoted is produced by rearrangement or breakdown of essential nutrients. This was first demonstrated by Wehmer (20, 21) who isolated oxalic acid from cultures of species of Aspergillus. He also isolated citric acid in high yields from species of Citromyces grown on sugar-containing media (22). Other simple acids including D-gluconic acid (23) glycollic and glyoxylic acids (24) fumaric acid (25) and malic acid (26) arise by the degradation of carbohydrate in the medium. Simple alcohols (including ethanol) have been isolated from widely distributed species, and simple aldehydes,

ketones and esters have also been reported frequently. Two interesting products obtained by the cyclic dehydration of a hexose molecule are the γ-pyrone kojic acid (I) and the furan derivative Sumiki's acid (II).



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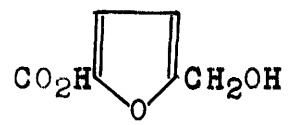

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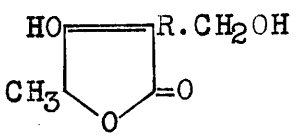
Kojic acid was first isolated by Saito (27) in high yield from cultures of Aspergillus oryzae, a micro-organism utilised in Japan to ferment rice in the production of the alcoholic beverage saké. It has subsequently been observed as a metabolic product of other members of the Aspergillus genus and also of Penicillium dalaea. No commercial use for this compound has yet been found. Sumiki's acid is produced from glucose or sucrose media by a number of species of Aspergillus (28).

Raistrick et al. (29) have isolated from Penicillium charlesii a series of acids (III-VI, R = $CO(CH_2)_{2-7}$) which were shown to be derivatives of tetronic acid (VII). Their interest lies in their similarity to ascorbic acid (vitamin C, VIII).



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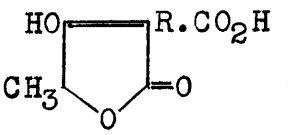

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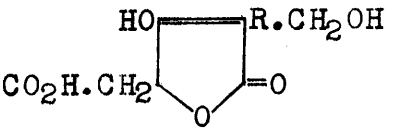

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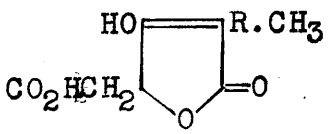

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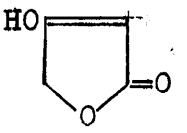

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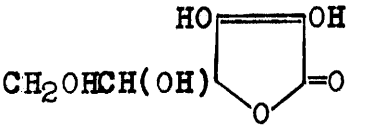
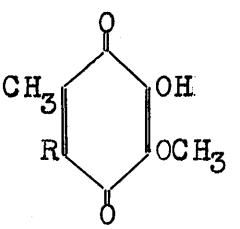


(VIII)

In addition to the production of fats (30), polysaccharides (31, 32, 33), polyenes (34, 35, 36, 37, 38) and sterols (39, 40, 41) from synthetic media containing sugars or polyhydric alcohols as the only source of carbon, a large number of pigments have also been isolated. These have been reviewed in many publications (42) and a few characteristic examples only will be mentioned.

The majority of naturally occurring pigments are derivatives of benzoquinone and anthraquinone, the simplest known being 2:6-dimethoxybenzoquinone, isolated by Karrer from Adonis vernalis L. (43). The maroon pigment fumigatin (IX, R = H) from Aspergillus fumigatus Fresenius (44) and spinulosin (IX, R = OH) from Penicillium spinulosum Thom (45)

are closely related to the yellow-brown pigment phoenicin (X) isolated from Penicillium phoenicoeum van Beyma (46). Typical of the anthraquinone series are the 2-substituted anthraquinone pigments which have been isolated from species of Helminthosporium and other genera by Raistrick and his co-workers in recent years.



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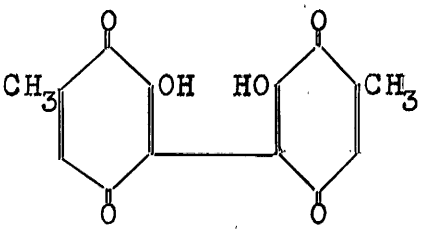


(X)

The work to be described in this Thesis involves the large-scale promotion of a strain of the mould Cordyceps militaris (Linn.) Link on a synthetic liquid medium and the subsequent isolation from the culture filtrates of a metabolic product to which the name cordycepin is given. This compound is shown to have antibiotic properties and the elucidation of its structure is described.

Previous work on the species Cordyceps militaris (Linn.) Link has been concentrated on its morphology, life history and relationships (47, 48, 49) and little

study has been made of its physiology (50, 51). The genus Cordyceps of the Ascomycetes class includes some sixty species which are found most commonly in semi-tropical climates, and which are generally parasites of the imagines, pupae and larvae of insects.

Cordyceps militaris attacks lepidopterous pupae and larvae and is very widely distributed. The mould usually enters the host orally and is propagated in the blood-stream with the subsequent destruction of all internal organs, the skin alone remaining intact. When the sclerotium is mature it is a compact mass of interwoven hyphae whose cells are rich in glycogen and oil, and from this mummified structure the stromata emerge through the surface of the host tissue. It is from the appearance of the stromata, which often stand erect as straight, red, cudgel-shaped bodies, that the name militaris was derived. Spores are scattered from the stromata to continue the life cycle of the mould.

That Cordyceps militaris might produce an antibiotic compound was deduced from the fact that the residue of host tissue incorporated in the pseudo-sclerotium is resistant to decay. A monoascospore culture from a sporophore collected at Tollymore Park, Co. Down (52) in the autumn of 1948 was promoted by

Dr. S. A. Hutchinson on a peptone-Lemco-glucose-agar medium in which the pH was adjusted to 7.0 before autoclaving. The culture grew vigorously when incubated at an arbitrary temperature of 24° and typical Cladosporium conidial stages (49) developed after 5 - 7 days. Preliminary tests of antibiotic activity were made by transferring agar discs out from petri-dish cultures of the fungus to the surface of agar plates seeded with test organisms. Positive results were obtained using Bacillus subtilis strain 288.

Subsequently a liquid medium (peptone 0.5%, Lemco 0.3%, glucose 1.0%) was employed with and without a phosphate buffer and the mould grew vigorously with the production of acidity in the medium. Maximum antibacterial activity of the same order in each case was observed in the culture filtrates after 3 - 4 weeks, the diffusion cup assay technique (3) being used. Employing the same media without Lemco the rate of production of activity was unaltered, but with a peptone-Lemco-phosphate medium only negligible mycelial development was observed and no antibiotic activity could be detected. Bacillus subtilis strain 6752 from the National Collection of

Type Cultures was found to give a slightly sharper response than the previous strain employed, and was used in these assays (Table I).

TABLE I.

Average results of 10 Cultures
(Medium depth 15 mm.)

		Peptone-Lemoo-glucose-phosphate.	Peptone-glucose-phosphate.	Peptone-glucose.	Peptone-Lemoo-phosphate.
Initial medium pH	:	8.0	8.2	5.8	8.2
8 days: Inhib.(mm.)	:	7-10(f)	7-10(f)	7(f)	-
pH	:	4.3	3.7	5.5	7.6
16 days: Inhib.(mm.)	:	26(s)	29(s)	28(s)	-
pH	:	7.3	7.0	3.5	7.4
24 days: Inhib.(mm.)	:	35(s)	36(s)	35(s)	-
pH	:	8.0	8.1	7.9	7.4

(f) = faint ill-defined inhibition.
(s) = clear well-defined inhibition.

Furthermore it was shown by Dr. Hutchinson that, over a wide pH range, variation in the initial acidity of the medium (peptone-glucose) has only a very slight effect on the rate of production of antibiotic activity by Cordyceps militaris. Maximum activity is

obtained from a medium which is initially neutral (Table II).

TABLE II.

Average Results of 3 Cultures.

Initial pH of Medium.	Days of Incubation. 5 pH	5 Inhib. (mm.)	9 pH	9 Inhib. (mm.)	13 pH	13 Inhib. (mm.)	16 pH	16 Inhib. (mm.)	21 pH	21 Inhib. (mm.)
3.4	3.0	-	2.8	-	3.9	10(f)	5.6	18(h)	7.7	25(s)
4.8	3.0	-	4.1	10(f)	7.6	22(s)	7.9	22(s)	8.2	24(s)
6.9	3.0	-	2.8	10(f)	6.9	24(s)	7.7	28(s)	8.1	28(s)

(s) and (f) are defined under Table I.
(h) = clear inhibition with hazy peripheral definition.

The data given in these tables are sufficiently comprehensive for the large-scale production of Cordyceps militaris (Linn.) Link to be undertaken. No attempts have yet been made to study the relationships between incubation temperature and antibiotic production, or the effects of trace concentrations of inorganic salts upon the promotion of the mould and the nature of the metabolic product.

THEORETICAL

THEORETICAL.

Promotion of Cordyceps militaris (Linn.) Link.

The choice of the most suitable synthetic medium on which a specific micro-organism should be promoted, with the object of isolating a particular metabolic product, may lead to a very protracted investigation. Slight alteration in the formula of a medium often causes considerable increase or decrease in yield of metabolic product, and on occasion, has been found to change the chemical configuration of the metabolic product. It was the experience of White and Hill (53, 54) that when a species of Aspergillus flavus was promoted on a variety of synthetic media, on all of which heavy mycelial growth was supported, several of the culture filtrates showed great activity against both Gram-negative and Gram-positive bacteria, while others exhibited negligible antibacterial activity. Furthermore, whilst a particular modification of a medium may yield culture filtrates of higher antibacterial activity, a concurrent increase in the difficulty of isolation of the active principle sometimes discounts this advantage (55).

In view of the natural habitat of the mould

Cordyceps militaris (Linn.) Link, and the investigation of Hutchinson described previously, it was decided that a high proportion of nitrogen should be incorporated in the medium to be employed. This condition was satisfied by the use of "Pronutrin", a commercially available enzymatic casein hydrolysate, which, together with glucose, was found to form a satisfactory medium. "Glaxo" type culture flasks were used throughout the investigation, and Bacillus subtilis strain 6752 of the National Collection of Type Cultures was employed as test organism. The response by this micro-organism to the activity produced in the mould culture filtrates, using the diffusion cup assay technique (3), was fairly sharp, but was not sufficiently sensitive to yield a method of quantitative assay.

An initial investigation was carried out to ascertain the most profitable medium depth in the culture flasks for antibiotic production. Arbitrary depths of 9 mm. (200 ml. medium/flask) and 18 mm. (400 ml. medium/flask) were employed, inoculation in each case being made from the same bulk aqueous conidial suspension of the mould, to ensure standardisation of the test. The flasks were incubated in the absence of light, and the development of acidity in the medium

(assessed by the Capillator method) and subsequent production of antibiotic activity were measured. From the results shown in Table III, it was found that the average rate-volume of production of antibiotic activity under each condition is almost constant, maximum activity in the shallower medium being attained after 12 - 15 days, and in the deeper medium after 24 - 30 days. The greater medium depth was therefore employed throughout, in the large scale production of the metabolic product.

TABLE III.

Average Results of 20 Cultures.

Days.	Shallow Medium (9 mm.)		Deep Medium (18 mm.)	
	pH	Inhib.(mm.)	pH	Inhib.(mm.)
1	5.8	-	5.8	-
3	5.8	-	5.8	-
6	5.8	-	5.8	-
9	3.2	27(h)	5.4	-
12	2.4	28(s)	4.5	28(f)
15	5.6	28(s)	3.9	30(h)
18	7.4	29(s)	3.5	28(h)
21	7.6	27(s)	4.6	26(s)
24	8.0	28(s)	6.4	29(s)
27	8.0	27(s)	7.4	29(s)
30	8.2	29(s)	7.8	28(s)

(f), (h) and (s) are defined under Table II.

It was also found that the mycelia maintain their capacity to produce active culture filtrates over a

prolonged period on repeated reflooding with fresh medium. The values in Table IV show that by reflooding established mycelia three to four times with fresh medium at intervals of approximately one week, the rate of production of active solutions can be greatly increased. This practice was not employed in the large scale production of cordycepin, due, not only to the impracticability of handling the large volume of active solution produced, but also to the difficulty of preventing contamination during large scale reflooding.

TABLE IV.

	Average Values of 10 Cultures.						
Reflood:	0	I	II	III	IV	V	VI
Age of Mycelia (Days)	22	28	34	41	47	61	92
Fermentation of Fresh Medium (Days)	22	6	6	7	6	14	31
Final pH	7.9	7.2	7.5	7.8	6.0	8.4	8.9
Inhibition Zone Diameter (mm.)	40	32	31	27	27	10	-
Inhibition Quality.	(s)	(s)	(s)	(s)	(s)	(f)	-

(f) and (s) are defined under Table I.

The maximum volume of medium which could be accommodated at one time for the surface production of the mould was 40 - 50 litres (100 - 110 flasks),

and the inoculant for this volume was prepared by promoting the mould in 8-ounce bottles on a peptone-glucose-agar medium. After incubation at 24° for 6 - 7 days, typical Cladosporium conidial stages developed (49), and the mycelium was then subcultured into "Thomson" culture flasks, containing the standard glucose-Pronutrin medium. These flasks were incubated until a heavy growth of mycelium was produced, and were then stored in a refrigerator until required. Finally, the mycelium was disintegrated by vigorous agitation to yield a bulk spore suspension, each "Thomson" flask thus yielding sufficient inoculant for 20 - 25 litres of medium.

Submerged growth was frequently observed in the culture medium some 24 - 48 hours after inoculation, and was quickly superseded by vigorous surface growth, a complete white mycelial "felt" being produced after 5 - 8 days. Inhibition assays of the medium were carried out at regular intervals during the second, third and fourth weeks of incubation, maximum inhibition (of diameter 35 mm. approximately) being almost invariably observed early in the fourth week. At this stage, incubation was discontinued, and the bulked active culture solutions were filtered free of

myoelia.

Isolation of Metabolic Product.

The problem of the isolation of the metabolic product from the culture filtrates was overcome by employing a modification of the method originally used in the isolation of penicillin from cultures of Penicillium notatum. The method has been described in detail by Whitmore et al. (56), and involves the absorption of the active metabolic principle on finely divided charcoal, followed by elution of the air-dried charcoal with aqueous acetone. The method has since been employed in the isolation of other antibiotics, for example, in the isolation of aspergillic acid from cultures of Aspergillus flavus (55). It was found that activated carbon (1%, weight/volume), absorbed the total anti-bacterial activity of the filtered broth on which Cordyceps militaris had been promoted, when stirred for 48 hours. The exhausted medium, which no longer showed any inhibitory action towards the control organism, was discarded and the air-dried charcoal was continuously extracted in a Soxhlet apparatus with warm acetone. On the first occasion on which this

method was employed, the concentrated, cooled extract yielded the metabolic product as a white high-melting crystalline solid. To this compound the name cordycepin is given. On subsequent occasions, however, the metabolic product was isolated as a dark oily residue, which on treatment with decolourising charcoal invariably yielded cordycepin in a crystalline condition; final purification was effected by recrystallisation from ethanol. The yield of pure cordycepin varied between 40 mg. and 80 mg. per litre of culture filtrate, the loss in weight during purification being approximately 30%.

Medium Variation and Submerged Promotion.

The replacement in the standard medium of the aldohexose D-glucose by the ketohexose D-fructose (laevulose) caused an increased yield of the metabolic product, cordycepin. A small number of culture flasks were inoculated and incubated under the standardised conditions. The mycelial development was vigorous with the rapid production of acidity in the medium. Due to a contamination in the test strain of B. subtilis the culture filtrate could not be assayed, but the development of acidity in the medium and the subsequent

return to neutrality followed closely the conditions observed when D-glucose was employed. After an arbitrary period of 24 days, incubation was discontinued and the culture filtrate was extracted to yield pure cordycepin at a level of 95 mg./litre, characterised by its melting point and mixed melting point with an authentic sample of the metabolic product, and by its ultraviolet light absorption (Fig. 1). The cost of D-fructose prohibits its use in the preparation of large volumes of culture media, but it is of interest to note that this alteration in the medium did not affect the nature of the metabolic product.

From the observation that in surface culture promotion, Cordyceps militaris invariably grows below the medium surface during the first 24 - 48 hours of incubation, it was deduced that a favourable method of production of the metabolic product might exist in deep culture promotion of the mould. An apparatus was constructed in which compressed air was blown through a bacterial filter into warm sterile water. The moist preheated air was then released through perforated rubber leads as a vigorous stream of fine bubbles at the bottom of the inoculated medium,

contained in large aspirators. The air was allowed to escape through bacterial filters and mercury air-flow gauges. Variations in the depth of medium and rate of air-flow were employed, and it was found that a high volume ratio of air-flow to medium was required to produce appreciable submerged growth. Under the most satisfactory conditions which could be obtained, the rate of submerged growth was slow, the first inhibition of Bacillus subtilis being shown by the medium after 30 days.

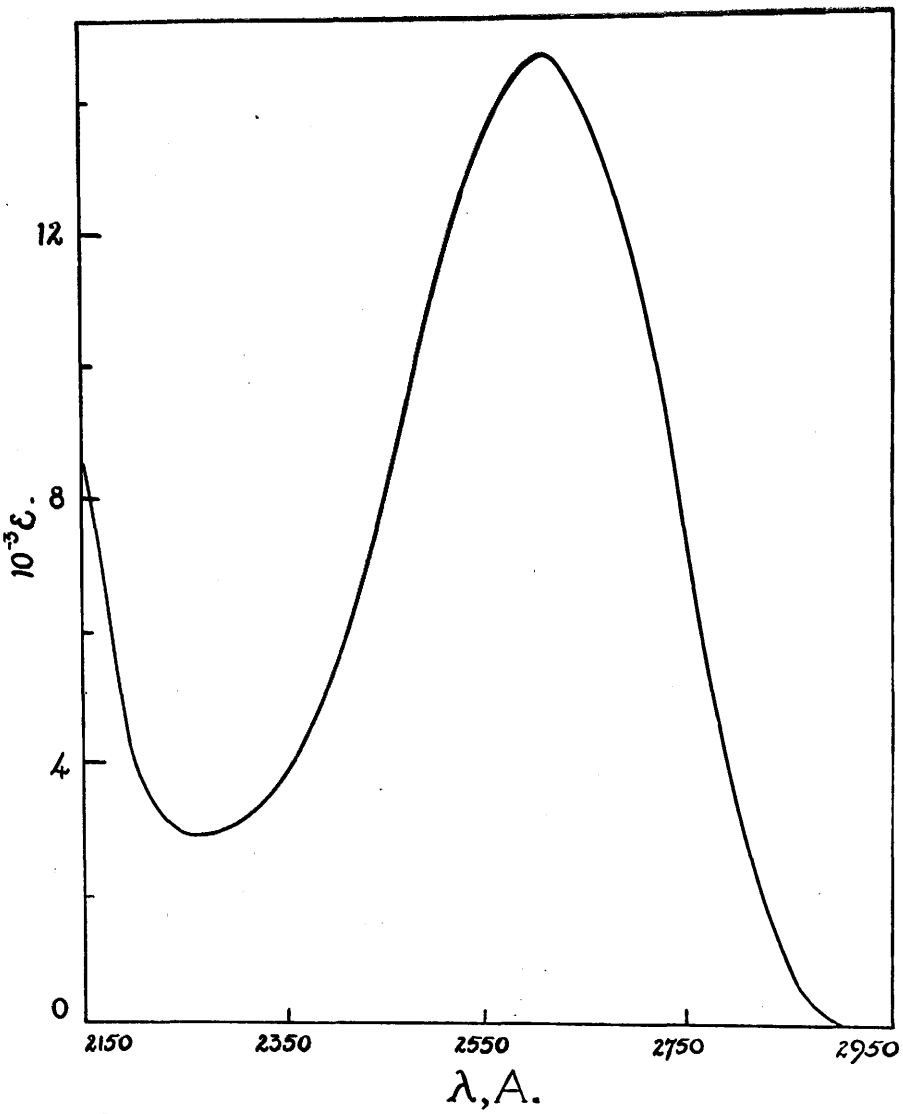
In aqueous solution at a concentration between 10 μg. and 100 μg. per ml., cordycepin was found to inhibit the growth of the Gram-positive Bacillus subtilis (strain 6753 N.C.T.C.) in Bouillon broth. This antibacterial activity is sufficient to classify cordycepin as an antibiotic under the conditions defined by Oxford (5). Cordycepin does not inhibit the following Gram-positive organisms: Staphylococcus aureus Oxf. H, Sarcina lutea, Escherichia coli, Bacillus welchii, Streptococcus haemolyticus; Streptococcus faecalis; nor is it active against the Gram-negative organisms, Bacillus proteus, Shigella flexneri and Pasteurella septica. It does inhibit an avian tubercle bacillus at a concentration of

0.1 mg./ml. using Youman's medium at 37°, and with Dubos-Tween-albumin medium it inhibits a bovine tubercle bacillus at a dilution of 1:60,000. The toxicity of cordycepin is of a very low order.

Cordycepin.

The metabolic product crystallises readily from ethanol and n-propanol (in either of which it is sparingly soluble at normal temperatures), to yield colourless lustrous needles. From n-butanol, cordycepin crystallises as needles or plates, and from water it separates as dull matted needles. In all cases the crystals contain solvent of crystallisation, which is difficult to remove. The pure compound, m.p. 225-6°, shows an optical rotation of $[\alpha]_D^{20} = -47°$, and an intense ultraviolet light absorption with a maximum at 2600 A, (ethanol, Fig. 1). The latter physical property was employed to check the purity of each crop of the metabolic product isolated, an arbitrary molecular weight being used to evaluate $\varepsilon_{max.}$ as a constant, until the empirical formula of cordycepin was elucidated. Initial investigation showed cordycepin to contain the elements carbon, hydrogen, oxygen and nitrogen only.

Fig I



Cordycepin in ethanol.

The Empirical Formula of Cordycepin.

Analyses of a number of samples of cordycepin crystallised from ethanol gave values in agreement with those required by the formula $C_{10}H_{13}O_3N_5$ or with those of a hydrate $C_8H_{10}O_2N_4.\frac{1}{3}H_2O$. Furthermore, the analysis of a sample which was dried intensively suggested that the formula $C_8H_{10}O_2N_4$ is the correct one. Crystallisation from n-propanol and n-butanol gave samples of unaltered analytical values, whilst a sample crystallised from water gave results in excellent agreement with the formula $C_8H_{10}O_2N_4.H_2O$. The balance of this evidence favours the formula of lower molecular weight.

In aqueous solution, cordycepin reacts with picric acid to yield a product the analyses of which were found to be in agreement with the values required by the monopicrate of either base $C_8H_{10}O_2N_4$ or $C_{10}H_{13}O_3N_5$. The picrolonate of cordycepin was also prepared and three independent analyses gave results in support of either formula. Finally, an attempt to prepare the flavianate of cordycepin yielded a compound which, after purification, furnished analytical values incompatible with those required by a mono- or diflavianate of either of the postulated empirical

formulae. The product was ultimately shown to be the flavianate of a degradation product of cordycepin (p.37).

At this stage it was evident that the choice between the formulae $C_8H_{10}O_2N_4$ and $C_{10}H_{13}O_3N_5$ for cordycepin could not be made from analytical data available on the metabolic product, or simple derivatives of it. Since these formulae have molecular weights of 194 and 251 respectively, a molecular weight determination within limits of accuracy of 5 - 10% would prove which, if either, is correct. On the other hand, the solubility properties of cordycepin are such that the cryoscopic or ebullioscopic method of molecular weight determination could not readily be employed. A simple and rapid method of molecular weight determination which could be carried out on bases such as cordycepin was therefore devised, and was proven, against a wide variety of known compounds, to afford values of molecular weight with an accuracy of the order ± 2%. The method is discussed in detail in an appendix to this Thesis.

The value obtained (p.125) for the molecular weight of cordycepin (254) is in close agreement with that required by the empirical formula $C_{10}H_{13}O_3N_5$ (251) and precludes the empirical formula $C_8H_{10}O_2N_4$.

During the course of an X-ray crystallographic

study carried out by Mrs. Dorothy Hodgkin and Dr. G. J. Pitt (Laboratory of Chemical Crystallography, Oxford) on a sample of cordycepin crystallised from n-propanol, it was observed that the unit cell dimensions resemble closely those of certain purine derivatives, in particular the hydrochlorides of adenine and guanine. The evidence also suggests the presence of solvent of crystallisation in the specimen. However, a second sample, which crystallised from n-butanol as orthorhombic crystals, showed no loss of weight on prolonged drying in vacuo. This suggests that the compound exists in different crystallographic modifications which may or may not be solvent-containing. In the case of the orthorhombic crystals, the following data was obtained.

Unit cell dimension a) 4.81 ± 0.08 A.
b) 10.13 ± 0.14 A.
c) 22.65 ± 0.33 A.

Density by flotation = 1.48_3 Space group is $p2_12_12_1$.

The dimension "a" is so small that the atoms appear to be resolved in projection, while the space group indicates the presence of 4 molecules in the unit cell.

From this data the unit cell volume = 1.103×10^{-21} c.c. Since this volume of density 1.48_3 g./c.c. is associated with 4 molecules, the mass of one molecule = 4.09×10^{-22} g.,

and the molecular weight of cordycepin = 248 (tolerance ± 10). This value is in close agreement with the previously found value and with that required for the formula $C_{10}H_{13}O_3N_5$ (251).

The Structure of Cordycepin.

The cordycepin molecule was found to contain three active hydrogen atoms (Zerewitinoff), but has neither a carbon-methyl group (Kuhn-Roth) nor a methoxyl group (Zeisel); a Herzig-Meyer estimation showed the absence of a nitrogen-methyl group.

A preliminary study of the chemical properties of cordycepin suggested the absence of ethylenic unsaturation, whilst neither a carbonyl nor a carboxyl reaction was detected by the standard tests. Attempts to hydrogenate solutions of cordycepin in ethanol and acetic acid, using Adams' catalyst (57) at normal temperature and pressure, failed; an aqueous ethanolic solution of cordycepin was not reduced by aluminium amalgam. A warm solution of cordycepin in n-butanol however reacted very vigorously upon the addition of small fragments of sodium. Ammonia was evolved (characterised by its reaction with Nessler's solution, and by the physical properties of its hydrochloride)

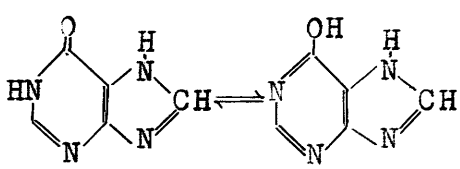
and the reaction mixture darkened very quickly. From this solution a small quantity of a white crystalline solid was finally isolated, but no deduction could be drawn from the analysis of this compound.

The first definite indication of the nature of cordycepin was obtained from a consideration of its ultraviolet light absorption (Fig. 1) which exhibits a well-defined maximum at 2600 A. (ε = 14,600) and this wave-length lies within a range which is characteristic of certain substituted pyrimidine compounds (58). The postulated presence of a pyrimidine ring in the cordycepin molecule is supported by the fact that in aqueous solution cordycepin was found to react with mercuric chloride, yielding a dimercurichloride. Many pyrimidine compounds exhibit double-salt formation with this reagent. Furthermore, many purines (59) exhibit intense absorption in the wavelength range 2550-2700 A. and the high nitrogen content of the cordycepin molecule supports the possibility of its containing a second heterocyclic ring system fused to a pyrimidine ring.

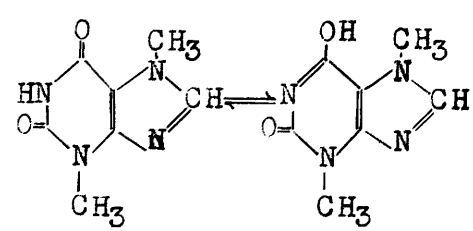
The maximum value of the ultraviolet light absorption of cordycepin is not altered appreciably in wavelength or intensity when measured in acid and alkaline solution (Table Vb) and the values obtained show a remarkable

similarity to those of adenine, adenosine and 9-methyladenine (Table Va) reported by Gulland and Holiday (60) but differ markedly from those reported for hypoxanthine, 9-methylhypoxanthine and 7-methylhypoxanthine. The alteration of absorption characteristics with pH in a molecule such as hypoxanthine (XI) is caused by keto-enol tautomerism of the grouping

and a study of the ultraviolet light absorption characteristics of theobromine (XII) and theophylline (XIII) (each of which can exist in tautomeric forms) in acid and alkaline solutions, confirmed that the presence of an amide grouping in a conjugated system causes a considerable variation in absorption characteristics with pH. The absorption of caffeine (XIV) (for which no tautomeric form can be written) was found to be unaltered in acid and alkaline solution. As in the cases of theobromine and theophylline, the absorption of caffeine is weaker and occurs at longer wavelength than in the case of cordycepin.



(XI)



(XII)

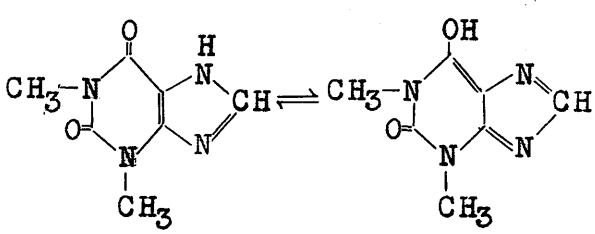
TABLE V(a).[*]

	A.		C	
	N/20 NaOH	N/20 HCl	N/20 NaOH	N/20 HCl
Adenine [⊖]	2,580	2,600	13,600	13,200
Adenosine	2,600	2,600	14,300	14,200
9-Methyladenine	2,600	2,600	14,700	14,200
Hypoxanthine	2,620	2,480	10,000	9,600
9-Methylhypoxanthine	2,550	2,480	12,100	10,300
7-Methylhypoxanthine	2,610	2,500	10,100	9,800
7-Methyladenine	2,690	2,690	11,400	14,600

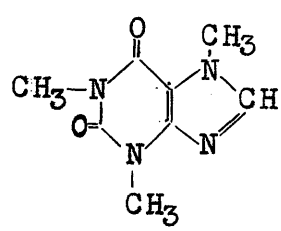
TABLE V(b).[∓]

	N/20 NaOH	N/20 HCl	N/20 NaOH	N/20 HCl
Cordycepin	2,600	2,600	14,600	14,400
Theobromine	2,750	2,730	10,100	9,300
Theophylline	2,750	2,700	8,500	7,000
Caffeine	2,720	2,730	9,300	9,200

[*] Gulland and Holiday (60)
[⊖] Compare Loofbourow and Stimson (61)
[∓] Present work.

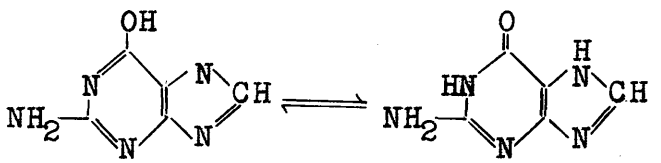


(XIII)



(XIV)

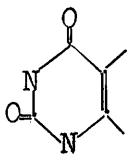
From this physical evidence it may be concluded that, although X-ray crystallographic evidence proves a close similarity between the unit cell dimensions of cordycepin and guanine (XV) the possible presence of a functional amide group in the nucleus of cordycepin, even if it be N-substituted to prevent tautomerism, is not supported by the ultraviolet light absorption data available.



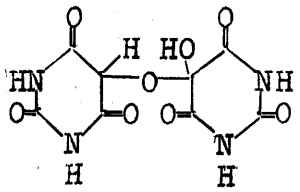
(XV)

The murexide test was carried out on cordycepin using uric acid, caffeine and theobromine as control materials. Each compound was evaporated to dryness with concentrated nitric acid, and on the addition of ammonia, the control purines gave the purple colouration of murexide (ammonium purpurate) whilst the cordycepin test was negative. Since uric acid, caffeine and theobromine all contain the grouping (XVI), their oxidation to a molecule of the alloxantin type (XVII) and hence conversion to murexide (XVIII) is facilitated by the presence of oxygen atoms at the 2 and 6 positions. The failure of cordycepin to give the murexide test is in

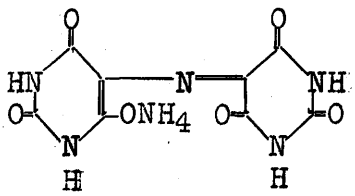
agreement with the previous evidence that, if the compound is a purine, it does not have a carbonyl group at the 2- and 6-positions.



(XVI)



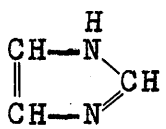
(XVII)



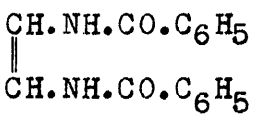
(XVIII)

A sample of cordycepin was dried <u>in vacuo</u> and was benzoylated in dry pyridine using a large excess of benzoyl chloride. The product crystallised from ethanol to yield cordycepin tetrabenzoate. Since a Zerewitinoff determination showed the presence of only three active hydrogen atoms in the cordycepin molecule, the isolation of a tetrabenzoate rather than a tribenzoate suggested an abnormal reaction. It has been shown by Bamberger and Berlé (62) that glyoxaline (XIX) and substituted glyoxalines can react with excess benzoyl chloride in pyridine solution with resultant fission of the ring, loss of the carbon atom at the 2-position and benzoyl-

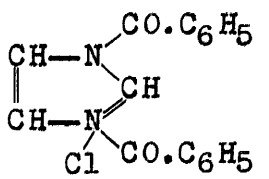
ation of each nitrogen atom to yield a compound of the type (XX). A tentative explanation of this reaction was made by Gerngross (63) who suggested that an intermediate of the type (XXI) was formed. Since the evidence regarding the structure of cordycepin is in favour of a purine nucleus, the possibility of degradation of the fused glyoxaline ring could not be overlooked as an explanation for tetrabenzoate formation. Assuming this to be the case, it was considered that hydrolysis of the poly-ester by hydrochloric acid might yield the hydrochloride of a diaminopyrimidine.



(XIX)



(XX)



(XXI)

Cordycepin tetrabenzoate was hydrolysed with warm concentrated hydrochloric acid and the benzoic acid which separated was removed by ether extraction. The concentrated mother liquor deposited a colourless crystalline product, the analysis of which approximates to that calculated for the empirical formula $C_5H_6N_5Cl$ and is in close agreement with a hemihydrate of that formula. Adenine hydrochloride ($C_5H_6N_5Cl$) in common

with many purines separates with water of crystallisation (64) and the hydrolysis product, m.p. 285° (decomp.) was undepressed in melting point when mixed with an authentic sample of adenine hydrochloride hemihydrate.

A second sample of the benzoate was hydrolysed and an aqueous solution of the product was converted into its picrate. The purified picrate [m.p. 290° (decomp.) alone or when mixed with an authentic sample of adenine picrate] was dissolved in spectroscopically pure ethanol and from the ultraviolet light absorption of the solution at 3800 A., the molecular weight of the picrate was shown to be 362 (p. 124). The molecular weight of adenine picrate is 364.

The isolation and characterisation of adenine from a derivative of cordycepin accounts for the total nitrogen content of the cordycepin molecule, and proves that the compound is a derivative of adenine. It also shows that cordycepin tetrabenzoate is not formed by fission of an imidazole ring. The nucleus of cordycepin does not contain oxygen, and on the assumption that the residue of the molecule is present as a single fragment, this side-chain must contain 3 oxygen atoms and have at least one optically active centre to accommodate the optical activity of the metabolic product. It has the

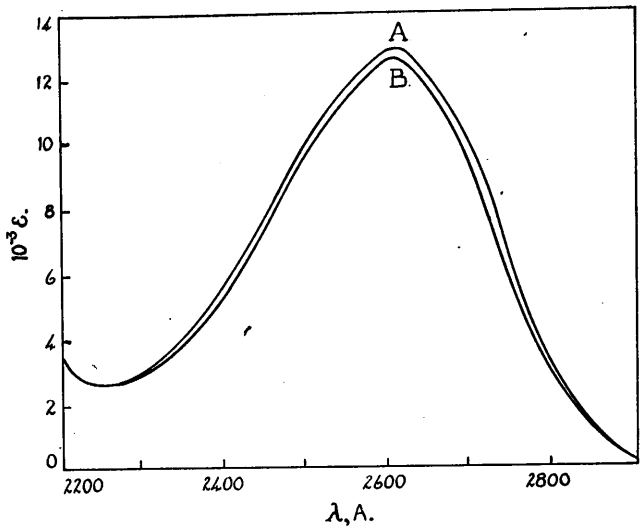
molecular formula -$C_5H_9O_3$ and has at least two active hydrogen atoms in the form of primary or secondary hydroxyl groups, the amine group on the 6-position of the pyrimidine ring accounting for the third active hydrogen atom in the cordycepin molecule. If the 6-amino group were substituted then all three oxygen atoms would have to be present in hydroxyl groups in the side-chain to account for the active hydrogen content of the molecule; but the bond between adenine and the side-chain is unstable to acid and this suggests that oxygen is associated with this linkage (for example, ester, urethane, amide or glycosidic bonding would split under the vigorous hydrolysis conditions employed) and it is therefore concluded that the 6-amino group is unsubstituted. Finally, an ester or urethane linkage requires 2 atoms of oxygen, and this is incompatible with the Zerewitinoff determination.

That cordycepin itself is unstable to dilute mineral acid was proven when a solution of the compound in dilute hydrochloric acid was warmed on a steam bath for a short period. The concentrated reaction solution deposited crystalline adenine hydrochloride hemihydrate, the identity of which was proven by its melting point of 285° (decomp.) and mixed melting point with an authentic

sample, by analysis, and by a comparison of its ultra-violet light absorption (Fig. 2) and infra-red light absorption (Fig. 3, carried out by Dr. I. A. Brownlie, 65) characteristics with those of an authentic sample of adenine hydrochloride hemihydrate. The hydrolysis product was also converted to its picrate, m.p. 290° (decomp.) which was undepressed on admixture with adenine picrate . (The sensitivity of cordycepin to mild acid accounts for the failure to obtain the flavianate of cordycepin, since flavianic acid furnishes sufficient hydrogen ion concentration to hydrolyse the molecule. Reinvestigation of the reaction product showed it to be adenine flavianate.)

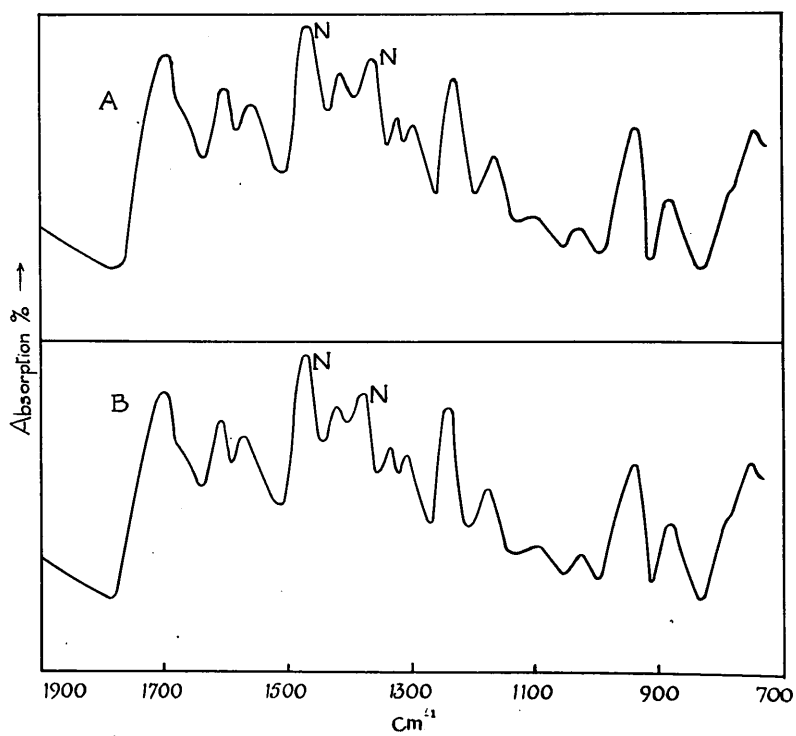
Addition of a saturated solution of 2:4-dinitro-phenylhydrazine in dilute hydrochloric acid to the mother liquor from the acid hydrolysis of cordycepin yielded a small quantity of an amorphous orange-red precipitate. The hydrolysis of a larger quantity of cordycepin was carried out and the cooled reaction solution was filtered free of adenine hydrochloride. The addition of an acid solution of 2:4-dinitrophenylhydrazine caused the precipitation over a very prolonged period of an amorphous red material. A dilute ethanolic solution of this compound, when treated with aqueous sodium

Fig. 2



A. Adenine hydrochloride.
B. Adenine hydrochloride from Cordycepin.,

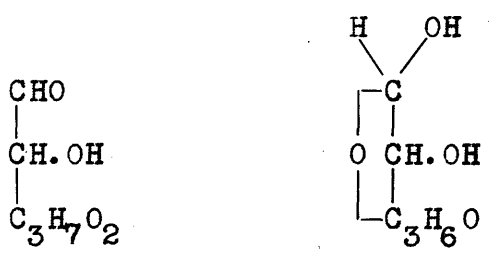
Fig. 3



A. Adenine hydrochloride
B. Adenine hydrochloride from Cordycepin
N. Nujol bands

hydroxide solution, gave the blue colouration characteristic of nitrophenylosazones. The purified product analysed for the empirical formula $C_{17}H_{16}O_{10}N_8$, which is that required by the 2:4-dinitrophenylosazone of a compound $C_5H_{10}O_4$.

The isolation of an osazone from the side-chain of cordycepin suggests that the liberated 5-carbon molecule has a structure related to that of a sugar and it is therefore designated cordycepose. The molecule must contain a free, or potentially free, carbonyl group adjacent to a primary or secondary hydroxyl group and is tentatively postulated as (XXII) an aldose structure being more probable than a ketose.

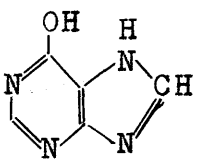


(XXII)

The negative result obtained in the Kuhn-Roth determination of carbon-methyl groups in the cordycepin molecule precludes the possible presence of the grouping $CH_3.CHOH.-$ or $CH_3.CO.-$ in the glycosidic side-chain. This was confirmed by the fact that the iodoform test of

Lieben (66, using the modified procedure of Fuson and Tullock, 67) gives a negative result in the case of cordycepin.

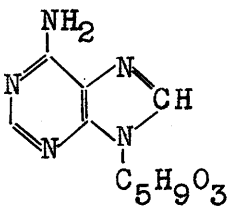
Deamination by means of nitrous acid has found application in studies of the stereochemical configuration of some purine nucleosides, in particular in the conversion of adenosine into inosine (68, 69, 60) and guanosine into xanthosine (70, 68, 71). In order to prove by positive evidence that the 6-amino group of the cordycepin molecule is unsubstituted, a sample of the metabolic product was deaminated using sodium nitrite and glacial acetic acid. The reaction conditions are sufficiently drastic to split the glycosidic linkage of cordycepin, and the liberated adenine was deaminated to hypoxanthine (XXIII) which was isolated from the reaction solution. It was identified by comparison of its picrate with that of the authentic purine in respect of melting point and mixed melting point. The molecular weight of this picrate (p. 124) was found to be 371, (hypoxanthine picrate requires 365).



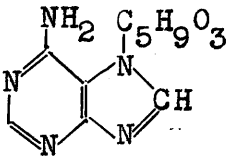
(XXIII)

Only the 7- and 9-positions of the adenine nucleus remain as possible points of attachment of the glycosidic residue in the cordycepin molecule. This same conclusion was made regarding the structure of the four purine nucleosides. Levene (71) confirmed experimentally that the glycosidic bond in xanthosine (ribosyl-xanthine) occurs at the 7- or 9-position, but no chemical method of distinguishing between these was available when Gulland et al. (72, 73, 74) evolved a physical solution to this problem. As a result of a study of the ultraviolet light absorption spectra of the purine nucleosides and a comparison of them with various authentic monomethylated purines, they showed, for example in the case of xanthosine, that the absorption of the nucleoside is very similar to that of 9-methylxanthine, but is unlike that of 1-methyl-, 3-methyl- or 7-methylxanthine. Hence, knowing that the effect of the carbohydrate group on such spectra is negligible (75) they deduced that xanthosine is a 9-ribosylpurine. Similarly, adenosine (adenine 9-D-ribofuranoside) and 9-methyladenine show maximum absorption at a wavelength of 2600 A. while the value for the 7-methyl derivative is 2690 A. The absorption of cordycepin (Table Vb) is identical, within the limits of experimental error, with that of adenosine

and 9-methyladenine, and the metabolic product is therefore ascribed the structure (XXIV) rather than (XXV).



(XXIV)

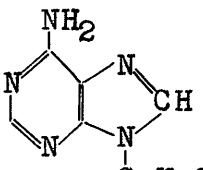


(XXV)

It is of interest to note that no purine nucleoside has been isolated in which the glycosidic bond is attached to the 7-position of the nucleus; all are in fact 9-ribosylpurines.

p-Toluenesulphonyl chloride reacts readily with primary hydroxyl groups in the presence of pyridine (76) the reaction with secondary hydroxyl groups occurring much more slowly. In the case of starch for example, it was shown by Hess and Pfleger (77) that tosylation of all three hydroxyl groups (of which one alone is primary) was achieved only after 9 days. Cordycepin was found to react with an excess of p-toluenesulphonyl chloride in dry pyridine, to yield an ester which could not be crystallised. It has been established that a tosyl (p-toluenesulphonyl) group attached to the primary

alcoholic group of aldohexoses (78) and aldopentofuranoses (79) is readily substituted by iodine when the ester is treated under pressure with an acetone solution of sodium iodide and under these conditions cordycepin p-toluene-sulphonate was decomposed to form sodium p-toluenesulphonate, the yield being high if based upon a mono-ester. The halogen derivative of cordycepin (XXVI) was also isolated as a glass which could not be crystallised.



$C_4H_6O_2 \cdot CH_2I$

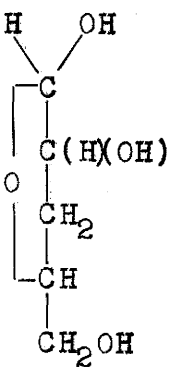
(XXVI)

Several attempts to prepare a mono-trityl (triphenylmethyl) ether of cordycepin were unsuccessful. Although the reagent reacts preferentially with a primary hydroxyl group in a sugar (80) it was shown by Levene and Tipson (81) that secondary hydroxyl groups will also react, a ditrityl ether of adenosine having been isolated. No explanation can be given at this stage for the failure of cordycepin to yield a trityl ether.

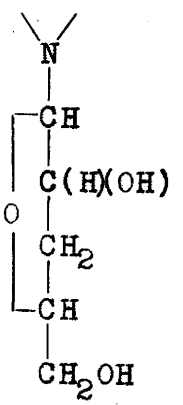
The tosylation reaction suggests that cordycepose must be a pentofuranose containing a primary alcoholic

group and this substantiates the concept that the side chain of cordycepin contains a terminal hydroxymethyl group. From this evidence the formula (XXII) for cordycepose can be expanded to (XXVII) and hence cordycepin is partially represented by (XXVIIIa). This molecule does not contain a α-glycol system and if previous deductions are correct, the metabolic product must be resistant to periodate oxidation (Malaprade, 83). Using the conditions of Lythgoe and Todd (84) a sample of cordycepin was not oxidised by sodium metaperiodate after 48 hours; the oxidation of a control sample of adenosine was completed after 4 hours.

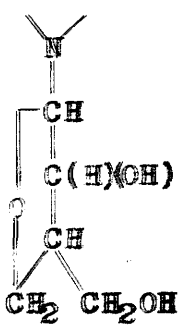
Three other formulae can be postulated (XXVIIIb, XXVIIIc, XXVIIId) for cordycepin (the last two being derived from a ketose structure) which are in agreement with the evidence regarding osazone formation, stability to periodate and possession of a hydroxymethyl group.



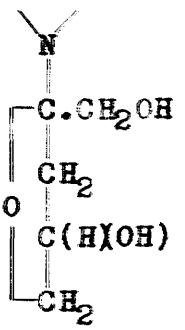
(XXVII)



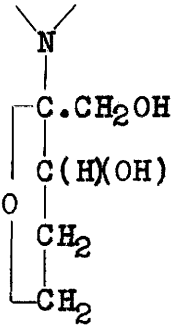
(XXVIIIa)



(XXVIIIb)

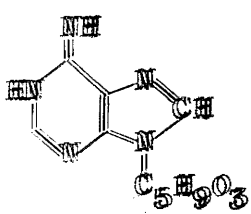


(XXVIIIc)

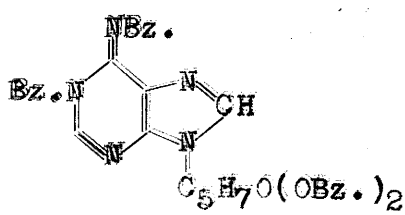


(XXVIIId)

These formulae for cordycepin are in complete agreement with the structural evidence, and account for the physical and chemical properties of the molecule, with the exception of the failure to obtain a trityl ether and the isolation of a tetrabenzoate of cordycepin. The molecule might be expected to yield a tribenzoate (in agreement with the active hydrogen determination) and a tetrabenzoate can only be postulated as (XXX) due to tautomerism of the 6-amino group (XXIX) in cordycepin.



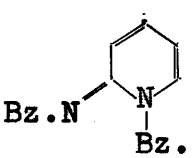
(XXIX)



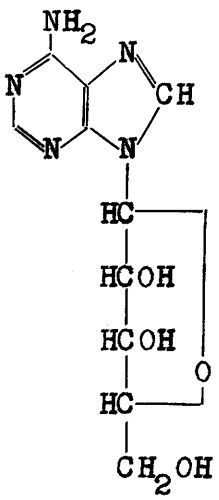
(XXX)

This is analagous with the formation of a dibenzoate of 2-aminopyridine (XXXI) (82). Adenosine (XXXII) the structure of which bears a remarkable resemblance to the

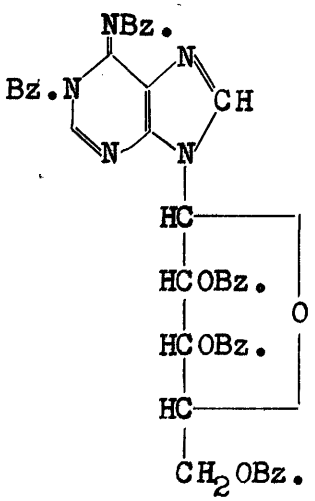
proposed formula (XXVIIa) for cordycepin and which contains four active hydrogen atoms, was benzoylated in pyridine solution using a large excess of benzoyl chloride. Adenosine pentabenzoate, to which the structure (XXXIII) must be ascribed, was isolated as the only reaction product. Thus the formulae postulated for cordycepin are not contradicted by the isolation of a tetrabenzoate of cordycepin.



(XXXI)



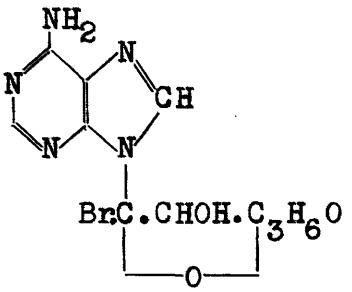
(XXXII)



(XXXIII)

A warm suspension of cordycepin in an inert solvent was found to react with bromine to yield a high-melting compound which appeared to be monobromocordycepin. The product could not be crystallised and was unstable in aqueous solution, being spontaneously degraded to adenine. A tentative explanation of this reaction is possible if

the structure (XXVIIIa) or (XXVIIIb) is assumed for cordycepin. Monobromocordycepin probably has a bromine atom on the 1'-position (XXXIV) which is so weakly bonded that in presence of water it is split out and the molecule is immediately degraded by this reaction, or by subsequent acid hydrolysis, to yield adenine. No previous reference has been made to a reaction of this type by purine nucleosides.



(XXXIV)

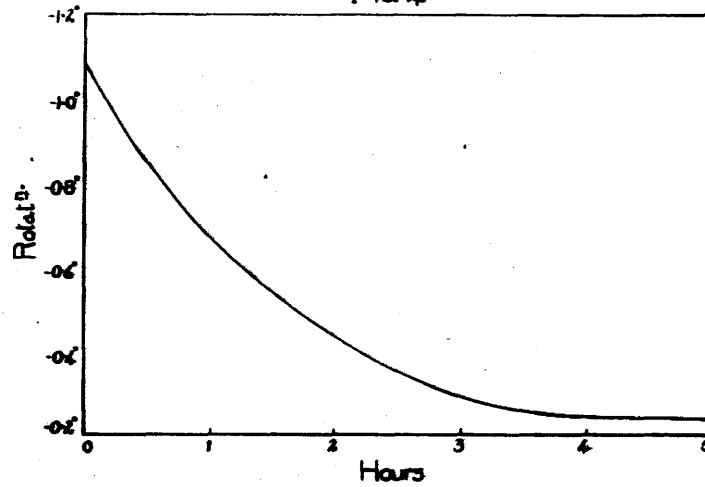
The isolation of pure cordycepose from cordycepin was carried out after experience had been gained with a model experiment in which D-ribose was isolated from adenosine. The nucleoside is more stable towards dilute mineral acid than is the metabolic product, and the progress of the hydrolysis of adenosine was observed by treating samples of the reaction solution with aqueous picric acid until pure adenine picrate was precipitated. Adenine was removed from the hydrolysis

solution by "Zeocarb 215" cation-exchange resin and the acid effluent was shown to contain less than 5 mg. of adenine and/or unchanged adenosine per 100 ml. of solution. This assay was made by a study of the ultraviolet light absorption of the reaction solution at a wavelength of 2600 A., assuming the value ε_{2600} = 14,000 for the purine and the glycoside. Hydrochloric acid was removed from the solution using freshly precipitated silver carbonate and hydrogen sulphide, and the neutral solution gave D-ribose in high yield.

The hydrolysis of cordycepin and the isolation of cordycepose was carried out under very similar conditions to those described above. The optical rotation of the hydrolysis solution (Fig. 4) was employed as a measure of the progress of hydrolysis, and the solution attained a constant value (α = -0.236, $[\alpha]_D^{22} \doteqdot -8^o$) after 5 hours. Cordycepose was isolated in good yield as a pale, laevorotatory syrup which could not be crystallised and which analysed for a deoxypentose. The sugar readily reduced Fehling's solution.

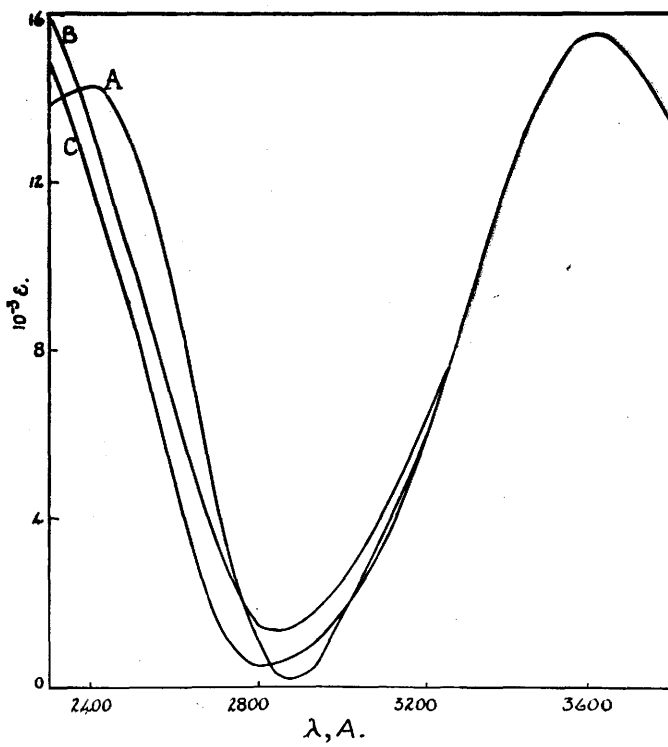
Attempts to prepare the p-nitrophenylosazone of cordycepose under conditions similar to those used by Mukherjee and Todd (91) for osazone formation gave a very poor yield of a product which showed the

Fig. 4.



Hydrolysis of Cordycepin.

Fig 5



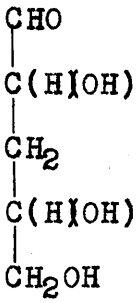
A 2-Aminothiazole picrate.

B,C Unidentified products.

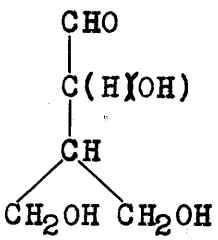
characteristic osazone colour reaction. Similarly the method of Kent, Stacey and Wiggins (85) was unsatisfactory in the case of cordycepose. The derivative was finally obtained by the hydrolysis of cordycepin in the presence of p-nitrophenylhydrazine hydrochloride. The tri-3:5-dinitrobenzoate (colourless needles) and anthraquinone-β-carboxylate (pale yellow needles) of cordycepose were also isolated.

The conversion of aldose sugars into the corresponding aldonic acids is generally accomplished by the action of chlorine or bromine on an aqueous solution of the sugar. Ketoses are much more stable under these conditions (86, 87, 88). Following a model reaction in which D-ribose was oxidised to D-ribonolactone and hence converted to D-ribonic acid phenylhydrazide, cordycepose was oxidised under identical conditions by the action of bromine on an aqueous solution of the sugar. Removal of free bromine and mineral acids, and evaporation of the residual solution, gave cordyceponolactone as a pale brown, dextrorotatory, hygroscopic syrup which liberated carbon dioxide from an aqueous solution of sodium bicarbonate. The isolation of a pentonic acid lactone by this oxidation proves conclusively that cordycepose is an aldopentose sugar, and cordycepin is an optical

isomer of (XXVIIIa) or (XXVIIIb). Cordycepose can now be postulated in the open-chain form (XXXVa) or (XXXVb).



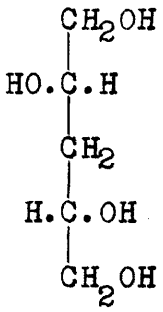
(XXXVa)



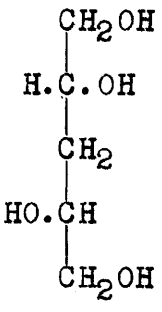
(XXXVb)

Several attempts to reduce cordycepose to cordycepitol (XXXVI - XXXVIIIa) by high-pressure hydrogenation using a Raney nickel catalyst (89, 90) under conditions similar to those used by Kent, Stacey and Wiggins (85) for the reduction of 3-deoxyxylose, yielded only a white high-melting solid which contained a large amount of inorganic material and which could not be crystallised or characterised. The isolation of cordycepitol would aid the elucidation of the structure of cordycepose, since, on the straight chain formula for cordycepose (XXXVa), cordycepitol can exist in the optically active D- (XXXVI) and L-(XXXVII) forms, or as the internally compensated mesocordycepitol (XXXVIII), whilst on the branch-chain structure for the sugar (XXXVb) the reduction product must be optically

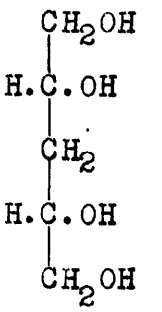
active (XXXVIIIa).



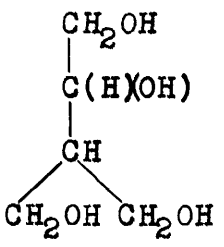
(XXXVI)



(XXXVII)

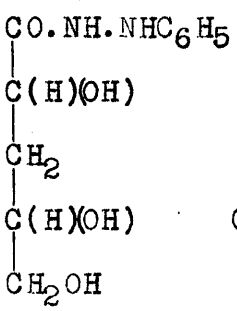


(XXXVIII)

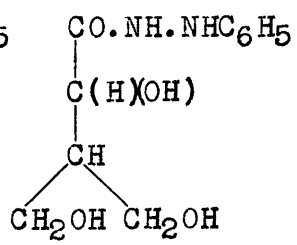


(XXXVIIIa)

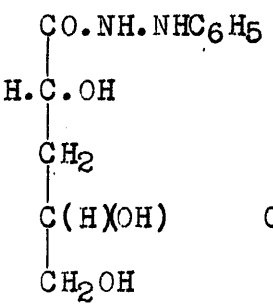
The conversion of cordycepanolactone to a crystalline acid phenylhydrazide (XXXIXa) or (XXXIXb) was carried out under standard conditions. The optical rotation of the product is $[\alpha]_D^{20} + 26^\circ$. Application of the phenylhydrazide rule of Hudson (92) leads to the conclusion that since cordycepanic acid phenylhydrazide is dextrorotatory the configuration (XLa) or (XLb) shows the correct spacial arrangement about C_2.



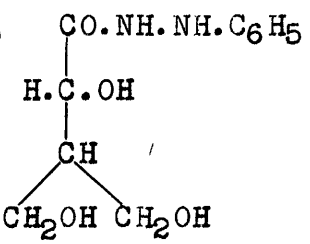
(XXXIXa)



(XXXIXb)



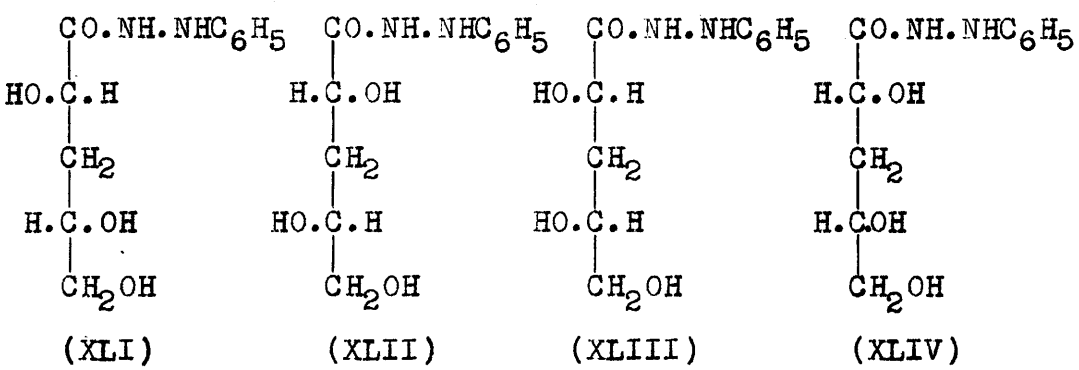
(XLa)



(XLb)

The formula (XLa) contains two optically active centres

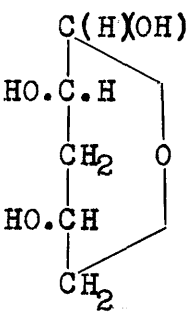
and hence can exist as four isomers comprising two pairs of enantiomorphs. Each of these compounds has been prepared by Nef (93) and they were named (according to rotation) D- and L- threo-1:3:4-trihydroxyvaleric acid phenylhydrazides [(XLI) and (XLII) m.p. 110°, $[\alpha]_D \pm 26°$] and D- and L- erythro-1:3:4-trihydroxyvaleric acid phenylhydrazides [(XLIII) and (XLIV) m.p. 150°, $[\alpha]_D \pm 9°$].



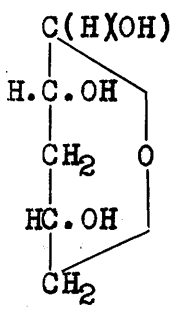
(XLI) (XLII) (XLIII) (XLIV)

Cordyceponic acid phenylhydrazide, m.p. 151°, differs greatly in melting-point from the D- and L- threo- derivatives of Nef, although the optical rotation is of a similar magnitude. On the other hand it exhibits a very similar melting-point to both the D- and L- erythro- derivatives but differs widely in optical properties. Furthermore, Mukherjee and Todd (91) have prepared L(+) erythro-1:3:4-trihydroxyvaleric acid phenylhydrazide from 3-deoxy-L-xylose (3-deoxy-L-ribose XLV) the structure of which is also confirmed by comparison

with 3-deoxy-D-xylose (3-deoxy-D-ribose, XLVI), subsequently prepared by Kent, Stacey and Wiggins (85). The product of Mukherjee and Todd [m.p. 194°, $[\alpha]_D$ +5°] is considered to be identical with the D-erythro-1:3:4-trihydroxyvaleric acid phenylhydrazide of Nef. A comparison in melting point and mixed melting point between cordyceponic acid phenylhydrazide and L(+) erythro-1:3:4-trihydroxyvaleric acid phenylhydrazide (kindly supplied by Professor A. R. Todd, F.R.S.) showed the two isomers to be different.



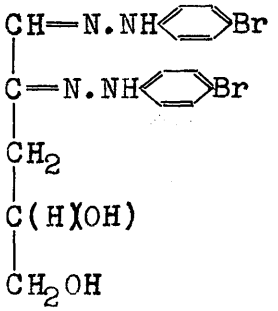
(XLV)



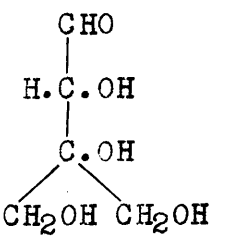
(XLVI)

An attempt to prepare the benzylphenylhydrazone of cordycepose (using the method by which Vongerichten and Muller (94) prepared the corresponding derivative of apiose) failed to yield a solid product. However, further evidence that the formula for cordycepin (XXVIIIa) and hence for cordycepose (XXXVa) are incorrect was obtained when cordycepose p-bromophenylosazone was

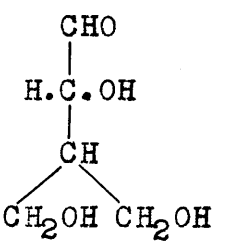
prepared according to the method by which Vongerichten prepared apiose p-bromophenylosazone (94, 95). No optical rotation could be observed in an ethanolic solution of the purified product, even at a high concentration and this suggests that osazone formation has removed the asymmetry of the cordycepose molecule. According to the structure (XXXVa) for cordycepose the p-bromophenylosazone (XLVII) must have an asymmetric carbon atom (C_4) and this supplies additional proof that cordycepose is, in fact, (XXXVb) and cordycepin (XXVIIIb). This formula is in agreement with the requirements that cordycepose is an aldopentofuranose in which C_2 carries a secondary hydroxyl group, C_3 is deoxy-, C_4 is hydroxylated to form the furanose ring and C_5 is in the form of a hydroxymethyl group; the resultant molecule yielding an optically inactive osazone. Cordycepose is therefore closely related to the only branched chain aldopentose known, viz., apiose (XLVIII) isolated by Vongerichten from parsley seed (95).



(XLVII)



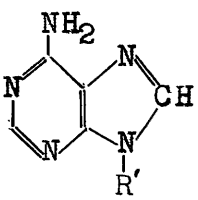
(XLVIII)



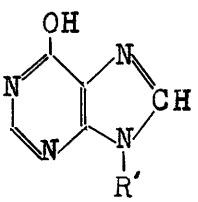
(XLIX)

Cordyceponic acid phenylhydrazide has one asymmetric carbon atom only (C_2) and the evidence of Hudson's phenylhydrazide rule regarding the configuration about this optical centre supports the formula (XLIX) for cordycepose.

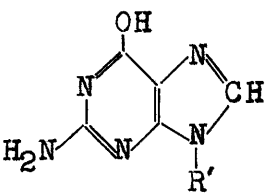
As in the case of the four ribosyl purine nucleosides, adenosine (L), inosine (LI), guanosine (LII) and xanthosine (LIII), (R' = D-ribose), no attempt has been made to elucidate the stereochemical disposition of the sugar-base link in cordycepin.



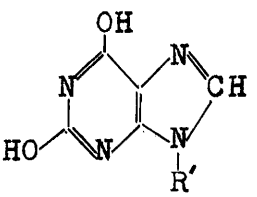
(L)



(LI)

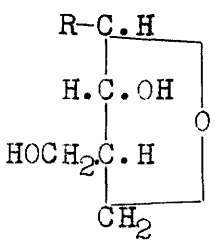


(LII)

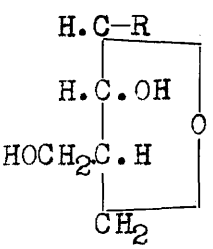


(LIII)

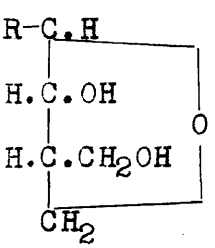
Furthermore, when cordycepose is written in the furanose form a new optical centre is generated at the C_3 position and cordycepin can therefore be represented by one of the four structures [(LIV) - (LVII) where R = adenine-9-.]



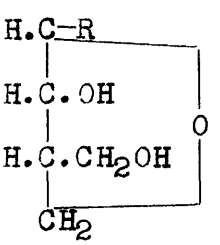
(LIV)



(LV)



(LVI)



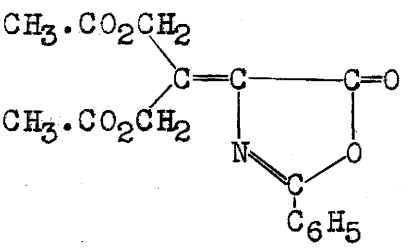
(LVII)

It is of interest to note that no naturally occurring 3-deoxyaldopentose has been described previously, while the only naturally occuring adenine deoxypentoside to have been reported is adenine-9;2'-deoxy-D-riboside isolated by Levene and London (98) from thymus nucleic acid.

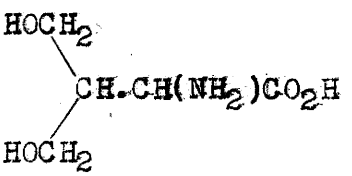
Vongerichten (96) and subsequently Schmidt (97) showed that the amorphous calcium salt of apionic acid could be reduced with hydriodic acid and phosphorus to yield a volatile acid which they characterised through its p-bromophenacyl ester as isovaleric acid. Thus the carbon skeleton of apionic acid and apiose was conclusively proven. The yield of this reaction is extremely small and insufficient cordycepin was available to attempt the corresponding degradation of cordycepose. Proof of the proposed structure for cordycepose was therefore undertaken by a synthetic route.

The first synthetic approach to the confirmation of the proposed structure for cordycepose was an attempt to prepare 1:3-diacetoxy-4'-isopropylidene-2'-phenyl-5'-oxazolone (LVIII) by the condensation of 1:3-diacetoxypropan-2-one (diacetoxyacetone, prepared by acetylation of dihydroxyacetone dimer by a method

similar to that described by Fischer and Mildbrand, 99) with hippuric acid or with 2-phenyl-5-oxazolone. Reduction followed by hydrolysis might be expected to yield 2-amino-4-hydroxy-3-hydroxymethylbutyric acid (LIX). Resolution of this compound and treatment of each isomer with nitrous acid should yield the two stereoisomeric forms of cordyceponic acid.



(LVIII)



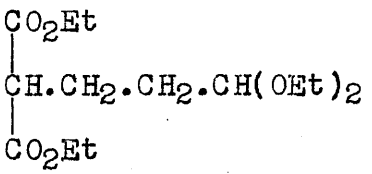
(LIX)

The condensation of the ketone with hippuric acid was attempted in boiling acetic acid in the presence of sodium acetate (100) but no product was isolated; nor could the ketone be condensed with preformed 2-phenyl-5-oxazolone in benzene using piperidine and magnesium sulphate as dehydrating agents, in acetic anhydride, or in benzene with a trace of phosphorus pentoxide. 2-Phenyl-5-oxazolone was recovered unchanged on every occasion.

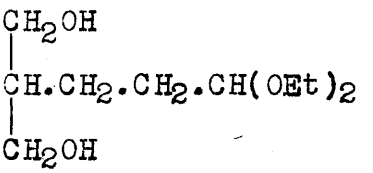
As in the case of apiose, the p-bromophenylosazone

of cordycepose does not contain a centre of asymmetry. Furthermore the derivative is a highly crystalline compound of sharp m.p. suitable for comparison with a synthetic material. The reactions to be discussed now were directed towards the synthesis of cordycepose p-bromophenylosazone.

In a recent publication it has been shown by Marvel and Hill (101) that 4:4-dicarbethoxybutan-1-al diethylacetal (LX) can be converted into 5-hydroxy-4-hydroxymethylpentan-1-al diethylacetal (LXI) by the action of lithium aluminium hydride. This reaction suggests a straightforward approach to the synthesis



(LX)

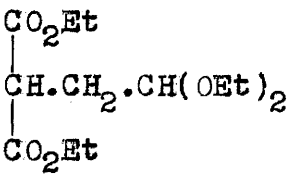


(LXI)

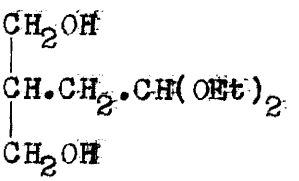
of cordycepose, since the reduction of 3:3-dicarbethoxypropan-1-al diethylacetal (LXII) will yield 4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal (LXIII) which is 2-deoxycordycepose diethylacetal.

3:3-Dicarbethoxypropan-1-al diethylacetal was prepared according to the method of Perkin and Sprankling (102), the yield being increased by the

use of a rocking-type autoclave in place of sealed glass tubes, and by a slight increase in the reaction temperature. Analyses of the acetal and of the corresponding 2:4-dinitrophenylhydrazone confirmed the structure of the product.



(LXII)

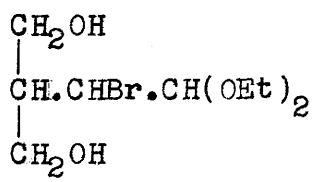


(LXIII)

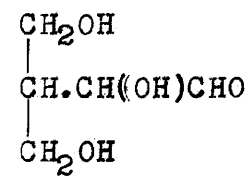
The reaction conditions of Marvel and Hill (loc. cit.) were followed closely in the reduction of 3:3-dicarbethoxypropan-1-al diethylacetal, the reaction product being distilled finally to yield two fractions each of which was shown by analysis to be 4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal. The product, unlike the starting material, is readily water-soluble. The corresponding 2:4-dinitrophenylhydrazone could not be obtained from this acetal by the action of Brady's solution, nor did a dilute mineral acid solution of the product yield the derivative when treated with an aqueous solution of 2:4-dinitrophenylhydrazine hydrochloride. 4-Hydroxy-3-hydroxymethylbutan-1-al 2:4-dinitrophenylhydrazone was finally prepared by

hydrolysis of the acetal in an acetic acid-sodium acetate buffer solution (pH 4) followed by the addition of a solution of 2:4-dinitrophenylhydrazine in dilute hydrochloric acid.

In order to convert 4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal into racemic cordycepose (LXV) a hydroxyl group must be introduced on the 2-position with the subsequent liberation of the aldehyde group. The most obvious method to complete this reaction is through the intermediate 2-bromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal (LXIV), but the action of bromine on the acetal in the presence of calcium carb--onate yielded a polymerised glass which could not be distilled. Marvel and Joncich (103) have shown that many simple aliphatic acetals can be converted to the



(LXIV)

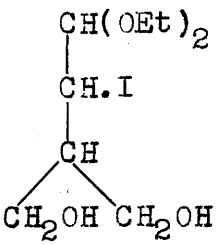


(LXV)

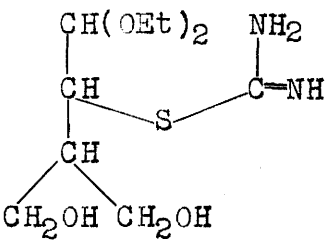
corresponding 2-bromo derivatives in high yield by N-bromosuccinimide. An ethereal solution of 4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal was allowed to react with a molar equivalent of N-bromosuccinimide

and the reaction product was isolated as a pale yellow oil. Micro-distillation of a small quantity of the product yielded a fraction the analysis of which suggested that it was 2-bromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal. On attempting to distil the bulk of the product, polymerisation and decomposition occurred and 50% was obtained in three fractions. The higher boiling fractions were fractionally redistilled and a sample from each was analysed. The values obtained showed that the distillates were mixtures of the monobrominated product and a dibrominated compound which is probably 2:3-dibromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal. That the desired 2-halogenated product was present in the mixture was proven by its conversion to the corresponding 2-iodo- compound (LXVI) (with sodium iodide in acetone) which reacted with thiourea to yield 2-amino-5-(2'-hydroxy-1'-hydroxymethyl)-ethylthiazole (LXVIII) isolated as its picrate, the analysis and molecular weight determination (p.124) of which are in agreement with the required formula. The reaction may be postulated as passing through the intermediate (LXVII). Using the same reaction conditions bromoacetal was converted into 2-aminothiazole, also characterised as

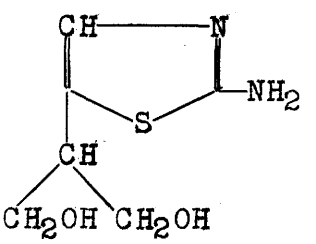
its picrate the solubility properties of which are very similar to those of the first product. (Chloroacetal is employed industrially in the preparation of 2-aminothiazole hydrochloride by the same reaction, 104).



(LXVI)



(LXVII)



(LXVIII)

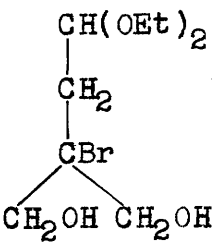
In an attempt to improve the yield of 2-bromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal the bromination was catalysed by strong ultraviolet irradiation. The product was less viscous than previously and reacted readily with sodium iodide in acetone to yield a dark brown oil. Conversion of a sample of this material through the isothiourea derivative yielded a picrate, the solubility properties of which were markedly different from those of 2-aminothiazole picrate and from those of the compound considered to be 2-amino-5-(2'-hydroxy-1'-hydroxymethyl)-ethylthiazole. The new product exhibits a higher m.p. than the latter picrate and is considerably

depressed in m.p. when mixed with it. On the other hand analysis and molecular weight determination (p.124) of this product showed it to be isomeric with the previous picrate. In the first N-bromosuccinimide reaction it was considered that the reaction product contained some 2:3-dibromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal in addition to the 2-brominated material. It would now appear that in the presence of strong ultraviolet light the 3-position is more active than the 2-position so that the only monobrominated product is 3-bromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal (LXIX). The corresponding 3-iodo-compound reacts with thiourea through the intermediate (LXX) to give 2-amino-6:6-di(hydroxymethyl)-1:3-thiazine (LXXI), isomeric with (LXVIII).

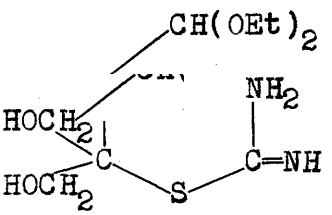
Although these products have been postulated as a thiazole and a thiazine (these being the only formulae compatible with the analyses and molecular weight determinations) there is only the evidence of comparative solubility to suggest which compound does in fact have the structure (LXVIII). A comparison of the ultraviolet light absorption of these compounds with that of 2-aminothiazole picrate (Fig. 5, 'C' is the compound postulated as (LXVIII), 'B' as (LXXI) offers

no aid to the identification of the products.

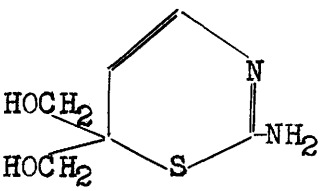
Bromination of the acetal was repeated using a tungsten-filament lamp to catalyse the reaction. The reaction was very slow to proceed and the product, after treatment with sodium iodide in acetone, condensed with thiourea to yield the high-melting picrate (LXXI) instead of the product isolated previously.



(LXIX)



(LXX)



(LXXI)

From this evidence it is apparent that bromination of 4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal with N-bromosuccinimide yields at least three products which cannot be separated or purified by distillation, and of which one is the required 2-bromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal. This approach to the synthesis of a cordycepose derivative was therefore discontinued.

EXPERIMENTAL

EXPERIMENTAL.

All melting points are uncorrected.
Analyses are by Drs. Weiler and Strauss, Oxford, and by W. McCorkindale, Royal Technical College, Glasgow.

Promotion of Cordyceps militaris (Linn.) Link.

A slope of the master culture of the mould was flooded with sterile water (5-10 ml.) and was agitated with a nickel spatula. The aqueous conidial suspension produced was distributed in 8-ounce bottles containing a medium of glucose (1.0%), peptone (0.5%) and agar (2.0%) and was incubated at 24°. After 8-12 days the mould from one bottle was sub-cultured into 3 culture flasks containing a medium (400 ml./flask) of glucose (1.0%) and "Pronutrin", a commercial enzymic casein hydrolysate (0.5%) and the flasks were incubated at 24° for a further 10-12 days. Finally the mycelium was disintegrated by vigorous agitation to yield a bulk spore suspension sufficient for the inoculation of 40-50 l. of medium (glucose 1.0%, "Pronutrin" 0.5%). The medium was dispensed in "Glaxo" surface culture flasks or in standard "penicillin" flasks (380 ml./

flask) and after sterilisation (15 lbs./sq. in., 15 minutes) was inoculated (10-15 ml. inoculum/flask). After 24 hours a submerged growth of the mycelium was observed, and after a further 24-48 hours numerous separated "islands" of surface production of the mycelium were observed. By the seventh day the surface was completely covered with a heavy, white felt and at this stage the acidity of the medium commenced to increase, reaching a maximum after 18-21 days (pH 2.0-3.0) coincidental with the first well-defined inhibitions (30-35 mm.). Incubation beyond this period resulted in a gradual decrease of acidity, neutrality being attained after 26-28 days, with little alteration in assay results.

Culture Filtrate Assay.

An agar slope of the test organism (Bacillus subtilis strain 6752 of the National Collection of Type Cultures) was flooded with distilled water (10 ml.) and scraped with a nickel spatula to yield a bacterial suspension which was used to inoculate the medium (90 ml.) consisting of "Lab. Lemco" (1.0%), peptone (1.0%), sodium chloride (0.5%) and agar (1.5%). The medium was dispensed in Petri dishes (20 ml./dish)

and after it had set, equidistant discs of ½" diameter were removed. The cavities were filled with the solution or filtrate to be tested, and the dishes incubated for 12 hours at 28°. The area and sharpness of definition of the inhibition zones afforded an estimate of the activity of the test solution. Cordycepin (10.0 mg.) was dissolved in distilled water (10.0 ml.) and portions of this solution were successively diluted to yield solutions of concentration 1.0 mg./ml., 0.2 mg./ml., 0.1 mg./ml., 0.01 mg./ml. and 0.001 mg./ml. Each solution was assayed against the test strain of Bacillus subtilis. The solution of highest concentration showed a very clear, well-defined inhibition zone of 35 mm. diameter. At a concentration of 0.2 mg./ml. and 0.1 mg./ml. the inhibition zones were 29 mm. and 32 mm. in diameter and were also clear and sharply defined. Inhibition area and definition decreased rapidly at lower concentration, and at 0.001 mg./ml. concentration the zone diameter was 5-10 mm. and was scarcely visible.

Isolation of Cordycepin (I)

Standard medium (22 l.) dispensed in 58"Glaxo" flasks was inoculated from a bulk conidial suspension

of C. militaris and incubated for 23 days at 24°. The exhausted medium was filtered from the mycelium under reduced pressure, and was then stirred mechanically for 4-5 days with activated animal charcoal (B.D.H. decolourising quality, 250 g.). The charcoal was allowed to settle for 24 hours before the supernatant liquor was removed by a syphon. The charcoal was collected by filtration and was air-dried at 24° for 5 days; it was then extracted in a Soxhlet apparatus with acetone (1,500 ml.) for 6 days. During the extraction cordycepin separated from the acetone solution as a white micro-crystalline incrustation, which was collected and dried in vacuo (320 mg.), m.p. 215-220°. The product was recrystallised from n-butanol (prisms, m.p. 221-222°) and finally separated from ethanol as lustrous needles (250 mg.), m.p. 225-6°. The bulked n-butanol and ethanol liquors were concentrated (7 ml.) to yield cordycepin as prisms (45 mg.), m.p. 225° (alone or mixed with a specimen from the initial crop). The pale yellow acetone solution (1,500 ml.) was concentrated to small bulk (250 ml.) and on cooling deposited cordycepin as a mat of colourless needles, m.p. 219-221°, associated with globules of a viscid oil; The mixture was

collected and air-dried (2.02 g.). Recrystallisation from n-butanol (100 ml.) yielded cordycepin as colourless plates (790 mg.), m.p. 224-225° (undepressed on admixture with a sample from the first crop). Concentration of the filtrate (20 ml.) yielded a further crop of the metabolic product (135 mg.), m.p. 222-224°. The acetone and n-butanol mother liquors were concentrated and heated under reflux with activated charcoal to yield, on filtration, a colourless solution from which cordycepin separated slowly as colourless plates, m.p. 217-220°. This material was pure after one recrystallisation from ethanol, from which it separated as needles (100 mg.), m.p. 225° (alone or when mixed with an authentic sample). The identity and purity of each crop was also established by a study of its ultraviolet light absorption (ethanol):

$\varepsilon_{maximum}$ = 14,600 at 2600 A. (Fig. 1)

The total yield of pure cordycepin was 1.320 g. (60 mg./l. medium); $[\alpha]_D^{20}$ -47° (c = 0.43, in water).

Isolation of Cordycepin (II).

The filtered, exhausted medium (44 l.) from a culture of the mould was extracted with charcoal (500 g.),

and the dried charcoal was continuously extracted with acetone (6 l.) as described previously. The brown acetone extract was concentrated to small bulk (200-300 ml.) and was maintained at 0° overnight. No solid material separated from the solution. The residual acetone was evaporated under reduced pressure to yield a dark gum which was dissolved in boiling water (50 ml.) and was heated under reflux with activated charcoal (0.1 g. approx.). The hot solution was filtered and on cooling overnight deposited cordycepin as dull matted needles (1.90 g.) m.p. 223-224°, undepressed in m.p. when mixed with an authentic specimen. Concentration of the aqueous mother liquor yielded a solution (5 ml.) from which cordycepin separated on prolonged cooling as pale brown needles, m.p. 220-222°. Recrystallisation from ethanol gave colourless lustrous needles (310 mg.) m.p. 224°. The extinction coefficient of the maximum ultra-violet light absorption for each crop (14,400, 14,500 at 2600 A.) confirmed the purity of the products. Total yield, 2.21 g. (50 mg./l. medium).

Promotion of C. militaris on Laevulose Medium.

A medium consisting of laevulose (D-fructose) (1%), and Pronutrin (0.5%) was dispensed in 20 "penicillin"

type flasks (400 ml./flask) and was sterilised, inoculated and incubated as before. Growth of the mould was very vigorous and after 24 days the mycelium was discarded and the medium was extracted to yield crude cordycepin (750 mg., 95 mg./ml.) m.p. 190-210°. The product was recrystallised from water and finally from ethanol, from which pure cordycepin separated as colourless needles (400 mg.) m.p. 224-5°, undepressed when mixed with an authentic specimen. The ultraviolet absorption of the compound showed the value ε = 14,300 at 2600 A. (ethanol).

Submerged Culture Promotion of Cordyceps militaris (Linn.) Link.

In place of an incubator a thermostatically controlled water tank was employed, maintaining a temperature of 24° ± 1° in each of two 20 litre aspirators containing glucose (1.0%), "Pronutrin (0.5%) medium, (10 l./flask). Air supplied by a rotary pump was passed through a sterile wool filter (30 cm. x 7 cm.) and through a 15 litre aspirator containing sterile water (12 l.) at 24°. The preheated moist air was released through perforated rubber leads giving a flow of fine bubbles in the inoculated medium. The air was finally allowed to escape through a mercury flow-meter, which

gave a reading of 100 l./hour. Growth of mycelium was very slow, except at the points of most vigorous aeration and after 28 days maximum acidity was attained pH 4.7) without the production of measurable activity.

The unit was altered to give an air-flow rate of 500 l./hour, using $3\frac{1}{2}$ l. of standard medium in each of two 10 litre aspirators. The rate of submerged growth was greatly increased while a heavy felt formed on the walls of the vessel at the medium-air interface. Maximum acidity (pH 5.2) was attained after 11 days and the medium was neutral after 27 days. After 30 days (pH 7.2) the medium showed the first inhibition of B. subtilis (5-10 mm. hazy inhibition). The experiment was discontinued at this stage.

Analyses of Cordycepin.

For analysis a sample of cordycepin recrystallised from ethanol as needles, m.p. 225°, was dried for 6 hours over phosphoric oxide at 78°/0.1 mm.

Found:	C,47.9, 47.8;	H,5.2, 5.2;	N,27.6, 28.3.
$C_{10}H_{13}O_3N_5$ requires	C,47.8;	H,5.2;	N,27.9.
$C_8H_{10}O_2N_4 \cdot \frac{1}{3}H_2O$ requires	C,48.0;	H,5.4;	N,28.0%.

A further sample crystallised from ethanol and dried for

150 hours, 80°/0.5 mm. was analysed using the standard technique for a hygroscopic compound.

Found: C,49.7; H,4.7.

$C_8H_{10}O_2N_4$ requires C,49.5; H,5.2%.

Cordycepin separates from n-propanol as fine needles, m.p. 225°, containing solvent of crystallisation which is difficult to remove. A sample was dried over phosphoric oxide for 8 hours at 100°/0.5 mm.

Found: C,48.1; H,4.9%.

From n-butanol the metabolic product crystallises in the form of lustrous plates, m.p. 224-5°. A sample was dried for 8 hours at 100°/0.5 mm.

Found: C,48.2; H,5.4%.

From water cordycepin separates as dull matted hydrated needles, m.p. 225°. A specimen was dried over phosphoric oxide for 2 hours at 78°/0.5 mm.

Found: C,45.3; H,5.9.

$C_{10}H_{13}O_3N_5\frac{2}{3}H_2O$ requires C,45.6; H,5.5.

$C_8H_{10}O_2N_4.H_2O$ requires C,45.3; H,5.7%.

Cordycepin Picrate.

To a warm solution of cordycepin (15 mg.) in water (1.0 ml.) was added an excess of a saturated aqueous solution of picric acid. The yellow flocculent

precipitate was collected and was dissolved in hot water. On cooling cordycepin picrate separated as needles (12 mg.) m.p. 195° (decomp.).

Found: C,39.7; H,3.2; N,23.4,23.2.

$C_{10}H_{13}O_3N_5 \cdot C_6H_3O_7N_3$ requires C,40.0; H,3.4; N,23.3.

$C_8H_{10}O_2N_4 \cdot C_6H_3O_7N_3$ requires C,39.7; H,3.1; N,23.2%.

Cordycepin Picrolonate.

To a warm solution of cordycepin (15 mg.) in water (1.0 ml.) was added a cold saturated aqueous-ethanolic solution of picrolonic acid. The precipitate of yellow prismatic needles which separated on cooling was collected and recrystallised from water to yield cordycepin picrolonate as yellow needles (10 mg.) m.p. 240° (decomp.).

Found: C,46.7,47.0; H,4.2,4.3; N,24.2,24.2.

$C_{10}H_{13}O_3N_5 \cdot C_{10}H_8O_5N_4$ requires C,46.6; H,4.1; N,24.5.

$C_8H_{10}O_2N_4 \cdot C_{10}H_8O_5N_4$ requires C,47.3; H,4.0; N,24.4%.

Attempted Preparation of Cordycepin Flavianate.

To a warm solution of cordycepin (20 mg.) in water (1.0 ml.) was added excess of a warm aqueous-ethanolic solution of flavianic acid (2:4-dinitro-1-naphthol-7-

sulphonic acid). The solution was concentrated (2 ml.) and after prolonged cooling deposited yellow needles, m.p. 275-280° (decomp.). Recrystallisation from a concentrated aqueous-ethanol solution yielded adenine flavianate as bright yellow needles, m.p. 283° (decomp.) alone or when mixed with an authentic sample.

Found: N,21.6.

$C_5H_5N_5 \cdot C_{10}H_6O_8N_2S$ requires N,21.8%.

Sodium-Butanol Reduction of Cordycepin.

Finely cut sodium (100 mg.) was added to a solution of cordycepin (96 mg.) in boiling n-butanol (7 ml.) heated under reflux over a period of 30 minutes. The non-condensible vapour which escaped from the top of the condenser was alkaline to litmus and gave a yellow colour to Nessler's solution. It was absorbed in dilute hydrochloric acid and the acid solution on evaporation yielded ammonium chloride (10 mg.) which did not melt on heating, but yielded a white sublimate above 200°. The reaction solution was cooled and extracted with hydrochloric acid ($\frac{N}{3}$,3 x 5 ml.). The extract was made alkaline with sodium hydroxide solution and was extracted with ether (5 x 10 ml.). The dried (sodium sulphate) extract was concentrated and treated with dry

hydrogen chloride to yield a white precipitate which did not melt below 250°. The product crystallised from ethanol-ether as micro-prisms (3 mg.).

Found: N,21.9%.

Cordycepin Di-mercurichloride.

A warm solution of cordycepin (7.0 mg.) in water (1.0 ml.) was treated with a saturated aqueous solution of mercuric chloride (2.0 ml.). The mixture was warmed to 100° to redissolve a gelatinous precipitate. On slow cooling a white amorphous granular precipitate of cordycepin di-mercurichloride separated.

Found: N,8.1.

$C_{10}H_{13}O_3N_5.2HgCl_2$ requires N,8.8.

$C_8H_{10}O_2N_4.2HgCl_2$ requires N,7.6%.

The Murexide Test.

Caffeine (0.025 g.) was evaporated to dryness with concentrated nitric acid (80%) and heating was continued until the yellow residue had turned pink. On the addition of a drop of concentrated ammonia solution (sp.gr. 0.88) the purple coloration of murexide was observed. The test was repeated using uric acid (0.03 g.) and theobromine (0.02 g.), a purple colour being

obtained in each case. Cordycepin (0.20 g.) yielded a dull red-brown colour.

Cordycepin Tetrabenzoate.

Cordycepin (50 mg.) was dried over phosphoric oxide for 3 hours at 78°/0.1 mm. and was suspended in pure dry pyridine (3.0 ml.). Benzoyl chloride (1.0 ml.) was added and the mixture was shaken to complete solution. The reaction mixture was kept at room temperature for 18 hours and was then warmed on a steam bath for 5 minutes to dissolve the precipitated pyridine hydrochloride. The dark red solution was added to crushed ice (20 g.) with vigorous stirring and after all the ice was melted the supernatant liquor was decanted from a dark-red oil. The oil was washed with water (10 ml.) and was dissolved in warm ethanol (3.0 ml.). On cooling the solution deposited pale orange needles (100 mg.) m.p. 170-175°. Recrystallisation from ethanol gave cordycepin tetrabenzoate as colourless lustrous needles, m.p. 179-180°.

Found: C,68.2,68.5; H,4.2,4.2; N,10.6,10.5.

$C_{38}H_{29}O_7N_5$ requires C,68.4; H,4.4; N,10.5%.

Hydrolysis of Cordycepin Tetrabenzoate.

Cordycepin tetrabenzoate (0.3 g.) was suspended in

concentrated hydrochloric acid (3.0 ml.) and the mixture was warmed on a water bath at 80° with occasional shaking. After 10 minutes the ester was completely dissolved, benzoic acid being precipitated as a white gummy mass. The cooled reaction mixture was extracted with ether (4 x 10 ml.) and the dried (sodium sulphate) extract was concentrated to yield benzoic acid (180 mg., 80% of theoretical) m.p. 120°. The hydrochloric acid solution was concentrated to 1.5 ml. and on cooling deposited colourless clustered needles (30 mg.) m.p. 280° (decomp.). The product recrystallised from a concentrated aqueous ethanol solution to yield adenine hydrochloride hemihydrate as colourless needles, m.p. 285° (decomp.) undepressed when mixed with an authentic specimen of adenine hydrochloride hemihydrate, m.p. 285° (decomp.). The ultraviolet light absorption of an ethanolic solution of the product showed a maximum at 2610 A., ε = 13,600. An analytical sample was dried for 3 hours at 80°/0.1 mm.

Found: C,33.6; H,4.1; N,39.3.

Calc. for $C_5H_6N_5Cl.\frac{1}{2}H_2O$: C,33.3; H,3.9; N,38.8%.

The hydrolysis of cordycepin tetrabenzoate (0.15 g.) was repeated and the product (15 mg.) was dissolved in warm water (1.0 ml.) and treated with an excess of a

saturated aqueous solution of picric acid (2.0 ml.). The yellow flocculent precipitate which separated was collected and crystallised from water. It separated as felted needles, m.p. 290° (decomp.) alone or when mixed with an authentic specimen of adenine picrate, m.p. 290° (decomp.). By a study of the ultraviolet light absorption of the picrate at a wavelength of 3800 A., the molecular weight of the picrate was found to be 362 (p. 124, $C_5H_5N_5.C_6H_3O_7N_3$ requires 364).

Hydrolysis of Cordycepin.

A solution of cordycepin (0.15 g.) in hydrochloric acid (2N, 3.0 ml.) was warmed on a steam bath for 30 minutes. The solution was concentrated (1.0 ml.) under reduced pressure, and cooled. The crystalline mass which separated was collected, washed with ethanol (0.5 ml.) and dried to yield adenine hydrochloride hemihydrate (60 mg., 59%) m.p. 285° (decomp.) alone or mixed with an authentic specimen. A sample was recrystallised from ethanol from which it separated as fine colourless needles, m.p. 285-286° (decomp.) and was dried for 3 hours at 78°/0.1 mm.

Found: C,33.2, 33.4; H,3.7, 4.1;
N,39.0, 37.8; Cl,19.4, 19.5.

Calc. for $C_5H_6N_5Cl.\frac{1}{2}H_2O$: C,33.3; H,3.9;
N,38.8; Cl,19.6%

The ultraviolet light and infra-red light absorption spectra of this product were found to be identical with those of an authentic specimen (Figs. 2, 3).

To a warm solution of the product (15 mg.) in water (1.0 ml.) was added a cold saturated aqueous solution of picric acid (2.0 ml.). The yellow precipitate which separated recrystallised from water to yield adenine picrate as yellow needles, m.p. 290° (decomp.), undepressed when mixed with an authentic sample. For analysis a sample of the picrate was dried for 3 hours at 100°/0.1 mm.

Found: C,36.3,36.4; H,2.3,2.2; N,30.9.

Calc. for $C_5H_5N_5.C_6H_3O_7N_3$: C,36.3; H,2.2; N,30.8%.

The acid filtrate after the removal of adenine hydrochloride was treated with a saturated solution of 2:4-dinitrophenylhydrazine in hydrochloric acid (2N, 10 ml.). After standing at room temperature for 10 days the amorphous orange precipitate (10 mg.) which formed was collected and washed with a small volume of water. The product showed m.p. 250-253° (decomp.) and a sample (<1 mg.) dissolved in ethanol (2 ml.) and treated with

dilute sodium hydroxide solution gave a deep blue-violet colouration.

Cordycepose 2:4-dinitrophenylosazone.

Cordycepin (0.40 g.) was hydrolysed with hydrochloric acid (2N, 5.0 ml.) as described previously. After the removal of adenine hydrochloride the residual solution was added to a saturated solution of 2:4-dinitrophenylhydrazine in hydrochloric acid (2N, 40 ml.). The amorphous flocculent precipitate which deposited during the first 24 hours was discarded and the solution was allowed to stand at room temperature for 10 days, the precipitated material being collected periodically. The bulked orange-red precipitates (50 mg.) were dried and crystallised twice from a small volume glacial acetic acid to yield cordycepose 2:4-dinitrophenylosazone as a reddish-brown microcrystalline powder, m.p. 254-258° (decomp.). The product was very sparingly soluble in ethanol. A dilute solution of cordycepose 2:4-dinitrophenylosazone in ethanol when treated with aqueous sodium hydroxide gave a deep blue-purple colouration. An analytical sample was dried for 4 hours at 100°/0.1 mm.

Found: C,41.0,41.6; H,3.2,3.4; N,22.6,22.7.

$C_{17}H_{16}O_{10}N_8$ requires C,41.5; H,3.3; N,22.7%.

The Iodoform Test.

Solutions of cordycepin (50 mg.) in dioxan (5.0 ml.) and isopropanol (50 mg.) in dioxan (5.0 ml.) together with a blank of dioxan (5.0 ml.) were all treated with a 10% aqueous solution of sodium hydroxide (0.5 ml.). To the solutions was added dropwise a solution of potassium iodide (20.0 g.) and iodine (10.0 g.) in distilled water (80 ml.) with vigorous shaking, until a slight excess was shown in each solution by the presence of a brown colour which did not disappear after shaking occasionally for 5 minutes. The reaction solutions were warmed on a water-bath at 60° for 2 minutes, the potassium iodide-iodine solution being added dropwise to maintain the colour of free iodine in the solutions. Excess iodine was finally removed by the addition of aqueous sodium hydroxide and each reaction solution was diluted with water (20 ml.). After standing for 15 minutes the blank and cordycepin solutions carried no precipitates, nor did they have the characteristic odour of iodoform. The isopropanol control solution gave a bulky yellow precipitate of iodoform, m.p. 119°.

Deamination of Cordycepin.

Cordycepin (0.10 g.) was dissolved in hot water

(8.0 ml.) and sodium nitrite (0.3 g.) and glacial acetic acid (0.5 ml.) were added. The reaction solution was maintained at 70° for 30 minutes and the resultant solution was made alkaline to brilliant yellow with aqueous sodium hydroxide. Evaporation of the solution on a steam bath under reduced pressure gave a white residue which was extracted with boiling ethanol (30 ml.) and the alcohol solution was cooled and filtered free of suspended sodium acetate. The solution was again concentrated, cooled and filtered, and finally evaporated to dryness under reduced pressure. The white residue was dissolved in concentrated hydrochloric acid (2.0 ml.) and warmed on a steam-bath for 10 minutes. Evaporation of the solution *in vacuo* gave a light brown amorphous residue which was extracted with boiling ethanol (20 ml.). The filtered extract was refluxed with activated charcoal and concentrated to small bulk (3 ml.). On prolonged standing the solution deposited hypoxanthine hydrochloride as colourless needles (20 mg.) m.p. 300° (decomp.). The compound (10 mg.) was dissolved in a warm aqueous solution of picric acid. On cooling the solution deposited yellow needles which were collected and recrystallised from water to yield hypoxanthine picrate as bright yellow needles, m.p. 240-250° (decomp., after

sintering with loss of water of crystallisation at 200-220°) alone or mixed with an authentic specimen.

Tosylation of Cordycepin.

Cordycepin (200 mg.) was dried for 1 hour at 78°/0.1 mm. and was dissolved in boiling anhydrous pyridine (5 ml.) in the absence of atmospheric moisture. The solution was cooled and p-toluenesulphonyl chloride (500 mg., 3 moles.) was added. After standing in a closed vessel for 3 days at room temperature, distilled water (0.5 ml.) was added to the reaction mixture and after 30 minutes a further addition of distilled water (25 ml.) was made. The solution was extracted with chloroform (3 x 8 ml.) and the chloroform extract was shaken with ice-cold normal sulphuric acid and then with a saturated aqueous solution of sodium bicarbonate. The dried (sodium sulphate) extract was concentrated, final traces of chloroform being removed under reduced pressure, to yield cordycepin p-toluenesulphonate as a pale brown glass (400 mg., 70%) which could not be obtained in a crystalline form. The product (400 mg.) was dissolved in dry acetone (5 ml.) and a solution of sodium iodide (450 mg.) in dry acetone (5 ml.) was added. After heating in a sealed tube for 24 hours at 100°, the crystalline

sodium p-toluenesulphonate which separated was collected and air dried (82 mg. 80%). The mother liquor was evaporated to yield a pale brown gum which was dissolved in ethyl acetate (20 ml.) and washed with water (3 x 10 ml.). The solution was dried (sodium sulphate) and the solvent evaporated to yield a glass which could not be crystallised.

Attempted Tritylation of Cordycepin.

Cordycepin (0.465 g.) was dried for 1 hour at 78°/0.1 mm. and dissolved in dry pyridine (40 ml.) by heating under reflux in the absence of atmospheric moisture. Freshly prepared trityl chloride (triphenylmethyl chloride, 0.570 g., 1.15 mole.) was added to the cooled solution and the reagents were maintained at 40° for 5 days, free from moisture. Addition of the reaction solution to ice-water (100 ml.) with stirring yielded a white precipitate which was removed by filtration and air-dried to yield tritanol (triphenylmethyl carbinol, 340 mg., 60%) m.p. 161-162° undepressed in m.p. when mixed with an authentic specimen, m.p. 163-164°. No other compound was isolated.

Attempted Periodate Oxidation of Cordycepin.

Approximately 0.4N sodium metaperiodate was prepared by dissolving sodium metaperiodate (4.28 g.) in distilled water (100 ml.). Standard sodium arsenite solution was prepared by dissolving pure arsenious oxide (2.4523 g.) in the minimum volume of a solution of sodium hydroxide in distilled water. The solution was neutralised with dilute hydrochloric acid and was made alkaline with aqueous sodium bicarbonate solution. The solution was diluted with air-free distilled water to 500 ml., the factor of this solution being 0.0992N. Solutions of cordycepin (0.0988 g., 0.394 millimole.) and adenosine (0.1040 g., 0.390 millimole.) in 50 ml. air-free distilled water were prepared. To each solution and to a blank solution was added the periodate solution (10.0 ml.) and a sample of each solution (5 ml.) was removed and diluted with saturated aqueous sodium bicarbonate solution. To each sample was added aqueous potassium iodide solution (N, 10.0 ml.) and the liberated iodine was titrated with the standard arsenite solution, using a freshly prepared starch indicator. Further aliquots were titrated after 2 hours, $2\frac{1}{2}$ hours, $3\frac{1}{2}$ hours, 21 hours and 27 hours.

Titration (ml. arsenite)

Time	:	0 hrs.	2 hrs.	3½ hrs.	21 hrs.	27 hrs.
Blank	:	3.45	3.42	3.30	3.27	3.26
Cordycepin:		3.30	3.25	3.15	3.13	3.13
Adenosine	:	3.42	3.41	2.40	2.37	2.36

The initial reading of the sodium arsenite solution against the blank solution gave to the diluted periodate solution the factor 0.0678N.

The periodate decomposed in 27 hours per 5 ml. of blank solution = 0.19 ml. standard arsenite solution.

The periodate decomposed in 27 hours per 5 ml. of cordycepin solution = 0.17 ml. standard arsenite solution.

Therefore no oxidation of cordycepin by periodate has occurred.

The periodate decomposed in 27 hours per 5 ml. of adenosine solution = 1.06 ml. standard arsenite solution.

Therefore the periodate reduced by adenosine in 5 ml. of solution = 0.87 ml. standard arsenite solution.

This represents the absorption of 1.4 moles. of oxygen per mole. of adenosine.

Adenosine Pentabenzoate.

To a suspension of adenosine (0.30 g.) in dry

pyridine (5.0 ml.) was added benzoyl chloride (4.0 ml.). The solution was kept at room temperature with the exclusion of atmospheric moisture for 18 hours and was subsequently warmed to 100° for 5 minutes. The hot solution was added carefully to crushed ice (20 - 30 g.) with vigorous agitation and the ice was allowed to melt slowly. The supernatant liquor was decanted and the residual red oil was washed with water (2 x 10 ml.) and dissolved in boiling ethanol (5 ml.). The solution was refluxed with activated charcoal, filtered and cooled to yield a colourless solution from which adenosine pentabenzoate separated as clustered prismatic needles, m.p. 180-182°. Recrystallisation from aqueous-ethanol gave colourless needles, m.p. 183-184°. An analysis sample was dried at 80°/0.1 mm. for 3 hours.

Found: C,68.6; H,4.3; N,8.6.

$C_{45}H_{33}O_9N_5$ requires C,68.6; H,4.2; N,8.9%.

Bromination of Cordycepin.

Bromine (50 mg.) was added to a suspension of cordycepin (50 mg.) in dry redistilled chloroform (10 ml.) and the reaction mixture was warmed under reflux for 5 minutes. Solvent and excess bromine were evaporated under reduced pressure to yield monobromocordycepin as

a white amorphous powder, m.p. 290° (decomp.) which could not be crystallised, but which, after drying for 2 hours at 80°/0.1 mm., gave a positive Beilstein test for halogen.

Found: N,21.8.

$C_{10}H_{12}O_3N_5Br$ requires N,21.2%.

The product (20 mg.) was dissolved in warm water (2 ml.) and excess of cold saturated aqueous picric acid solution was added. The reaction mixture was warmed to redissolve the yellow amorphous precipitate and the yellow needles which separated were recrystallised from water to yield adenine picrate, m.p. 290° (decomp.) undepressed in mixed m.p. with an authentic sample. A sample was dried for 3 hours at 100°/0.1 mm.

Found: C,36.1; H,2.2; N,30.6.

Calc. for $C_5H_5N_5.C_6H_3O_7N_3$: C,36.3; H,2.2; N,30.8%.

Isolation of D-Ribose from Adenosine.

Adenosine (2.0 g.) was dissolved in warm hydrochloric acid (0.1N, 10 ml.) and the solution was refluxed for 4 hours. To a sample of this solution (0.1 ml.) was added excess saturated aqueous picric acid solution and the amorphous yellow precipitate which separated immediately, crystallised from water to yield adenosine

picrate as yellow needles, m.p. 190° (decomp.). The acid concentration was increased (0.5N) and the solution was boiled under reflux until a sample on treatment with aqueous picric acid solution yielded adenine picrate, m.p. 285-290° (decomp.). The additional time required for complete hydrolysis was 3 hours. "Zeocarb 215" resin was finely ground and washed by decantation until the wash water was colourless. A column of the resin (15 cm. x 0.5 cm.) was activated by repeated washing with ammonia (N), hydrochloric acid (2N) and distilled water, and the reaction solution was passed through the column under slight positive pressure. The ultraviolet light absorption of the resultant solution at 2610 A. showed the concentration of adenine (plus any unchanged adenosine) to be 0.0003 g./10 ml. of solution. Freshly prepared, well washed, silver carbonate (from silver nitrate 4.0 g.) was added to the hydrolysis solution and after the reaction was completed the precipitate of silver salts was removed by filtration. The filtrate was saturated with hydrogen sulphide and the finely divided silver sulphide precipitate was coagulated by freezing the mixture (in an acetone-carbon dioxide bath) and allowing it to thaw slowly. The precipitate was removed by filtration through "Hyflo Supercel" and

activated charcoal, and the clear colourless filtrate was evaporated under reduced pressure to yield D-ribose as a pale yellow sweet-smelling syrup (900 mg., 80%).

$[\alpha]_D^{20}$ -20° (c = 0.59 in ethanol).

Levene and Jacobs (Ber., 1909, 42, 1198) reported the value $[\alpha]_D$ -19.5° (water) for D-ribose.

Cordycepose.

Cordycepin (3.0 g.) was dissolved in warm dilute hydrochloric acid (N/10, 100 ml.) and the solution was filtered. Polarimetric examination of the solution gave the value $[\alpha]_D^{25}$ = -38°. The solution was heated under reflux, the optical rotation of the solution being examined at intervals of 30 minutes (Fig. 4). After 5 hours the specific rotation of the solution was constant ($[\alpha]_D^{22}$ = -8°) and a sample of the solution (0.2 ml.) on treatment with saturated aqueous picric acid solution, yielded a flocculent yellow precipitate which recrystallised from water to give adenine picrate as yellow needles, m.p. 285-290° (decomp.). Dilute hydrochloric acid (N/10, 10 ml.) was added and the hydrolysis solution was allowed to stand for 30 minutes. The cooled solution was passed through a column of pre-activated "Zeocarb 215" resin (30 cm. x 0.5 cm.) to yield a

solution which was shown by ultraviolet light absorption measurement to contain less than 1 mg. of adenine and which yielded an orange-red precipitate when treated with a solution of 2:4-dinitrophenylhydrazine in hydrochloric acid. Chloride ions were removed from the solution by treatment with freshly prepared silver carbonate, and hydrogen sulphide (as described in the isolation of D-ribose from adenosine). The colourless reaction solution was evaporated under reduced pressure and the residual gum was dissolved in methanol (15 ml.) and filtered free from a trace of insoluble inorganic material. The solution was evaporated, finally under high vacuum, to yield cordycepose as a pale yellow, laevorotatory, sweet-smelling, hygroscopic syrup (1.064 g., 66%). The product in ethanol showed the value $[\alpha]_D^{19} = -26.6^\circ$ (c = 0.638). In dilute hydrochloric acid (N/20) the sugar gave the value $[\alpha]_D^{20} = -18.7^\circ$ (c = 0.536) and after warming the solution under reflux for 4 hours gave $[\alpha]_D^{20} = \pm 0.0^\circ$. This figure was unchanged on standing for 4 days. A sample for analysis was dried at 20°/0.1 mm. for 6 hours.

Found: C,44.5: H,7.9.

$C_5H_{10}O_4$ requires C,44.8; H,7.5%.

Attempted Preparation of Cordycepose p-Nitrophenylosazone (I).

(cf, Mukherjee and Todd, J.,1947,969.)

Cordycepose (130 mg.) was dissolved in water (4 ml.) and a solution of p-nitrophenylhydrazine (recrystallised from ethanol, m.p. 157°, 345 mg.) in glacial acetic acid (1.5 ml.) was added. The reaction mixture was warmed on a steam-bath for 90 minutes and the resulting solution was cooled. The red precipitate which separated was collected (200 mg.) and a portion of it (1 mg.) was dissolved in ethanol (1 ml.). The addition of a few drops of dilute aqueous sodium hydroxide caused the development of a brown colour characteristic of unchanged p-nitrophenylhydrazine. The mother liquor on standing deposited a red precipitate (3 mg.), an ethanolic solution of which gave a deep violet colour with aqueous sodium hydroxide. The mother liquor was diluted with water (10 ml.) but no further product separated.

Attempted Preparation of Cordycepose p-Nitrophenylosazone (II).

(cf. Kent, Stacey and Wiggins, J.,1949,1232.)

Cordycepose (40 mg.) was dissolved in glacial

acetic acid (1.5 ml.) and p-nitrophenylhydrazine (85 mg.) was added. The mixture was warmed on a steam-bath for 45 minutes. On cooling, the solution deposited unchanged p-nitrophenylhydrazine (m.p. 156°). The mother liquor was diluted with water (5 ml.) and deposited a red gum which could not be crystallised and which, in ethanolic solution, gave a red colour with aqueous sodium hydroxide.

Cordycepose p-Nitrophenylosazone.

Dry freshly recrystallised p-nitrophenylhydrazine (750 mg.) was dissolved in hydrochloric acid (N, 25 ml.) and the solution was kept for 15 minutes at room temperature before being filtered free of a dark red precipitate. It was then added to a solution of cordycepin (300 mg.) in hydrochloric acid (N, 8 ml.) and the reagents were kept at room temperature overnight. No product separated from the pale yellow solution which was then warmed to reflux. A flocculent red precipitate commenced to separate immediately and after refluxing for 5 minutes the solution was set aside to cool. The product was separated and air-dried (107 mg.) m.p. 250-255° (decomp.) and gave a deep blue colouration in dilute ethanolic solution when treated with aqueous sodium hydroxide. Crystallisation from ethyl acetate

yielded cordycepose p-nitrophenylosazone as a red microcrystalline powder, m.p. 260° (decomp.) and recrystallisation from ethanol gave dark red prisms, m.p. 261° (decomp.).

Found: C,49.8; H,4.6; N,20.5.

$C_{17}H_{18}O_6N_6$ requires C,50.7; H,4.5; N,20.9%.

Cordycepose Tri-3:5-Dinitrobenzoate.

3:5-Dinitrobenzoyl chloride was prepared and purified according to the method of Stacey and Saunders (Biochem. J., 1942, 36, 368). To a solution of dried cordycepose (80 mg.) in dry pyridine (5 ml.) was added 3:5-dinitrobenzoyl chloride (400 mg.). After 12 hours the reaction mixture was warmed on a steam-bath and added to ice-water (20 g.). The emulsion which was obtained was coagulated by freezing to yield a gum which could not be crystallised. A solution of the gum (100 mg.) in dry benzene (30 ml.) was passed through a short column of alumina and the column was eluted with benzene until the effluent was orange in colour. The benzene solution was concentrated to small volume and on spontaneous evaporation deposited orange needles, m.p. 70° (30 mg.) which crystallised from aqueous ethanol to yield cordycepose tri-3:5-dinitrobenzoate as colourless needles,

m.p. 74°.

Found: N,11.2.

$C_{26}H_{16}O_{19}N_6$ requires N,11.7%.

Cordycepose Anthraquinone-β-carboxylate.

Anthraquinone-β-carboxylic acid chloride (800 mg.) was added to a solution of cordycepose (100 mg.) in dry pyridine (5 ml.). The reaction mixture was kept overnight at room temperature and was then warmed on a steam-bath for 5 minutes. The cooled solution was diluted with benzene (15 ml.) and was washed with dilute hydrochloric acid (2N, 2 x 20 ml.), aqueous potassium hydroxide (50%, 5 ml.) and water (3 x 20 ml.). The dried (sodium sulphate) solution was concentrated under reduced pressure to yield a gum which crystallised from aqueous-ethanol to give cordycepose anthraquinone-β-carboxylate as pale yellow micro-needles, m.p. 120°.

Found: C,64.4; H,4.4.

$C_{20}H_{16}O_7$ requires C,65.2; H,4.4%.

D-Ribonolactone.

D-Ribose (241 mg.) was dissolved in water (3.0 ml.) and bromine (0.15 ml.) was added. The reaction mixture was shaken in a closed vessel in the absence of light

for 56 hours. Excess bromine was removed by drawing a vigorous stream of air through the solution and the volume was increased to 5 ml. by the addition of distilled water. The solution was treated with freshly prepared silver carbonate (from silver nitrate 0.50 g.) and the precipitate of silver salts was removed by filtration. The filtrate was saturated with hydrogen sulphide and the solution was filtered through "Hyflo Supercel" and activated animal charcoal until a colourless filtrate was obtained. Concentration of this solution under reduced pressure yielded a pale brown gum which was extracted with ethanol (10 ml.), filtered free of insoluble inorganic material and concentrated, finally under high vacuum, to yield D-ribonolactone as a pale gum (200 mg., 83%).

D-Ribonic Acid Phenylhydrazide.

D-Ribonolactone (200 mg.) was dissolved in absolute ethanol (0.5 ml.) and freshly distilled phenylhydrazine (0.2 ml.) was added. The reaction mixture was warmed under reflux for 1 hour. The crystals which separated during the reaction were collected and recrystallised from ethanol to yield D-ribonolactone phenylhydrazide as colourless

plates, m.p. 162-163° (decomp.).

Found: N,11.3.

Calc. for $C_{11}H_{16}O_5N_2$: N,10.9%.

Cordycepononolactone.

Cordycepose (600 mg.) was dissolved in water (7.0 ml.) and bromine (0.3 ml.) was added. The oxidation of the sugar and the removal of excess bromine and bromide ions was completed according to the method employed in the isolation of ribonolactone from D-ribose. The crude product, which evolved carbon dioxide from an aqueous solution of sodium carbonate, was dissolved in methanol (10 ml.) and filtered free from a trace of inorganic material. Solvent was removed, finally in vacuo, and the residue was maintained at 80°/0.1 mm. for 30 minutes to yield cordycepononolactone as a pale brown, hygroscopic syrup (347 mg., 59%).

Found: C,45.8; H,6.4.

$C_5H_8O_4$ requires C,45.5; H,6.1%.

$[\alpha]_D^{20}$ +32.2° (c = 0.433 in ethanol).

Catalytic Reduction of Cordycepose.

(cf. Kent, Stacey and Wiggins, J.,1949,1234.)

Cordycepin (1.03 g.) was hydrolysed with hydrochloric acid (N/10, 50 ml.) and cordycepose (426 mg., 77%) isolated as described previously, was dissolved in water (240 ml.); Raney nickel catalyst (c. 2.5 g.) in water (10 ml.) was added and the mixture was hydrogenated at 100° and 30 atmospheres pressure for 10 hours in a rocking autoclave. The catalyst was removed from the reaction mixture by filtration through activated animal charcoal and evaporation of the filtrate, finally in vacuo, yielded a gum which on the addition of ethanol solidified to form a white amorphous cake. The product did not melt below 360°; it was hygroscopic and contained a large amount of inorganic material.

Found; C,6.3; H,1.3%.

The reaction was repeated using freshly prepared catalyst; the same product was isolated.

Cordyceponic Acid Phenylhydrazide.

Cordyceponolactone (54 mg.) was dissolved in ethanol (0.2 ml.) and freshly distilled phenylhydrazine (0.1 ml.) was added. The solution was warmed on a

steam-bath under reflux for 30 minutes during which time it darkened slowly. After cooling at 0° for several hours, the product commenced to crystallise and finally set to a solid mass of clustered needles. The product (40 mg., 41%) was dissolved in boiling ethyl acetate and cordyceponic acid phenylhydrazide separated as colourless, clustered needles, m.p. 150-151° (decomp.). A sample for analysis was dried overnight at 20°/0.1 mm.

Found: C,54.8; H,6.6; N,11.6.

$C_{11}H_{16}O_4N_2$ requires C,55.0; H,6.7; N,11.7%.

$[\alpha]_D^{20}$ +26° (c = 0.300 in ethanol).

Attempted Preparation of Cordycepose Benzylphenylhydrazone.

(cf. Vongerichten and Muller, Ber.,1906,39,235.)

Benzylphenylhydrazine hydrochloride (2.0 g.) was added to a solution of sodium (200 mg.) in ethanol (25 ml.). The mixture was shaken and filtered through kieselguhr. Evaporation of the solvent yielded a brown oily residue which was distilled under 1 mm. of pressure to yield benzylphenylhydrazine as a pale yellow oil. Dry cordycepose (175 mg.) was dissolved in absolute ethanol (3 ml.) and a solution of benzyl-

phenylhydrazine (300 mg.) in ethanol (2 ml.) was added. The reaction solution was kept at room temperature for 12 hours and was then warmed to reflux on a steam-bath. The solvent was removed under reduced pressure to yield a brown gum which on cooling set to a glass, and which could not be obtained in a crystalline form.

Cordycepose p-Bromophenylosazone.

(cf. Vongerichten, Ann.,1901,318,128.)

Cordycepose (400 mg.) was dissolved in dilute acetic acid (15%, 5 ml.) and p-bromophenylhydrazine hydrochloride (500 mg.) and fused sodium acetate (600 mg.) were added. The reaction mixture was warmed to complete solution and was kept at 100° for 90 minutes. The yellow gum which separated solidified partially on cooling and scratching and oily material was removed by washing with a small volume of ice-cold ethanol. The solid residue was collected and dried in vacuo, (220 mg.) m.p. 158-160°. Crystallisation from ethyl acetate yielded cordycepose p-bromophenylosazone as pale yellow clustered prisms, m.p. 163°; recrystallisation from water (charcoal) yielded colourless blades (95 mg.) m.p. 163-164°.

Found: C,43.1; H,3.8; N,12.0; Br,35.2.

$C_{17}H_{18}O_2N_4Br_2$ requires C,43.4; H,3.9; N,11.9; Br,34.0%

$[\alpha]_D^{20}$ 0 ± 1° (c = 0.820 in ethanol)

3:3-Dicarbethoxypropan-1-al Diethylacetal.

(cf. Perkin and Sprankling, J.,1899,12.)

Ethyl malonate (125 g.) and bromoacetal (100 g., prepared according to the method of Adkins and Harting, J. Amer. Chem. Soc.,1927,49,2517.) were added to a solution of sodium (17.8 g.) in ethanol (212 ml.) and the mixture was heated to 150° for 4 hours in a rocking autoclave. The ethanol was removed by distillation and the residue was treated with water (100 ml.) and extracted with ether. The dried (sodium sulphate) extract was concentrated and distilled under reduced pressure to yield a low-boiling fraction, b.p. 110°/2mm. which was discarded, and a fraction of crude 3:3-dicarbethoxypropan-1-al diethylacetal (85 g., 40%), b.p. 116-121°/2 mm. (n_D^{20} 1.4309). The product was redistilled and a fraction (55 g.), b.p. 117-119°/2 mm. was collected (n_D^{20} 1.4312).

Found: C,56.4; H,9.0.

Calc. for $C_{13}H_{24}O_6$: C,56.5; H,8.8%

A fraction (9 g.), b.p. 116-117°/2 mm. was also pure

3:3-dicarbethoxypropan-1-al diethylacetal (n_D^{20} 1.4320).

Found: C,57.0; H,8.7%.

Excess of a saturated solution of 2:4-dinitrophenylhydrazine sulphate in ethanol (Brady's solution) was added to a warm solution of 3:3-dicarbethoxypropan-1-al diethylacetal (0.2 g.) in ethanol (1 ml.). The yellow prisms which separated, m.p. 85-87°, recrystallised from ethanol to yield 3:3-dicarbethoxypropan-1-al 2:4-dinitrophenylhydrazone as lustrous orange-yellow plates, m.p. 98°.

Found: C,47.4; H,4.5.

$C_{15}H_{18}O_8N_4$ requires C,47.1; H,4.7%.

4-Hydroxy-3-hydroxymethylbutan-1-al Diethylacetal.

(cf. Marvel and Hill, J. Amer. Chem. Soc., 1951, 73, 481.)

A solution of 3:3-dicarbethoxypropan-1-al diethylacetal (60 g., 0.2175 gm.-mole.) in anhydrous ether (100 ml.) was added over a period of 2½ hours to a well-stirred mixture of lithium aluminium hydride (11.07 g., 0.2925 gm.-mole.) and anhydrous ether (225 ml.) at such a rate that the solvent refluxed gently. Stirring was continued for 30 minutes and excess lithium aluminium hydride was destroyed by the cautious addition of water. The mixture was diluted with ether and stirred to

hydrazine in dilute hydrochloric acid (50 ml.) was added and the reaction solution was decanted from a gummy residue which separated during 24 hours. After standing for a further 48 hours at room temperature the pale yellow precipitate which separated was collected and crystallised from aqueous methanol (50%) from which 4-hydroxy-3-hydroxymethylbutan-1-al 2:4-dinitrophenylhydrazone separated as pale yellow micro-needles, m.p. 149-150°.

Found: C,44.2; H,4.6; N,18.9.

$C_{11}H_{14}O_6N_4$ requires C,44.3; H,4.7; N,18.8%.

A very dilute ethanolic solution of the product gave a deep red colouration on the addition of aqueous sodium hydroxide solution.

Attempted Preparation of 2-Bromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal (I).

N-Bromosuccinimide (19 g., freshly prepared according to the method of Ziegler et al., Ann., 1942, 551, 80) was added in small portions over a period of 1 hour to a well-stirred solution of 4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal (20 g.) in anhydrous ether (100 ml.). The reaction was catalysed by exposure to a tungsten filament lamp and a deep-red

colouration developed in the solution as the reaction proceeded. Stirring was continued until the reaction mixture was pale brown in colour and the succinimide which separated was removed by filtration. The filtrate was shaken with a saturated aqueous solution of sodium bicarbonate (25 ml.) for 30 minutes and the ether layer was separated. The aqueous solution was extracted with ether (25 ml.) and the combined, dried (sodium sulphate) ether liquors were concentrated to yield a viscous yellow oil (19 g.) n_D^{20} 1.4919. The product (1.0 g.) was micro-distilled under high vacuum to yield a fraction of 4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal (50 mg.) b.p. $70^{\circ}/10^{-3}$ mm., and a principal fraction of 2-bromo-4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal (100 mg.) b.p. $120^{\circ}/10^{-3}$ mm. as a colourless viscous oil, n_D^{20} 1.4975.

Found: Br,29.0.

$C_9H_{19}O_4Br$ requires Br,29.5%.

The residue polymerised to a coloured glass which could not be distilled.

The crude bromo compound (18 g.) was distilled *in vacuo* to yield fraction A (4.5 g.), b.p. 76-92°/10^{-3} mm., n_D^{20} 1.485; fraction B (2.9 g.) b.p. 92-94°/10^{-3} mm., n_D^{20} 1.4970; fraction C (1.3 g.) b.p. 114°/10^{-3} mm., n_D^{20} 1.4995, and a polymerised residue (8.5 g.).

Fraction A (2.0 g.) was distilled to yield four fractions and a polymerised residue, of which the third fraction, n_D^{20} 1.4936 was analysed.

Found: C,36.6; H,5.7; Br,39.0.

$C_9H_{19}O_4Br$ requires C,39.9; H,7.1; Br,29.5.

$C_9H_{18}O_4Br_2$ requires C,30.9; H,5.2; Br,45.7%.

Fraction B (2.0 g.) was distilled to yield two fractions n_D^{20} 1.4969 and n_D^{20} 1.4975 and a polymerised residue. The fraction of higher refractive index was analysed.

Found: C,35.2; H,5.2; Br,39.1%.

Thiourea Condensation Product.

To a solution of sodium iodide (0.490 g.) in acetone (15 ml.) was added fraction B (0.883 g.) described above. The solution was warmed under reflux for 1 hour and the precipitated sodium bromide (380 mg., 60%) was removed from the cooled reaction mixture by filtration. The solution was concentrated under reduced pressure and the residual brown oil was separated from a precipitate of sodium halide by ether extraction (3 x 10 ml.). Concentration of the dried ethereal solution yielded a brown oil (0.91 g.) n_D^{20} 1.5150. A mixture of this product (150 mg.), thiourea (200 mg.) and ethanol (3 ml.) was warmed under reflux for 1 hour and the reaction solution,

on the addition of excess of a saturated ethanolic solution of picric acid, yielded 2-amino-5-(2'-hydroxy-1'-hydroxymethyl)-ethylthiazole picrate as acicular blades, m.p. 195° (decomp.). The product recrystallised from water as glistening yellow needles, m.p. 196° (decomp.).

Found: C,35.7; H,3.3; N,17.9.

$C_{12}H_{13}O_9N_5S$ requires C,35.7; H,3.2; N,17.4%.

The molecular weight of this compound was found to be 397 (p. 124, $C_{12}H_{13}O_9N_5S$ requires 403).

2-Aminothiazole Picrate.

A warm solution of bromoacetal (100 mg.) and thiourea (100 mg.) in ethanol (3 ml.) was heated under reflux for 30 minutes. Treatment of this solution with excess saturated ethanolic picric acid solution yielded 2-aminothiazole picrate as yellow needles, m.p. 220-221° (decomp.) which recrystallised from water as clustered needles, m.p. 222° (decomp.).

Found: C,32.7; H,2.4; N,21.0.

Calc. for $C_9H_7O_7N_5S$: C,32.8; H,2.1; N,21.3%.

The molecular weight of this compound was found to be 328 (p. 124, $C_9H_7O_7N_5S$ requires 329). The same product, m.p. 220° (decomp.) was obtained from bromoacetaldehyde,

the m.p. being undepressed on admixture with the above product.

Attempted Preparation of 2-Bromo-4-hydroxy-3-hydroxymethylbutan-1-al Diethylacetal (II).

The addition of N-bromosuccinimide (9.5 g.) to a solution of 4-hydroxy-3-hydroxymethylbutan-1-al diethylacetal (10 g.) in anhydrous ether (50 ml.) was carried out over a period of 1 hour, the reaction mixture being exposed to strong ultraviolet light. The reaction proceeded rapidly to completion and the cooled, filtered reaction mixture was washed with a saturated aqueous solution of sodium bicarbonate (15 ml.), dried and concentrated to yield a pale brown oil (8.5 g.) n_D^{20} 1.4990 of lower viscosity than the product previously isolated.

The product (0.81 g.) was added to a solution of sodium iodide (0.45 g.) in acetone (15 ml.) and after warming to reflux for 1 hour the reaction mixture was filtered free of precipitated sodium bromide. The solution was concentrated in vacuo and the residue extracted with ether (3 x 10 ml.). Evaporation of the ethereal solution yielded a dark brown oil (0.83 g.). A mixture of this product (100 mg.), thiourea (130 mg.)

and ethanol (2 ml.) was warmed under reflux for 1 hour and excess of a cold saturated solution of picric acid in ethanol was added. No product separate from the cooled concentrated solution on prolonged standing, but on the drop-wise addition of water an amorphous yellow precipitate deposited and was collected and crystallised from aqueous ethanol from which it separated as yellow prismatic needles, m.p. 208-210° (decomp.). A mixture of this product (2-amino-6:6-di-(hydroxymethyl)-1:3-thiazine picrate) and 2-amino-5-(2'-hydroxy-1'-hydroxymethyl)-ethylthiazole picrate, m.p. 196° (decomp.) had a m.p. of 180-183° (decomp.).

Found: C,35.3; H,3.5.

$C_{12}H_{13}O_9N_5S$ requires C,35.7; H,3.2%.

The molecular weight of this picrate was found to be 400 (p.124, $C_{12}H_{13}O_9N_5S$ requires 403).

APPENDIX.

The Micro-determination of Molecular Weight by a Spectrophotometric method.

INTRODUCTION.

The science of quantitative analysis of organic compounds on the micro-scale has been and is a key factor in the progress of organic chemistry, particularly in fields such as those of hormone and vitamin study where the amount of material available is frequently of a very low order. Closely related to this work is the subject of molecular weight determination, the various approaches to which have, in recent years, received considerable attention. Many comprehensive papers and reviews have been published on this subject and only a brief mention of the more important methods shall be given here.

The ebullioscopic method of molecular weight determination, which is dependent upon the elevation of the boiling point of a pure organic solvent by a solute (the sample) with which the solvent does not react, has been studied by Pregl (105), Reiche (106) and Sucharda, Bobranski and Schmitt (107, 108). The method of Pregl requires a sample of 10 mg., while the other workers employed 15-25 mg., solvents of the highest possible degree of purity being used in every case. Unsatisfactory results have been reported for all

these methods (109, 110). The Schmitt (107) modification of the Suoharda-Bobranski method is probably the most widely used.

The cryoscopic method of molecular weight determination depends upon the depression in melting point of a solid organic solvent by a solute (the sample) which must neither react with the solvent nor suffer thermal decomposition at the m.p. of the latter. By using solvents which exhibit a high molecular melting point depression constant (111, 112, 113), the technique is greatly simplified. Camphor and borneol are two very widely employed solvents but values frequently reported in the literature for the molecular melting point depression constant of these compounds show wide variations and this is a reflection upon the accuracy of the method.

In the vaporimetric method the principle involved is the vaporisation of the sample in a closed system in such a manner that its vapour displaces an equal volume of mercury. The displaced mercury is estimated gravimetrically and the volume of vapour is calculated, corrections being made for temperature pressure and expansion of the mercury. Two types of apparatus are employed, one for low-boiling liquids and the other for

high-boiling liquids and solids. It has been shown by Schmitt (114) that a slightly enlarged apparatus can be used in which a sample of 10 - 20 mg. is employed, the tolerance being not less than ±10%.

The isothermic method of molecular weight determination (115) was developed by Barger (116, 117) from the observation of Errara that in a closed system containing droplets of a salt solution and of the pure solvent, the former increase in volume at the expense of the latter. Employing this fact, a solution of known concentration of a compound of unknown molecular weight can be compared with various standard solutions of slightly varying molarity until one solution is found with which the unknown is in or near the isopiestic state. Thus the molarity of the unknown solution, and hence the molecular weight of the unknown compound can be deduced. The method demands very precise working and is tedious, while the greatest accuracy obtained by the method is of the order ±10%.

Apart from these purely micro-analytical methods, a very accurate evaluation of the molecular weight of a crystalline solid can be obtained from X-ray crystallographic examination which ultimately evaluates the volume occupied by, and hence the weight of

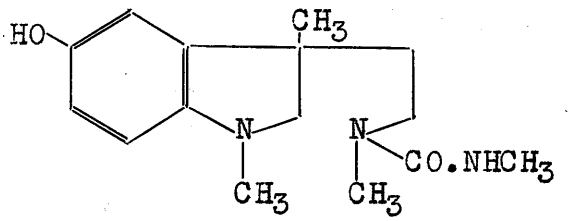
1 molecule of the unknown substance.

THEORETICAL.

The photoelectric spectrophotometer is used for the measurement of the ultraviolet or visible light absorption of a compound and carries two cells of quartz or glass in one of which is placed a standard solution of the specimen under observation and in the other the pure solvent. The measure of the absorption by the latter cell and its content is used to cancel all absorption by the former other than that caused by the specimen in solution, the result being the evaluation of the absorption of the specimen only.

In the course of a comparative study of the ultraviolet light absorption of a number of bases in the eserine series a compound was isolated (dihydro-isoeserine, tentatively postulated as LXXII) which could not be obtained in a crystalline form, the picrate of which was however readily purified. A standard solution of the picrate in ethanol was placed in the solution cell of the spectrophotometer and in the solvent cell was placed a compensating solution of picric acid in ethanol, the concentration of which was calculated to be identical with the concentration

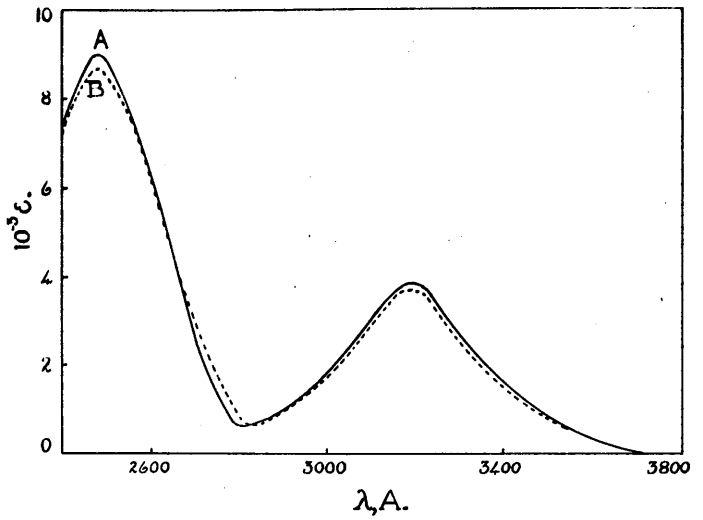
of picric acid in the picrate solution. Absorption readings were taken in the normal manner and a graph of absorption was thus obtained (calculated on the concentration and molecular weight of the base) which was found to be in close agreement with that of a sample of the crude base (Fig. 6).



(LXXII)

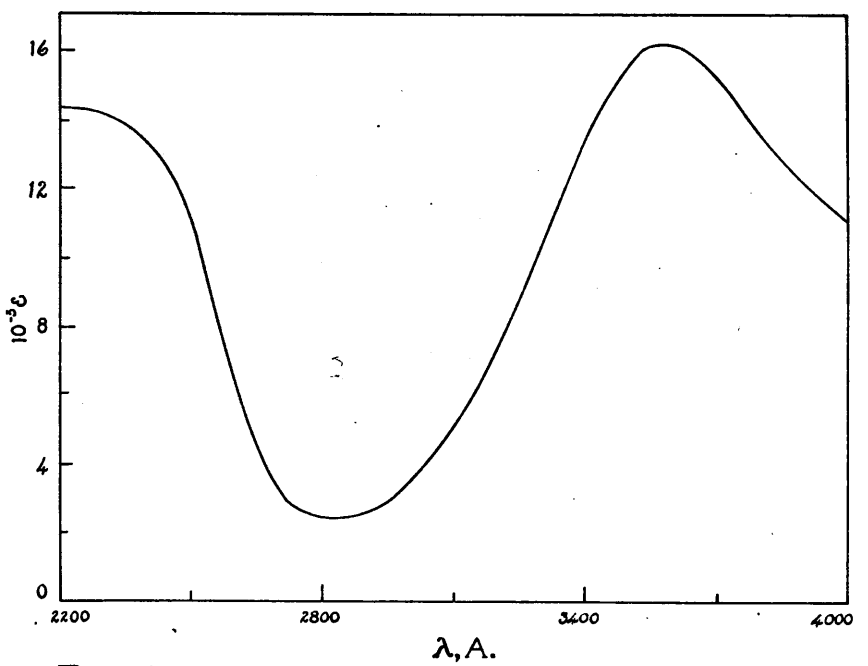
An examination of the ultraviolet light absorption graph of picric acid (Fig. 7) shows that although this compound exhibits two bands of high-intensity absorption, one being at the near-visible end of the ultraviolet spectrum, these are not in evidence in Fig. 6 and it was therefore concluded that the absorption of dihydroiso-eserine picrate can be considered to consist of two parts, the absorption due to the base and that due to picric acid, such that the latter can be cancelled quantitatively by an equivalent solution of picric acid to reveal the absorption of the base only.

Fig. 6



A Dihydroisoeserine Picrate ~ Picric Acid.
B Dihydroisoeserine.

Fig. 7



Picric Acid in ethanol.

The Molecular Weight of Picrates (Method I).

Very few organic compounds other than polycyclic hydrocarbons show appreciable light absorption in the region 3500 - 4000 A. and it was therefore deduced that the absorption of a picrate in this region of the spectrum is usually entirely due to the picric acid in the picrate. If this be the case it should be possible to cancel completely the absorption of a picrate in the region 3500 - 4000 A. by placing in the compensating cell of the instrument an ethanolic solution of picric acid of concentration equivalent to that of picric acid in the picrate solution. Not only was this found to be true in the cases examined, but also it was observed that a small alteration in the concentration of picric acid (even of the order ±2%) caused a large alteration in the absorption graph. The graphs on Fig. 8 show the results obtained in a typical case (papaverine);

(a) Absorption due to papaverine.

(b), (c) and (d) Absorption due to papaverine picrate balanced by theoretical, 98% theoretical and 102% theoretical concentration of picric acid, respectively.

Conversely the procedure must be applicable to

the determination of the amount of picric acid in an unknown picrate since the presence of the theoretically equivalent concentration of picric acid in the compensating cell will completely cancel the absorption at the near-visible end of the ultraviolet range, or will reproduce the light absorption of the free base in the rare cases in which this is appreciable at high wavelength. Employing arbitrary concentrations of picric acid and interpolating to evaluate the exact equivalent, the results of 25 estimations are shown in Table VI. These compounds are representative of the principal classes of picrate-forming compounds including hydrocarbons.

Every compound examined has been a monopicrate. In the case of a di- or tripicrate the result obtained will be one half or one third respectively of the correct value of the molecular weight of the picrate and the corresponding correction factor must be applied.

TABLE VI.

Compound picrate	Molecular Weight.[x] Found	Calc.	Error %.
Hydrocarbons:			
Acenaphthene	385	383	+0.5
Chloronaphthalene	394	391.5	+0.6
2-Methylnaphthalene	372	371	+0.3
Primary amines:			
Ethanolamine	290	290	0.0
Chloroaniline	361	356.5	+1.3
Dichloroaniline	396	391	+1.3
β-Naphthylamine	369	372	-0.8
Secondary amines:			
Ethylaniline	354	350	+1.1
N-Methylphenetidine	379	380	-0.3
Heterocyclic bases:			
Piperidine	313	314	-0.3
8-Hydroxyquinoline	375	374	+0.3
Adenine	366	364	+0.5
2-Methylpyridine	320	322	-0.6
N-Methylmorpholine	330	330	0.0
2-Aminopyrazine	323	324	-0.3
Indole	345	346	-0.3
4-Methylimidazole	308	311	-1.0
2-Ethoxy-4-methyl-quinoline-1-oxide	430	432	-0.5
Cordycepin	477	480	-0.6
Alkaloid bases:			
Cocaine	531	532	-0.2
Papaverine	565	568	-0.5
Brucine	622	623	-0.2
Vomicine	611	609	+0.3
Narcotine	649	642	+1.1

x 'Whole number' atomic weights have been employed.

The calculation of the molecular weight of 8-hydroxyquinoline will be discussed as a typical example. Calculations are based in every case on values of log (I_o/I) at 3800 A., this wavelength being chosen arbitrarily as one remote from the region of general absorption (2000 - 3500 A.) but at which picric acid still exhibits a high molecular extinction coefficient (ε_{3800} = 13,450)

Concentration of 8-hydroxyquinoline = 0.04890 mg./ml.
Concentration of 8-hydroxyquinoline picrate
= 0.01388 mg./ml.

Table VII and Fig. 9 show:

(a) Absorption (log I_o/I) of picrate solution with picric acid of concentration 0.00838 mg./ml. in the solvent cell.

(b) Absorption (log I_o/I) of picrate solution with picric acid of concentration 0.007905 mg./ml. in the solvent cell.

(c) Absorption (log I_o/I) of 8-hydroxyquinoline solution.

(d) Absorption (log I_o/I) of 8-hydroxyquinoline solution corrected for concentration after initial calculation.

TABLE VII.

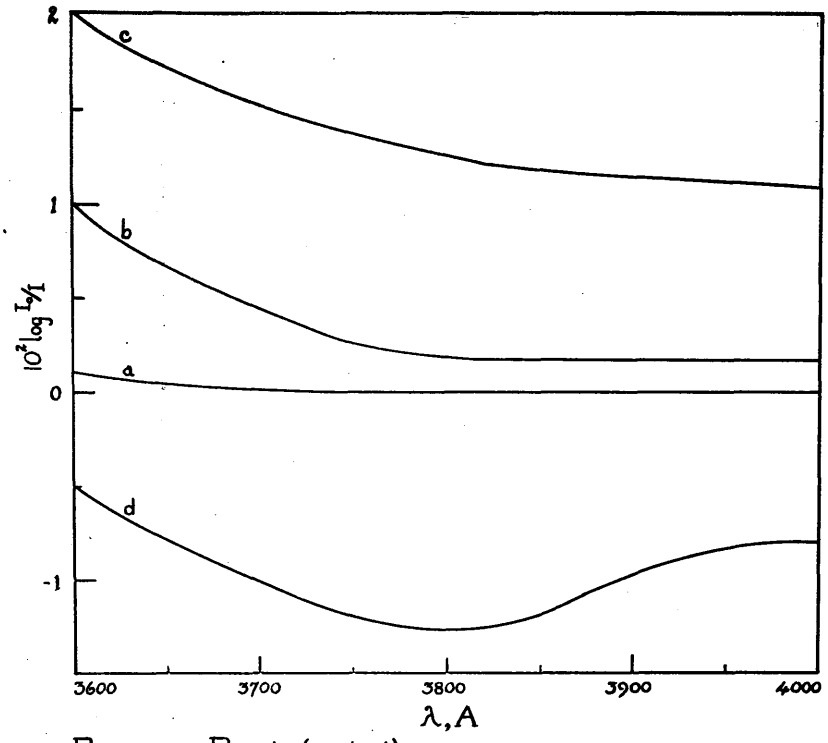
Wavelength	a	b	c	d
3500	0.015	0.047	0.185	0.021
3550	0.010	0.045	0.110	0.012
3600	0.007	0.042	0.044	0.005
3650	0.007	0.042	0.033	0.004
3700	0.007	0.041	0.010	0.001
3750	0.006	0.039	0.006	0.001
3800	0.006	0.036	0.002	0.000
3850	0.005	0.035	0.002	0.000
3900	0.004	0.034	0.001	0.000
3950	0.003	0.032	0.001	0.000
4000	0.002	0.029	0.001	0.000

The approximate molecular weight is based on the value at 3800 A. assuming no absorption by the base or hydrocarbon at this wavelength.

By interpolation $\log ({}^{I_o}/_{I})_{3800} = 0.000$ at a picric acid concentration = 0.008475 mg./ml. Therefore 0.01388 mg. picrate contains 0.008475 mg. picric acid, and the approximate molecular weight of the picrate is 375. The concentration of base in the picrate solution = $\frac{375 - 229}{375} \times 0.01388 = 0.00540$ mg./ml. The factor to correct values of $\log ({}^{I_o}/_{I})$ for the base to those for the concentration of base in the picrate solution = $\frac{0.00540}{0.04890} = 0.110$.

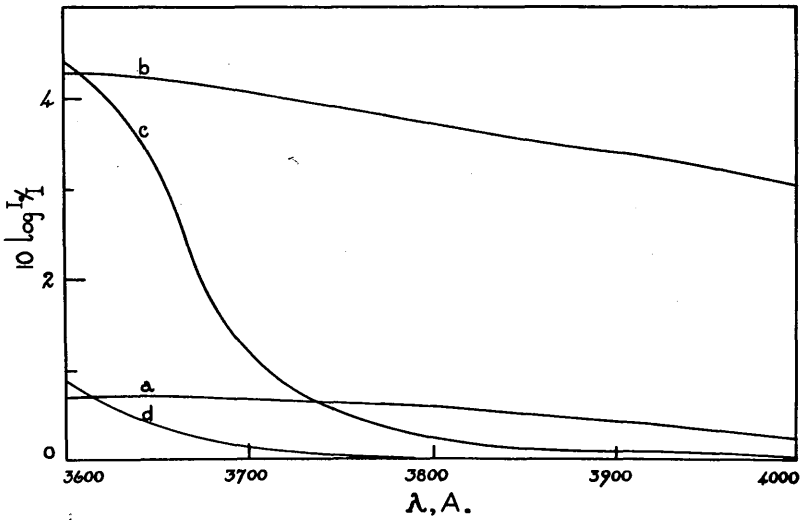
The base corrected for concentration (d) shows no absorption at 3800 A. and the approximate molecular weight is therefore the same as the corrected molecular

Fig.8



Papaverine Picrate (see text)

Fig.9



8-Hydroxyquinoline Picrate (see text)

weight (375).

It has been found that in place of picric acid solution in the solvent cell it is satisfactory to employ solutions of the picrate of a base which does not absorb in the region 3500-4000 A. (morpholine picrate has been used with success) and from theoretical considerations it can be argued that this is, in fact, preferable. The results suggest that the method is accurate within the limits of ±2% , but it is tedious in that it requires the preparation of a range of standardised solutions.

The Molecular Weight of Picrates (Method II).

From the arguements employed and the results obtained in the work described it is obvious that the picrates of those bases which do not absorb appreciably at a wavelength of 3800 A. must all show the same molecular extinction coefficient as picric acid at this wavelength. The results shown in Table VIII substantiate this deduction within narrow limits of experimental error.

TABLE VIII

Picrate	c(mg./100 ml.)	$\log(I_o/I)_{3800}$	ε_{3800}
Ethanolamine	1.109	0.512	13,390
Piperidine	1.090	0.469	13,510
Morpholine	1.462	0.620	13,400
2-Aminopyrazine	1.137	0.472	13,450
N-Ethylaniline	1.196	0.459	13,430
Picric Acid	1.107	0.650	13,450

As is required by the constancy of the values observed for ε_{3800} for the picrates shown in Table VIII, it was found that the bases corresponding to these picrates show no appreciable absorption at this wavelength, nor was the absorption of the base of any of the large number of picrates subsequently examined sufficient to cause appreciable error in the results obtained.

The molecular extinction coefficient of a compound is evaluated by employing the formula $= \frac{M. \log(I_o/I)}{C}$ where M is the molecular weight of the compound and C is the concentration in g./l. If, for a compound of unknown molecular weight, a value of ε be established

for a particular wavelength, then $M = \frac{\varepsilon . C}{\log(I_o/I)}$.

Assuming the value $\varepsilon_{3800} = 13,440$, the molecular weights of the monopicrates listed in Table IX were estimated. From the results obtained the method appears to be accurate within the limits ±2%.

The case of adenosine picrate is of interest since the sample prepared [m.p. 182° (decomp.)] was assessed to have a molecular weight of 508 whilst that of adenosine picrate is 496. Gulland and Holiday (60) quote the melting point of this compound to be 192° (decomp.) for the anhydrous picrate whilst Levene and Jacobs (118) give the value 182° (decomp.) together with an analysis which, although correlated to the formula of the anhydrous picrate, is in much closer agreement with that of the monohydrate. That our sample was hydrated was proven by the fact that on prolonged drying the m.p. rose to 191° (decomp.) and the product (the molecular weight of which was assessed to be 500) analysed in excellent agreement with the values required for the anhydrous salt.

TABLE IX.

Picrate.	c.	l.	M. Found	Calc.	Error %.
Acenaphthene	2.801	0.990	380	383	-0.8
1-Bromonaphthalene	1.353	0.420	433	436	-0.7
2-Methoxynaphthalene	1.161	0.401	389	387	+0.5
2:5-Dichloroaniline	1.421	0.485	394	391	+0.8
Quinoline	1.319	0.491	361	358	+0.8
8-Hydroxyquinoline	1.388	0.498	375	374	+0.3
Adenine	1.361	0.500	366	364	+0.5
Adenine (from cordycepin)	1.240	0.460	362	364	-0.5
Adenosine.H_2O	1.845	0.488	508	514	-1.2
Adenosine	2.270	0.610	500	496	+0.8
Hypoxanthine	1.411	0.511	371	365	+1.6
2-Methylpyridine	1.165	0.490	320	322	-0.6
N-Methylmorpholine	1.300	0.525	333	330	+0.9
4-Methylimidazole	1.959	0.850	310	311	-0.3
Carbazole	1.327	0.443	403	396	+1.8
Cocaine	2.159	0.550	528	532	-0.8
Narcotine	2.938	0.615	642	642	0.0
Strychnine	2.481	0.598	558	563	-0.9
Vomicine	2.530	0.558	609	609	0.0
2-Aminothiazole	1.250	0.512	328	329	-0.3
2-Amino-5-(2'-hydroxy-1'-hydroxymethyl)-ethylthiazole	1.379	0.467	397	403	-1.5
2-Amino-6:6-di-(hydroxymethyl)-1:3-thiazine	1.631	0.548	400	403	-0.7

'c' represents the concentration in mg./100 ml. (and also the weight in mg. of sample taken) and 'l' the value of $\log(I_o/I)_{3800}$. 'Whole number' atomic weights are used throughout.

A determination of the molecular weight of cordycepin was carried out using its monopicrate with the following results:

	c.	l.	M.
Cordycepin picrate	1.036	0.288	483

This value corresponds to a molecular weight of 254 for cordycepin which is in close agreement with that of 248 ± 10 obtained by the X-ray crystallographic method by Mrs. Dorothy Hodgkin and Dr. G. J. Pitt, and with that of 251 required by $C_{10}H_{13}O_3N_5$ subsequently shown by analytical and degradative examination to be the molecular formula of the metabolic product.

Although only mono-picrates have been studied the method is applicable to any type of picrate using the modified formula:

$$M = \frac{13,440 \times C \times n}{\log(I_o/I)}$$

where 'n' is the molar ratio of picric acid:base or picric acid:hydrocarbon in the picrate.

The accuracy of this method of molecular weight determination is dependent upon the accuracy with which the spectroscopic solution is prepared and upon the accuracy with which the value of $\log(I_o/I)$ is

determined. It has been found that the photoelectric spectrophotometer is capable of giving results reproducible within the limits ± 1%. In the wave-length region 3700 - 4000 A. the variation of ε with wave-length is considerable in the case of picrates, so that a slight error in the wave-length determination mechanism of the instrument employed may lead to an appreciable alteration in the value of the constant ε_{3800}. (That such a variation can occur was shown when the average value of ε_{3800} for 12 picrates measured on a second spectrophotometer was 13,200.) For this reason it is essential that the instrument to be employed should be standardised against a number of picrates of known molecular weight, using matched quartz cells in the same aspect.

Although several properties of picrates make them unique in their applicability to spectrophotometric molecular weight determination, the method is so attractive in virtue of its simplicity and accuracy, that its possible extension to include compounds other than bases and hydrocarbons must not be overlooked. For example anthraquinone-2-carboxylic acid shows considerable light absorption at the near-visible end of the ultra-

TABLE X.

Ester	Solvent	c.	l.	ε.
Ethyl	ethanol	1.241	0.217	4,900
Ethyl	chloroform	1.327	0.237	5,000
n-Butyl	ethanol	1.274	0.207	5,000
n-Butyl	chloroform	1.421	0.229	4,970
Bornyl	ethanol	1.569	0.203	5,020
Bornyl	chloroform	1.284	0.168	5,080
p-Methylcyclohexanyl	ethanol	1.500	0.210	4,870
β-Amyrin	chloroform	1.836	0.144	5,180
Cholesteryl	chloroform	2.002	0.165	5,110
Anthraquinone-2-carb. acid	ethanol	1.388	0.301	5,460
Anthraquinone-2-carb. acid	chloroform	1.520	0.326	5,400

'c' represents the concentration in mg./100 ml. (and also the weight in mg. of sample taken) and 'l' the value of $\log(I_o/I)_{3270}$.

violet spectrum (fig. 10) while the esters of this acid are crystalline compounds; it has been observed (Table X) in the limited number of alcohols examined, that the corresponding anthraquinone-2-carboxylic esters exhibit

remarkably similar absorption properties. On the other hand, the solution to the problem of spectrophotometric molecular weight determination of alcohols may lie in esterification with an acid such that the resulting ester will be capable of forming a picrate. This is similar to the approach which has already been employed in the case of alkyl halides (of which the S-alkyl iso-thiourea picrates have been used) and its development may ultimately embrace several types of compound.

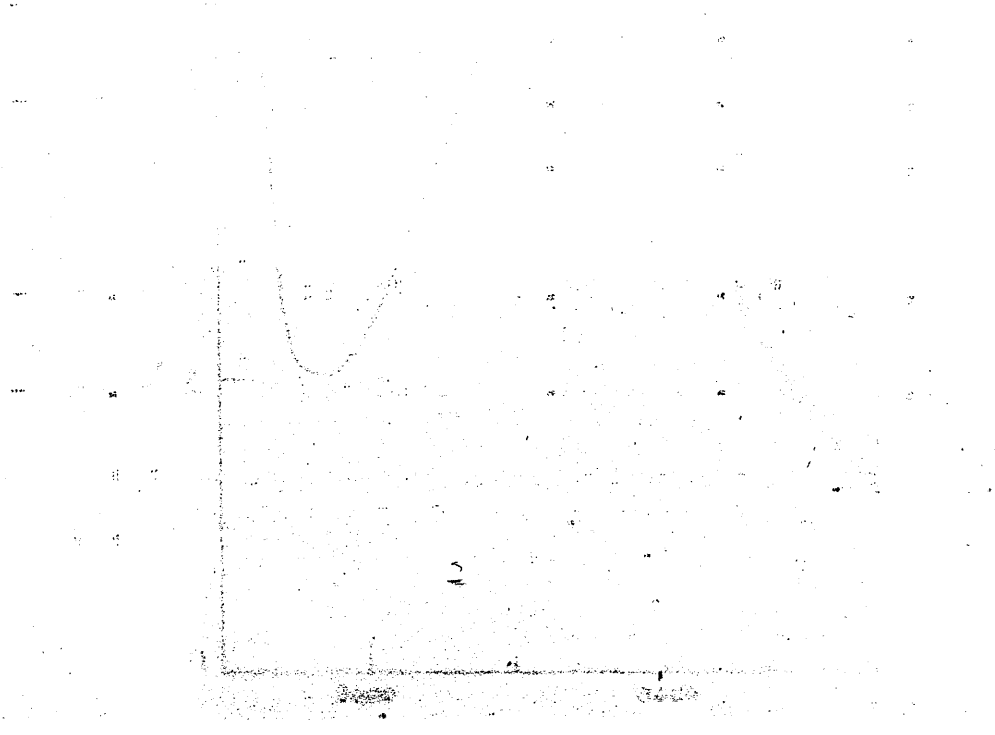
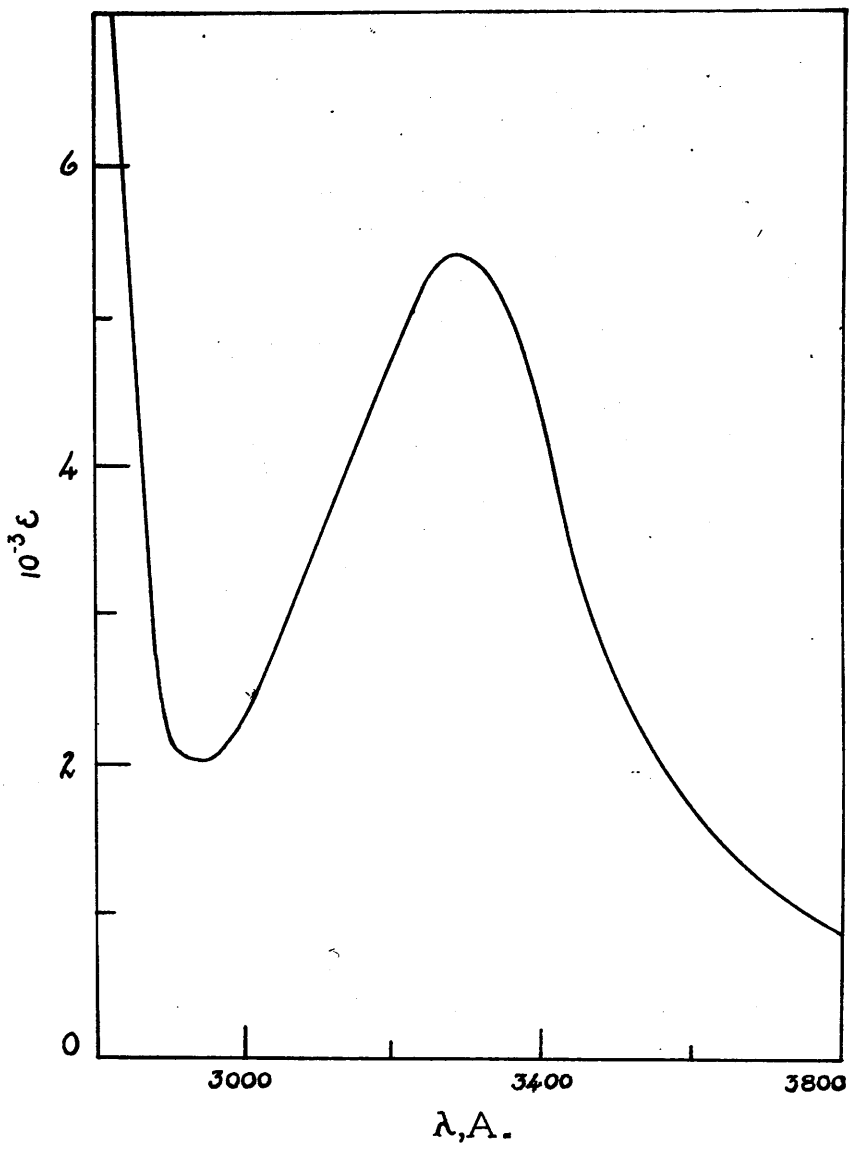


Fig. 10



Anthraquinone-2-carboxylic acid.

EXPERIMENTAL.

All melting points are uncorrected.

These picrates which have been described previously were prepared and purified according to the literature methods; the preparations of the others are given in the text of the previous Experimental section, or are described below. Absorption determinations were made using a Unicam S. P. 500 Spectrophotometer with matched 1 cm. quartz cells. Unless stated otherwise, spectroscopically pure ethanol was employed as solvent. The various concentrations of picric acid were obtained by diluting to 50 ml. the theoretical volumes of a standard solution of picric acid (or morpholine picrate in the cases in which the latter was employed), Grade 'B' volumetric equipment being used throughout.

Dihydroisoeserine Picrate.

(cf. Polonovski and Nitzberg, Bull. Soc. Chim., 1916, 19, 33.)

Adams' catalyst (25 mg.) was added to a solution of isoeserine (0.3 g., prepared by the reaction of eseroline and methylisocyanate in benzene at 20°) in absolute ethanol (25 ml.) Hydrogenation was complete

after 4 hours at normal temperature and pressure, and the filtered reaction solution was concentrated to yield a colourless, light-sensitive glass. The product was dissolved in warm ethanol (10 ml.) and excess of a cold saturated ethanolic picric acid solution was added. The product which separated was crystallised from absolute ethanol to yield dihydroisoeserine picrate as bright- yellow needles, m.p. 173-174°.

Found: C,50.1; H,5.5; N,16.3.

$C_{21}H_{26}O_9N_6$ requires C,49.8; H,5.2; N,16.6%.

2-Ethoxy-4-methylquinoline 1-Oxide Picrate.

A solution of 2-ethoxy-4-methylquinoline (3.3 g., Knorr, Ann., 1886, 236, 102) in peracetic acid (80 ml.) was maintained at 50° for 48 hours. The reaction mixture was evaporated (reduced pressure) and the cooled residue was made alkaline with 3N-potassium hydroxide solution. The mixture was extracted with chloroform (6 x 35 ml.), the dried (sodium sulphate) extract was evaporated and the viscous residue was dissolved in the minimum quantity of absolute ethanol. The addition of excess of a cold saturated ethanolic solution of picric acid yielded 2-ethoxy-4-methyl-quinoline 1-oxide picrate (2.1 g.) which separated from

ethanol as prismatic needles, m.p. 144-145°.

Found: C,50.0; H,3.9; N,12.9.

$C_{18}H_{16}O_9N_4$ requires C,50.0; H,3.7; N,13.0%.

2:5-Dichloroaniline Picrate.

Excess of a cold saturated aqueous picric acid solution was added to a warm aqueous solution of 2:5-dichloroaniline hydrochloride (30 mg.). The product which separated (25 mg.) recrystallised from water to yield 2:5-dichloroaniline picrate as yellow needles, m.p. 86°.

Found: C,37.0; H,2.3; N,13.9.

$C_{12}H_8O_7N_4Cl_2$ requires C,36.8; H,2.1; N,14.3%.

Adenosine Picrate.

(cf. Levene and Jacobs, Ber., 1909, 42, 2703.)

Excess of a cold saturated aqueous solution of picric acid was added to a warm aqueous-ethanolic solution of adenosine (40 mg.). The product which separated (30 mg.) recrystallised from aqueous-ethanol to yield adenosine picrate as yellow needles, m.p. 182° (decomp.). After drying for 8 hours at 80°/0.1 mm. the compound exhibited m.p. 191° (decomp.)

Found: C,38.6; H,3.2; N,22.9.

Calc. for $C_{16}H_{16}O_{11}N_8$: C,38.7; H,3.2; N,22.6%.

Anthraquinone-2-carboxylic Acid Chloride.

2-p-Toluylbenzoic acid was prepared by the reaction of toluene (100 g.), phthalic anhydride (25 g.) and aluminium chloride (50 g.) according to the method of Fieser (Org. Synth. Coll., Vol. I, 503) and the product (35 g.) m.p. 138°, was cyclised with concentrated sulphuric acid (cf. Fieser, Org. Synth. Coll., Vol. I, 345) to yield 2-methylanthraquinone as almost colourless needles (20 g.) m.p. 172-173°. Oxidation of a solution of the product (20 g.) in concentrated sulphuric acid (120 g.) and water (60 ml.) with sodium dichromate (60 g.) at 90° (after the method of Ilinskii and Kazakova, J. Gen. Chem. (U.S.S.R.), 1941, 11, 16) gave an almost quantitative yield of anthraquinone-2-carboxylic acid, m.p. 291-292°. The acid (10 g.) was converted to the corresponding acid chloride by warming to 100° with phosphorus oxychloride (20 g.) and phosphorus pentachloride (9 g.) until the evolution of hydrogen chloride ceased. Ligroin (100 ml.) was added to the reaction mixture and the product was separated by filtration, washed with ligroin, dried under reduced

pressure and distilled to yield anthraquinone-2-carboxylic acid chloride as a yellow oil (6.0 g.) b.p. 210°/0.2 mm. which solidified to a pale yellow crystalline mass, m.p. 147°. (Liebermann and Glock, Ber., 1884, 17, 889, gave m.p. 147°.)

Found: C,66.4; H,2.7; Cl,14.0.

Calc. for $C_{15}H_7O_3Cl$.: C,66.6; H,2.6; Cl,13.1%.

Ethyl Anthraquinone-2-carboxylate.

Anthraquinone-2-carboxylic acid chloride (400 mg.) was added to a solution of ethanol (0.1 ml.) in dry benzene (3.5 ml.) and pyridine (1.5 ml.). The reaction mixture was allowed to stand for several hours and was then warmed on a steam-bath for 15 minutes and was added to crushed ice. The solid which separated was crystallised from aqueous ethanol to yield ethyl anthraquinone-2-carboxylate as pale yellow needles, m.p. 146-147° (Liebermann and Glock, loc. cit., quote m.p. 147°).

Found: C,72.6; H,4.6.

Calc. for $C_{17}H_{12}O_4$: C,72.8; H,4.3%.

n-Butyl Anthraquinone-2-carboxylate.

The ester of n-butanol was prepared according to the method described above. n-Butyl anthraquinone-2-

carboxylate separates from petroleum-ether (b.p. 60-68°) as pale yellow clustered rods, m.p. 122-123°.

Found: C,74.3; H,5.2.

$C_{19}H_{16}O_4$ requires C,74.0; H,5.2%.

Bornyl Anthraquinone-2-carboxylate.

Anthraquinone-2-carboxylic acid chloride (500 mg.) was added to a solution of borneol (250 mg.) in dry benzene (3 ml.) and pyridine (1.5 ml.). The reaction solution was heated under reflux for 15 minutes, cooled, diluted with ether (15 ml.) and washed successively with dilute hydrochloric acid, alkali and water. Solvent was removed under reduced pressure and the residue crystallised from chloroform-petroleum ether (b.p. 40-60°) to yield bornyl anthraquinone-2-carboxylate as small pale yellow prisms, m.p. 185°.

Found: C,77.7; H,6.2.

$C_{25}H_{24}O_4$ requires C,77.3; H,6.2%.

p-Methylcyclohexanyl Anthraquinone-2-carboxylate.

The p-methylcyclohexanyl ester was prepared according to the method described for the bornyl ester. p-Methylcyclohexanyl anthraquinone-2-carboxylate separates from petroleum ether (b.p. 40-60°) as pale

yellow clustered needles, m.p. 146-147°.

Found: C,76.0; H,5.8.

$C_{22}H_{20}O_4$ requires C,75.8; H,5.8%.

β-Amyrin Anthraquinone-2-carboxylate.

The product isolated from the reaction according to the conditions described in the case of the bornyl ester could not be obtained in a crystalline form. After chromatographic purification (alumina-benzene) however, β-amyrin anthraquinone-2-carboxylate separated from chloroform-methanol as pale yellow needles, m.p. 308-310°.

Found: C,81.7; H,8.3.

$C_{45}H_{56}O_4$ requires C,81.8; H,8.5%.

Cholesteryl Anthraquinone-2-carboxylate.

The crude cholesteryl ester which was isolated under the standardised conditions could not be obtained in a crystalline form. A chloroform-benzene solution of the ester was passed through a chromatographic (alumina) column, solvent was removed and the yellow residue was dissolved in the minimum volume of boiling petroleum ether (b.p. 60-80°). The cooled solution set to a gel from which cholesteryl anthraquinone-2-carboxylate slowly separated as hard, yellow prisms,

m.p. 172-176°.

Found: C,81.1; H,8.5.

$C_{42}H_{52}O_4$ requires C,81.2; H,8.4%.

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