STUDIES IN THE CHEMISTRY OF VIOLACEIN.

A THESIS

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by

STEPHEN FARDLEY

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CONTENTS

SUMMARY.

This work is concerned with the chemistry of the dark purple bacterial pigment Violacein, produced by Chromobacterium Violacein.

In the first section, a general classification of the Chromobacterium Group is made and the properties exhibited by the bacteria in this class surveyed. The chemical and antibiotic properties of the pigment Violacein are mentioned and the results of the earlier investigators discussed. The section is concluded with an account of recent investigations into the structures of two major degradation products from Violacein, the hydrogen-iodide product and the C_{20} Acid.

The theoretical discussion is divided into several sub-sections each of which is concerned with different aspects of the investigation. The first of these discusses the methods which have been employed in the cultivation of the bacterium by previous workers and describes the superior method of growth on solid medium which is at present used in these laboratories. The work carried out on the C_{20} acid is then discussed and the presence of a

β-acylindole system and of a γ-keto acid system in the molecule demonstrated. During this work full use was made of ultra-violet and infra-red spectroscopic analyses and the spectra of several model compounds were measured for comparative purposes. It has therefore been found possible to eliminate a number of structures and there would appear as a result to be only two possible structures which could represent this acid. A discussion of the peculiar behaviour of this acid and its trimethyl derivative upon acetylation then follows, and a number of possible explanations are suggested and critically discussed in the light of degradational, synthetical and spectroscopic evidence.

The problem of the molecular formula of violacein is next discussed and the results of the analyses of important derivatives closely examined. Methylation of the pigment with dimethyl sulphate, potassium carbonate in acetone is shown to give rise to two distinct methyl derivatives and the nature of violacein as a molecular addition compound of two molecules, $C_{21}H_{15}N_3O_3$ and $C_{21}H_{13}N_3O_4$ demonstrated for the first time.

From a study of the properties of the two methyl ethers on acetylation, a re-investigation into the molecular

formula for acetylviolacein was made. This tended to indicate that this molecule is not a hexa-acetate as suggested by earlier workers, but rather a hepta-acetate.

The properties of the two methyl ethers are then described and on degradation with hydrogen iodide only the higher melting ether is shown to undergo a similar transformation to acetylviolacein. On reductive alkaline degradation both methyl ethers are shown to give rise to methyl derivatives of the same C_{20} acid and thus both molecules present in violacein must have the same fundamental structure.

The results of a re-investigation into the action of caustic soda on violacein are then described when both the red and yellow solids described by Wrede, were prepared. The statement by this worker, claiming to have obtained the same acetyl derivative from these two solids, namely acetylviolacein, has been shown to be incorrect. Whilst the red solid has been shown on acetylation to give an acetyl derivative having an identical infra-red spectrum with acetylviolacein, the acetyl compound derived from the yellow solid, has been shown to be quite different and to have a lower acetyl content. The investigations have been extended to the trimethyl

derivative from violacein but because analyses have not at present been performed, discussion of the nature of the reaction is reserved.

The theoretical discussion is concluded with a section describing the possible structural formulae for violacein put forward by previous workers. It demonstrates how violacein does not contain a trisubstituted methene structure like Prodigiosin, and goes on to discuss in the light of the discovery of the two C_{21} molecules present in violacein and the structures thought most probable for the two major degradation products, possible structural formulae to represent these molecules.

HISTORICAL and INTRODUCTION

THE CHROMOBACTERIUM GROUP.

The organisms of the chromobacterium group are usually defined as small, non sporing, aerobic rods usually motile and usually gram-negative producing a yellow, red, or violet pigment which is generally insoluble in water. They are saprophytic and are commonly found in water or soil.

The classification of organisms of the chromobacterium group presents some difficulty. The American Committee of Bacteriologists[I] differentiates between those organisms producing red or pink pigment and those producing violet pigment. It refers to the former as Erythrobacillus and reserves Chromobacterium for the latter. Because of the relatively little attention paid to these organisms by bacteriologists and consequently incomplete study of properties, it was thought advisable in this country to group all which fit the definition under the single genus Chromobacterium.

All members of the group are markedly aerobic, growing best in the presence of an ample supply of oxygen and growth under anaerobic conditions besides being poor, results in lack of pigmentation. The

production of pigment also depends upon the temperature and the optimum temperature for pigment formation does not necessarily correspond with that for growth. Pigment is developed best on the surface of solid media and potato is a medium which may be recommended for the study of its production. The formation of pigment is most abundant on primary isolation and after subculture for some time the power to produce it may diminish seriously or be lost altogether. With regard to their pathogenicity, organisms of this group are essentially saprophytic and although a few doubtful cases of suppuration in man have been described, generally speaking they do not give rise to natural disease in man or in animals. On experimental innoculation into laboratory animals they proved harmless except in very large doses.

General Classification of the Chromobacterium Group.[2]

Group I. Producing a violet pigment.

Chromobacterium Violaceum.

" Janthinum.

" Amethystium.

" Coeruleum.

Group 2. Producing a pink or red pigment.

(a) Chromobacterium Prodigiosum.
" Indicum.
" Kielense.

(pigment red at first, later becoming darker.)

(b) Chromobacterium Rubricum.
" Ruber.

(pigment orange-red or yellow-red.)

(c) Chromobacterium Mycoides Roseum.

(pigment salmon pink.)

(d) Chromobacterium Lactis Erythrogenes.
" Rubefaciens.

(rose-red soluble pigment.)

Group 3. Producing yellow or orange pigment.

Chromobacterium Aquatile.
" Typhi-flavum.
" Ochraceum.
" Fuscum.
" Aurantiacum.
" Denitrificans.

Chromobacterium Violaceum.

Chromobacterium Violaceum, the chief member of the group producing a violet pigment, was first described

by Bergonzini[3] in 1881. It is a common inhabitant of water and has been found in an abcess of a decaying tooth.

The organism grows in rods (1.5-3.0)x0.6 which are arranged singly or linked together in long chains and which are motile by peritrichate flagella. It is non-sporing, gram-negative, non-acid fast and has the ability to liquify gelatine. It is also aerobic and pigment formation is best at 25-30°C., but it does not depend on light for growth. The organisms are killed when maintained at a temperature of 55°C. for one hour. The organism is non-pathogenic to man and animals, but its isolation from a fatal human infection by Black and Shahan[4] in 1937 aroused renewed interest in its possible use as an antibiotic.

H.C.Lichstein and V.F.Van de Sand[5] examined the pigment produced by the organism for antibiotic activity. Strains from pathological sources were found to produce rich violet pigment upon cultivation and they were found to exert an inhibitory action against Staphylococcus Aureus and Staphylococcus Albus. A saprophytic strain which produced little pigment

during growth was found to have no effect on the growth of these organisms. For the purpose of a systematic study of the action of the pigment on the growth of various micro-organisms, aqueous-dioxan solutions varying in strength from .001-.03% were prepared. The results showed the pigment in these concentrations exerted marked inhibitory action on the growth of gram-positive bacteria but had little effect on the members of the gram-negative group. The most resistant of the former group was Clostridium Welchii, which grew well in the presence of the pigment until a concentration of 0.01% was reached. Of the gram-negative group only the meningococcus showed marked susceptibility. Four strains of molds were tested but only one, Blastomyces dermatiditis, showed marked susceptibility, exhibiting limited growth even at a concentration of 0.005%. The growth of the other three strains, Epidermophyton floccosum, Tricophyton rubrum, and penicillium notatum, was inhibited by a concentration of 0.01% of the pigment. The only strain of yeast studied was Saccharomyces cerivisiae and it was found to be very susceptible to the action of the pigment. The activity of the pigment was found to be influenced only slightly

by the number of bacteria in the test inoculum but was markedly diminished by the presence of serum.

Violacein, the dark violet pigment produced by Chromobacterium Violaceum, is insoluble in water, chloroform, carbon tetrachloride, carbon disulphide, benzene and petroleum ether. It is very sparingly soluble in ether, ethyl acetate, glacial acetic acid, ethyl alcohol and methyl alcohol but somewhat more soluble in acetone the best of the common solvents for dissolving the pigment. These properties serve to distinguish violacein from another pigment investigated by Friedheim[6] and by Sartory, Meyer and Waeldele[7] also referred to as violacein. The material studied by these workers was found to be soluble in chloroform and other common organic solvents and was given the molecular formula $C_{10}H_{12}NO_3$ which is considerably smaller than the violacein molecule is known to be. The earlier workers, Hartley[8], Lasseur and Girardet[9], Reilly and Pyne[10], were unable to crystallise violacein and it was not until 1932 that Kögl[11] first isolated the pigment as small deep violet needles with a greenish sheen which charred on heating without melting. From the analytical results he was able to put forward two possible empirical

formulae for the molecule: (a) $C_{35}H_{25}N_5O_6$,
(b) $C_{42}H_{30}N_6O_7$.

Wrede[12] who later also investigated the pigment was able to provide evidence supporting the C_{42} formula. He first crystallised violacein from pyridine and found that it crystallised with two molecules of pyridine of crystallisation, the pyridine being determined by treating the crystals with hydrochloric acid and estimating the pyridine as its aurichloride. The analytical values for this compound however did not distinguish between the two formulae $C_{35}H_{23}N_5O_6$ and $C_{42}H_{28}N_6O_7$ which he had proposed. Wrede also prepared the acetyl derivative of violacein, the analysis of which indicated the presence of five acetyl groups if violacein were a C_{35} molecule, or six acetyl groups if the molecule were C_{42}. It did not however differentiate between the two formulae. When Wrede showed that Violacein could be crystallised with chlorinated solvents such as 2-chloropyridine or o-chloroaniline this difficulty was resolved because the chlorine analysis of the addition compounds clearly indicated the C_{42} formula. There was an interesting difference between the addition compounds formed by violacein and the two

chlorinated solvents. Whilst the violacein 2-chloropyridine compound analysed for one molecule of violacein to two molecules of 2-chloropyridine, the o-chloroaniline compound analysed for one molecule of the pigment to one molecule of o-chloroaniline and one molecule of alcohol, the solvent used for the preparation of these compounds. Violacein was shown to form a similar crystalline complex with aniline, one molecule of the pigment, one molecule of aniline and one molecule of alcohol separating as black violet needles. The alcohol could be removed from these complexes by drying in vacuum for periods up to 148 hours. Because of the extremely low solubility of violacein in suitable solvents, accurate molecular weight determinations were precluded and thus no decision was reached as to the molecular formula of the pigment.

Wrede next made a study of the chemical properties of violacein. He showed that on digestion with hydrochloric acid a mono-hydrochloride was formed the pigment appearing to behave as a mono-acid base and with sulphuric acid similar behaviour was adhered to. The pigment did not dissolve in sodium carbonate solution, but was found to be soluble in sodium hydroxide solution

giving a bright green colour. This solution soon changed however to red after passing through a transitory brown colour. Saturation of the red solution with carbon dioxide caused the precipitation of a red microcrystalline solid whilst acidification of the colourless filtrate with hydrochloric acid resulted in the precipitation of a yellow solid, which soon changed to a green and then to a much darker colour.

Both these products on treatment with pyridine gave the same crystalline complex as did violacein and both on acetylation gave acetylviolacein. Wrede suggested that the red solid was the enol form of the yellow one which he thought might be a lactone or a lactam. Wrede also showed that upon hydrogenation in acetic acid solution with a palladium-charcoal catalyst, violacein gave a colourless solution which on exposure to the air slowly oxidised back to the original colour. From the amount of hydrogen absorbed he deduced the presence of seven or eight reducible double bonds in the molecule.

A series of fusions on violacein using zinc dust, soda lime and alkali carried out by Wrede and Tobie[13], a later worker, resulted only in the isolation of oily

products which gave positive colour tests for pyrroles. Tobie claimed by use of a colour reaction to have identified one of the products as anthranilic acid. It was at this stage that the investigation of violacein was commenced in these laboratories. Subramanian, by zinc dust fusion of violacein, was able to isolate a crystalline product, m.p.112°C., but was unable to prove its identity. This product was later identified by Beer and Clarke[14] as a mixture of isomeric indole derivatives, namely oxindole and 5-hydroxy indole. The effect of hydrogenation on acetylviolacein was investigated by Clarke[15] who found that in acetic acid solution using Raney nickel as catalyst, pale coloured material was obtained which could not be crystallised. This material, however, when dissolved in acetone and exposed to the air became red and on recrystallisation from acetic anhydride yielded acetylviolacein. This seemed to indicate a leuco compound and the discovery was of interest because Tobie had previously suggested that violacein might be indigoid in type. When reduced under more strenuous conditions acetylviolacein yielded two crystalline products in very low yield which had melting points of 218°C and 242°C. The lower melting

material was thought to be a more fully reduced form of the higher melting compound since it was formed using freshly prepared catalyst. An interesting point was that although insoluble in water both products dissolved easily in strong hydrochloric acid and in cold caustic soda solution.

Oxidation of acetylviolacein using chromic oxide in boiling glacial acetic acid was shown by Clarke to give N-acetylanthranilic acid and 5-acetoxy N-acetylanthranilic acid, the latter being produced in far better yield by Khorana[16] when cold glacial acetic acid was used. From oxidations in acetone using potassium permanganate Clarke obtained two other acids, one of which he discovered to be 5-hydroxy-N-acetyl-anthranilic acid and the other which had a molecular formula $C_{10}H_9NO_5$ he failed to identify. From the neutral fraction of the reaction products isatin was isolated.

Acetylviolacein was found by Beer and Parker when subjected to degradation with hydrogen iodide-acetic acid to give rise to a red crystalline hydriodide derived from a sparingly soluble yellow crystalline base having the formula $C_{13}H_{10}N_2O_2.H_2O$.

When refluxed with alcoholic hydrogen chloride a deep red solution was produced which upon standing deposited the hydrochloride, $C_{13}H_{10}N_2O_2.HCl$, in very dark red needles. The base on high vacuum sublimation was found to yield a little 5-hydroxyindole.

The degradation base was studied by Jennings[17] who showed that it had properties very similar to dipyrrylmethenes. With acids, for instance, it formed only a mono acid salt, indicating that only one of the nitrogen atoms was basic. It also formed metallic derivatives and when a proton was accepted the pale yellow base was changed to a bright red colour indicative of a highly resonating structure.

Upon subtracting the elements of 5-hydroxy indole from the empirical formula of the free base there remained C_5H_3ON the carbon and nitrogen of which were readily accounted for as a methene bridge and a pyrrole nucleus. Jennings from these considerations tentatively put forward an indole-pyrryl methene structure. The remaining oxygen atom was assumed to be in the form of a second hydroxy group and, in view of the production of 5-hydroxy indole by sublimation, was considered not to be in the indole nucleus. Of

the remaining positions, the methene bridge carbon atom and the pyrrole nucleus, the latter was considered most likely.

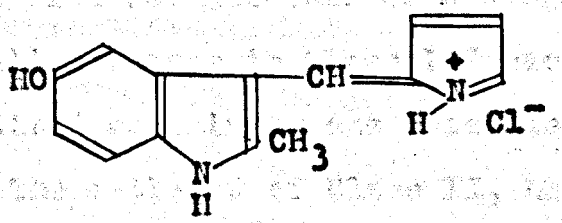
With these considerations in mind, Jennings synthesised a number of indole-pyrryl methenes, a system not previously synthesised, from which by a study of properties and subsequent comparison with those of the degradation product he was able to arrive at certain conclusions. He divided the indole-pyrryl methenes which he had synthesised into three classes:-

(i) The ones which did not contain a hydroxy pyrrole nucleus.

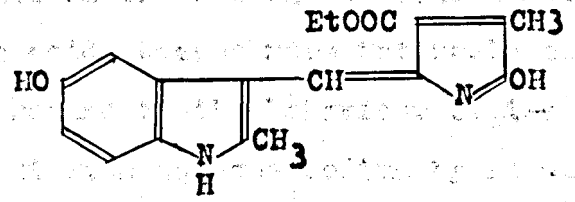
(ii) The ones which contained an α-hydroxy pyrrole nucleus.

(iii) The ones which contained a β-hydroxy pyrrole nucleus.

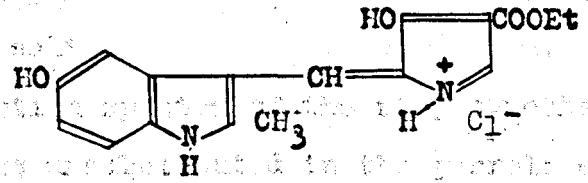
e.g. Class 1



Class II



Class III



With regard to the basicity of the methenes, all in Class I were found to be highly basic giving deep red solutions in very dilute alcoholic hydrogen chloride and those obtained in the solid state crystallised with one molecule of hydrogen chloride. Similarly, all methenes in Class III were highly basic and crystallised containing one molecule of hydrogen chloride. The methenes of Class II, however, were found to be only weakly basic and crystallised as the free base from acid solutions. These methenes also gave much paler solutions in dilute alcoholic hydrogen

chloride than the methenes of Classes I and III but the solutions, on increasing the concentration of the hydrochloric acid, became more intensely coloured.

With regard to the behaviour of the methenes upon treatment with aqueous sodium hydrogen carbonate, it was shown that methenes of Classes I and III formed sodium salts in a similar manner to the degradation product, whilst methenes of Class II showed no tendency to form such salts.

Absorption spectra of the simple methenes of Class I, those unsubstituted in the pyrrole nucleus, resembled that of the degradation base. In Class II both the methenes synthesised differed very considerably from that of the degradation base hydrochloride. In Class III the spectrum of one methene resembled that of the degradation product. The other having an ethoxcarbonyl group in the 2-position of the indole nucleus had a dissimilar spectrum.

From his work on the hydrogen iodide degradation product Jennings was able to arrive at the following conclusions:-

(1) The original theory that the degradation product was the hydriodide of an indole-pyrryl-methene was most

probably correct.

(II) The hydroxyl group in the pyrrole ring was in one of the β-positions. This arises from consideration of the highly basic nature of the degradation product and also its property of forming a sodium salt and the fact that neither of these properties occurred in the α-hydroxy compounds synthesised.

(III) The methene linking in the indole nucleus was probably in the 3- position, although the evidence for this was not very strong.

Jennings was not able to arrive at any conclusion concerning the position of the methene linking in the pyrrole nucleus.

This work on the indole-pyrryl-methene was undertaken concurrently with a study by Boggiano of another major degradation product of violacein, the C_{20} acid. This work will now be described in detail.

Reductive Alkaline Hydrolysis of Violacein.

Towards the end of his work on violacein, Clarke[15] discovered that by heating violacein or acetylviolacein with 10% sodium hydroxide in the presence of zinc dust it was possible to isolate a colourless acidic material, m.p.252-254°C (decomp.). A potentiometric titration

carried out on this material gave a molecular weight of ca. 390 which, with its analysis, indicated a C_{21} molecule. Boggiano, who repeated this work, discovered that the yield and purity of the acid were vastly improved when the neutral and phenolic impurities were first removed by selective precipitation at pH 6.5-7. A series of potentiometric titrations carried out on the acid and its trimethyl derivative by Boggiano consistently indicated a value of ca. 365 for the molecular weight which was in very close agreement with that required by the empirical formula obtained from analyses, namely $C_{20}H_{16}N_2O_5$.

The acid was found to be quite stable to alkali and prolonged reaction time for the degradation tended in no way to decrease the yield. The alkaline gas liberated during this degradation was shown by Boggiano by analysis of the p toluene-sulphonyl derivative to be ammonia. This meant that during the degradation two carbon atoms and two nitrogen atoms had been lost if violacein were considered a C_{42}molecule, or only a single carbon atom and a single nitrogen atom if it is a C_{21} molecule.

Boggiano showed that the acid was stable to

alkali, did not give a hydrochloride nor did it give ketonic derivatives. It was recovered unchanged after attempted hydrogenation using palladium charcoal or platinum as catalyst and also after mild treatment with alkaline hydrogen peroxide. The acid gave negative reactions with ferric chloride and with Ehrlich reagent but gave a positive fluorescein reaction. On methylation with methyl iodide and potassium carbonate in acetone the C_{20} acid was converted into a mono methyl derivative which was no longer acidic but which did dissolve in sodium carbonate. Methylation of the acid with dimethyl sulphate and alkali resulted in the formation of a trimethyl derivative which was still acidic. It was found, however, that if the reaction mixture in this methylation was allowed to become acid then a non-acidic compound separated which was subsequently shown to be a tetramethyl derivative. These experiments showed that besides the acidic group present in the C_{20} acid there were also three hydrogen atoms capable of being replaced by methyl groups and since the violacein molecule had been shown to contain the 5-hydroxy indole and oxindole nuclei, Boggiano considered the possibility of their presence in the C_{20} acid. He

compared the absorption spectrum of the acid with that of an equimolecular mixture of oxindole and 5-hydroxy indole and found an obvious similarity which suggested that both systems were in the acid, probably linked by some saturated system which insulated them from each other. Boggiano then interpreted the results of the methylation experiments on this basis. He considered that the methyl iodide-potassium carbonate method had esterified the acidic group but had not affected the hydroxyl group in the indole nucleus; this was rather surprising until he subsequently showed that 5-hydroxy indole itself was recovered unchanged from such a methylating mixture. Dimethyl sulphate and alkali had methylated the nitrogen atoms in the indole nuclei and the 5-hydroxy group, resulting in a trimethyl derivative, and when the reaction mixture had become acid, esterification had also occurred yielding the tetramethyl derivative. It was later found that esterification of the acid group could be effected with diazomethane and diazoethane; and both the methyl and ethyl esters were prepared in this manner. Jennings later demonstrated the presence of the two indole nuclei in the C_{20} acid when by

oxidation of the trimethyl derivative of the acid with potassium permanganate in acetone he isolated N-methyl isatin and 5-methoxy-N-methyl isatin. He thus proved that Boggiano's suggestions concerning the methylation experiments were correct.

Clarke had found that on acetylation with acetic anhydride and sodium acetate the C_{20} acid gave a bright red crystalline acetate. Boggiano continuing this work showed that the acetate was non-acidic and would not regenerate the acid on attempted deacetylation. Analyses indicated that four acetyl groups had been introduced, dehydration and possibly oxidation (loss of two hydrogen atoms) having also occurred. When the acetylation was carried out using pyridine and acetic anhydride in the cold, a colourless compound (m.p.235°C) mixed with a considerable amount of the red acetate was obtained. Acetylation of the methyl ester proved difficult due to partial de-esterification, but when the ethyl ester was used a colourless triacetate was formed. On acetylating the trimethyl acid, however, Boggiano found that a magenta coloured compound was formed which crystallised very readily and was shown to be non-acidic. Analysis

of this compound indicated the addition of one acetyl group and loss of a molecule of water, and possibly one of hydrogen. Acetylation when attempted on the tetramethyl derivative resulted in recovery of unchanged material.

Boggiano arrived at certain conclusions concerning the behaviour of the C_{20} acid and its derivatives on acetylation. The presence of a free carboxyl group was necessary for the production of colour and on acetylation of the compounds containing it, the carboxyl group was involved in a cyclisation because the red acetate had one more acetyl group than the acetate of the ethyl ester. Boggiano concluded that this must be due to the production, during acetylation, of a fourth acetylateable group which could only conceivably have occurred by enolisation of a keto group formed by a cyclisation involving the carboxyl group. He also noted that apart from the carboxyl group all the groups which could be methylated with dimethyl sulphate could also be acetylated.

Oxidation of the red acetate with potassium permanganate in acetone yielded only N-acetyl anthranilic acid and isatin, but a similar oxidation carried out on

the magenta acetate gave N-methyl isatin, 5-methoxy-N-methyl isatin and 5-methoxy N-methyl-3-indolylglyoxylic acid.

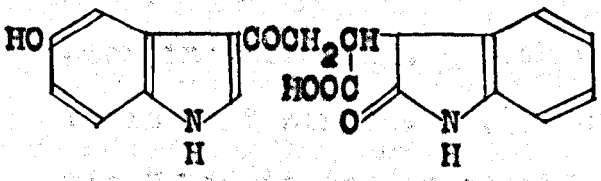
Boggiano showed that on attempting to decarboxylate the C_{20} acid by heating the acid in glycerol at 260-270° in the presence of copper bronze two products were obtained. One of these was shown to be 5-hydroxyindole and therefore proved conclusively that this nucleus was present intact in the C_{20} acid whilst the other product was shown to be a non-acidic dehydration product of the C_{20} acid. This material was shown to be recovered unchanged after heating with dilute sodium hydroxide and was therefore considered not to be a lactone or lactam. It also was found to give a 2:4-dinitrophenylhydrazone thereby indicating the presence of a carbonyl group and was in fact referred to by Boggiano as the "Dehydration Ketone". Acetylation with acetic anhydride-sodium acetate occurred readily to give a pale buff acetate, m.p.193°C, analyses of which suggested the presence of four acetyl groups in the acetate.

Boggiano found that the C_{20} acid did not give any ketonic derivatives but that the tetramethyl derivative did from a 2:4-dinitrophenylhydrazone.

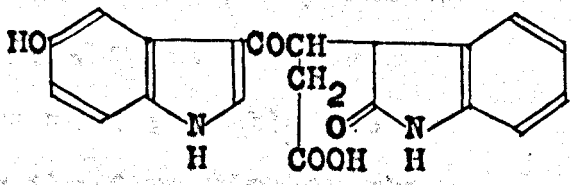
In the light of these experiments Boggiano was

able to suggest possible formula for the C_{20} acid.

If the acid were considered to be a keto acid then there were three types to be considered, namely, α, β and γ-keto acids. Boggiano did not consider that an α-keto acid system was present when after treatment with hydrogen peroxide in alkali the acid was recovered unchanged, nor did he think a β-keto acid system probable after attempted decarboxylation experiments had shown that the carboxyl group preferred to cyclise rather than to be eliminated. Left only with the possibility of a γ-keto acid he considered the only four possibilities which could be written were :-



(I)



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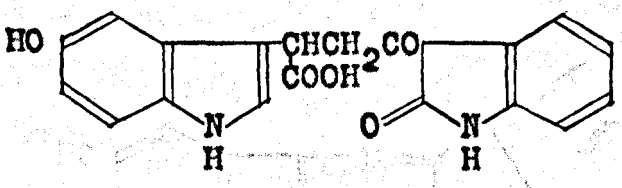

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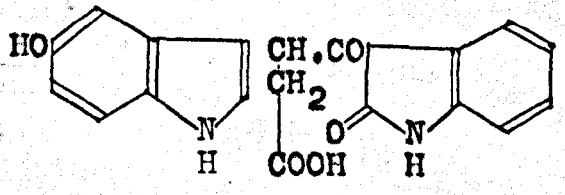
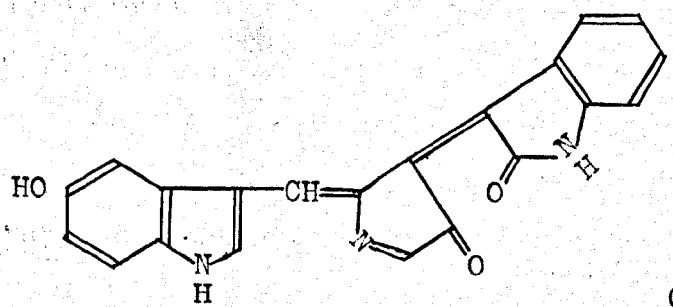


(IV)

Of these four, spectroscopic evidence did not favour possibilities (III) and (IV) as strongly as (I) and (II). Also β-acyloxindoles are easily split by alkali whereas the C_{20} acid had been shown to be very stable to alkali.

Boggiano and Jennings attempted to reconcile the formation of the C_{20} acid and of the indole-pyrryl methene from violacein. They thought that the four carbon atoms in the C_{20} acid not present in the indole nuclei must have been derived from the methene carbon atom and from the opening of a pyrrole ring. If this were so it would not be possible to accommodate structure (II) since the carbon atom adjacent to the carbonyl group would have been derived from a quaternary carbon atom in violacein. Both workers thought on this basis that structure (I) was the most probable one for the C_{20} acid and on considering the formation of the methene suggested the following structure for violacein:-



$C_{21}H_{13}N_3O_3$.

Whilst the analytical evidence suggested that violacein is a C_{42} molecule this structure requires only a molecular formula $C_{21}H_{13}N_3O_3$. Both workers considered that the degradational evidence favoured a C_{21} molecule and were not able to explain why the molecule analysed for $C_{42}H_{28}N_6O_7$.

THEORETICAL DISCUSSION

GROWTH AND EXTRACTION OF VIOLACEIN.

The first recorded growth of Chromobacterium Violaceum in the laboratory was that by Hartley[8] in 1913 who grew the pigment on slices of potato. The first attempt to grow it on a synthetic medium was made by Girardet[9] in 1926 who used asparagin as the nutrient. Later Reilly and Pyne[10] found that violacein could be grown in a solution of lactose (5gm.), peptone (5gm.) and beef extract (3gm.) in water (1L.) and this medium with minor modifications was used by Kögl[11] who was the first worker to obtain the pigment in the crystalline form. The same medium was used by later workers and Strong[19], who is amongst the most recent workers in this field also used a lactose broth but improved the isolation by use of centrifuge obtaining a yield of violacein of 710mg. from 65L. of broth.

During the present researches in these laboratories the yields of violacein and the methods of growth and isolation have been considerably improved. Subramanian found that violacein, as might be expected, being an aerobic pigment grew well on solid agar slopes. He found that good results were obtained using a medium

containing glycerine and peptone, but experienced some diffivulty in removing the bacterial mass from the solid medium in each bottle. Clarke[15], who took up the work at this stage, experimented with various peptones present in the culture medium and found that Difco Proteose Peptone gave the best results. Glucose was tried as a substitute for glycerine but was found to retard the growth and resulted in a lower yield.

Since little improvement could be made in the conditions of growth as used by Subramanian, Clarke attempted to improve the technique of growth and isolation of the pigment. He improved the preparation of each bottle, and in the isolation of the bacterial mass found that the layer of pigment could be washed from the surface of the agar by shaking with warm brine solution. After filtering off any pieces of broken agar the bacterial mass was absorbed on to kieselguhr, dried and extracted with acetone. Upon concentration of the acetone extract the impure pigment was obtained which was defatted by treatment with (i) petroleum ether, (ii) chloroform, (iii) carbon tetrachloride, leaving the crude violacein. The yield of the pigment obtained by this method was 7.0-8.0gm. per 1000bottles equivalent

to 3gm. per 65L. as compared with Strong's yield of 0.71gm. for the same volume of broth. During the last stages of his researches Clarke evolved a vastly superior method for growing violacein. Because of the aerobic nature of the organism he considered the possibility of its growth on open trays and discovered that under reasonably sterile conditions it could be grown very well on a solid medium. The adoption of this method of growth made the harvesting and extraction of violacein a very much simpler process than it had previously been. The yields of violacein were considerably improved by the introduction of this new procedure and Clarke obtained 2.3gm. of the pigment from eleven trays which is equivalent to 6.7gm. from 65L. The pigment however was obtained by precipitation with water and acetic acid from the acetone extraction liquors and was thus mixed with considerable quantities of lipoid material which had to be removed by extraction with defatting solvents. This procedure for the growth of violacein was that used by Boggiano[18] and the author and consisted of three main stages:-

(i) The growth of the pigment on test-tube slopes or Petri dishes.

(ii) The growth of innoculation broths.

(iii) The growth on trays.

Certain modifications were introduced which did not improve the yield of violacein (2.0gm. from eleven trays), but the purity of the pigment was considerably superior, 75% of the material crystallising from the acetone during the extraction.

A more recent modification of this procedure was introduced by the author and his co-worker, Barrett, which eliminated the second stage involving growth of the pigment in liquid medium and substituted growth on solid medium in oblong dishes fitted with sealed lids. Although the yield of violacein was not improved, being the same as that obtained by Boggiano, the whole procedure became far easier to operate and was considerably less time consuming.

An interesting feature during the present researches and one previously observed by Boggieno was the appearance from time to time of a pale grey form of the pigment mixed with the usual deep violet form. Attempts to separate this grey material from the violet pigments were made by Boggiano but were not successful, whilst with chromatogtapgic methods no success was obtained

due to the insolubility of the material in suitable solvents. He showed that the grey material must have a similar structure to violacein because on acetylation and degradation with zinc dust and alkali it gave the same products as violacein in comparable yields. Because of this Boggiano assumed that the grey material was some sort of precursor of violacein and suggested that it may be a reduced form of the violet pigment resulting from insufficient oxygen supply during the growth of the bacteria.

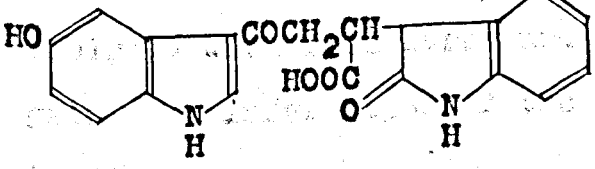
The appearance in cultures of Chromobacterium Violacein of a poorly pigmented form is not new and has been previously observed. Tobie[13] in one of his papers comments on its appearance. Ramchandani (1930) discusses the colourless and poorly pigmented form which occasionally arises in cultures of B.Violaceus and states that such changes may be caused by growth on different media. Bessenowa and Zochow (1930) described a strain of plague bacillus, which formed a violet pigment after two month's culturing. It is quite possible that this may be related to the smoke-grey to inky-black pigment which Preisz (1926) found in old cultures of a plague bacillus. In 1936 Dmitrevskia found that the loss

of pigmentation by cultures of B. Violaceus could be prevented by the addition of potassium or sodium nitrate to the medium and restoration of the fully pigmented form could be achieved by cultivation on potato.

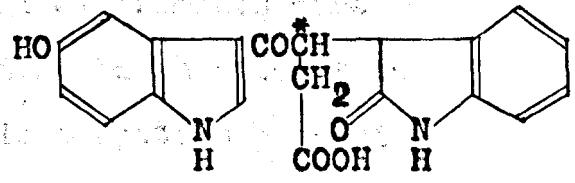
During the present work when it was deemed necessary to revitalise the strain, cultivation on potato or on the usual medium containing water in which potatoes had been boiled was found to be effective.

THE C_{20} ACID

On considering the evidence accumulated by Boggiano[18] for the structure of the C_{20} acid, the structures considered most probable for this substance were :-

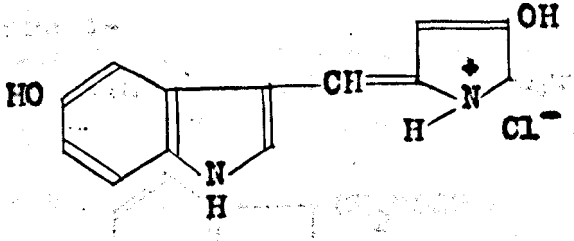


(I)



(II)

On attempting to reconcile these with the formation of a methene of the type :-



(III)

only structure (I) would satisfy the necessary conjugation requirements since the carbon atom marked C* in structure (II) would, besides being a member of the pyrrole ring have two external bonds and therefore of necessity be quaternary. Quaternisation at this

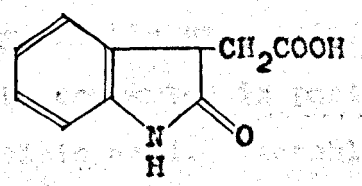
centre would quite clearly destroy conjugation throughout the violacein molecule and make explanation of the pigment's deep purple colour even more difficult.

On this basis the structure (I) was thought the more probable for the C_{20} acid and experiments were designed with this structure in mind in an attempt to gain some information of the various systems present in it.

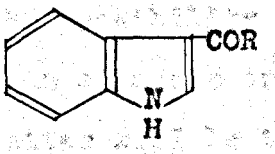
Examination of structure (I) shows that it contains three distinct chemical systems whose properties are rather characteristic.

These are :-

(a) An oxindole-3-acetic acid system.



(b) A β-acyl-indole system.



(c) A γ-keto acid system.

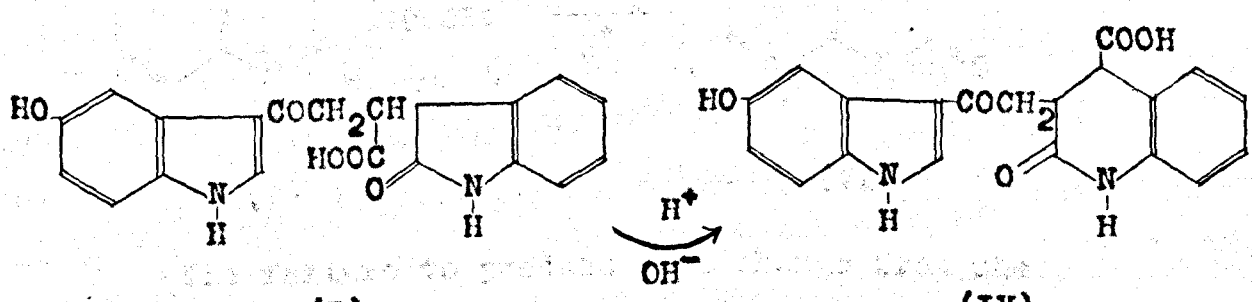
and an investigation into the chemistry of the C_{20} acid was conducted in an attempt to prove the presence of each of these separate systems.

Oxindole-3-acetic Acid.

Oxindole-3-acetic acid has been the subject of considerable controversy during its long history in the literature[9]. Many workers have attempted to synthesise it but not until 1953 when Julian[20] appreciated the mistakes of former workers was its synthesis first achieved. All previous attempts to isolate this substance involved vigorous hydrolysis procedures which resulted in the formation of 2-oxo-1,2,3,4-tetrahydroquinoline-4-carboxylic acid. Because of the vigorous hydrolytic step oxindole-3-acetic acid, if formed, would be converted in part at least, to o-aminophenylsuccinic acid. Aeschlimann[21], in a study of the relative stability of the quinolone and oxindole rings, came to the conclusion that in any reactions where the possibility exists that ring closure to either an oxindole or quinolone ring can take place, the latter will be the preferred course

of the reaction.

Because the structure (I) for the C_{20} acid contains an oxindole-3-acetic acid system, it suggested the possibility that on hydrolysis with acids and alkalis the acid may undergo the transformation from the oxindole to the quinolone ring system.

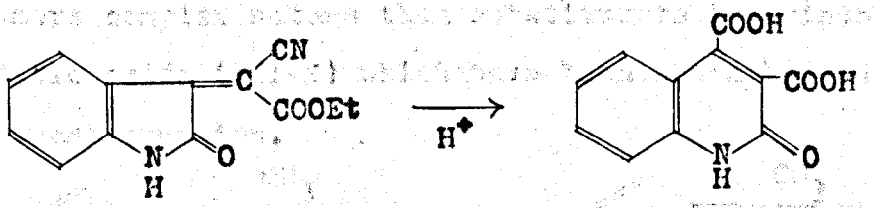


(I) (IV)

Experiments of this nature were conducted under a series of conditions, but in no case could a transformation of this kind be effected. In all cases when the C_{20} acid was heated in acid solution a dark red coloured solution was obtained but no crystalline material was ever isolated. Boggiano had previously shown that the acid was stable to heating with 10% and 30% caustic soda solution and when the trimethyl derivative of the C_{20} acid was heated with 50% caustic soda solution it was recovered unchanged.

Similar experiments conducted by Barrett (private

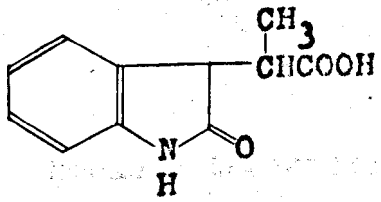
communication) in these laboratories on known oxindole-3-acetic acid derivatives resulted in the smooth transformation of the 5-membered oxindole system (V) into the 6-membered quinolone ring (VI).



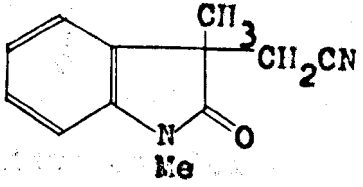
(V) (VI)

The failure to produce this change from the oxindole ring to the quinole ring in the C_{20} acid might tend to suggest that an oxindole-3-acetic acid system is not present but the general change of colour of the solution to red in all cases is felt to be significant. It appeared feasible that another change involving the carboxyl group, not altogether dissimilar from the change occurring during acetylation of the C_{20} acid might actually be taking place but at a very much slower rate. Since the oxindole-quinolone change is dependent on both carboxyl groups being available for cyclisation, any other competing reaction might tend to operate against the formation of a six membered ring by

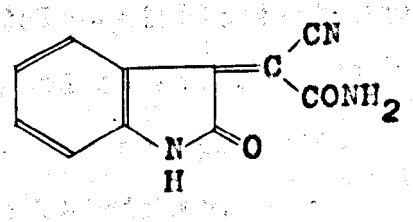
involving the carboxyl group necessary for the cyclisation to that system. It must also be remembered that should the C_{20} acid actually be substituted oxindole-3-acetic acid the substituent is of a larger and more complex nature than substituents in oxindole-3-acetic acids (VII-X) which have been shown to undergo the transformation.



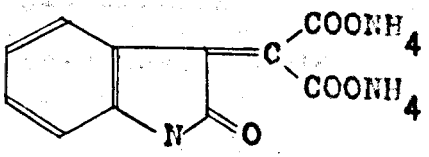
(VII)



(VIII)



(X)

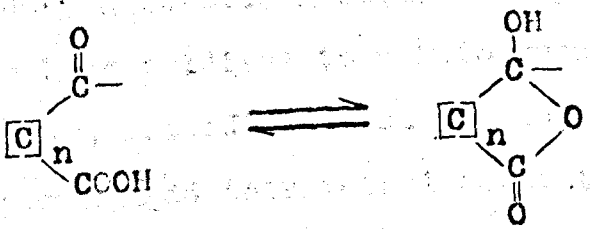


(IX)

This might suggest that the transformation is not only dependent upon the nature of the substituent but also that in larger molecules stereochemical factors may have some influence.

Furthermore should the carboxyl group not be available for cyclisation by virtue of its interaction or assocaition with other centres in the molecule then

the transformation would not readily occur. Since a keto group is known to be present in the molecule then the possibility of keto-lactol tautomerism is worthy of consideration.

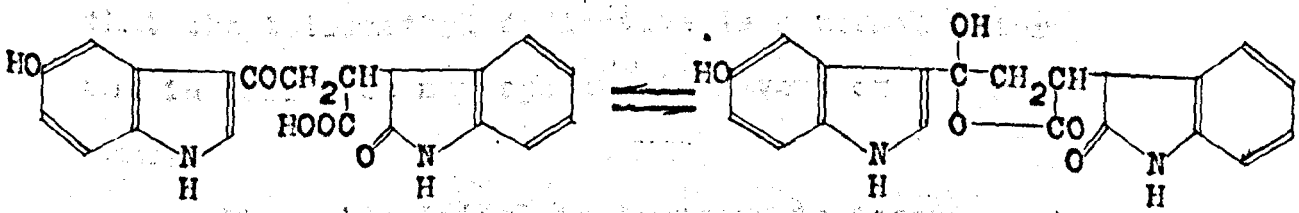


Because therefore of the relative complex nature of the C_{20} acid its failure to undergo the oxindole-quinolone change cannot be justifiably accepted as evidence that the oxindole-3-acetic acid system is not present.

β-Acyl-Indoles.

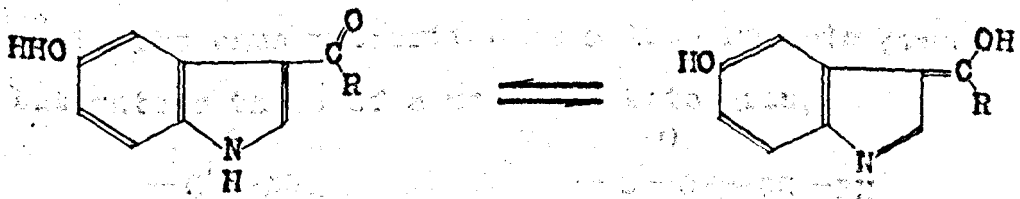
The presence of a keto group in the C_{20} acid was first shown by Boggiano[18] when he was able to prepare a 2 : 4-dinitrophenylhydrazone of the tetramethyl derivative. Because of the failure of the acid itself to exhibit carbonyl properties the presence of a keto group had been thought unlikely and Boggiano had previously considered the molecular formula of the C_{20} acid to be $C_{20}H_{18}N_2O_5$ thereby

accommodating a secondary alcoholic grouping. When the product from an Oppenauer oxidation of the tetramethyl derivative was shown to form a 2 : 4-dinitrophenylhydrazone it did appear most probable that a secondary alcoholic grouping was present and that this had been oxidised to a keto group. Boggiano, however, showed that the Oppenauer product was actually unchanged tetramethyl derivative and it became apparent that a keto group was originally present but whilst exhibiting itself in the fully methylated product it was in someway being masked in the C_{20} acid itself. It was later shown by a systematic examination that the formation of a ketonic derivative only occurred in the fully methylated compound and that with the C_{20} acid and partially methylated derivatives no simple ketonic derivative could be obtained. Examination of structures (I) and (II) for the C_{20} acid shows clearly two possible tautomeric forms which the molecule could assume and which would diminish the carbonyl activity. The first of these is the keto-lactol tautomerism previously mentioned due to the presence of a carboxyl group in a conveniently spaced position to the carbonyl group.



(I) (XI)

and the second is the amido-imidol tautomerism due to a 1 : 5-prototropic shift in the β-acyl-indole system.

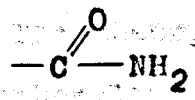


Replacement of the hydrogen by methyl on the indole nitrogen atom would in this second tautomeric change clearly prevent formation of the imidol form and therefore leave the molecule entirely in the keto form. It would appear in this particular case therefore that methylation would decidely favour carbonyl activity and studies of this system in the infra-red substantiate this view.

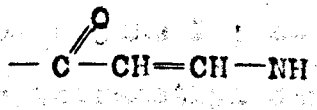
In the case of keto-lactol tautomerism, methylation need not necessarily prevent the formation of a pseudo ester but it has been shown (vide infra).

that the tetramethyl derivative is a normal ester and in this case may operate in favour of the keto form.

The amido-imidol tautomerism is important in so much that it draws attention to the fact that the keto group as written in formulae (I) and (II) is not a true keto group but rather the vinylogue of an amide. It therefore cannot be expected to have quite the same properties as a true ketonic grouping but rather those of a modified keto group.



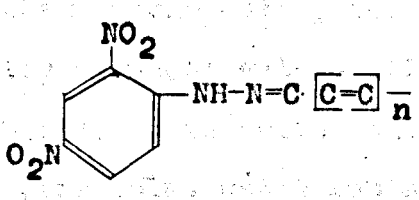
amide



vinylogue of amide

When the 2 : 4-dinitrophenylhydrazone of the tetramethyl derivative was prepared the dark red colour of the crystals was felt to be significant. Braude and Jones[22] have correlated data relating the maximum absorption band in the absorption spectrum of a 2 : 4-dinitrophenylhydrazone with the type of carbonyl group forming the derivative. Data for the light absorption maxima of some fifty 2 : 4-dinitrophenylhydrazones was collected and found to lie between the values λ_{max}=348 mμ for formaldehyde and λ_{max}=410 mμ for $CH_3(CH{=}CH)_3CHO$. It was shown that λ_{max} varied with

the number (n) of ethylenic bonds in the system,

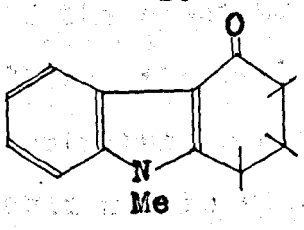


and with the degree of alkyl sustitution.

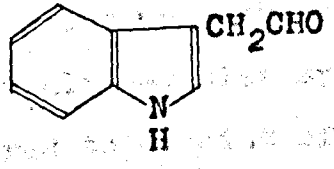
The bathochromic effect of a conjugated ethylenic bond was found to be of the order $+\Delta\lambda = 100\text{-}150\text{A}^{\circ}$ whilst that of an alkyl sustituent was found to be $+\Delta\lambda$ 50-100A°.

The absorption spectrum of the 2 : 4-dinitrophenylhydrazone of the tetramethyl derivatives was thus measured in chloroform solution and the value of λ_{max} was found to be 431 mμ. This value was rather higher than any observed by Braude and Jones and suggested that the keto group was somewhat different in character from the ordinary saturated or αβ-unsaturated types. The high value certainly suggested that the keto group was at least αβ-unsaturated but its unusually high value was considered perhaps to be characteristic of a β-acyl indole system. Subsequent measurement of the absorption spectrum of the 2 : 4-dinitrophenylhydrazone of a β-acyl indole, that of

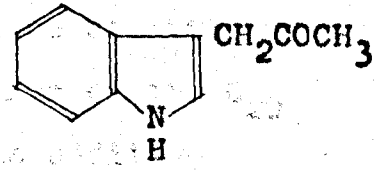
N-methyl-4-keto-1,2,3,4-tetahydrocarbazole (VII) gave a value for the maximum absorption at 436 mμ. The close proximity of these values allowing for an alkyl substituent bathochromic shift of $\Delta\lambda$ = 50A in the λ_{max} of the carbazole, was taken as indicative of the presence of a carbonyl system, namely a β-keto-indole, in the C_{20} acid and its derivatives.



(XII)



(XIII)

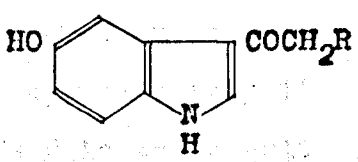


(XIV)

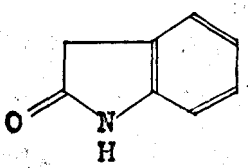
Had the carbonyl group in the tetramethyl derivative been separated from the indole nucleus by a $-CH_2-$ group as in 3-indolylacetaldehyde (XIII) or 3-indolylacetone (XIV) the absorption maximum of the 2 : 4-dinitrophenylhydrazone would have been expected to have been in the region 350-375 mμ, 3-indolylacetaldehyde having a maximum absorption at 358 mμ. Further, as would be expected the colour of the 2 : 4-dinitrophenylhydrazones of these compounds is considerably lighter than those of the β-acyl indoles.

Ultra-Violet Spectroscopic Evidence for the Presence of a β-Acyl Indole System in the C_{20} Acid.

An examination of structures (I) and (II) for the C_{20} acid shows that they contain three distinct chromophoric systems namely, (i) A β-acyl indole, (ii) Oxindole, (iii) A carboxyl group. In the part of the spectrum considered, that between 240 mμ and 310 mμ, the carboxyl group as such makes no significant contribution and the ultra-violet spectrum of the C_{20} acid may be considered to consist of the additive contributions of the other two systems.



(i)



(ii)

The ultra-violet spectrum of the C_{20} acid has been shown to contain two peaks in the region considered, one at 253 mμ Log.ε 4.28 the other at 301 mμ Log.ε 3.99 whilst on total methylation a slight ba bathochromic shift occurs and they then appear at 255 mμ Log.ε 4.35 and 308 mμ Log.ε 4.05.

A comparison of the absorption spectrum of

the C_{20} acid with that of an equimolecular mixture of oxindole and 5-hydroxy indole was made by Boggiano[18]. He found an obvious similarity between the curves suggesting that both oxindole and 5-hydroxy indole nuclei were present but the absence of the peak at 301mμ suggested that some other chromophoric system must be present in the C_{20} acid. It was considered that this peak at 301mμ was most probably attributable to the β-acyl indole system since the spectrum of oxindole shows no absorption at this wave length. When therefore it was found that in the spectra of a series of β-acyl indoles measured by Boggiano a peak occurred in each member in the region 301-303mμ and at approximately the same intensity this consideration was felt to be quite justified.

2-Methyl-3-phenacetylindole.....244mμ 268 mμ 303 mμ

2-Methyl-3-acetylindole.....241"" 266 " 300 "

5-Methoxy-2-methyl-3-phenacetylindole..255 " 278 " 303 "

5-Methoxy-2-methyl-3-acetylindole.....254 ""278 ""301 "

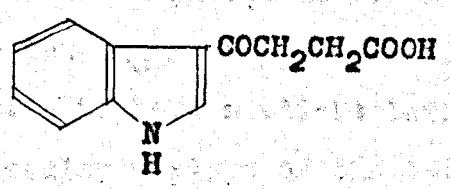
The appearance of a peak in the 266-278mμ region is characteristic of the β-acyl indole system and might be expected to appear in the C_{20} acid series. Its non-appearance in this series however is due simply to

the effect of a minimum chromophoric contribution at this wave length by the oxindole nucleus.

Thus on analysing the additive contributions of the two systems it can be shown:-

5-Methoxy 2-methyl- 3-acetylindole	254mμ	278mμ	301mμ
Oxindole	248 mμ	low absorption	no absorption
Mixture	248-255 increased intensity	no peak but possibly an inflection	301mμ same intensity
C_{20}Acid	253 mμ		303mμ

A significant contribution substantiating these views was made by Barrett (private communication) who prepared γ(3-indolyl)- γ-keto-butyric acid (XV) and examined its ultra-violet spectrum



(XV)

The spectrum, as expected, was found to be similar to the spectra of the β-acyl indoles previously considered. The peak at 266mμ. was not so clearly defined and appeared as an inflexion at 262mμ. The other two peaks were however very similar, one occurring at 242mμ(log.ε.4.07) and the other at 298mμ(log.ε.4.06).

It was thus expected that the ultra-violet absorption spectrum of an equimolecular mixture of this acid and oxindole should be very similar to that of the C_{20} acid and only differ because of the bathochromic shift introduced by the hydroxyl group in the 5-position of the indole nucleus. This was indeed found to be the case and the two spectra were identical in shape differing only in a wavelength shift caused by the indole 5-hydroxyl group. The addition of oxindole increased the intensity of the peak at 244mμ from log.ε.4.07 to log.ε.4.45 and had very little effect on the peak at 298mμ thereby proving the above suggestions, regarding the additive contributions of the chromophoric systems present in the C_{20} acid. Furthermore, methylation was shown to have a slight bathochromic effect, an equimolecular mixture of the γ-(N-Methyl-indolyl)-γ-keto butyric acid and N-methyloxindole

absorbing at 245mμ , 302mμ.

Infra-Red Spectroscopic Evidence for the Presence of a β-Acyl Indole System in the C_{20} Acid.

Consideration of the structures (I) and (II) for the C_{20} acid quite clearly indicates that the infra-red spectrum would be complex and rather difficult to interpret. Because of the difficulty in assigning bands below 1500cm^{-1} to any one vibration in a molecule of such complexity, studies in this series were confined to attempting to interpret bands appearing in the 1550-1800cm^{-1} region and in the 2900-3300cm^{-1} region. All spectra determinations were obtained using a Nujol suspension and a Grubb-Parsons double-beam spectrometer.

When the C_{20} acid and its methyl derivatives were examined, the following bands were found in the carbonyl stretching frequency region.

Compound	*1700-1750-	1650-1700-	1620-1650-	1580-1620-
C_{20} Acid.		1695		1605
Methyl Ester of C_{20} Acid.		1695		1605
Trimethyl Deriv. of C_{20} Acid.	1730	1670	1639	1613
Tetramethyl " " " "	1736	1695	1631	1610

*All units in this and the following table are cm^{-1}

Of these spectra only that of the tetramethyl derivative contained clear and well defined bands. The spectra of the others contained broader bands not nearly so well defined, indicative of inter and/or intra-molecular association.

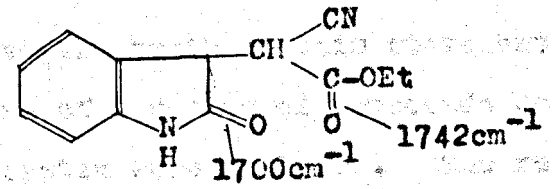
The first significant feature in the spectra of these compounds is the appearance in all of the two bands in the region 1695cm^{-1} and 1605cm^{-1}-1613cm^{-1}. When the spectra of oxindole and N-methyl oxindole were studied and found to contain only two bands in this region at these same wave numbers the presence of these bands was considered to be due to the presence of the oxindole nucleus in the C_{20} Acid.

Compound	1700-1750	1650-1700	1620-1650	1580-1620
Oxindole.		1695		1610
N-Methyloxindole.		1695		1605
Oxindolyl-3-Cyanacetic Ester.	1742	1700		1613

Randall et. al.[23] have attributed the band at 1695cm^{-1} to the carbonyl stretching frequency in the oxindole nucleus while that at 1605-1613cm^{-1} they attribute to the oxindole ring system.

Because the saturated carboxylic acid and ester groups are known to have a carbonyl stretching frequency in the 1700-1750cm^{-1} region, bands in this region were expected to occur in the C_{20} acid and its derivatives. The spectra of the C_{20} acid and its methyl ester contained no bands in this region although shoulders did appear in each spectrum in the 1720-1740cm^{-1} range. Attempts to resolve these shoulders into separate bands have so far been unsuccessful probably due to the occurrence of strong inter and ontra molecular association. The spectra of the trimethyl and tetramethyl derivatives did however each contain a band in this region. In the trimethyl derivative this band occurred at 1730cm^{-1} and was assigned to the carbonyl stretching frequency of a saturated aliphatic acid. In the tetramethyl derivative the frequency had risen to 1736cm^{-1} and was attributed to the carbonyl stretching vibration of a normal saturated ester. Oxindolyl-3-cyanacetic ester (XVL), as is to be expected, exhibits a similar band at 1742cm^{-1}.

The infra red spectra thus provides evidence for the presence of the oxindole nucleus and for the



(XVI)

carboxyl group in the C_{20} acid and consideration must now be given to the interpretation of the two bands occurring in the trimethyl and tetramethyl derivatives at 1639cm^{-1} and 1631cm^{-1} respectively. If either of the structures (I) or (II) for the C_{20} acid were to be correct, the carbonyl group in the β-acyl indole system should be responsible for this remaining band. Because the introduction of a double band $\alpha\beta$ to a carbonyl group may lower the carbonyl stretching frequency from 1725-1705cm^{-1} to 1685-1665cm^{-1} it was thought likely that substitution of nitrogen on the β-carbon atom as found in a β-acyl indole system may produce an even greater shift to the lower frequency region. That such an effect was considered likely was due to the abnormally high wave length absorption of the maximum intensity band in the ultra-violet absorption spectra of the 2 : 4-dinitrophenylhydrazone of a β-acylindole, (vide supra).

In order to prove the truth of this statement the infra-red spectra of a series of compounds containing an acyl-indole system were analysed. The results strongly supported these views and showed that the carbonyl stretching frequency of the keto group in a β-acyl indole occurs at an unusually low wave number. It was found that Cromwell[24] had shown that the carbonyl frequency in a β-amino-α,β-unsaturated ketone occurred at between 20-80cm^{-1} lower than the usual value. The carbonyl stretching frequency of the keto group in N-methyl-4-keto-1,2,3,4-tetrahydrocarbozole, for example, was found to be 1626cm^{-1} which is in very good agreement with the values assigned to the frequency of this system in the trimethyl and tetramethyl derivatives of the C_{20} acid. The assignment of this band in these derivatives to the carbonyl stretching frequency of a β-acyl indole system was thus considered justified.

The table shows the acyl indoles and related compounds which were analysed with the frequency of the bands found in the N-H and C=O stretching frequency regions of their infra-red spectra. All the bands quoted unless otherwise stated were of strong intensity.

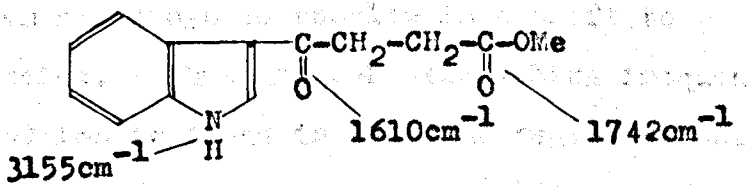
Name of Compound	System Under Consideration.	N-H Stretching Frequency.	C=O Stretching Frequency.
1,2,3,4-Tetrahydrocarbazole		3333cm	
1-Keto-1,2,3,4-Tetrahydrocarbazole	α-acyl indole	3247cm	1629cm
4-Keto-1,2,3,4-Tetrahydrocarbazole	β-acyl indole	3165cm (shoulder)	1592cm
N-Methyl-4-Keto-1,2,3,4-Tetrahydrocarbazole	N-Methyl β-acylindole		1626cm
2-Methyl-3-acetyl indole	β-acyl indole	3135cm	1597cm
2-Methyl-3-acetyl-5-methoxyindole	β-acyl indole	3134cm	1601cm (1618cm methoxylated benzene ring)
5-Methoxyindole	Indole ring	3360cm	1618cm (m) 1582cm (m)
2-Methyl indole	Indole ring NH	3311cm	
2-Acetyl-3-methyl indole	α-acyl indole	3311cm	1631cm
2-Methyl-3-benzoyl indole	β-acyl indole	3165cm	1600cm
2-Methyl-3-benzoyl-5-methoxy indole	β-acyl indole	3175cm	1595cm 1618cm (m)
Oxindole	NH frequency	3165cm	

Consideration of the values quoted for the tetrahydrocarbazoles makes it immediately evident that the band in the 1592-1629cm^{-1} region must be assigned to the acyl indole system. It is shown also that it is possible to distinguish between α and β-acyl indoles, the α-acyl indoles absorbing at a frequency of approximately 35cm^{-1} higher than their β-isomers. Thus whilst 1-keto-1,2,3,4-tetrahydrocarbazole absorbs at 1629cm^{-1}, 4-keto,1,2,3,4-tetrahydrocarbazole absorbs 37cm^{-1} lower at 1592cm^{-1}. Similarly 2-acetyl-3-methyl-indole absorbs at 1631cm^{-1} whilst 2-methyl-3-acetyl indole does not absorb until 1597cm^{-1}, 34cm^{-1} lower.

An important observation is that replacement of hydrogen by methyl in a β-acyl indole system results in the carbonyl stretching frequency being raised from 1592cm^{-1} to 1626cm^{-1}. Thus in the C_{20} acid and its methyl ester in which the indole nitrogen atom is not attached to a methyl group the stretching frequency of the carbonyl group in the β-acyl indole system would be expected to absorb in the 1592-1601cm^{-1} region. Since it has already been demonstrated that in the spectrum of these two compounds a band occurs at

1605cm^{-1} which has been assigned to the oxindole ring system it is not difficult to appreciate that the two bands have such similar frequencies that they would be coincident and only one would be observed in this wave number region. Therefore it was considered that in the spectrum of the C_{20} acid the band occurring at 1605cm^{-1} was due to the oxindole ring system and to the carbonyl stretching frequency of the β-acyl indole system both absorbing at the same frequency and only on methylation of the indole system and consequent increase in frequency of the β-acyl indole carbonyl group to 1626cm^{-1} could this band be specifically assigned to the oxindole ring.

These views were shown to be correct when Barrett (private communication) examined the infra-red spectrum of γ-indolyl-γ-keto-butyric ester (XVII) and found as expected two bands in the carbonyl stretching frequency, one at 1742cm^{-1} due to the saturated ester group and the other at 1610cm^{-1} due to the β-acyl indole system.



(XVII)

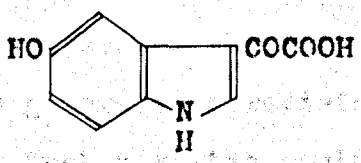
When a synthetical mixture of this keto-ester and oxindole were examined in the infra-red the band attributed to the carbonyl stretching frequency of the keto group in the β-acyl indole system at 1610cm^{-1} and the band attributed to the oxindole ring system 1605-1610cm^{-1} appeared together as one band at 1613cm^{-1} thus proving the above contention. Due most probably to inter-molecular association, the band attributable to the ester grouping was not clearly defined, appearing as a shoulder at 1736cm^{-1} whilst the band assigned to the carbonyl stretching frequency in oxindole appeared at a slightly higher frequency than usual (1695cm^{-1}) at 1708cm^{-1}.

The table also shows the effect on the N-H stretching frequency of the introduction of a keto group into the 3-position of an indole nucleus. The usual value for this vibration is of the order of 3330cm^{-1} but introduction of the keto group in the 3-position and consequent change of character of the N-H group from a normal heterocylic to the N-H in the vinylogue of an amide system results in a shift to lower frequencies. Thus the N-H stretching frequency in β-acyl indoles is found in the same region as the

N-H stretching frequency of oxindole, namely $3165cm^{-1}$.

Evidence Supporting the Presence of a γ-Keto Acid System in the C_{20} Acid.

Having demonstrated the presence of a keto group and of a carboxyl group in the C_{20} acid, it is of interest to consider the exact location of these groups in the molecule with respect to each other. Because the molecular formula $C_{20}H_{16}N_2O_5$ after satisfying the requirements of the two indole residues $(C_8H_6ON)_2$ allows only a residue COC_2H_3COOH, the C_{20} acid must be a keto acid of the α, β or γ type. However since the keto group has been shown to be present in a β-keto indole system, a α-keto acid of the type (XVIII)

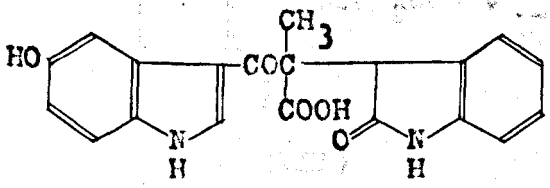


(XVIII)

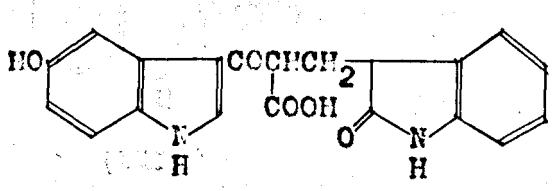
clearly cannot be envisaged because there remains no point of attachment in this system, to accommodate the remainder of the molecule. Substitution of the

2-position of this system is not suggested because there is no evidence of any kind to support this view.

Consideration of the presence of a β-keto acid system in the C_{20} acid leads to the formulation of two possible structures, (XIX) or (XX).



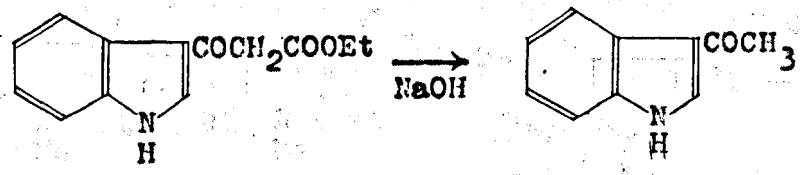
(XIX)



(XX)

Of these, structure (XIX) may be immediately eliminated because it contains a -C-Me group the absence of which in the C_{20} acid has been shown by a negative Kuhn-Roth determination. With regard to structure (XX), such a structure is extremely improbable for the C_{20} acid because the latter displays none of the properties generally associated with a β-keto acid system. Such a system would be expected to decarboxylate quite readily whereas the C_{20} cannot be decarboxylated, the carboxyl group preferring to be involved in a cyclic dehydration reaction. Evidence against the formulation of a β-keto acid system is demonstrated by the effect of alkali on an indolyl

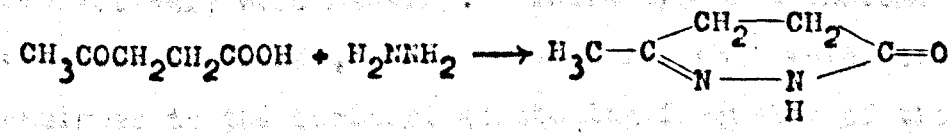
β-keto ester. On heating ethyl-(γ-indolyl)- γ - keto propionate (XXI) with alkali[26] saponification of the ester group, followed by ready decarboxylation resulted in the formation of 3-acetyl indole (XXII).



(XXI) (XXII)

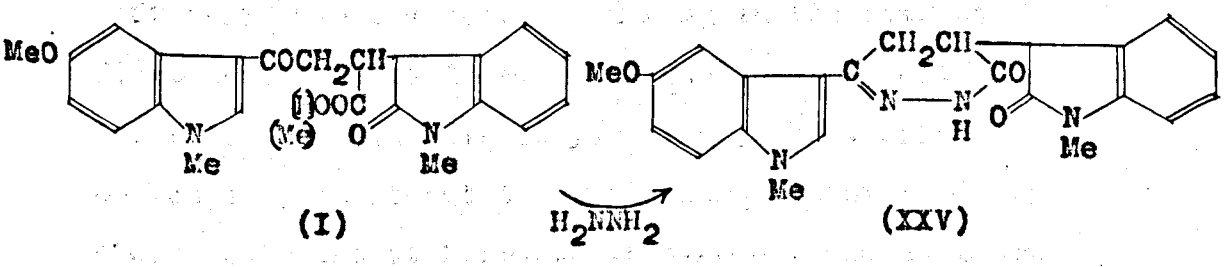
Since the C_{20} acid and its derivatives have been shown to have been recovered unchanged even after heating with 50% alkali the presence of a β-keto acid must be considered extremely unlikely.

There thus remains only a γ-keto acid and the formulation of the original structures (I) and (II) for the C_{20} acid. If such a system is present then on reaction with hydrazine it should form, as for instance does laevulinic acid (XXIII) and benzoyl propionic acid, a substituted pyridazinone derivative (XXIV).



(XXIII) (XXIV)

This was indeed shown to be the case because both the trimethyl and the tetramethyl derivative of the C_{20} acid condensed with hydrazine to form the same indolyl-oxindolyl-pyridazinone (XXV).

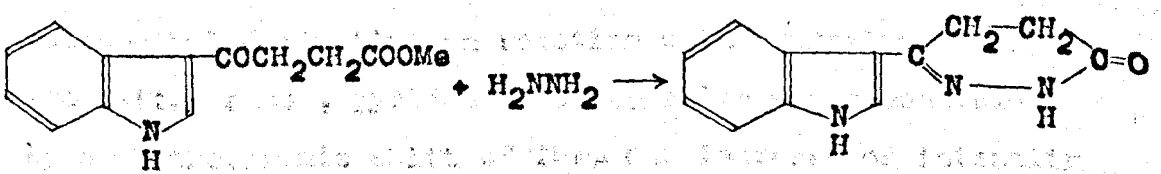


That reaction with hydrazine had proceeded in this manner was confirmed by analytical data the infra-red and ultra-violet spectra of the product and by the analogous behaviour of a known indole-δ-keto acid with hydrazine.

The infra-red spectra showed that in the hydrazine product, the bands at 1736cm^{-1} and 1626cm^{-1} found in the spectrum of the tetramethyl derivative and assigned to the carbonyl stretching frequencies in the ester group and in the β-acyl indole system r respectively were missing. There appeared instead of these two bands, a new band at 1665cm^{-1} which is assigned to the carbonyl stretching frequency of the keto group in the pyridazinone system.

The ultra-violet spectrum of the pyridazinone was as expected quite different from that of the tetramethyl derivative. The most significant difference was the shift occurring in the peak at 308mμ log.ε4.05 to 328mμ log.ε 4.3 together with the large increase in intensity. The peak at 255mμ was no longer present but the pyridazinone contained two other peaks one at 266mμ log.ε 4.28 and one at 280 mμ log.ε 4.26. These changes are due to the disappearance in the tetramethyl derivative of the β-acyl indole system and its replacement by the -C=N group with the possibility of extended conjugation into the pyridazinone system.

Reaction of γ(β-indolyl)- γ-keto-butyric ester with hydrazine was shown by Barrett (private communication) to occur in an analogous manner to the derivatives of the C_{20} acid.



(XVII) (XXVI)

The hydrazine product analysed correctly for an indolyl-pyridazinone (XXVI) and the infra-red and ultra-violet spectra were similar to the pyridazinone from the C_{20} acid and its derivatives.

Thus in the carbonyl stretching frequency of the infra-red spectrum, the ester band 1742cm^{-1} and the β-acyl indole band at 1610cm^{-1} were missing and instead a new band at 1650cm^{-1} occurred. The close proximity of this pyridazinone carbonyl stretching frequency with that found in the pyridazinone of the tetramethyl derivative 1665cm^{-1} was taken as indicative of the same cyclic system.

The ultra-violet spectrum of this indolyl pyridazinone, as in the C_{20} acid series, was found to be quite different from the parent keto-acid. The significant feature was the shift in wave length of the peak at 294mμ to 316mμ accompanied by an increase in intensity thus displaying exactly parallel behaviour to the changes occurring in the spectrum of the tetramethyl derivative on reaction with hydrazine. In the latter case, pyridazinone formation was accompanied by a bathochromic shift of 20mμ and increase of intensity

from log. ε 4.05 to log. ε 4.3 whilst in this case the bathochromic shift is 22 mμ and the intensity rises from log. ε 4.16 to log. ε 4.32. Because of the oxindole contribution in the lower parts of the spectrum and the bathochromic effect of the 5-hydroxyl group no fair analogies can be drawn regarding the 240-280 mμ region.

The above evidence was considered to support strongly the presence of a γ-keto acid system in the C_{20} acid and its derivatives.

ACYLATION OF THE C_{20} ACID AND ITS DERIVATIVES.

One of the more interesting features of the chemistry of the C_{20} acid and its derivatives is their behaviour upon acetylation. When acetylated the C_{20} acid, depending on the conditions employed can form two different acetyl derivatives. Acetylation by heating with sodium acetate/acetic anhydride forms a red crystalline, non-acidic, tetra-acetate with the loss of the elements of water. When acetylated by the pyridine/acetic anhydride method at low temperature the red acetate is formed together with a colourless crystalline, non-acidic acetate the analysis of which also indicates that it is a tetra-acetate.

The trimethyl derivative of the C_{20} acid appears to behave in an analogous manner. With sodium acetate/acetic anhydride it forms a magenta, non-acidic trimethyl mono-acetate with loss of the elements of water whilst with pyridine/acetic anhydride at low temperature together with the magenta acetate is formed a colourless crystalline non-acidic material the analysis of which has not at present been determined.

The behaviour of the two ester derivatives of

the C_{20} acid on acetylation was shown by Boggiano to be quite normal. The methyl ester forms a colourless triacetate, acetylating as would be expected the two nitrogen atoms of the indole nuclei and the phenolic hydroxyl group of the 5-hydroxy-indole system. The tetramethyl derivative does not acetylate and can be recovered unchanged in very high yield.

That the production of highly coloured compounds from the C_{20} acid is not specifically due to acetylation but rather to general acylation was demonstrated by the formation of a highly coloured, non-acidic benzoyl derivative.

Two important conclusions may be drawn from these experimental results:-

(i) The free carboxyl group in the C_{20} acid and the trimethyl derivative may undergo more than one sequence of reactions when reacted upon by acylating agents. The production of highly coloured acetates is due to the effect of one such reaction sequence.

(ii) When this reaction occurs a group, determinable as acetyl is introduced into the molecules because the acetate obtained from the C_{20} acid analyses for a tetra-acetate whereas the acetate from the methyl ester is

shown to contain only three acetyl groups. The same conclusion is arrived upon by considering the parallel behaviour of the trimethyl and tetra methyl derivatives upon acetylation.

It is necessary to consider the possible mechanisms by which reaction of the carboxyl group in the C_{20} acid with acylating agents might occur.

There appears to be three possible intramolecular mechanisms by which this reaction might occur.

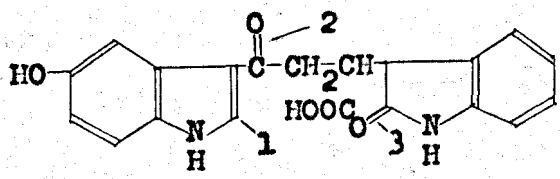
(i) The carboxyl group might first under the influence of acylating agents, be involved in a cyclisation, with loss of a molecule of water, on to some suitable centre in the molecule. A fourth acetylateable centre could then be introduced by enolisation of the newly formed keto group.

(ii) The carboxyl group might first react with the acylating agent to form an intermediate which could then cyclise with elimination of a molecule of water on to a suitable centre in the molecule.

(iii) The acylating agent might attack some centre in the C_{20} acid other than the carboxyl group to form an intermediate product which could then react, by elimination of water, with the carboxyl group.

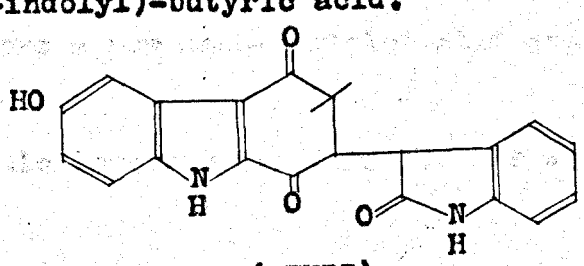
This third possibility can clearly be disregarded since such a mechanism would not explain why on attempted acetylation, the tetramethyl derivative is recovered unchanged.

With regard to the first possible mechanism, consideration of structure (I) for the C_{20} acid leads to the conclusion that there are three centres within the molecule which could conceivably enter into cyclisation with the carboxyl group. These are marked 1,2 and 3.



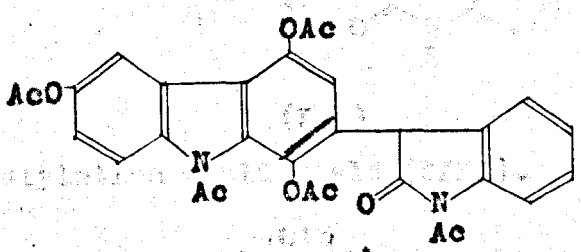
(I)

Cyclisation of the carboxyl group on to the carbon atom marked 1 would involve the formation of a keto-carbazole system (II) (XXVII). Such a cyclisation has been previously observed by Jackson and Manske[27] who isolated 1-keto-1,2,3,4-tetrahydrocarbazole during the preparation of γ-(3-indolyl)-butyric acid.



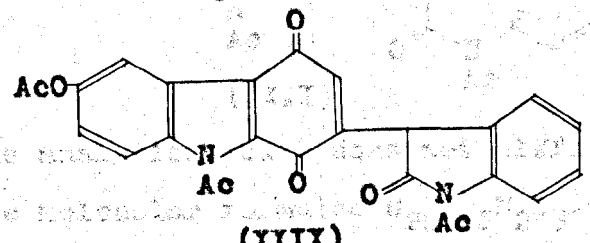
(XXVII)

However, a system such as (XXVII) would be expected to aromatise by diacetylation of the keto system to (XXVIII),



(XXVIII)

or to oxidise to the quinonoid state (XXIX),

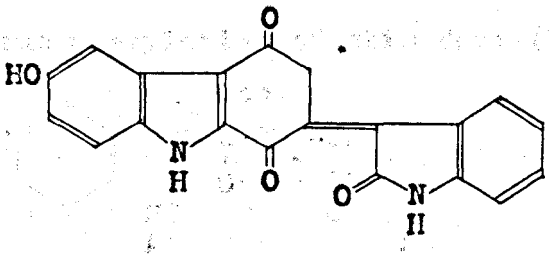


(XXIX)

in which there remains no acetylateable centre for the introduction of a fourth acetyl group.

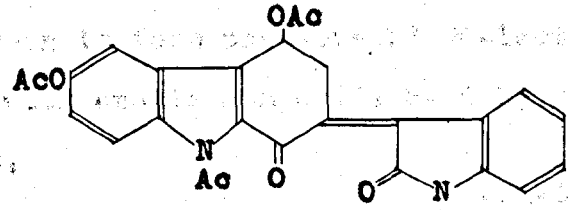
Neither (XXVIII) nor (XXIX) however explains the experimental results and a cyclisation of this nature would have to be followed by a primary oxidation of the oxindolyl system to an oxindolidene type before a new mono-acetylateable centre is introduced.

This involves the formation of an intermediate (XXX)



(XXX)

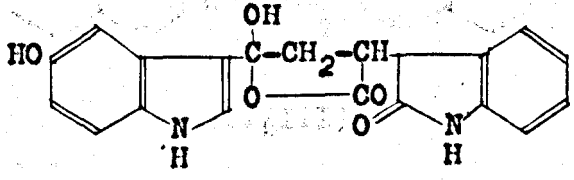
which on acetylation would yield (XXXI).



(XXXI)

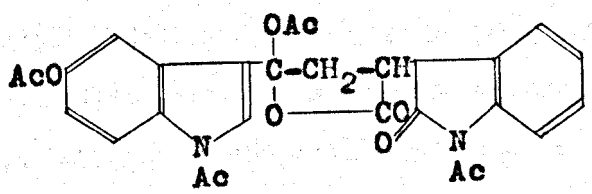
Because the analytical data does not differentiate between the molecular formulae $C_{20}H_{16}N_2O_5$ + $4C_2H_2O-H_2O$ and $C_{20}H_{16}N_2O_5$ + $4C_2H_2O-H_2O-H_2$ the compound (XXXI) would also satisfy the analytical requirements.

Cyclisation of the carboxyl group on to the carbon atom marked 2 has been previously discussed in connection with keto-lactol tautomerism and would in this case involve the formation of the lactol form (XI).



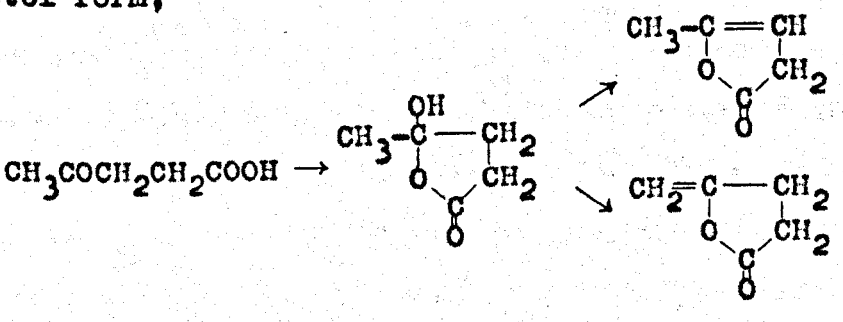
(XI)

and subsequent acetylation of this form (XXXII).

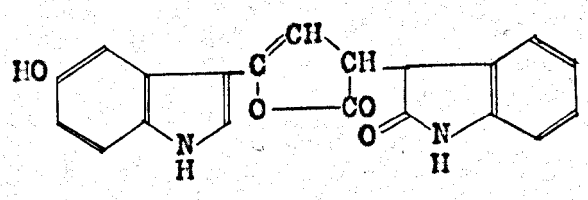


(XXXII)

However, as certain γ-keto acids, e.g. laevulinic acid are known to form unsaturated γ-lactone systems on heating with acetic anhydride by dehydration of the lactol form,



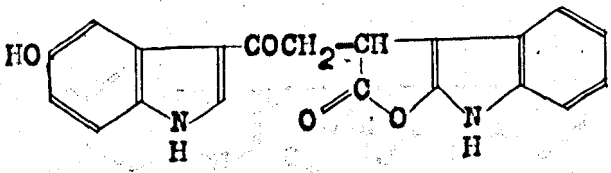
and dehydration is known to occur during this acetylation a similar reaction might be envisaged. This however would lead to the structure represented by (XXXIII),



(XXXIII)

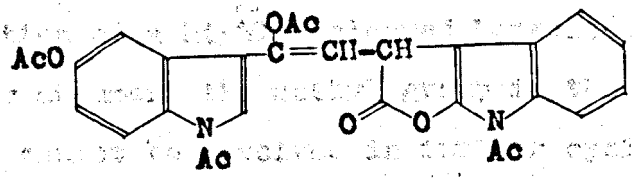
which contains no centres for introduction of the fourth acetyl group and is thus an unlikely possibility.

Cyclisation of the carboxyl group onto the oxygen atom marked 3 would involve the formation of a system (XXXIV)

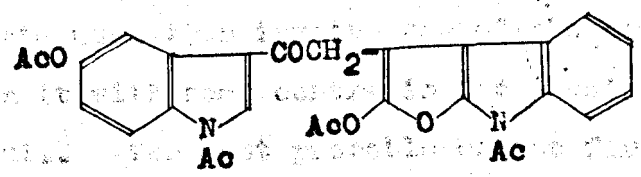


(XXXIV)

which would, in order to account for the fourth acetyl group, have to undergo an enolisation to one of the systems (XXXV) or (XXXVI) when it would then



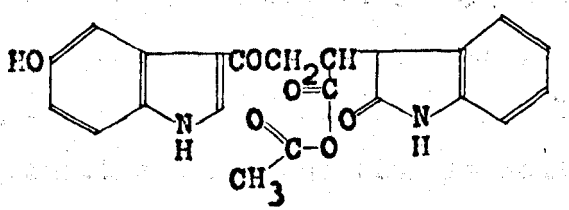
(XXXV)



(XXXVI)

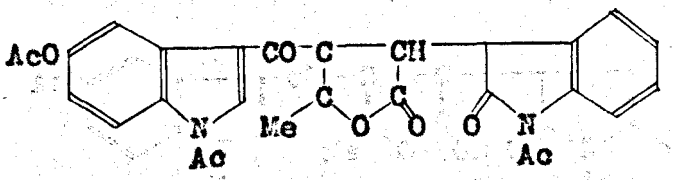
represent the red acetate.

With regard to the second postulated mechanism for the acetylation, involving first the formation of an intermediate the most likely reaction to occur between the carboxyl group and the acylating agent is that leading to the formation of a mixed anhydride (XXXVII).



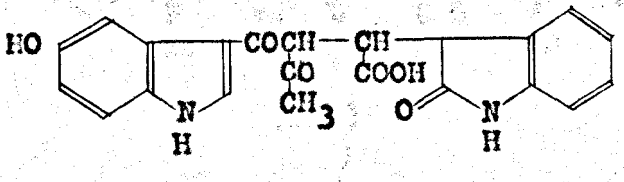
(XXXVII)

That reaction between the carboxyl group and the methyl group in acetic anhydride does not occur is shown by the formation of a highly coloured benzoate. Moreover, by similar argument the methyl group in the mixed anhydride cannot be involved in further cyclisation. Elimination of a molecule of water from the anhydride intermediate must thus involve one of the carbonyl groups present in it with some centre in the remaining part of the molecule. The most probable course for such a reaction would involve the methylene group and lead to the formation of compound (XXXVIII).



(XXXVIII)

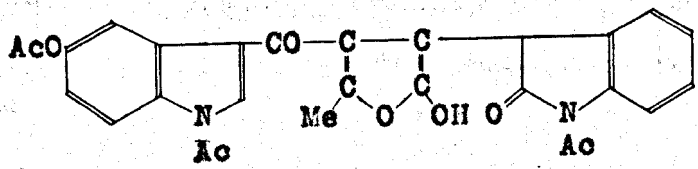
The presence of a fourth group in this compound determinable as acetyl can be appreciated on considering the effects of alkaline hydrolysis. Since the system conatins a lactone ring it would , on heating with alkali, be expected to open and yield a β-ketonic system (XXXIX) which on further alkaline hydrolysis



(XXXIX)

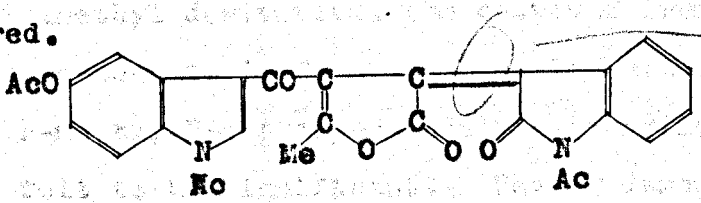
should split off a molecule of acetic acid.

It is difficult to state with any certainty just what colour such a system as (XXXVIII) might possess. It might be argued that because the conjugation does not extend through the molecule the colour cannot be expected to be red but it is possible that be existing in the enol form, the extent of conjugation will be increased and the molecule may assume a darker colour.



(XL)

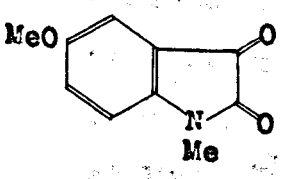
Clearly if one envisages a primary oxidation of the oxindolyl system as previously considered the resulting oxindolidine (XLI) compound would be expected to be red.



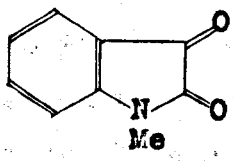
(XLI)

Before considering the arguments in favour and against these various structures it is necessary to consider first, further evidence regarding degradational, synthetical and spectroscopic studies on these acetates.

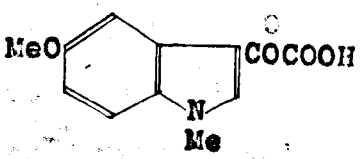
Boggiano by oxidation of the magenta acetate was able to isolate three products, (XLII-XLIV).



(XLII)



(XLIII)



(XLIV)

The appearance of the isatins (XLII) and (XLIII) from an oxidation of this kind is not unexpected since they had also been isolated from a similar oxidation of the trimethyl derivative, the compound from which the magenta acetate is formed. The isolation of (XLIV), N-methyl-5-methoxy-3-indolyl-glyoxylic acid is however felt to be significant. Having demonstrated the presence of a β-acyl indole system in the acid, the isolation of this system intact from the oxidation of the magenta acetate is considered as evidence that during the formation of the magenta acetate it is not in any way affected. Had cyclisation involved the 2-position of the 5-hydroxy-indole nucleus the indole-3-glyoxylic acid (XLIV) would not have been expected to occur but rather a derivative substituted in the 2 and 3-positions of the indole ring.

The infra-red spectrum of the magenta acetate

when examined shows no bands of any kind in the -O-H stretching frequency region but in the carbonyl stretching frequency region there appears four bands at 1767cm^{-1},1680cm^{-1}, 1629cm^{-1} and 1606cm^{-1} respectively. The bands at 1680cm^{-1} and 1606cm^{-1} are assigned to the N-methyl oxindole system whilst that at 1629cm^{-1} is considered to be due to the carbonyl group in the N-methyl β-acyl indole system, (vide supra). Because these three bands occur, with minor frequency shifts, in the precursor of the magenta acetate, the trimethyl derivative, their appearance in the spectrum of the magenta acetate is considered to indicate that these systems as such are not affected by the changes which occur on acetylation. The disappearance of the carbonyl stretching frequency band in the carboxyl group of the trimethyl derivative at 1730cm^{-1} and the appearance of a band at 1767cm^{-1} is considered to represent the major change occurring during the acetylation. The assignment of this band at 1767cm^{-1} is therefore of some importance in assisting to elucidate the structure of the magenta acetate. Because the magenta acetate analysis for the presence of one acetyl group, the most probable explanation

of the occurrence of this band at 1767cm^{-1} is that it originates from the carbonyl stretching frequency of an acetate group.

A survey of the literature shows that the carbonyl stretching frequency in acetates is dependent to some considerable extent on the environment of this group. Jones and co-workers [18] have investigated the carbonyl stretching frequencies of many sterol acetates and shown that the saturated materials absorb in the range 1742-1735cm^{-1}. Barnes et.al.[29] however found that vinyl esters, those containing the system -CO-O-C=C showed a marked enhancement of the carbonyl stretching frequency, regardless of whether the double bond is normal or part of an aromatic ring. They found that vinyl acetate for example absorbed at 1776cm^{-1} whilst Hartwell et.el.[30] found a similar effect with phenyl acetate.

The only other possible systems which were found to exhibit carbonyl stretching frequencies in this region were saturated, $\alpha\beta$ -unsaturated and $\beta\gamma$ -unsaturated γ-lactones.[31]

γ-lactones saturated	1780-1760cm^{-1}
γ-lactones, $\alpha\beta$ -unsaturated	1760-1740cm^{-1}

γ-lactones, βγ-unsaturated 1800-1780cm^{-1}

These results would tend to indicate that the new system present in the magenta acetate contains an enol acetate or an equivalent group. The formation of an enol acetate can be envisaged by cyclisation of the carboxyl group and acetylation of the enol form of the newly formed carbonyl group. An alternative explanation is that the enol acetate system is present in a βγ-unsaturated γ-lactone ring, formed by the mechanism leading to the formation of compound (XXXVIII). That the band at 1767cm^{-1} is somewhat lower than the usual value, 1790cm^{-1}, found for this system may be due to the rather unusual substituents.

The effects of acetylation upon an indolyl γ-keto acid, γ(3-indolyl)-γ-keto butyric acid were investigated by Barrett (private communication) who showed that deep red solutions are formed on attempting to acetylate either by the sodium acetate/acetic anhydride or by the pyridine/acetic anhydride method. A red solid which was non-acidic was obtained but which unfortunately resisted all attempts to crystallise it. Thus this solid was not analysed and it is not possible to state whether the acid undergoes on acetylation a

similar change to the C_{20} acid. If it were found to do so then the result would be significant because it would indicate that the oxindole nucleus in the magenta acetate is in no way involved in the reaction.

A series of experiments designed to study the effect of reductive acetylation of the C_{20} acid and the trimethyl derivative provided interesting results. On heating the C_{20} acid with zinc dust, acetic acid and acetic anhydride the deep red colour developed and only after heating in this medium for a considerable period did the colour lighten to yellow. However, when the trimethyl derivative was added to a boiling mixture of zinc dust, acetic acid and acetic anhydride no colour developed and on heating the mixture for five minutes and leaving overnight the solution remained colourless. Although it is appreciated that in this latter case the presence of a reducing medium might in some way prevent the normal acetylation reaction from occurring it is thought a rather more satisfactory explanation that it does in fact operate against some facile oxidation inherent in the formation of the coloured acetates. In the former acetylation it appears probable that this oxidation had occurred

before the reduction medium became operative and thus the formation of the coloured acetate first occurred. Thus in the formation of the coloured acetates, the evidence favours:-

1. The presence of the β-acyl indole system as it occurs in the C_{20} acid and its derivatives.

2. The presence of the oxindole system in either an oxindolyl or in an oxindolidene form.

3. The presence of an enol acetate or equivalent system.

On reconsidering the various possible structures proposed for the magenta acetate it is seen that (XXXI), (XXXV) and (XXXVI) involve either the β-acyl indole system or the oxindole carbonyl group.

Of the remaining structures (XXXIII) clearly does not explain the formation of the oxidation products and only the structure (XXXVIII) can be reconciled with the experimental evidence. The main arguments against this structure are that as written it cannot be expected to be highly coloured and that attempted deacetylation with alkali should lead to the original trimethyl acid whereas Boggiano was not able to identify

any diacetylation products.

The postulation of an oxidation of the oxindole system to an oxindolidine (XLI) would explain both the colour of this compound and the failure to isolate the trimethyl acid on diacetylation. This structure must therefore at present be considered the most probable although it is not completely satisfactory.

With regard to the colourless compounds which can be obtained from the C_{20} acid and its trimethyl derivative by acetylation at low temperature, no structures can at present be proposed, because complete analyses have not been performed. An acetyl analysis on the solid obtained from the C_{20} acid indicated that it is a tetra-acetate but until a complete analysis is performed no molecular formula can be ascribed. The infra-red spectrum of the colourless solid obtained from trimethyl derivative of the C_{20} acid indicated that it has quite a different structure from the magenta acetate because quite unlike the spectrum of the latter it contains bands in the -OH region and also is different in the carbonyl stretching frequency region. The bands in the -OH region occur at 3400cm^{-1} and 3106cm^{-1} whilst in the carbonyl region three sharp bands at 1778cm^{-1},

1692cm^{-1} and 1606cm^{-1} occur. The oxindole nucleus accounts for the bands at 1692cm^{-1} and 1606cm^{-1} whilst the disappearance of the bands at 1730cm^{-1} and 1639cm^{-1} in the trimethyl derivative and the appearance of a band at 1778cm^{-1} indicates that both the carboxyl group and the β-acyl indole carbonyl group are involved in the reaction which occurs. The band at 3400cm^{-1} would seem to suggest the presence of a hydroxyl group and if so it must have resisted acetylation. However, until this compound has been analysed and the acetyl content determined, possible structures cannot profitably be discussed.

THE MOLECULAR FORMULA OF VIOLACEIN.

The first workers to propose an empirical formula for Violacein were Reilly and Pyne in 1927 but because it was derived from analyses of the amorphous pigment it cannot be seriously considered. The first recorded analysis of crystalline Violacein was made by Kögl in 1932 who suggested two alternative empirical formulae, $C_{35}H_{25}N_5O_6$ and $C_{42}H_{30}N_6O_7$. A noteworthy series of experiments by Wrede who showed that the pigment formed comparatively stable crystalline addition compounds with a number of bases pointed to the empirical formula $C_{35}H_{23}N_5O_6$ or $C_{42}H_{28}N_6O_7$.

Found for violacein crystallised several times from pyridine dried over P_2O_5 at room temp. 5 hours.	C 70.32 70.26	H 4.53 4.39	N 12.86 12.64
$C_{35}H_{23}N_5O_6 + 2C_5H_5N$ requires	70.37	4.33	12.74
$C_{42}H_{28}N_6O_7 + 2C_5H_5N$ requires	70.40	4.32	12.61

However by crystallisation from a chlorinated base e.g. 2-chloro-pyridine and analysis of the chlorine content he was able to distinguish between these two

formulae.

	C	H	N	Cl
Violacein freed from pyridine crystallised from 2-chloro-pyridine, dried over P_2O_5, 5 hours room temp.	65.60 65.44	3.94 3.87	11.76	7.11 7.44
$C_{35}H_{23}N_5O_6 + 2C_5H_4NCl$ requires	64.57	3.74	11.73	8.48
$C_{42}H_{28}N_6O_7 + 2C_5H_4NCl$ "	65.32	3.80	11.73	7.42

The analytical values for specimens of violacein prepared in these laboratories are in close agreement with those of Wrede and are listed for comparison purposes.

	C	H	N
$C_{42}H_{28}N_6O_7 + 2C_5H_5N$ requires	70.40	4.32	12.61
Wrede	70.32 70.26	4.53 4.39	12.86 12.64
Subramanian	70.10 70.06	4.17 4.28	10.43
Beer	70.70 70.80	4.30 4.30	13.00 12.20
Clarke	70.10	4.30	11.80

An interesting observation is that with violacein and with acetylviolacein the results of nitrogen estimations are unexpectedly variable (cf. Wrede loc. cit.).

Because of the extremely low solubility of violacein in suitable solvents, molecular weight determinations based on one of the colligative properties have not so far been possible. Further more, determination of the molecular weight by the Rast method is not applicable, for although solution in camphor occurs the melting point of the solution cannot be accurately ascertained due to the extremely dark colour obtained.

The analytical values for acetylviolacein found by Wrede also favoured the C_{42} formula and suggested that six acetyl groups had been introduced. Clarke in these laboratories synthesised the propionyl derivative which on anlaysis gave results in agreement with the accepted formula.

Thus a decision regarding the molecular formula of violacein could not be profitably made at this stage of work because of the various difficulties precluding accurate molecular weight determinations.

The first indication that the violacein molecule

is not as large as the C_{42} formulation would suggest was made by Clarke[15] as a result of degradative investigation of the pigment. In a series of quantitative oxidations of acetylviolacein, Clarke isolated acetylanthranilic acid in theoretical yield based on the assumption that there is one nucleus in the C_{42} molecule which can give rise to this acid. Since experimantal losses and side reactions in a degradative experiment of this nature must be quite considerable, there must almost certainly be two nuclei per 42 carbon atoms which can give rise to acetylanthranilic acid. Clarke also felt that the fact that he was not able to isolate any other fragments together with the C_{20} acid from the reductive alkaline degradation of violacein was significant. He therefore pointed out as a result of these experiments the possibility that the violacein molecule may consist of two identical portions joined by some early ruptured bond or that it may only be a C_{21} molecule.

This view seems to be supported by the high yields of the C_{20} acid (720mg. from 1gm of violacein) obtained by Boggiano[18] and during the present researches. Boggiano, like Clarke, also considered the possibility

that the violacein molecule may consist of similar halves joined loosely together but thought it a more acceptable theory that violacein is really a C_{21} molecule, ie $C_{21}H_{14}N_3O_3$ or $C_{21}H_{14}N_3O_4$.

X-ray diffraction analysis of acetylviolacein could not, due to the extremely small size of the crystal, afford results of the expected accuracy to enable a decision between the $C_{42}H_{28}N_6O_7$ formula and one of the C_{21} formulae to be made.

As a result of the degradative work previously discussed and the work carried out by Jennings[17] on the hydrogen iodide product from violacein both Doggiano and Jennings thought the $C_{21}H_{13}N_3O_3$ formula the most probable molecular formula.

Thus when the present researches began the analytical evidence favoured the C_{42} formulation for the violacein molecule whilst the degradative evidence suggested that the molecule is actually only half this size.

Efforts were directed towards recrystallising violacein from simple organic solvents and obtaining analytical results for specimens of the pure pigment without the formation of addition compounds. It was found that by use of a Soxhlet apparatus, violacein could be

recrystallised from acetone, and specimens thus obtained, after drying, because violacein is known to hold on tenaciously to acetone, were submitted for analysis. The results obtained are rather interesting and are shown in the table.

	C%	H	N
Found for violacein which was recrystallised from acetone. Dried 45 mins. at 60C.	68.97 69.52 69.23	4.06 4.26 4.09	11.7
Found for violacein left in Soxhlet apparatus not extracted by acetone, dried 30 mins. at 60C.	67.47	3.71	
$C_{42}H_{28}N_6O_7$ requires	69.23	3.85	11.5
$C_{21}H_{13}N_3O_3$ "	70.98	3.66	11.83
$C_{21}H_{15}N_3O_3$ "	70.59	4.20	11.76
$C_{21}H_{13}N_3O_4$ "	67.92	3.50	11.32
$C_{21}H_{15}N_3O_4$ "	67.56	4.02	11.26

They clearly indicate that for the violacein extracted and recrystallised from acetone the $C_{42}H_{28}N_6O_7$ formula is to be preferred to either of the C_{21} formulae.

It is not however quite so clear just what significance can be attached to the values obtained for

the material which remained in the Soxhlet apparatus and was not extracted. This material though quite crystalline had not been recrystallised and therefore cannot be considered to be as pure as the extracted material. However in view of the period allowed for the extraction this particular material would appear to be considerably less soluble in acetone than the material extracted and could therefore be a slightly different variety than the usual violacein having the formula $C_{21}H_{15}N_3O_4$ or $C_{21}H_{13}N_3O_4$.

This interesting posibility made the problem of the molecular weight all the more important and efforts were made to determine whether this could not be found by the use of a mass-spectrographic method. As a result of discussion with Dr.Waldron it was learned that the general properties of violacein e.g. its low volatility (Clarke reported that violacein slowly sublimed when heated at 350°C at a pressure of .00001mm.) and high molecular weight, would make a mass spectrographic analysis extremely difficult. Furthermore the number of instruments in this country capable of making such determination was only of the order of one or two.

The solution to this problem of the molecular

formula of violacein came from a study of the products obtained by methylation of violacein with dimethyl sulphate, potassium carbonate in acetone. Methylation of the pigment by this method was found to give rise to two methylated derivatives. The relative amounts in which these derivatives were formed depended on the state of the purity of the violacein used. With the purer crystalline pigment, the two methyl derivatives were formed in equal amounts but with the not so pure material their relative amounts were somewhat variable. Because of their different properties the two methyl derivatives could be easily separated. One exhibited appreciable solubility in acetone whilst the other resembling more the characteristics of violacein was only very sparingly soluble and thus separated out from the methylation mixture. The more soluble methyl derivative could be crystallised from a variety of solvents such as benzene, chloroform, ethyl acetate, acetone and ethyl alcohol and could be obtained in a high state of purity by chromatography in benzene. Some difficulty was initially encountered in preparing specimens of this compound for analysis because it was found that it retained solvent of crystallisation which

besides affecting the melting point quite appreciably, resulted in irreproduceable analytical results. The importance of characterising this compound was increased when it was learned that Simpson (private communication) had obtained a very similar compound as the sole product from the methylation of a purple pigment which he had isolated from a marine bacterium and was growing on a synthetic medium in the laboratory. Infra-red and ultra-violet spectroscopic studies later showed that these two methyl derivatives are identical and their analysis indicated that they are a trimethyl derivative of a $C_{21}H_{15}N_3O_3$ molecule.

	C	H	N	OMe
Lower melting Methyl deriv. obtained from violacein. (Eardley)	71.83 71.94	5.14 5.25	10.75 10.42	8.19
Methyl deriv. obtained from purple pigment as sole product (Simpson)	71.80 72.36	5.33 5.43	10.27 10.23	8.04
$C_{21}H_{15}N_3O_3 + 3(CH_2)$ requires	72.18	5.26	10.53	7.7

A molecular weight determination of this compound by Simpson gave a value of 380 which is in good agreement

with that of 399 demanded by the formula.

Having decided the molecular formula of this compound efforts were directed towards establishing the molecular formula of the second methylation product from violacein.

This unfortunately exhibited none of the solubility properties of the trimethyl derivative. It was very sparingly soluble in all organic solvents and attempts to purify it by recrystallisation from acetone in a Soxhlet apparatus similar to the method which had proved successful for violacein ended in complete failure due to its extreme insolubility. That methylation had occurred was demonstrated by the fact that it was no longer soluble in cold caustic soda solution and that it had a positive value when analysed for methoxyl. Failure to purify this methyl derivative which because it did not melt but decomposed slowly over 300°C was referred to as the high melting methyl ether, led to attempts to prepare a more suitable derivative of it. Acetylation was found to give a highly crystalline compound which although still very insoluble could be recrystallised from acetic abhydride after prolonged treatment and which on

analysis gave a positive value for acetyl. It thus differed in this respect from the low melting trimethyl derivative which was recovered unchanged after attempted acetylation experiments. Before discussing the analytical results obtained for this high melting methyl-acetyl derivative it is felt advisable to consider first the general significance of the isolation of these different methyl ethers andtheir different behaviour towards acetylating agents.

On considering the accepted empirical formula for violacein $C_{42}H_{28}N_6O_7$ and attempting to reconcile this with the isolation of a methyl derivative of a molecule $C_{21}H_{15}N_3O_3$ it is quite clear that violacein is either a mixture of the two molecules, $C_{21}H_{15}N_3O_3$ and $C_{21}H_{13}N_3O_4$ or it is a C_{42} molecule in which these two molecules are held together by some very easily ruptured bond. The fact that the two different methyl ethers are obtained from such a mild methylation experiment and that they are not always obtained in equimolecular amounts strongly suggests that violacein is in fact a mixture of two C_{21} molecules. Since the lower melting trimethyl ether has been shown to be a derivative of $C_{21}H_{15}N_3O_3$ the high melting methyl ether must then be a

a derivative of $C_{21}H_{13}N_3O_4$. An observation which at the time had no definite significance ie. that the material which during the recrystallisation of violacein remained insoluble analysed for $C_{21}H_{15}N_3O_4$ or $C_{21}H_{13}N_3O_4$, now acquires rather more significance because the high melting methyl derivative similarly could not be recrystallised from acetone and this , if the argument is correct is a derivative of that particular insoluble $C_{21}H_{13}N_3O_4$ molecule. It was therefore expected that because Wrede had shown acetylviolacein to contain six acetyl groups and because the trimethyl ether had been recovered unchanged after attempted acetylation that the number of methyl plus acetyl groups in this high melting methyl-acetyl derivative would be three. Further since it had been shown that at least one methyl and one acetyl group was present the number of possible formulae was considered to be restructed to two namely a dimethyl-monoacetyl or a monomethyl-diacetyl derivative of the molecule $C_{21}H_{13}N_3O_4$. The analytical results are shown in the table.

High melting methyl-acetyl derivative found	C 67.14	H 4.55	N 8.84	OAc 17.64

	C	H	N	OAc
$C_{21}H_{13}N_3O_4$ + $2CH_2$ + $1(CH_2CO)$	67.45	3.98	9.8	10.07
$C_{21}H_{13}N_3O_4$ + $1CH_2$ + $2(CH_2CO)$ requires	66.52	4.05	8.9	18.3

The acetyl value in the methyl-acetyl derivative clearly indicates that two-acetyl groups have been introduced, during the acetylation. The carbon and hydrogen values however are higher than those demanded by the monomethyl-diacetyl formula suggesting more the introduction of a second methyl group. Contrary to the argument previously discussed concerning the number of functional groups present and based on Wrede's consideration of acetylviolacein to be a hexaacetate, a molecular formula assuming dimethylation and diacetylation to have occurred was envisaged in an attempt to accommodate more satisfactorily the analyses found.

High melting methyl-acetyl derivative	C 67.14	H 4.55	N 8.84	OAc 17.64
$C_{21}H_{13}N_3O_4$ + $2CH_2$ + $2(CH_2CO)$ requires	67.1	4.34	8.7	17.8

Such a formula is indeed more satisfactory and all four analyses are in good agreement with those found

for the methylacetyl derivative. However this postulation of a dimethyl-diacetyl derivative cannot be reconciled with Wrede's view that acetylviolacein is a hexa-acetate. The introduction of four groups into the $C_{21}H_{13}N_3O_4$ molecule together with the three methyl groups known to be present in the $C_{21}H_{15}N_3O_3$ molecule would necessitate that acetylviolacein be a hepta-acetate. It was thus considered advisable that because previous workers had not subjected acetylviolacein to analysis for acetyl content, an acetyl determination should be made in order to ensure that this compound does in fact contain six acetyl groups.

The results of such an analysis are extremely interesting and are listed in the following table. It can be readily appreciated from the table that the major difficulty in this analysis is the removal of the last traces of acetic anhydride from the specimen. The carbon content is seen to increase as the drying conditions become more drastic and the difficulty is in regulating these conditions so that removal of all solvent occurs without decomposition of the molecule commencing. Because, as previously mentioned, the nitrogen estimations are found to be unexpectedly variable with

violacein and acetylviolacein this analysis mainly depends upon the result of the acetyl determination.

Acetylviolacein dried in high vac. 1mm. 16hrs. dried to constant weight 130-140°C 30 mins	C 65.67	H 4.18	N 8.59	OAc 29.10 (alkali) 30.00 (CrO_3/ H_2SO_4)
Acetylviolacein same sample redried at 180°C for 1hr. slight loss in weight (30 + 50 ɣ)	66.22	4.41		
Acetylviolacein (Wrede)	66.10 66.15	4.20 4.06	8.79	
$C_{42}H_{28}N_6O_7$ + 6(CH_2CO) requires	66.11	4.08	8.57	26.32
$C_{42}H_{28}N_6O_7$ + 7(CH_2CO) "	65.75	4.11	8.2	29.45

In the sample dried to constant weight at 130-140°C for 30 mins. this determination was performed by two independent methods which both gave values in very close agreement to that demanded by the hepta-acetate formula. The carbon and hydrogen analysis of this specimen were also found to be in very good agreement with the values

demanded by the hepta-acetate and would suggest that acetylviolacein is a hepta-acetate rather than a hexa-acetate.

Accepting this evidence, the problem of the molecular formula of violacein can be simply explained. Violacein when pure is an equimolecular mixture of two similar purple pigments having the molecular formula $C_{21}H_{15}N_3O_3$ and $C_{21}H_{13}N_3O_4$ respectively. It thus analyses as the molecular addition compound $C_{42}H_{28}N_6O_7$.

On acetylation, a molecular addition compound, acetylviolacein is formed containing seven acetyl groups. It is composed of an equimolecular mixture of two similar acetyl derivatives, the triacetate of the $C_{21}H_{15}N_3O_3$ molecule and the tetra-acetate of the $C_{21}H_{13}N_3O_4$ molecule. The tri-acetate of the $C_{21}H_{15}N_3O_3$ molecule has been prepared independently by Simpson by acetylation of the purple pigment $C_{21}H_{15}N_3O_3$ and been shown to have very similar properties to acetylviolacein. This clearly explains why on acetylation of violacein, the two acetates are not independently isolated, their close similarity of properties eg. high melting point, very low solubility in organic solvents does not allow a separation to be made.

The methylation of violacein however is quite different because the two methyl derivatives which are formed have quite distinct properties and allow an easy separation to be made. In the case of the $C_{21}H_{15}N_3O_3$ molecule its three reactive groups are all methylated and a highly soluble, highly crystalline trimethyl ether is formed quite different in properties from the parent pigment. In the case of the $C_{21}H_{13}N_3O_4$ molecule this does not occur. For some reason not at present known, only two of the groups in this molecule which can be acetylated are methylated and a dimethyl ether is formed. This substance quite unlike the trimethyl ether is exceptionally insoluble in organic solvents and shows no sign of melting below 320°C. Because in the trimethyl ether all reactive groups had been methylated it was recovered unchanged after attempted acetylation but because the dimethyl ether still contains reactive groups acetylation resulted in the formation of a dimethyl-diacetyl compound.

PROPERTIES OF THE TWO METHYL ETHERS OBTAINED FROM VIOLACEIN.

The Low Melting Trimethyl Ether.

The low melting trimethyl ether is obtained from violacein as the acetone soluble product of the methylation with dimethyl sulphate and potassium carbonate. After filtering off the potassium carbonate and high melting dimethyl ether, the trimethyl ether may be obtained as a dark blue crystalline solid by evaporation of the acetone under reduced pressure. It can be recrystallised from a number of solvents including benzene, chloroform, ethyl acetate, pyridine and ethyl alcohol giving royal blue solutions and crystallising in the form of long needles. It is best purified by chromatography on a neutral alumina column using pure benzene as the eluent and is in this way obtained in dark blue needles with a shiny black sheen first melting at 127°C resolidifying and finally melting 190°C. These crystals have been shown to contain benzene of crystallisation and their analysis approximates to one mole of benzene to one mole of the ether. The melting point of this material on recrystallisation from ethyl acetate rises to 220-221°C and the crystals after drying

analyse correctly for the trimethyl ether $C_{21}H_{15}N_3O_3$ + $3CH_2$. The ultra-violet spectrum of this material measured in 95% alcohol is very similar to that of violacein. It contains three distinct peaks at 270 mμ log.ε . 4.31, 382 mμ log.ε . 3.88 and 600 mμ log.ε . 4.29. This last peak is at a slightly higher wave length than the corresponding peak in the violacein spectrum.

The trimethyl ether is totally insoluble in water, sodium bicarbonate and sodium carbonate solution. It is also insoluble in cold and in hot caustic soda solution and thus does not exhibit any acidic properties. It does not dissolve in dilute acids but does so in concentrated sulphuric acid to give an orange red solution. Addition of dilute acid to a blue alcoholic solution of the trimethyl ether causes the colour to change to olive green whilst on addition of alkali it changes to orange-red. These changes are reversible and indicate that when in solution the molecule can exist in different forms depending on the pH value of the solution.

The trimethyl ether can be hydrogenated at room temperature and ordinary pressure using Adams catalyst in glacial acetic acid solution. The absorption of hydrogen was found to be slow, 4-5 hours being required

before it was complete. During the hydrogenation the royal blue solution slowly lightened first to dark green then to an olive green. The solution remained this colour from the solution which although chromatographed on a alumina column in benzene resisted all atempts to crystallise it.

Attempts to obtain a reduced form of the trimethyl ether were made using a number of reducing agents. With zinc and acetic acid and sodium or potassium borohydride inhot methanol the blue solutions were changed to light yellow but in no case could the product be induced to crystallise.

On attempting to degrade the trimethyl ether with hydrogen iodide in glacial acetic at 130°C for 1½ hours a yellow-brown amorphous solid was obtained. When treated with caustic soda solution this solid changed its appearance and an amorphous blue material was obtained. The solution gave a positive test for iodide ion and it was thought most likely that the yellow-brown material was a hydrogen-iodide salt of the amorphous blue solid which was considered to be a partially demethylated derivative of the trimethyl ether. The blue solid was found to be quite soluble in acetone but totally insoluble in water

or in alkali and resembled in no way the indole-pyrryl methene, the product obtained by treating violacein with hydrogen iodide. It is difficult to explain why this blue solid, if a partially demethylated product, does not dissolve in alkali because demethylation with hydrogen iodide would have been expected to result in the formation of a free phenolic hydroxyl group in the 5-position of the indole nucleus. Simpson (private communication) also investigated the effect of hydrogen iodide in acetic acid on this trimethyl ether and obtained identical results. He did however remethylate the blue solid with dimethyl sulphate, potassium carbonate in acetone which resulted in the formation of the original trimethyl ether.

This result is extremely interesting because it demonstrates that the changes which occur on heating violacein or acetylviolacein with hydrogen iodide resulting in formation of the indole-pyrryl-methene do not occur with this particular methyl derivative. Two probable explanations why this reaction does not occur may be suggested:-

(1) The transformation from the violacein type molecule to the indole-pyrryl-methene may occur in only

one of the C_{21} molecules namely $C_{21}H_{13}N_3O_4$.

(2) The change to the highly resonating system found in the indole-pyrryl-methene may not occur so readily when the nitrogen atom in the 5-hydroxy indole nucleus is attached to methyl (vide infra).

The first explanation does not become tenable on considering the yield of indole-pyrryl methene which Jennings[17] obtained from acetylviolacein. The maximum theoretical yield of methene from 0.2gm. of acetylviolacein is, if only one of the C_{21} molecules undergoes the transformation 0.068gm. whilst a yield of 0.125gm. was reported.

It would thus appear that methylation in the trimethyl ether operates in some way against the formation of a methene.

When an attempt was made to degrade the trimethyl ether with zinc dust and alkali it was found that the insolubility of the ether in alkali prevented the reaction from proceeding. However, by adding an ethanolic solution of the ether to the zinc dust and alkali, reaction proceeded smoothly and the trimethyl derivative of the C_{20} acid was obtained in good yield. This established at once the position of the three methyl

groups in this trimethyl ether, two being present in the N-methyl-5-methoxy indole system and one in the N-methyloxindole.

The High Melting Dimethyl Ether.

The high melting dimethyl ether is obtained as the insoluble product from the methylation of violacein. Filtration of the methylation reaction mixture results in the dimethyl ether and the potassium carbonate being left as residue and this when washed free from potassium carbonate leaves the dimethyl ether as a black micro-crystalline solid.

This ether has none of the solubility properties found in the trimethyl ether and is very sparingly soluble in all the common solvents except pyridine. It is rather more soluble in pyridine but does not crystallise out from the solvent. Its solubility in the common solvents is rather less than that of violacein and when an attempt to recrystallise it from acetone using a Soxhlet apparatus was made, a method which had proved successful with violacein, very little material was extracted and none separated from the solution.

The ether, like violacein, does not melt and decomposes slowly above 320°C. It is totally insoluble

in water, sodium bicarbonate and sodium carbonate solutions. It is also insoluble in cold caustic soda solution but in hot caustic soda solution it dissolves to give a red coloured solution similar to that formed when violacein is dissolved in hot alkaline solution. It is insoluble in dilute acids but gives an orange-red solution with concentrated sulphuric acid. On heating with sodium acetate/acetic anhydride, the high melting ether acetylates to form a diacetyl-dimethyl compound which is blue-black crystalline solid with a green reflex. This acetyl derivative though still very sparingly soluble in organic solvents can be recrystallised from acetic anhydride in the form of needles after prolonged treatment with the boiling solvent.

The behaviour of the dimethyl ether on attempted propionylation is rather interesting and is not at present completely understood. When heated with propionic anhydride and sodium propionate at 130°C the ether forms a propionyl derivative which is a blue crystalline solid with a red reflex and which is very sparingly soluble in organic solvents. On heating with the same reagents at 170°C (the boiling point of propionic anhydride), two propionyl derivatives are formed. One is the derivative,

previously described formed at the lower temperature whilst the other is a blue crystalline solid which is very soluble in all the common solvents and can be crystallised from ethyl acetate. When crystallised from benzene this compound retains solvent of crystallisation which depresses the melting point to 254°(d) because both propionyl derivatives when free from solvent do not melt below 320°C.

An attempt to form a p-nitrobenzoate of the dimethyl ether with p-nitrobenzoyl chloride in pyridine resulted in the return of unchanged material.

When degraded with hydrogen iodide in acetic acid, the dimethyl ether was found to yield a dark red crystalline solid. This material was found to have a similar ultra-violet and infra- red spectrum to the indole-pyrryl-methene obtained from violacein. It dissolved readily in alkali to give a yellow solution which did not become green and only darkened very slowly thus exhibiting very similar behaviour to the methylated indole-pyrryl-methene. This material was not obtained in sufficient yield to enable it to be converted to the hydrochloride and analysed but it was considered to be a derivative of the same indole-pyrryl-methene as that

obtained from violacein.

On degradation with zinc dust and alkali, the dimethyl ether gave a colourless acidic material. This material was shown to be a derivative of the C_{20} acid because its ultra-violet and infra-red spectra were similar to the spectra of the latter and further more it gave on acetylation a magenta coloured non-acidic acetate. Unfortunately this acid could not be crystallised but on methylation with dimethyl sulphate and alkali the trimethyl and tetramethyl derivatives of the C_{20} acid were formed in a similar manner to their formation by methylation of the C_{20} acid.

THE ACTION OF ALKALI ON VIOLACEIN.

The action of alkali on violacein was first investigated by Wrede[12] who showed that the pigment dissolved in caustic soda solution to give initially a bright green solution which later changed to red after passing through a transitory phase. He then found that addition of CO_2 to this red solution precipitated a microcrystalline red solid whilst addition of mineral acid to the colourless filtrate resulted in the formation of a yellow solid. This solid was found to be rather unstable and rapidly changed to a much darker colour. Acetylation of the red and yellow solids, Wrede claimed, gave rise to the same crystalline acetyl derivative, namely acetylviolacein. He thus suggested that the two compounds were the keto and enol forms of either a lactone or lactam.

With the discovery that violacein actually consists of two C_{21} molecules it was decided to reinvestigate this reaction with alkali in the hope that it might possibly lead to separation of the two entities. Whilst such a separation has not at present realised, a preliminary study of this reaction has produced some rather

interesting results.

It was first shown that violacein on mild treatment with alkali and subsequent addition of CO_2 and mineral acid led to the production of the red microcrystalline solid in rather larger amounts than the yellow solid. On somewhat more drastic treatment, refluxing the alkaline solution for periods of up to 45 minutes subsequent treatment with CO_2 and later mineral acid produced the yellow solid in the larger amounts.

The yellow solid if filtered quickly and washed well with water could be obtained as a deepyellow powder but if allowed to remain for a short while in the acid medium rapidly darkened and appeared to have changed to a blue black solid. On solution in organic solvents however, th is solid gave wine coloured solutions.

The red solid on methylation with dimethyl sulphate potassium carbonate in acetone whilst giving an initial red solution changed slowly to purple and an insoluble methyl derivative which had an infra-red spectrum identical with the spectrum of the high melting ether was obtained in a yield of 65mg. from 100mg. of the red solid. The other product from the methylation (25mg.) was soluble in acetone, benzene and most of the

common solvents and thus exhibited similar behaviour to the trimethyl ether from violacein. It was not however obtained in the crystalline form and not identified as this particular methyl derivative.

On acetylating the red solid with sodium acetate/acetic anhydride a dark red crystalline acetate with a green reflex was obtained. This acetate has an infra-red spectrum identical with that of acetylviolacein and must therefore be considered to consist of a mixture of the tri-acetate of the $C_{21}H_{15}N_3O_3$ molecule and the tetra-acetate of the $C_{21}H_{13}N_3O_4$ molecule.

When the yellow solid was acetylated by the sodium acetate/acetic anhydride method a bright red mixture was obtained from which a bright red crystalline acetyl derivative separated. It was found also that when the dark solid obtained from this unstable yellow material was acetylated in a similar manner, the same bright red crystalline material was obtained. This suggests that the yellow material and the darker material are closely related, possibly as tautomeric forms. This acetyl derivative was shown not to be acetylviolacein, because it had a completely different infra-red spectrum.

It must be quite different in character from acetyl-violacein and is probably formed as a result of some fundamental change having occurred. The nitrogen analysis of this compound confirms this view, and suggests that degradation of the C_{21} molecules has occurred with loss of a nitrogen atom. If this is so, then a degradation similar to that leading to the formation of the C_{20} acid could quite possibly have occurred resulting in the loss of a carbon atom and the formation of the same compound from both C_{21} molecules.

In an attempt to obtain a clearer insight into any changes which might occur during this degradation, the behaviour of the two methyl ethers when treated with alkali was studied. Because of the nature of these derivatives, it was not possible to investigate the effect of mild alkali and only their behaviour when refluxed in alkaline solutions for periods up to one hour was studied. It was found that both these ethers after refluxing with alkali for one hour, gave red solutions which on acidification with mineral acids deposited unstable yellow solids. These solids, like the one obtained from violacein darkened rapidly to solids which gave magenta coloured solutions in organic solvents.

The material obtained from the trimethyl derivative was heated with sodium acetate/acetic anhydride and a red reaction mixture obtained. On cooling, this deposited a red crystalline solid with a bright green reflex. This solid, like the one similarly obtained from violacein, could be recrystallised from benzene and in the infra-red contained bands which also appeared in the spectrum of the latter. The nitrogen analysis of this compound suggested that the trimethyl derivative had undergone degradation with loss of nitrogen and the acetyl analysis showed that no acetyl groups were present. The loss of a nitrogen atom on refluxing with alkali would seem to suggest that a degradation similar to that producing the C_{20} acid has occurred and that the yellow solid is possibly a precurser of this acid which on standing in acid solution undergoes some change leading to the formation of a conjugated system. A band at 1764cm^{-1} in the infra-red spectrum of this compound obtained from the trimethyl derivative, which was originally thought to be due to the carbonyl stretching frequency in an acetyl group must be due to some newly formed carbonyl group. Such a group could quite possibly be that found in a lactone system formed as a

result of an intramolecular cyclisation.

Thus, although at present the evidence is not very strong, it does suggest that the yellow compounds obtained from violacein and the methyl derivative are acids formed by degradation of these molecules with loss of ammonia. Further these acids appear rather unstable and revert to some more stable conjugated system possibly by the formation of a lactone ring.

POSSIBLE STRUCTURAL FORMULAE FOR VIOLACEIN.

Prior to the investigation into the chemistry of violacein in these laboratories no structural formula was reported for the pigment. This was because none of the previous workers had been able to identify a major degradation product. Any suggestions as to the nature of the molecule were made solely as a result of colour reactions. Thus Tobie[13] in 1935, from one such reaction claimed to have obtained an indication of the presence of anthranilic acid and suggested that violacein is a derivative of indigo. From the empirical formulae put forward by Kögl he knew that the violacein molecule was considerably larger than the molecule of indigo and therefore suggested that the indigo molecule was attached to certain large substituents.

The first workers to consider possible structures for violacein were Beer and Clarke[14] after the identification of a number of products from oxidative and pyrolytic degradation. These degradation products were divided into two distinct classes:-

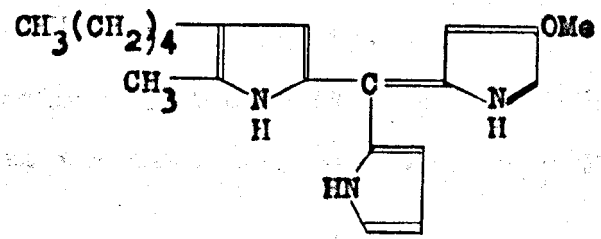
(i) Oxindole, acetylanthranilic acid and isatin.

(ii) 5-Hydroxyindole, 5-acetoxy-N-acetylanthranilic

acid and 5-hydroxy-N-acetyl anthranilic acid. All the members of one class were considered most probably to have been derived from the same nucleus in the violacein molecule. It was considered that the oxindole and 5-hydroxyindole nuclei were present as such in the original molecule and that the other members of each class were derived from the respective indole nucleus.

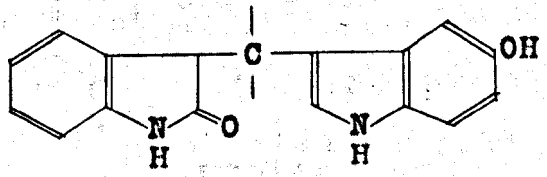
The isolation of the oxindole nucleus from violacein is extremely interesting because it was the first reported example of the isolation of this nucleus from a natural product. It has been shown more recently that the oxindole system does occur in certain species of alkaloids.

Clarke thought that several modes of linkage of the two nuclei could be envisaged but thought it interesting to consider the example of prodigiosin which has been investigated by Wrede[32] and assigned the structure (XLV).



(XLV)

He then suggested that an analogous arrangement in the case of violacein would result in its formulation as a tri-indolyl-methene (XLVI).

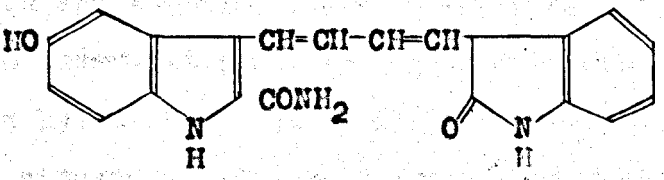


(XLVI)

Clarke next considered the reductive alkaline degradation of violacein and commented that the production of a basic gas suggested the presence of two amide or imide groups within a C_{42} molecule. Furthermore the fact that the oxidation products contained only one carboxyl group in the benzene ring was considered to imply that the amide groups are attached to the pyrrole ring in the 5-hydroxyindole nucleus or in the unknown part of the molecule.

Clarke then discussed the question of the molecular formula and noted that whilst the analytical data favoured the C_{42} structure the isolation of a "C_{21} acid" during the mild alkali treatment substantiated the view that the molecule is in fact only C_{21}. He attempted to incorporate the oxindole nucleus, the 5-hydroxyindole

nucleus and one amide grouping into a C_{21} structure and proposed the following formulation:-



(XLVII)

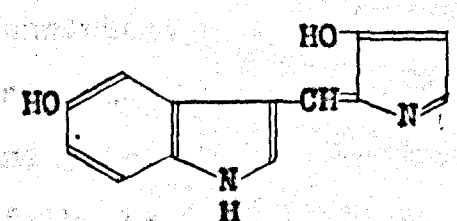
This compound, containing half an indigoid chromophoric grouping and also a conjugated side chain, Clarke considered, would be highly coloured. The formation of a leuco compound on mild reduction, he suggested, could be envisaged, as can that also of all the oxidation products.

On the basis of the above structure reductive alkaline hydrolysis would be expected to result in the formation of C_{21} acid plus a molecule of ammonia. When violacein is degraded in this manner, the acid obtained however has been shown to be only a C_{20} molecule, the formation of which would be difficult to explain on the above formula.

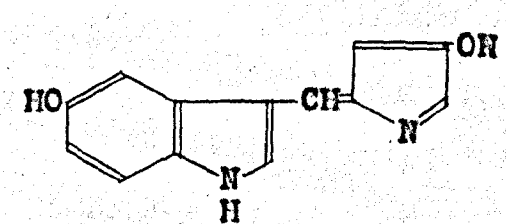
The next workers to suggest possible structural formulae for violacein were Boggiano[18] and Jennings[17] who were engaged on investigations of the structures of

two major degradation products. Jennings studied the product obtained from the hydrogen iodide degradation of violacein and suggested probable structures whilst Bo Boggiano undertook an investigation into the reductive alkaline hydrolysis product, the C_{20} acid and put forward certain structures to represent this molecule. Both workers then suggested the same structural formula to represent violacein after having discussed and reconciled thefformation of both degradation products from this structure.

From his investigation of the properties of the product $C_{13}H_{10}N_2O_2$ obtained from acetylviolacein with hydrogen iodide, Jennings suggested that this compound was an indolylpyrryl-methene a type of compound which had not been previously described. He thought that the most probable structures for this product were:-



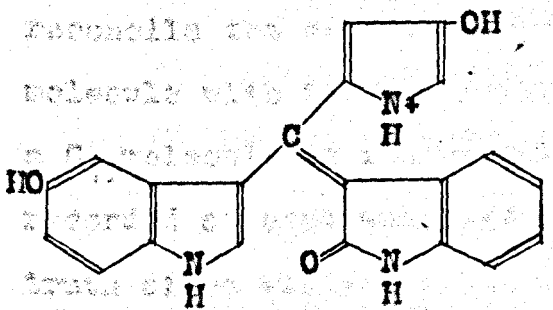
(XLVIII)



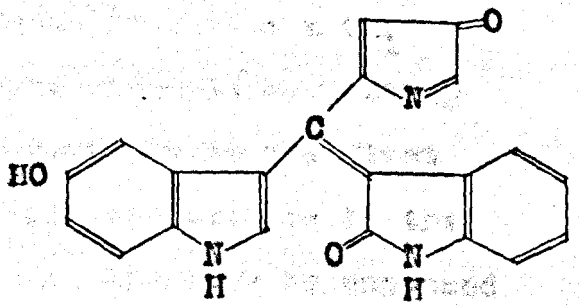
(III)

and also considered the presence of a β-hydroxypyrrylmethene in violacein.

In a paper describing this work of Jennings[17] it is pointed out that the β-hydroxypyrrylmethene system in (XLVIII) and (III) suggested the possibility that violacein may be a 5-hydroxyindolylhydroxypyrryloxindolymethene (eg.XLIX) $C_{21}H_{15}O_3N_3$ or an oxidised form of this eg. (L) $C_{21}H_{13}O_3N_3$ and that it had not been overlooked that these recalled the tripyrrylmethene structure (XLV) assigned to the bacterial pigment prodigiosin.



(XLIX)



(L)

It was also noted that structures of this type are consistent with the results of oxidative degradations and would account for the formation of 5-hydroxyindole and oxindole on pyrolysis and of an indolylpyrrylmethene of the type (XLVIII) or (III) with hydriodic acid.

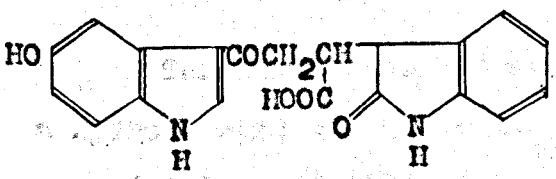
It was suggested that the empirical formula

$C_{42}H_{28}N_6O_7$ put forward by Wrede for violacein could perhaps be accommodated by supposing the crystalline pigment to be a molecular complex of two C_{21} molecules, eg. $C_{21}H_{15}N_3O_3$, $C_{21}H_{13}N_3O_3H_2O$. Further discussion of possible structures was however reserved until it had been found possible to interpret satisfactorily the results of the reductive alkaline hydrolysis investigated by Boggiano and later by the author.

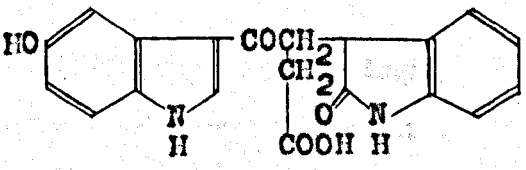
Although this type of formulation (XLIX) (L) is now known to be incorrect, the attempt to try and reconcile the degradative evidence favouring a C_{21} molecule with the analytical data strongly suggesting a C_{42}molecule is interesting because it is the first recorded attempt and does actually approximate to the truth since violacein has now been shown to be composed of two distinct C_{21} molecules.

The investigation of the reductive alkaline hydrolysis product of violacein by Boggiano soon showed that this compound was not a C_{21} acid as Clarke had suggested but a C_{20} acid of molecular formula, $C_{20}H_{18}N_2O_5$ or $C_{20}H_{16}N_2O_5$. He demonstrated the presence of the oxindole and 5-hydroxyindole nuclei in the acid and allowing for the requirements of the carboxylic group

accommodated the 5th oxygen atom as either a keto group or an alcoholic group. If the presence of a keto group was assumed he considered that the acid must be a γ-keto acid and suggested the two structures (I) or (II) as being the most probable.

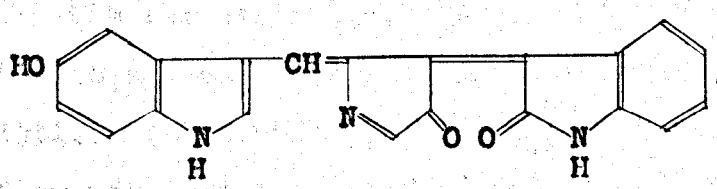


(I)



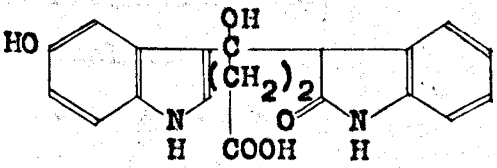
(II)

Boggiano and Jennings attempted to reconcile the formation of the methene (III) with these two structures from the C_{20} acid. They considered that the structure (II) could not be reconciled with the formation of the methene because the carbon atom to which the oxindole nucleus is attached would in the violacein structure be quaternary and would not allow the representation of a conjugated molecule. They therefore selected the structure (I) as being most probable for the acid and formulated the structure (LI) for violacein as the only one which could give rise to γ-keto acid and retain an indole-pyrryl-methene residue containing a β-hydroxy pyrrole residue.

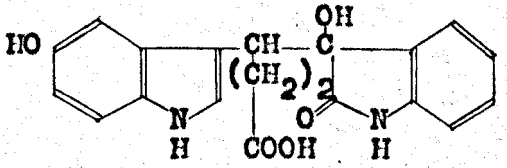


(LI)

Turning to the possibility of the C_{20} acid being a hydroxy acid the two most probable formulae suggested were (LII) and (LIII).

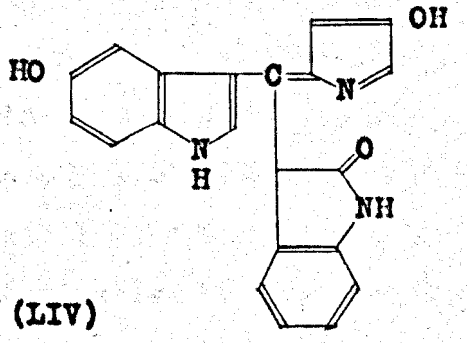


(LII)



(LIII)

The formulation of an acid of this type together with the indole pyrrole methene system was imagined to have been possibly derived from a structure (LIV) resembling that of prodigiosin (XLV.



(LIV)

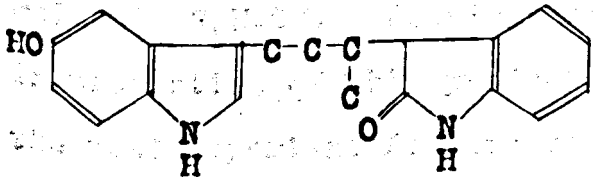
$C_{21}H_{15}N_3O_3$

The structures suggested both referred to C_{21} molecules and although both workers commented on the analytical data favouring the $C_{42}H_{28}N_6O_7$ molecule neither was able to reconcile this with conflicting degradational evidence.

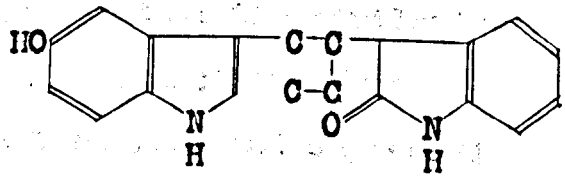
During the present researches it has been shown that violacein is an equimolecular mixture of two C_{21} molecules $C_{21}H_{15}N_3O_3$, $C_{21}H_{13}N_3O_4$, and the problem of reconciling the degradation evidence with the analytical data for violacein and acetylviolacein has been resolved.

The work on the C_{20} acid has shown that it is actually a γ-keto acid and not a hydroxy acid. Further more since the keto group has been shown to be present in a β-acyl indole system the oxindole nucleus cannot be attached to the same carbon atom as the 5-hydroxy indole nucleus. This immediately eliminates the possibility that violacein might be a di-indolyl-pyrryl methene and cannot therefore be of the same type as prodigiosin (XLV). The C_{20} acid has been shown to have one of the structures (I) or (II) and since the methyl derivatives of both $C_{21}H_{15}N_3O_3$ and $C_{21}H_{13}N_3O_4$ give rise to a methyl derivative of the same C_{20} acid both these

molecules must contain the partial structure (LV) or (LVI).



(LV)



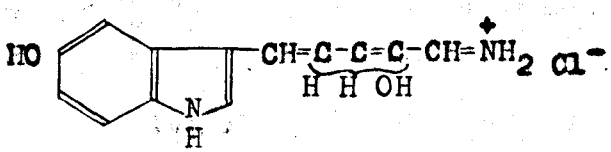
(LVI)

It is felt advisable at this stage before suggesting possible structural formulae for the two molecules present in violacein to discuss in more detail theoretical aspects concerning the structure of the hydrogen iodide degradation product investigated by Jennings, because this same product must be obtained from both the $C_{21}H_{15}N_3O_3$ and $C_{21}H_{13}N_3O_4$ molecules present in violacein.

The experimental evidence accumulated by Jennings for the hydrogen iodide product $C_{13}H_{10}N_2O_2$ suggests as probable structures (XLVIII) and (III) but does not allow an unequivocal decision in favour of these two structures to be made. The presence of the 5-hydroxy-indole system as such in this compound had been previously demonstrated by Beer when he obtained 5-hydroxyindole itself from a high vacuum sublimation of

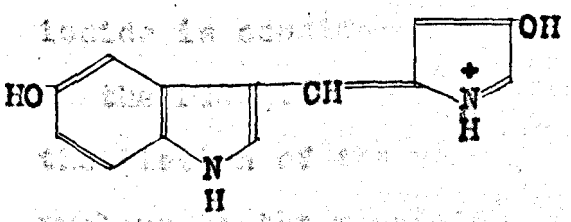
the product and the problem resolved itself into assigning the correct structure to the remaining part of the molecule C_5H_3ON. Jennings considered that the similarity in properties of this product to dipyrrylmethenes was the most important feature of its behaviour and suggested that in this remianing part of the molecule the carbon and nitrogen were readily accounted for as a methene bridge and a pyrrole nucleus. He thus put forward for this compound a tentative indole-pyrryl methene structure and did not consider any alternative formulations. It is now proposed to consider all possible structural formulae for this product and examine each critically.

The properties of this hydrogen-iodide product clearly indicate that it contains a structure easily capable of accepting a proton and forming a highly resonating cationic system. Furthermore this basic nature exhibited in other reactions indicated that the oxygen atom was not present in an amido system. These properties might possibly be contained in a straight chain structure of the type (LVII)

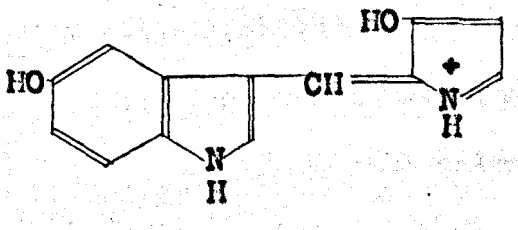


$C_{13}H_{12}N_2O_2$ HCl

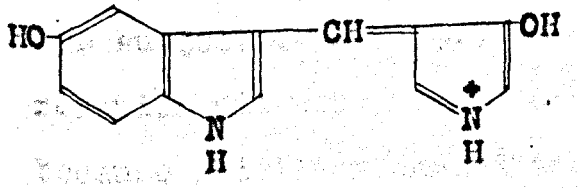
but the hydrogen content in such a structure is greater than that demanded by the analysis of the hydrogen iodide degradation product. The structure of the latter must therefore contain a second cyclic system of either five or six members since a ring containing less than five members is most unlikely on the grounds of stability. This degradation product must therefore be represented by one of the following formulae



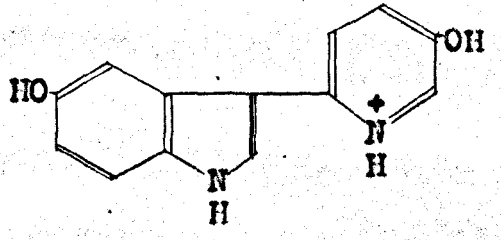
(III)



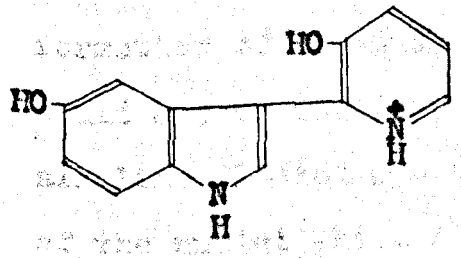
(XLVIII)



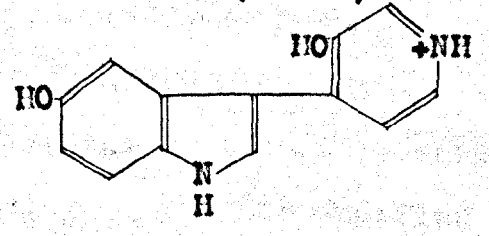
(LVII)



(LVIII)



(LIX)

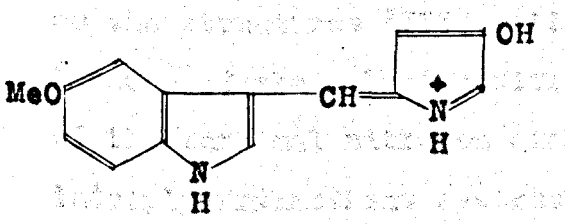


(LX)

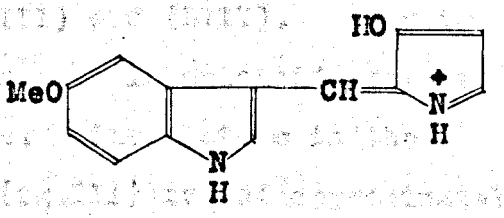
The possibility of the second nucleus being an α or γ hydroxy pyridine has not been overlooked but these systems like α-hydroxypyrroles have been shown to exist mainly in the amide form and exhibit properties which are not consistent with the behaviour of the degradation product. It is considered that this second cyclic system must be either a β-hydroxy- pyrrole or β-hydroxy-pyridine. The nature of the degradation with hydrogen - iodide is considered to be rather more complex than that of the reductive alkaline hydrolysis because it involves the fission of the carbon-carbon bond linking the oxindole nucleus to the remaining part of the molecule. Also in the case of the $C_{21}H_{13}N_3O_4$ molecule the loss of an oxygen atom is involved. When the hydrogeniodide product was subjected to reductive alkaline hydrolysis, the reaction did not proceed as in the case of violacein because acidification of the alkaline medium resulted in the formation of deep red colour attributed to the formation of a highly resonating cationic system. It would appear thus that this nucleus is stable to reductive alkaline fission and must be a considerably modified form of the nuclei which are present in violacein and from which it was derived. It is possible that this distinction in

properties may be due to the splitting off of the oxindole residue and conversion of the molecule to one of true aromatic character but it may be due to the formation of an essentially different system. Jennings and Boggiano having considered the most probable structure of this product to be the indolylpyrrylmethene (III) also inferred that this system is present in violacein. Although this may be so the above considerations regarding the nature of this degradation make it necessary to discuss other possibilities. The reaction might for instance proceed through the formation of a straight chain intermediate which after the fission of the oxindole residue then recyclised to form a new ring system containing one more or one less member than had originally been present.

The experimental evidence accumulated at present does not serve to distinguish between the structures (III) and (XLVIII) (LVII-LX) considered possible for the degradation product but the results of the methylation experiment are interesting. On methylating the degradation product with dimethyl sulphate and alkali, Jennings obtained a monomethyl derivative, which was thought most probably to be represented by (LXI) or (LXII) because on general grounds the hydroxyl



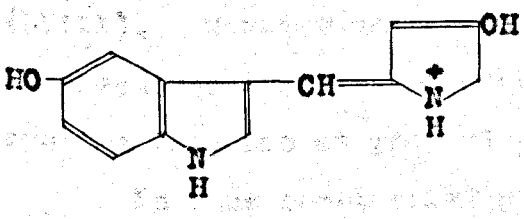
(LXI)



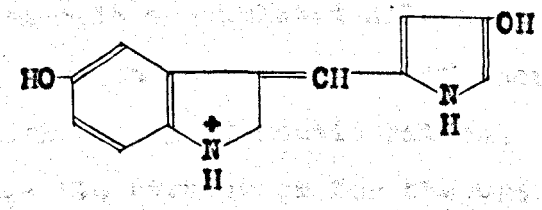
(LXII)

groups in the indole residue was expected to be preferentially methylated. The hydroxyl group in the second nucleus may therefore be one which resists methylation with these reagents. Because the hydroxyl group in a β-hydroxy pyrrole has been shown to resist methylation by any of the known methods whilst β-hydroxpyridine forms a methyl ether quite readily, suggests rather the presence of the former nucleus in the hydrogen iodide product and hence the formulation of a methene. This evidence thus tends to operate in favour of the structures (III) (XLVIII) (LVII) and against (LVIII-LX). Because also of the similarity to dipyrrylmethenes and other systems such as the cyanine dyes the methene formulation would appear the more probable. The resonating system involving the direct joining of the two heterocyclic nuclei as in the structures (LVIII-LX) is not described nearly.so frequently and might not on theoretical grounds be expected to be as highly coloured

as the structures (III), (XLVIII) and (LVII). This is because of the relative differences in anionoid activity of the terminal nitrogen atoms. These atoms in the indolylpyrrylmethene systems (eg.III) are of approximately equal basicity and when a positive charge is acquired the molecule will resonate between the two mesomeric forms (III) and (LXLLL) which will make approximately equal contributions.

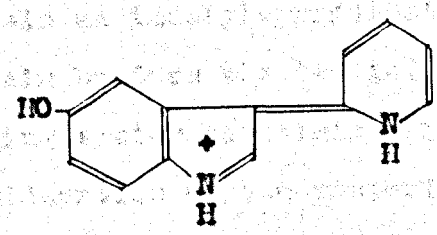


(III)



(LXIII)

However in the indolyl-pyridyl systems (eg.LVIII) the two nitrogen atoms are of different basicity, pyridine being a stronger base than indole. Any positive charge gained will thus tend to remain on the pyridine nitrogen atom and contribution from the mesomeric form (LXIV) will be small.



(LXIV)

The indolyl-pyridyl structures (LVIII-LX) will thus absorb at higher frequencies and will not be as highly coloured as the indolylpyrrylmethenes. Since the hydrogen iodide degradation product has been shown to form a highly coloured cation it would suggest that the two nitrogen atoms are of approximately equal basicity and would therefore favour the indolepyrryl-methene formulation (eg.III) rather than the indolyl pyridyl (LVIII). However until evidence is accumulated allowing an unequivocal decision to be made in favour of one of these systems they are at present both worthy of consideration.

In thus considering possible structures for the two molecules present in violacein and attempting to reconcile from these the formation of the two major degradation products, three distinct approaches must be employed. The C_{21} molecules may be considered:-

1. To consist of an open chain structure to which the two indole nuclei are attached.

2. To contain an indolyl-pyrrylmethene structure.

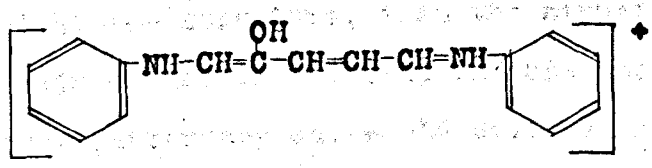
3. To contain besides the two indole nuclei a third heterocyclic ring system containing six members.

On consideration of the properties of these molecules

and on grounds of general stability the accommodation of the C_5H_3NO and C_5HNO_2 residues in a cyclic system of other than five or six members is thought unlikely.

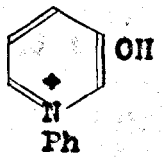
When an open chain formulation is considered for the C_5H_3NO and C_5HNO_2 residues present in the violacein molecules it is found that all structures which may be written for these molecules contain either a -C=N or -CH=NH group. The presence of such a group in the violacein molecules must be considered rather improbable because of the ready susceptibility to attack by acids reagents of these groups in contrast with the relative stability of violacein to such reagents.

Although conjugated chains involving -CH=NH linkages can be stabilised to some extent by addition of a proton and formation of a resonating system, as in the Stenhouse dyes[33], in the majority of cases the nitrogen atoms are attached tp an aromatic or substituted aromatic residue (LXV)

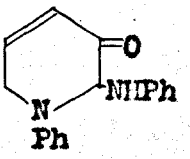


(LXV)

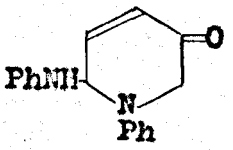
In systems such as these the open chain molecule is not particularly stable, and cyclises to the six membered ring system (LXVI) when heated in glacial acetic acid or alcoholic solution with loss of a molecule of aniline. On treatment with alkali, the resonance is largely destroyed and the open chain system cyclises to one of the systems (LXVII) or (LXVIII).



(LXVI)

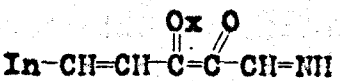


(LXVII)



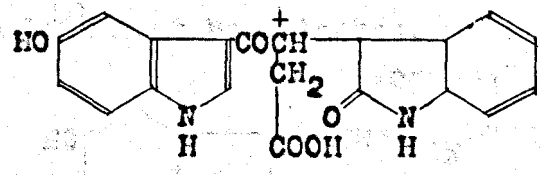
(LXVIII)

The presence therefore of an open chain system such as(LXIX) for the violacein molecule $C_{21}H_{15}N_3O_3$ must be considered unlikely.



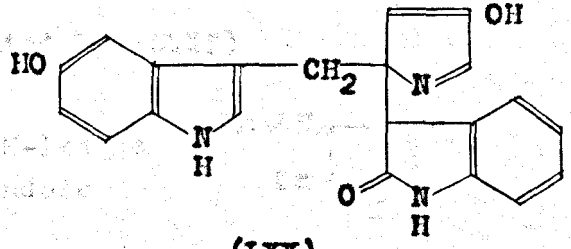
(LXIX)

If the violacein molecules are considered to contain an indole-pyrrylmethene structure, then the structure (II) for the C_{20} acid cannot be possible because the carbon atom C^+ becomes quaternary and would destroy conjugation throughout a large part of the molecule.



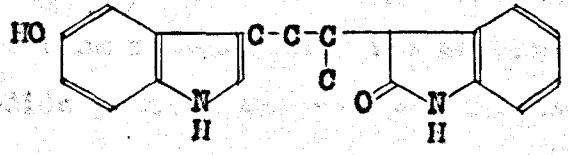
(II)

Only a structure (LXX) could then be written for the violacein $C_{21}H_{15}N_3O_3$ molecule, and this would not be expected to be coloured.



(LXX)

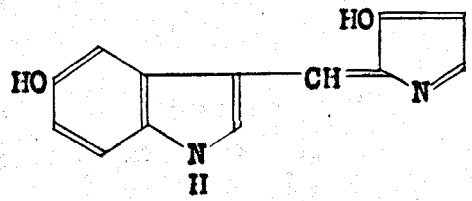
Any proposed structure for the violacein molecules must therefore explain the formation of the C_{20} acid (I) and possess a carbon skeleton (LV).



(LV)

The methene (XLVIII) thus will not be considered for the hydrogen-iodide product because it contains the

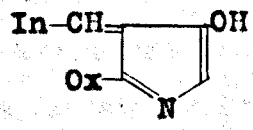
hydroxyl group attached to the carbon atom on which the oxindole nucleus must be attached.



(XLVIII)

Further the only type for the $C_{21}H_{15}N_3O_3$ molecule which can be reconciled with the formation of the methene (LVII) is represented by (LXXI)

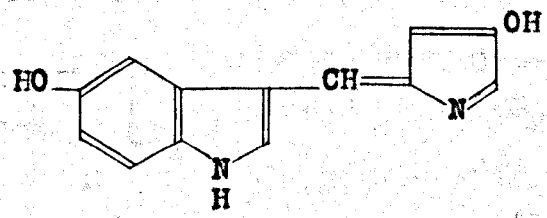
In = 5-OH-Indole
Ox = Oxindole



(LXXI)

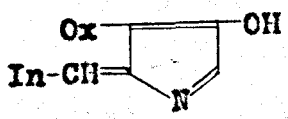
This type of structure cannot however give rise to the C_{20} acid (I) and therefore the methene (LVII) cannot represent the structure of the hydrogen iodide product.

There thus remains only the methene (III) for the hydrogen iodide product and reconciliation of this structure



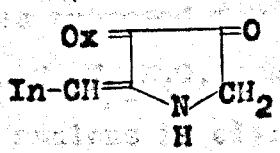
(III)

with the structure (I) for the C_{20} acid necessitates a structure of the type (LXXII) for the $C_{21}H_{15}N_3O_3$ violacein molecule.

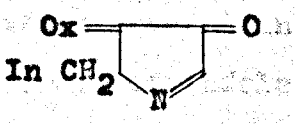


(LXXII)

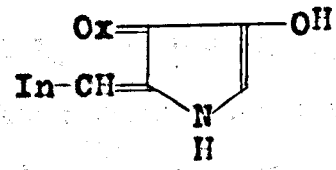
The molecular formula $C_{21}H_{15}N_3O_3$ does not allow the representation of a fully conjugated structure if three heterocyclic nuclei are considered to be present. The totally conjugated structure (LI) proposed by Boggiano and Jennings requires a molecular formula $C_{21}H_{13}N_3O_3$ but although it is not possible at present to rule out such a formula the analyses all favour the $C_{21}H_{15}N_3O_3$ molecule. It is thus possible to accommodate the extra two hydrogen atoms at different centres in the molecule and this allows other isomeric and tautomeric forms to be written, such that the molecule $C_{21}H_{15}N_3O_3$ may be represented by any of the following structures (LXXII-LXXVI)



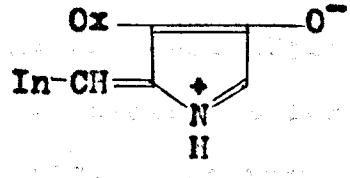
(LXXIII)



(LXXIV)

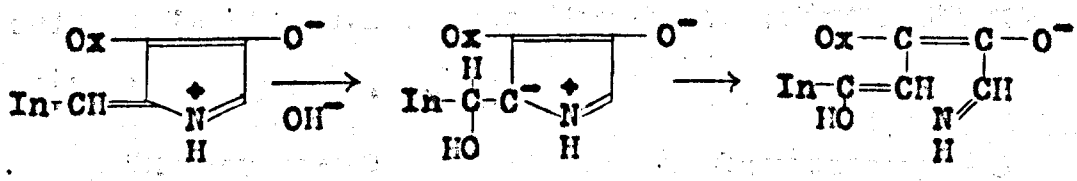


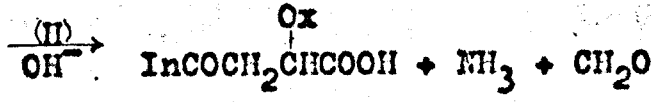
(LXXV)



(LXXVI)

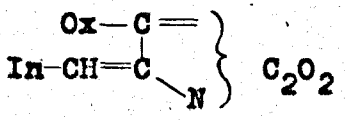
Whilst the formation of an indole pyrryl methene from these structures can be envisaged by a fission of the bond joining the oxindole nucleus to the remaining part of the molecule, the formation of an acid of the structure (I) is very much more difficult to explain. In such a degradation the methene system (-CH=) is transformed into a carbonyl (-C=O) group in a β-acyl indole system. A probable mechanism for such a transformation might approximate to the following scheme:-





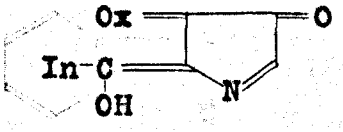
On the basis of an indole-pyrryl methene, any structures proposed for the other molecule present in violacein $C_{21}H_{13}N_3O_4$ must contain the 5-hydroxyindole or oxindole nucleus in either the respective indolenine or

oxindolidene form because the number of hydrogen atoms required by the 5-hydroxyindole, and oxindole nuclei and the methene carbon atom would otherwise account for the total hydrogen content of the molecule. The partial structure (LXXVII) cannot thus appear in structures for this molecule, because the pyrrole ring system cannot be

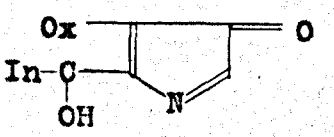


(LXXVII)

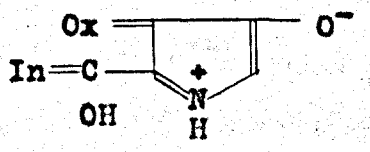
constructed without the presence of at least one hydrogen atom. Further there remain only two sites for the accommodation of the extra oxygen atom; either it must be attached to the methene carbon atom or to that carbon atom which is eliminated during the formation of the C_{20} acid. In the former case structures (LXXVIII-LXXX) might be suggested,



(LXXVIII)

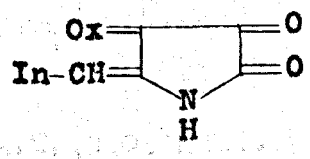


(LXXIX)

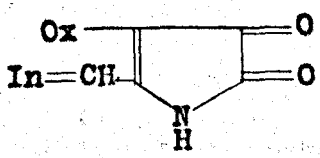


(LXXX)

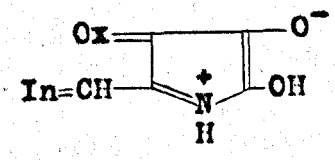
whilst in the latter, the structures (LXXXI - LXXXIII).



(LXXXI)



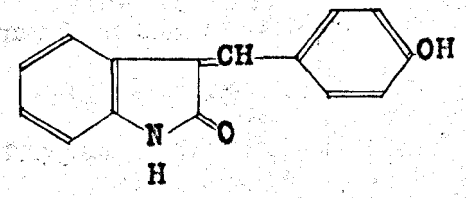
(LXXXII)



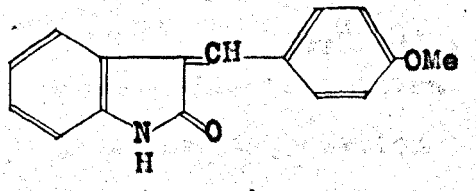
(LXXXIII)

The fact that only a dimethyl ether is formed when this molecule is methylated is very probable due to one of the indole nuclei existing permanently in either the indolenine or oxindolidene form.

It has been shown by Jennings that when p-hydroxy-benzylidene oxindole (LXXXIV) is methylated in a similar manner to that used to methylate oxindole, a mono-methyl derivative p-methoxy benzlidene oxindole (LXXXV) is formed.



(LXXXIV)



(LXXXV)

It is not known why the nitrogen atom in oxindole does not methylate; it may be due to a solubility effect

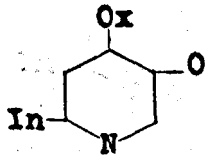
but could also be due to the presence of the oxindole system linked to a further conjugated system.

The extra oxygen atom is known to introduce into the $C_{21}H_{13}N_3O_4$ molecule an extra acetylateable group because on acetylation of the dimethyl ether a diacetate is formed. In the formulae considered (LXXVIII-LXXXIII) the extra oxygen group can be imagined to form an acetate either by direct acetylation when present in an enolic system or by enolisation followed by acetylation when present in a pyrollidone system. α-Hydroxypyrroles have been shown on acetylation to form α-acetoxy derivatives.

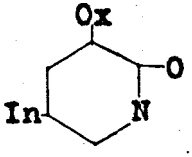
The accommodation of the extra oxygen atom on the methene carbon atom (LXXVIII-LXXX) certainly allows a simpler interpretation of the formation of the C_{20} acid to be made. It can be imagined that during the degradation, this enol form will change to the keto form and lead to the production of the β-acyl indole system in the C_{20} acid.

During the hydrogen iodide degradation this extra oxygen function must be reduced because the degradation product is the same as that obtained from the $C_{21}H_{15}N_3O_3$ molecule. The reduction of a α-hydroxy group in a pyrrole nucleus by hydriodic acid has been shown to occur during the degradation of the bilirubin pigments[34].

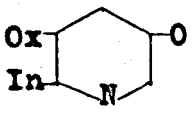
Consideration will now be given to the third possibility that of the presence of a six membered heterocyclic nucleus in the two molecules composing violacein. The partial structures (LV) and (LVI) which are known to be contained in these molecules allow only four partial formulae to be written (LXXXVI-LXXXIX).



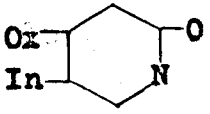
(LXXXVI)



(LXXXVII)



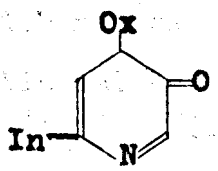
(LXXXVIII)



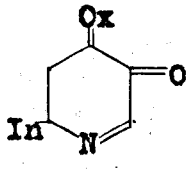
(LXXXIX)

The structures (LXXXVII) and (LXXXIX) are not however consistent with the formation of a hydrogen-iodide degradation product in which a cyclic amide system is not present nor is the loss of one carbon atom during the reductive alkaline hydrolysis likely on these structures and they will not be further considered.

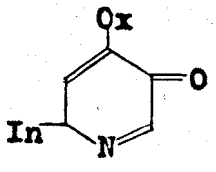
As previously discussed the molecular formula $C_{21}H_{15}N_3O_3$ does not allow the presence of a totally conjugated molecule in which three heterocyclic system are present and therefore based on the partial structure (LV), several isomers and tautomeric forms may be written for this molecule (XC-XCV)



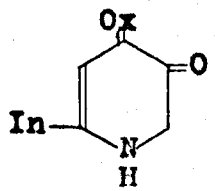
(XC)



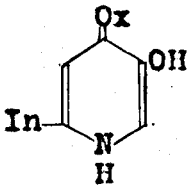
(XCI)



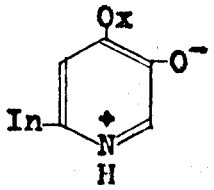
(XCII)



(XCIII)

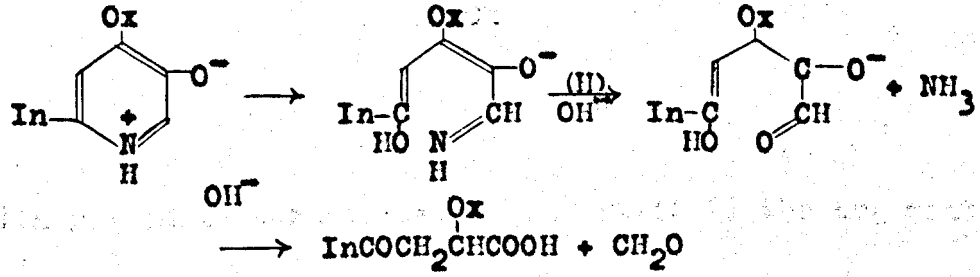


(XCIV)

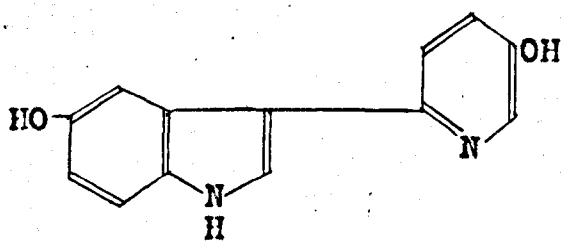


(XCV)

The formation of the C_{20} acid of structure (I) is more readily explained on any of the above formulations than in the case of the structures containing an indole pyrrylmethene system. Although the mechanism of this reaction may be at the moment rather vague it may not be altogether dissimilar from the formation of γ-keto acids by decomposition of pyridazinones with alkali and may be illustrated by the following tentative scheme.

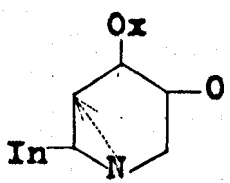


With regard to the formation of the hydrogen iodide product this mat be considered to occur either by fission of the oxindole carbon-carbon bond and formation of a system (LVIII),

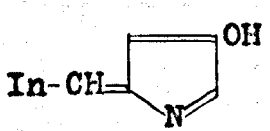


(LVIII)

or by the formation of an intermediate



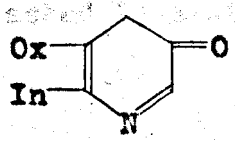
which after the oxindole nucleus had been detached, reverted through a 1:2 shift to an indole-pyrryl methene structure (III).



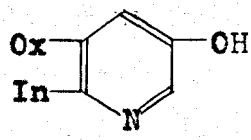
(III)

With regard to the partial structure (LVI) the arguments

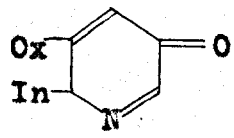
which prevented its adoption in the case of the five membered system do not in this case apply and structures for the $C_{21}H_{15}N_3O_3$ molecule which might arise from it are listed (XCVI-CI).



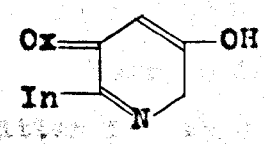
(XCVI)



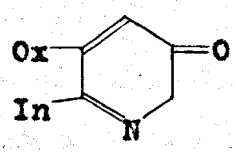
(XCVII)



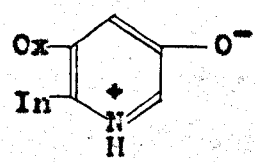
(XCVIII)



(XCIX)



(C)

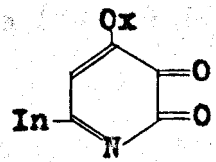


(CI)

The formation of the C_{20} acid of structure (II) can be explained on the above structures and the mechanism can be imagined to be the same as that previously described for the alkaline degradation of the six membered heterocyclic system. Also the formation of the hydrogen iodide product from one of the above structures is similar to that previously discussed for the structures (XC-XCV).

Upon considering the $C_{21}H_{13}N_3O_4$ molecule on the basis of the six membered heterocyclic system the arguments presented in the case of the indole pyrryl methene systems

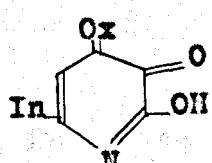
necessitating one of the indole nuclei to assume either the respective indolenine or oxindolidene form do not necessarily apply. A structure (CII) can be written for this molecule in which the indole and oxindole nuclei are both attached by a single carbon-carbon bond.



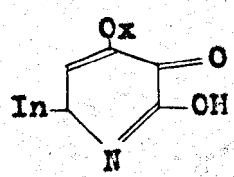
(CII)

However in all the other structures which may be written for this molecule the oxindole or indole nucleus has to revert to a modified form. In this system there are two carbon centres to which this extra oxygen function may be attached the more probable one being the carbon atom which is eliminated during the formation of the C_{20} acid (eg.CIII).

It is found that several structures can be written to represent this molecule, the most probable ones being (CIII) and (CIV).

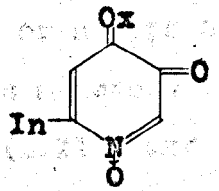


(CIII)

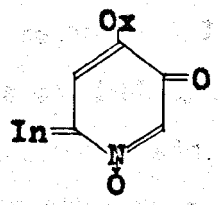


(CIV)

However it must be remembered that the nitrogen atom in pyridine can donate electrons to form a co-ordinate bond with an oxygen atom leading to the formation of a pyridine oxide. If the extra oxygen atom were accommodated in such a system, in this molecule, it would lead to the structure (CV) or (CVI).



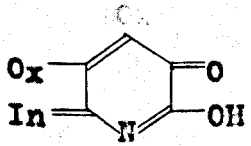
(CV)



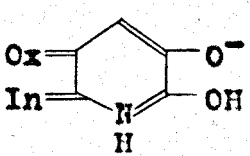
(CVI)

These oxides form 2-acetoxy derivatives when heated with acetic anhydride/sodium acetate and this would account for the presence of an extra acetyl group.

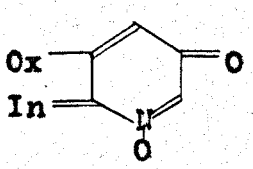
On the basis of the partial structure (LVI) the extra oxygen atom in $C_{21}H_{13}N_3O_4$ can likewise be accommodated in three different positions in the ring and a number of structures may be written. The most probable structures for this molecule are considered to be (CVII-CIX).



(CVII)



(CVIII)



(CIX)

This section may now be summarised:-

The two C_{21} molecules present in violacein give rise to the same degradation products and must be considered to have the same fundamental structure. This structure has been shown to contain a 5-hydroxyindole and an oxindole nucleus together with either a type of pyrrole or a type of pyridine system. In the former case the molecule will contain an indolyl pyrryl methene system (LXXII) and in the latter the two indole nuclei are attached directly to a pyridine system (eg.XC). Whilst the hydrogen iodide degradation product (III) is best explained in the basis of the structure containing the indolyl pyrryl methene, the C_{20} acid (I) is more easily explained when a pyridine nucleus is postulated.

EXPERIMENTAL

GROWTH OF CHROMOBACTERIUM VIOLACEIN AND EXTRACTION OF THE PIGMENT.

Revitalization of the Strain.

During the researches on violacein in these laboratories the medium found to give the best results (Clarke), was one containing glycerol-difco proteose peptone. Because of the aerobic nature of the organism, growth takes place entirely at the surface of the medium and a solid agar slope, 1.5cm in depth was found to give the best yield. Further more with regard to maintaining a good yield it is necessary to maintain a healthy strain of the bacterium. Thus when it was considered necessary the strain was revitalized in the following way.

Growth on Potato.

A slice of potato 1cm. in thickness and circular in shape was cut to fit into the base of a Petri dish. It was first sterilised in the autoclave and then inoculated from a stock culture. It was allowed to stand in a room maintained at 25°C for an incubation period of 36 hours and then transferred to the violacein room where it grew in the dark at room temperrrure. After some five to six days the surface of the potato was covered with the deep

shining purple pigment. The more vigorously growing colonies of this revitalised strain were selected to inoculate test tube slopes or petri dishes.

Preparation of the Petri Dishes and Test Tube Slopes.

The stock cultures and working slopes of the bacterium were kept on either test tube slopes or Petri dishes and were renewed at frequent periods.

The medium was composed of :-

Difco proteose peptone	1%
Glycerol	0.5%
Agar	1.8%
Tap water	ad 100%,

and was prepared in the following way. Agar (1.8gm.) was finely powdered and added to a solution of difco proteose peptone (1gm.) and glycerol (0.5gm.) in water (100ml.).

The medium was heated until all the agar had dissolved when it was poured into test tubes in 5ml. portions and each test tube plugged with cotton wool. The tubes were then sterilized in a steam autoclave at 140°C and at a pressure of two atmospheres for thirty minutes and then sloped at an angle of 10-20° until the agar had solidified.

For the preparation of Petri dishes the medium after

solution of the agar was poured into the base of each dish to a depth of 1cm. and each cover then placed in position. After sterilising in the autoclave as described, each dish was set aside to cool.

The slopes and Petri dishes were usually allowed to stand for two or three days, until any surface moisture had evaporated from the agar surface, before being inoculate from a healthy stock culture. They were inoculated by removing a small portion of a healthy bacterial colony from the potato culture by means of a sterile wire and spreading this over the surface of the medium. The slope was then plugged after flaming the plug and/or the dish, covered, and then incubated at 25°C for 2/3 days. At the end of this period songle colonies appeared and the more vigorous of these were removed and transferred to test tube slopes or Petri dishes as described. The surface of the slope became heavily pigmented after 3-4 days, and growth reached a maximum after 10 days. During these researches it was found that in general, test tube slopes were more satisfactory than Petri dishes and a constant supply of healthy slopes were maintained throughout the work and used as stock cultures and as working slopes for the second stage of

the growth procedure (vide infra).

Broths for Inoculation of Trays.

The bacterial mass from a test tube slope was brought into suspension by flooding the slope with sterile water and breaking up the surface growth with a sterile needle. This suspension was then used to inoculate Roux bottles each containing 150ml. of 1% difco-proteose peptone, 1% glycerol solution which had been sterilized in the autoclave. Each slope was found to be sufficient to inoculate four or five bottles and forty such bottles were inoculated in one period. The bottles were maintained at a temperature of 25°C and were ready for inoculation on a larger scale after a fortnight.

Large Scale Growth of Violacein on Trays.

The growth of violacein on trays was evolved by Clarke and Parker towards the end of their researches in this department and its success depends upon the fact that the organism is aerobic and strongly growing, reaching maximum degree of pigmentation after ten to twelve days. The method was developed by Boggiano and was that first used by the author for the production of the pigment.

The bacterium was grown on trays in a room which is

maintained in a reasonably sterile condition by frequent spraying with a bacteriostatic agent (thymol 2%, glycerol 2½%, in 50% alcohol). The room used for growing violacein, "the violacein room" is entered through two doors which form an air lock. In an effort to make the room as contamination proof as possible the air inlets and ventilation grills are plugged with cotton wool whilst the inner door is fitted with a rubber seal. The room contains no windows and is maintained in total darkness during the growth of the pigment. The trays used are of white enamelled metal measuring 53cm. x 40cm. and when inoculated are covered with similar inverted trays to act as covers. In one batch thirty-six trays were usually employed and two days before they were due to be flooded with the medium, were placed in position and the room thoroughly sprayed, with the bacteriostatic agent. The spraying was repeated the next day and on the day of flooding.

The medium used is:-

Difco proteose peptone	10gm.
Glycerol	20ml.
Agar	18gm.
Tap Water	to 1 litre.

Seventy two bottles, each containing 1 litre of this

medium were prepared and after sterilisation in the steam autoclave at 130°C were quickly transported, in two batches of thirty-six bottles, to the violacein room. The contents of each bottle were poured, whilst still hot, onto the previously flamed trays; each tray receiving two litres of this medium and then being covered with an inverted tray which had just previously been flamed.

As soon as the agar was considered to have hardened sufficiently, which was usually after four hours, the medium was inoculated. The reasons for this were:-

(1) The trays were still slightly warm and this helped to incubate the organism.

(2) Any possible contamination had less chance to begin growing.

The trays were inoculated from broths which had been growing for 14 days and one broth was used for each tray. The covers of the trays were removed, the necks of the bottles flamed and their contents poured onto each tray. The broth was spread evenly over the surface of the medium by gently tilting the tray and after allowing the organism to settle for about ten minutes, the excess liquid was carefully decanted off. The violacein room was then locked for three days and after this time was sprayed at regular

intervals until the harvesting of the pigment began.

The Harvesting and Extracting of Violacein.

After twelve days, growth of the pigment was considered to have reached its maximum and harvesting operations were then carried out. The moist deep-violet layer of the bacterial mass was carefully removed from the agar surface, by means of a broad spatula, and placed in small portions upon layers of filter paper, to dry. The mass took from five to six days at 30°C before it was considered sufficiently hard and dry to be ground in a mortar. The powdered mass was then extracted in a large Soxhlet apparatus with acetone for six to seven days after which time the extracts were running a very pale purple and negligable amounts of the pigment were being extracted. Most of the violacein (5-6gm.) crystallised out during the extraction as heavy granular crystals with a greenish sheen and was filtered off at intervals during the extraction to prevent violent bumping occurring. The violet filtrate was concentrated to 500ml. and glacial acetic acid (20ml.) followed by water (1.5-2L) added. The dark semi-solid lipoid material which was precipitated was allowed to stand for several days, during which time it slowly hardened and was then filtered. This material

which consisted of amorphous violacein mixed with a considerable quantity of lipoid material was allowed to dry before being defatted. The defatting process consisted of treatment of the violacein, wrapped in filter paper and placed in a Soxhlet thimble, with petroleum ether (40-60°C), chloroform and carbon tetrachloride for two days with each solvent. The precipitated material yielded amorphous violacein (1.5gm.) after defatting by this procedure.

Towards the end of his researches, Boggiano introduced an improved method of sterilization and preparation of the solid medium used on the trays. The method eliminated the necessity of transporting the bottles of hot medium and the rather unpleasant process of pouring the steaming medium on to the trays in a closed room.

An air tight container was designed and constructed from aliminium angle-bar and sheeting, so as to accommodate forty-six trays, stacked horizontally in two compartments. The external dimensions of this container were such (3'9" x 1'9" x 2'6"), that it could be run off a trolley which was used to transport it, into the autoclave. The trays set in position in the container, were charged with the medium, agar suspended in the difco proteose-peptone

glycerol solution, from a large carboy and the sliding panel closed. The container was then run off its trolley into the autoclave, care being taken to prevent undue splashing of the liquid, and the medium sterilised in the manner previously described. After being allowed to cool down in the autoclave overnight, the container was removed on to its trolley and transported to the violacein room. Boggiano terminated his researches at this stage and thus did not appreciate the difficulties which were encountered by the author and his co-worker C.B.Barrett in attempting to grow violacein by this method.

The most serious difficulty which arose was the inoculation of the trays prepared in this manner. Upon pouring the broths on to these trays it was perceived that the agar surface was not in the same horizontal plane as the bench, having set in a different horizontal position in the autoclave. As a result, the broth would tend to collect in one corner of the tray and although considerable attempts were made to spread the inoculum evenly over the whole surface, they were never completely satisfactory. Thus it was found that on only a few trays did the violacein layer completely cover the surface and on the majority, areas on which no violacein grew, appeared.

After some days there appeared evidence of some other growth on these areas which later began to contaminate the violacein. Many attempts to overcome this difficulty were made, the amount of broth used per tray was varied, the size of the particles of violacein present in the broths was varied, by different degrees of shaking, the time allowed for the broth to remain on the tray was varied but in none of these cases was the result satisfactory. Attempts were then made to inoculate the trays whilst they remained in the container, which was, by the use of thick paper wads, maintained in the same horizontal position as it had been in the autoclave. These resulted in distinct improvement in the growth and more trays were completely covered, but other difficulties which arose made the general procedure rather unsatisfactory. Amongst these, was encountered a contamination which occurred on some of the trays when too many were stacked in the container for charging. It was found that when more than twenty-five trays were used this contamination could not be prevented and seemed to be due in some way to incomplete sterilization, brought about by the increased amounts of metal and volume of medium, which the larger number of trays necessitated. Unfortunately an increase in the time

allowed for sterilization brought about slow hydrolysis of the agar with a result that the medium did not harden. Furthermore when more than twenty-five trays were used, many trays were found to be unsatisfactory for inoculation purposes because they contained too little agar and therefore did not set solid. This was due to the difficulty encountered in handling the large volume of medium which more than twenty trays involved and in particular ensuring complete homogeneity of the agar suspension. Because of this the medium on the last trays charged, contained insufficient agar to produce a solid medium suitable for inoculation purposes.

It was also found that Boggiano had not allowed sufficient clearance in the design of the width of the container, for its smooth transfer into the autoclave. Some difficulty was encountered in moving the fully charged container into the autoclave which invariably resulted in considerable splashing of the medium over the inside walls of the container. The container was thus dismantled and rebuilt two inches less in width when it fitted into the autoclave quite smoothly and with no less of medium.

From these experiences it was thus considered that for the preparation of twenty trays containing the medium

and suitable for the growth of violacein, the use of the trolley and container was distinctly superior to the old method. Nevertheless it was appreciated that the inoculation procedure was not completely satisfactory and that some new method of inoculation was necessary if the introduction of the container was to be overall superior to that previous method used for production of pigment.

The author and his co-worker C.B.Barrett thus investigated the growth of violacein on these trays after they had been inoculated from test tube slopes with a sterile needle and found that the method though quite tedious was highly successful, in that the violacein grew extremely strongly. The number of test tube slopes required to inoculate twenty trays was clearly far too great to make the method practical whilst the actual inoculation with a needle was considered to take too long a period to represent any improvement on previous methods. The first difficulty was overcome by substituting for the test-tube slopes, pyrex dishes measuring 23cm x 16cm x 3cm which were fitted with sealed covers. These were charged with 250ml. of the medium, sterilized in the autoclave and after cooling, inoculated from a test tube slope. The violacein grew extremely

well in these dishes and after ten to twelve days, contained sufficient to permit them to be used to inoculate the trays. It was also found that by using small pads of cotton wool which had been sterilized in a flame, the violacein from these dishes could be transferred quickly on to the trays in a very thin film and that one dish provided sufficient violacein to inoculate approximately ten trays. The trays inoculated by this method grew extremely well and yields of violacein were the same as reported by Boggiano.

Thus the technique for the growth of violacein without the use at any stage of liquid cultures was evolved and is the one at present used to grow the pigment. Instead of growing violacein in batches of thirty-six trays per fortnight it is grown in batches of twenty trays per week. The whole procedure now becomes healthier and far less time consuming than the old method whilst still producing the same quantities of violacein. The method demands that the violacein which is grown in the dishes must be healthy and strongly growing and in order to ensure this, the medium used in the dishes frequently contained water in which potatoes had been boiled.

REDUCTIVE ALKALINE DEGRADATION OF VIOLACEIN.

(Cf.Boggiano Ph.D.Thesis, Liverpool,1953.)

Crystalline violacein (1.0gm.) was mixed with twice its weight of zinc dust and the mixture gently heated under reflux in an atmosphere of nitrogen with 100ml. of 2N caustic soda solution. The violacein on dissolving, gave a brilliant green solution and if the mixture was continuously shaken, the intensity slowly decreased as the reduction occurred until finally a pale green colour was obtained. If however, the mixture was not continually shaken, the initial green colour changed to a bright red and after a short time the solution became brownish-yellow. The heating was continued for ¾ hour after which time the flask was cooled in a stream of cold water. The alkaline solution was decanted from the zinc which was washed with a little water. The combined liquids were then neutralized with concentrated hydrochloric acid pH 6.5-7, and filtered from the resulting zinc salts. Acidification of the yellow filtrate caused the precipitation of the pale yellow acid which was then allowed to ripen in the refrigerator. After filtration, the acid was washed with water and dried in an exsicator. The yield of pale yellow solid varied

depending on the purity of the violacein used but an average yield of 700mg. was obtained. The acid was recrystallised by dissolving in dry acetone (200ml.), concentrating to 75ml. and adding petroleum ether (40-60°C) until a faint cloudiness occurred. Small colourless needles M.P. 252-254°(d) separated on cooling. Yield-500mg.

The best yield of crystalline acid obtained from a number of such degradations was 660mg.

METHYL ESTER OF THE C_{20} ACID USING DIAZOMETHANE.

The C_{20} acid (120mg.) was dissolved in methanol (25ml.) and to the solution, added an excess of ethereal diazomethane. After standing for 30 minutes, a drop of acetic acid was added to destroy excess diazomethane and the solvent removed, under reduced pressure. The residue was triturated with sodium hydrogen carbonate solution and the solid filtered off. It was then washed with water and dried in an exsicator, 130mg.

The solid was dissolved in pure dry ethyl acetate and the solution chromatographed on a neutral alumina column, ethyl acetate being used as the only eluent. The solution was reduced to 15ml. and petroleum ether (60-80°C)

carefully added. This caused the ester to crystallise slowly as colourless needles.

Yield. 100mg. M.P. 257°C.

	N	CMe
Found	7.45	8.43
$C_{20}H_{16}N_2O_5 + CH_2$ requires	7.45	8.20

Infra-red Spectrum.

Bands appear at:-

1695cm^{-1} (carbonyl stretching frequency in oxindole system)
1605cm^{-1} (carbonyl stretching frequency in acylindole system plus oxindole ring system)

TRIMETHYL DERIVATIVE OF THE C_{20} ACID.

The C_{20} acid (250mg.) was dissolved in 2N caustic soda solution (17ml.) and the solution warmed on the steam bath in an atmosphere of nitrogen. Dimethyl sulphate (2.5ml.) was added in 0.5ml. portions over a period of 45 minutes, the mixture being thoroughly shaken at frequent intervals, during this period. The solution was then cooled and acidified with hydrochloric acid. The white precipitate of the trimethyl derivative was filtered off, washed well with water and dried in an exsicator 250mg.

Unchanged C_{20} acid was removed by washing with cold

acetone and the trimethyl derivative recrystallised from acetone as colourless plates. M.P.266-7°(d).

Above 240°C the crystals gradually assume a red colour and finally decompose to a deep red liquid.

	C	H	N
Found	67.7	5.5	6.8
$C_{20}H_{16}N_2O_5 + 3CH_2$ requires	68.0	5.4	6.9

Infra-Red Spectrum.

Bands appear at:-

1730cm^{-1} (carbonyl stretching frequency in carboxyl group)

1670cm^{-1} (carbonyl stretching frequency in oxindole group)

1639cm^{-1} (carbonyl stretching frequency in acylindole)

1613cm^{-1} (oxindole ring system).

TETRAMETHYL DERIVATIVE OF THE C_{20} ACID.

The C_{20} acid (1gm.) was dissolved in 2N caustic soda solution (30ml.) and the solution maintained in an atmosphere of nitrogen. Dimethyl sulphate (7.5ml.) was added in three portions at 10 minute intervals with shaking, to produce homogeneity. After the last addition, the solution slowly became acidic and the tetramethyl derivative was slowly precipitated. After standing for

15 minutes, 2N caustic soda solution was carefully added with shaking and gentle warming until the solution remained alkaline. The tetramethyl derivative was then filtered off and alkaline solution maintained under nitrogen, again treated with dimethyl sulphate in 0.5ml. portions until the solution once more became acid and more of the tetramethyl derivative slowly separated out. Again after standing for 15 minutes 2N caustic soda solution was carefully added with shaking and warming until the solution remained alkaline, and the tetramethyl derivative filtered off. The combined residues were then washed well with water and dried in an exsicator. Yield. 900mg.

This material was then recrystallised from absolute alcohol in colourless needles. M.P.181°C.

Acidification of the alkaline filtrate with hydrochloric acid yielded 50mg. of the trimethyl derivative.

	C	H	OMe
Found	68.7	6.0	
$C_{20}H_{16}N_2O_5 + 4CH_2$ requires	68.6	5.7	14.7

Molecular weight determined by the X-ray diffraction method found to be 423.

$C_{20}H_{16}N_2O_5 + 4CH_2 = 420$

Infra-Red Spectrum.

Bands appear at:-

1736cm^{-1} (carbonyl stretching frequency in ester group)

1695cm^{-1} (carbonyl stretching frequency in oxindole group)

1631cm^{-1} (carbonyl stretching frequency in acylindole)

1610cm^{-1} (oxindole ring system)

ATTEMPTED ALKALINE DEGRADATION OF THE TRIMETHYL DERIVATIVE OF THE C_{20} ACID.

The trimethyl derivative of the C_{20} acid (100mg.) was heated under reflux in an atmosphere of nitrogen with 50% caustic soda solution (20ml.). The acid did not immediately dissolve but formed a brown mass on the surface. This solid did however, soon go into solution, and the heating was continued. After one hour the solution was cooled and acidified with concentrated hydrochloric acid. The pale brown solid which was precipitated was filtered off, washed with water and dried.

Yield. 80mg.

This material was recrystallised from acetone as small colourless plates. M.P.M.P.266°(d)

Mixed melting point with trimethyl derivative undepressed.

ACTION OF GLACIAL ACETIC ACID ON THE C_{20} ACID.

The C_{20} acid (100mg.) was gently refluxed with glacial acetic acid (5ml.), because after 5 minutes refluxing, all the solid had not dissolved, more acetic acid (5ml.) was added and on further heating a yellow-orange solution was obtained. After refluxing this solution for 15 minutes the colour was observed to have darkened to red. The red solution was allowed to stand over-night but no solid material separated. The addition of water to a sample of the red solution did not result in any precipitation of solid. The solvent was removed under reduced pressure and a dark brown residue was obtained which was dried in an exsiccator. The solid was readily soluble in acetone but only tarry material separated on addition of petroleum ether (40-60°C). After filtering off the tarry material which separated on three occasions, after addition of the petroleum ether, a further addition caused the separation of a creamy white solid (30mg.) M.P.248-250°C.

This solid gave a red colour with acetic anhydride and a mixed melting point with the C_{20} acid was undepressed.

ACTION OF ACETIC ACID AND HYDROCHLORIC ACID ON THE C_{20} ACID.

The C_{20} acid (100mg.) was dissolved in glacial acetic acid (10ml.) and the brownish red solution refluxed under an atmosphere of nitrogen. Concentrated hydrochloric acid (10ml.) was added to the solution which changed to a yellow - brown, but on gently refluxing for 30 mins. reverted to a brownish-red. The solvent was removed under reduced pressure and the tarry residue extracted with dry ether but the ether extracts contained no solid material. On extraction of the residue with dry acetone, a brownish red solution was obtained but no crystalline material separated. The addition of petroleum ether (40-60°C) precipitated only tarry material which could not be removed from the filter paper. The solvent was removed under reduced pressure and the residue dissolved in methanol and treated with ethereal diazomethane. After standing for 30 minutes a drop of acetic acid was added to destroy the excess diazomethane and the solvent removed under reduced pressure. The residue was triturated with sodium hydrogen carbonate solution and the solid filtered off (40mg.). This was recrystallised from acetone-petrol (40-60°C). M.P.251°C, mixed melting point with methyl ester of C_{20} acid undepressed.

A small amount of material was formed which did not dissolve in the acetone but which was very soluble in absolute alcohol giving a deep red solution. No crystalline material however could be obtained from this solution.

The C_{20} acid (100mg.) was heated on the steam bath with a mixture of glacial acetic acid and concentrated hydrochloric acid (1:1) (5ml.) under an atmosphere of nitrogen. The colour of the solution slowly changed from yellow to a yellow-red but the solid did not completely dissolve. After one hour, a further 1 ml. of the acid mixture was added when the remaining solid dissolved. The heating was continued for a further ½ hour when the mixture was set aside and left over-night. No solid material separated from the solution and the solvent was removed under reduced pressure, to yield a yellow-orange residue which contained dark tarry material and which was dried in an exsiccator. This residue was then dissolved in methanol (2-3ml.) and ethereal diazomethane added over a period of ½ hour. The solvent was removed under reduced pressure and the residue triturated with sodium hydrogen carbonate solution. After filtering, a solid (30mg.) was

obtained whilst addition of dilute acid to the hydrogen carbonate solution resulted in the precipitation of an orange-brown solid (50mg.). This acidic material was dissolved in methanol and excess ethereal diazomethane added over a period of one hour. On destroying the excess diazomethane, removing the solvent under reduced pressure and triturating the residue with sodium hydrogen carbonate a brown solid was obtained (35mg.). These two solids were independently dissolved in ethyl acetate and chromatographed on a neutral alumina column but in neither case could any crystalline material be obtained from the fractions obtained.

ACTION OF CONCENTRATED HYDROCHLORIC ACID ON THE C_{20} ACID.

The C_{20} acid (40mg.) was gently refluxed under an atmosphere of nitrogen with concentrated hydrochloric acid (5ml.). The solid which dissolved gave initially a yellow solution but this changed to a brownish red after ½ hour refluxing. The material which did not appear to have dissolved became very dark and appeared rather tarry. The mixture was left overnight and filtered when a yellow filtrate was obtained. Addition of water to this caused no precipitation whilst continuous ether extraction resulted only in gummy material (5mg.) being obtained. The residue

was triturated with sodium bicarbonate solution, a small amount remained insoluble but after filtration, addition of dilute hydrochloric acid caused the precipitation of a reddish material (20mg.). This solid was soluble in ethanol, methanol and acetone giving red solutions but could not be obtained in a crystalline form from these solvents. It was insoluble in petroleum ether (60-80°C), benzene, ether, and ethyl acetate and did not melt below 330°C above which it slowly charred. A further 5 mg. of this material separated from the neutralised bicarbonate solution on standing for 2 days.

PROLONGED ACTION OF CONCENTRATED HYDROCHLORIC ACID ON THE C_{20} Acid.

The C_{20} acid (100mg.) was heated under an atmosphere of nitrogen, with concentrated hydrochloric acid (15ml.) in an oil bath maintained at 130°C. The colour of the solution slowly darkened from yellow to red during the first hour. At the end of this period the temperature was raised tp 150°C and the heating continued for a further seven hours. On cooling, no solid appeared to separate and on filtering only a negligible amount of tarry material was obtained. 5ml. of the filtrate were removed under vacuum and water (5ml.) added. On standing overnight the solution deposited a

small amount (2-3mg.) of a black solid. Most of the solvent was then removed under vacuum and the mixture neutralised with caustic soda solution. It was then made slightly acid with dilute hydrochloric acid and on standing deposited a little more of the black material. The total amount of this material precipitated was 10mg. and had a melting point greater than 290°C. Further it could not be sublimed at 340°C at 0.4mm pressure.

Continuous ether extraction of the filtrate yielded traces of colourless material which could not be crystallised.

2:4-DINITROPHENYLHYDRAZONE OF THE TETRAMETHYL DERIVATIVE OF THE C_{20} ACID.

(Cf. Boggiano, Ph.D. Thesis, Liverpool, 1953)

The tetramethyl derivative (20mg.) was dissolved in ethanol (4ml.) and 0.6% 2:4-dinitrophenylhydrazine solution (1.5ml. 0.9mole) added. The solution was left to stand at room temperature when after a short time a deep red colour developed. On leaving overnight a deep red precipitate formed which was filtered, washed with alcohol containing a little hydrochloric acid and dried. The 2:4-dinitrophenylhydrazone was recrystallised from benzene/

petrol (60-80°C) as red microcrystalline prisms.

Yield. 20mg. M.P. 230°(d)

Ultra-Violet Spectrum.

λ max.	Log ε.	λ min.	Log ε.
281 mu	4.00	276 mu	3.95
319 "	3.91	299 "	3.81
431 "	4.30	352 "	3.70

This spectrum was measured in chloroform solution.

2:4-DINITROPHENYLHYDRAZONE OF N-METHYL-1,2,3,4-TETRAHYDRO-CARBAZOLE.

(Cf. Jennings, Ph.D. Thesis, Liverpool, 1953.)

The method used for the preparation of this compound was the same as that previously employed for the 2:4-dinitrophenylhydrazone of the tetramethyl derivative. This derivative was however less soluble in the common solvents and was recrystallised from glacial acetic acid as the deep red prisms. M.P. 297-8°(d).

Ultra-Violet Spectrum.

λ max.	Log ε.	λ min.	Log ε.
267 mu	4.0386	263	4.0349
307 "	3.85	295	3.77
436 "	4.35	345	3.29

This spectrum was measured in chloroform solution.

ATTEMPTED REDUCTION OF THE TETRAMETHYL DERIVATIVE OF THE C_{20} ACID WITH LITHIUM ALUMINIUM HYDRIDE.

The tetramethyl derivative of the C_{20} acid (1gm.) in benzene (50ml.) was added over a period of 45 minutes, with stirring, to a slurry of lithium aluminium hydride (1gm.) in ether (150ml.). After 10-15 minutes, a white precipitate appeared which seemed to increase in amount as more of the solution was added. The stirring was continued for one hour after all the benzene solution had been added, when the excess hydride was decomposed with moist ether. With continued stirring, 2N hydrochloric acid (25ml) was added and most of the solid went into solution. The ether benzene layer was separated from the aqueous layer which appeared cloudy, and set aside. The addition of water (20ml.) followed by 2N hydrochloric acid (5ml.) to the aqueous layer, caused it to become clear and it was then shaken with ether (3 x 50ml.), and the ether extracts added to the ether from the reaction mixture. The combined ether extracts were then washed with 2N hydrochloric acid (2 x 100ml.) followed by water (2 x 20ml.) and then dried over anhydrous sodium sulphate. The solvent was

removed under vacuum and left a gummy residue, (0,57gm.). Extraction of this gummy material with hot petrol (60-80°C) (2 x ml.) yielded only the minutest traces of oily material but on extracting with benzene petrol (60-80°) (1:1) the material quite readily dissolved. This solution was then chromatographed on a neutral alumina column, most of the material (350mg.) being eluted with the benzene-petrol (1:1) mixture. Several other smaller fractions were collected by eluting with benzene/petrol (3:2), benzene/ petrol (2:1), benzene/petrol (5:2), benzene, chloroform, ether, acetone and alcohol but it was not found possible to crystallize the material obtained from any of these fractions. The infra-red spectra indicated that they contained essentially the same material.

The hydrochloric acid washings from the combined ether extract, on neutralisation with 2N caustic soda solution and extraction with ether, yielded a gummy residue (220mg.). This material did not appear to be soluble in cold 2N hydrochloric acid.

The two residues were combined and dissolved in a mixture of benzene (100ml.) and ether (50ml.). To this solution was added slowly and with stirring a slurry of lithium aluminium hydride (2gm.) in ether (150ml.). A

white insoluble solid separated, as soon as the addition began, and appeared in increasing amounts as the addition continued. The hydride was run into the reaction mixture over a period of 1½ hours and the mixture stirred for a further ½ hour. The excess hydride was then decomposed with moist ether followed by a little water. Dilute hydrochloric acid (50ml.) followed by water (100ml.) was added to the mixture and on stirring, resulted in clarification of the aqueous phase. The ether/benzene layer was then separated and the aqueous layer extracted with ether (3 x 50ml.) followed by benzene (2 x 25ml.). The combined ether/benzene extracts were then washed with water (50ml.) which was then shaken with ether (10ml.) and the combined extracts dried over anhydrous sodium sulphate. On removing the solvent, under reduced pressure an amorphous solid (0.56gm.) was obtained which resisted all attempts to crystallise it.

An attempt to prepare a p-nitrobenzoate of this material was made but no crystalline material could be obtained.

After the second reduction, the infra-red spectrum was the same as the spectrum of the material after the first

reduction and it appeared that only the ester group had been reduced. The attempt to reduce the other systems appeared to have failed due to the formation of an insoluble complex of the primary alcoholic group with the lithium hydride.

ATTEMPTED OXIDATION OF TETRAMETHYL DERIVATIVE WITH SELENIUM DIOXIDE.

The tetramethyl derivative (130mg.) was dissolved in absolute alcohol (10ml.) and 50mg. of selenium dioxide added to the solution. The selenium dioxide dissolved and a colourless solution was obtained. The solution was refluxed for 14 hours during which time the colour slowly darkened to red but no metallic selenium was precipitated. On cooling the solution a white solid crystallised out.

Yield. 95mg. M.P. 181°C.

Mixed melting point with tetramethyl derivative undepressed. The mother liquor on standing yielded a further 20mg. of the tetramethyl derivative.

The tetramethyl derivative (100mg.) was dissolved in dioxan (4.5ml.) and selenium dioxide (40mg.) added to the solution. On refluxing, the solution soon became red and a

black solid was precipitated. After two hours the flask was cooled and the black solid filtered off. The filtrate was allowed to stand overnight but no solid material separated. On pouring into cold water (5ml.) however, a yellow solid was obtained.

Yield. 50mg.

This solid was soluble in benzene, acetone, ethyl alcohol but insoluble in petroleum ether, it resisted however, all attempts to crystallise it.

CONDENSATION OF THE TETRAMETHYL DERIVATIVE OF THE C_{20} ACID WITH HYDRAZINE.

The tetramethyl derivative of the C_{20} acid (200mg.) was dissolved in absolute alcohol (15ml.) and to the colourless solution was added 90% hydrazine hydrate (0.5ml.). The mixture was refluxed for 1½ hours and then left to stand overnight during which a white solid crystalline separated.

Yield. 180mg. M.P. 181°C.

The mixed melting point with tetramethyl derivative 181°C.

The above experiment was repeated using the same amount of tetramethyl derivative but adding 1ml. of 90% hydrazine hydrate. After refluxing for 2½ hours, the solution was allowed to stand overnight when once more a

white crystalline solid separated. A test portion confirmed the view it was unchanged starting material and the reaction mixture was refluxed for a further 8 hours and then allowed to stand overnight. The tetramethyl derivative again separated but in a much smaller quantity than on the previous occasion. The refluxing was therefore continued until no solid material separated on cooling. A total period of 28 hours was required and the solution during this period had acquired a yellowy-brown colour. The solution was evaporated under reduced pressure to 4ml. when a solid began to crystallise out in pale yellow needles.

Yield. 100mg.

This material proved to be very sparingly soluble in all the common organic solvents but was recrystallised from 95% alcohol in colourless needles.

M.P. 236-7°C.

To effect solution a large amount of solvent was required and this had to be heated for a long period. The solid crystallised when the bulk of the solution was reduced to about 5ml. A second recrystallisation raised the melting point to 239-40°C. It was shown later that the period of refluxing could be considerably shortened to give a

purer product.

A solution of the tetramethyl derivative (200mg. in 15ml. absolute alcohol) was refluxed with 90% hydrazine hydrate (1ml.) for sixteen hours. On cooling a white crystalline solid was obtained.

Yield. 100mg. M.P. 239-40°C.

A further 20mg. of a less pure product M.P. 220°C separated on leaving the mother liquor to stand.

This compound proved to be identical with that obtained by condensing the trimethyl derivative of the C_{20} acid with hydrazine out for the analyses, see page 182.

CONDENSATION OF THE TRIMETHYL DERIVATIVE OF THE C_{20} ACID WITH HYDRAZINE.

A mixture of the trimethyl derivative of the C_{20} acid (200mg.) 90% hydrazine hydrate (1ml.) and absolute alcohol (15ml.) was refluxed gently. After five minutes a colourless solution was obtained and this was refluxed for four hours, when a white solid began to separate. The refluxing was continued for a further 4 hour period after which no more solid appeared to separate. The mixture was cooled and filtered when a white crystalline solid was obtained.

Yield. 200mg. long colourless needles. M.P. 242°C.

Recrystallisation from 95% alcohol did not raise the melting point. This material proved identical with that obtained from the condensation of the tetramethyl derivative with hydrazine.

	C	H	N
Found	68.5	5.6	13.8
Formation of hydrazide or hydrazone, $C_{23}H_{24}N_4O_4$ requires	65.7	5.7	13.3
Formation of intramolecular cyclised compound $C_{23}H_{22}N_4O_3$ requires	68.6	5.5	13.9
Formation of hydrazide and hydrazone $C_{23}H_{26}N_6O_3$ requires	63.6	6.0	19.3

Ultra-Violet Spectrum.

λmax.	Log ε.	λmin.	Log ε.
266 mu	4.28	245 mu	4.18
280 "	4.26	275 "	4.23
328 "	4.30	292 "	4.01

Infra-Red Spectrum.

Bands appear at:-

3250cm (NH stretching frequency)
1705cm (carbonyl stretching frequency in oxindole)

1665cm (carbonyl stretching frequency in pyridazinone or C=N stretching vibration)
1605cm (oxindole ring system).

ACETYLATION OF THE C_{20} ACID AT LOW TEMPERATURE.

The C_{20} acid (100mg.) was dissolved in pyridine (3ml.) in a test tube suspended in an acetone/CO_2 bath maintained at -50°C and nitrogen bubbled through the solution. Acetic anhydride (0.5ml.) dissolved in pyridine (0.5ml.) was cooled in the same bath and this solution added to the solution of the C_{20} acid whilst a stream of nitrogen was still directed into the tube. The mixing of the solutions caused no change in colour to occur but did result in the separation of a white crystalline material. The test tube was carefully stoppered and allowed to remain in the acetone/CO_2 bath which was then placed in the refrigerator. The mixture was kept in the refrigerator for five days, during which time the colour had changed to red, when it was removed, filtered and washed well with water.

Yield. 65mg. mixture of colourless and red crystalline material. The mixture dissolved in benzene

on warming giving a red solution, which on cooling deposited a colourless solid.

Yield. 40-45mg. M.P. 235°C.

Recrystallisation from benzene removed a slight pinkish tinge but did not raise the melting point.

The original benzene solution on longer standing deposited a red material which under the microscope proved to be a mixture of red and white needles, 10mg.

Found	C	H	OAc
	60.12	5.21	33.79
	57.66	5.22	
	57.10	5.07	

The acetyl value would tend to suggest that this molecule is a tetra-acetate.

ACETYLATION OF THE TRIMETHYL DERIVATIVE OF THE C_{20} ACID AT LOW TEMPERATURE.

The trimethyl derivative of the C_{20} acid (100mg.) was dissolved in pyridine (3ml.) in a test tube suspended in an acetone/CO_2 bath maintained at -50/60°C and a stream of nitrogen bubbled through the solution. Acetic anhydride (0.5ml.) dissolved in pyridine (0.5ml.) was cooled in the same bath and this solution added to the solution of the trimethyl derivative. The mixture was shaken and whilst

a stream of nitrogen was directed into the tube, it was carefully stoppered and left in the acetone/CO_2 bath which was then placed in the refrogerator. The addition of the anhydride did not cause any change in the colour of the solution but after standing in the refrigerator for 4 days a magenta coloured solution was obtained but no solid had separated. When after 6 days no solid material had separated from the solution, it was removed from the refrigerator and poured onto ice and hydrochloric acid. A mauve coloured solid was precipitated which was filtered washed well with water and dried.

Yield. 100mg. M.P. 105-110°C.

This material was soluble in benzene giving a red solution which on standing deposited a colourless crystalline solid contaminated with a little dark red material. On refluxing this material with chloroform a reddish solution was obtained but the colourless material did not appear to be very soluble. Filtration yielded a colourless crystalline solid.

Yield. 30mg. M.P. 190°C (d).

The crystals were in the form of rhomboids and on melting decomposed to a red liquid. The benzene solution

on longer standing deposited crystals of the magenta acetate.

Infra-Red Spectrum.

Bands appear at:-

3333cm^{-1}	(O-H stretching frequency)
3106 "	
1779 "	(carbonyl stretching frequency vinyl acetate?)
1692 "	(carbonyl stretching frequency in oxindole ring)
1605 "	(oxindole ring system).

REACTION OF THE C_{20} ACID WITH BENZOYL CHLORIDE IN PYRIDINE.

The C_{20} acid (100mg.) was dissolved in pyridine (2ml.) and benzoyl chloride (0.5ml.) added to the solution. This addition caused the precipitation of solid material and pyridine (2ml.) was added to the solid mass which was continuously shaken. The solid slowly dissolved and a red coloured solution was obtained. After leaving overnight, this solution deposited a red coloured solid which was filtered, washed well with water and dried. The solid on closer examination was found to be crystalline athe crystals being in the form of neddles.

Yield. 80-85mg.

The compound was recrystallised from benzene as red needles with a copperish tinge.

M.P. 312°(d).

PURIFICATION AND RECRYSTALLISATION OF VIOLACEIN.

Violacein (300mg.) which had crystallised out during its extraction with acetone and which had been subsequently defatted with chloroform, was recrystallised from acetone by the use of a Soxhlet extractor. After three days approximately half the violacein had been extracted and had crystallised out from the acetone. This was filtered, dried and any fat impurities removed by extraction in a Soxhlet apparatus with chloroform for three days followed by carbon tetrachloride for a similar period. Both the chloroform and carbon tetrachloride remained quite colourless throughout both extractions. The violacein thus obtained was in the form of dark purple needles with a greenish sheen.

		C%	H	N
Found for violacein which was		68.97	4.06	
recrystallised from acetone		69.52	4.26	11.7
Dried 45mins. at 60°C		69.23	4.09	
Found for violacein left in Soxhlet apparatus not extracted by acetone, dried 30 mins.		67.47	3.71	
$C_{42}H_{28}N_6O_7$	"	69.23	3.85	11.5
$C_{21}H_{13}N_3O_3$	"	70.98	3.66	11.83
$C_{21}H_{15}N_3O_3$	"	70.59	4.20	11.76
$C_{21}H_{13}N_3O_4$	"	67.92	3.50	11.32
$C_{21}H_{15}N_3O_4$	"	67.56	4.02	11.26

Ultra-Violet Spectrum.

λ max.	Log ε.	λ min.	Log ε.
244 mu	4.12	238 mu	4.11
268 "	4.17	251 "	4.11
380 "	3.78	320 "	3.59
570-600 "	4.35	440 "	3.38

ACETYLATION OF VIOLACEIN.

Violacein (0.5gm.) was intimately mixed with anhydrous sodium acetate (0.5gm.) and the mixture gently refluxed for 15 minutes with acetic anhydride (25ml.). On leaving to cool, the mixture set semi-solid and the solid was filtered off, washed with acetic acid, then with water and dried. The acetyl compound was shown to have separated in dark red needles with a green reflex.

Yield. 0.4gm. M.P. 320°C. Begins to decompose slowly above 280°C.

The mother liquor on standing deposited a further 50mg. of the acetyl compound.

	C	H	N	OAc
Found for acetylviolacein dried in high vacuum 1mm. 16 hours dried to constant weight 130-140°C 30 mins.	65.67	4.18	8.59	29,10 (alkali) 30.00 (CrO_3/H_2SO_4)

	C	H	N	OAc
Found for acetylviolacein (same sample) redried at 180°C for 1hr. slight loss in weight (30 + 50)	66.22	4.41		
$C_{42}H_{28}N_6O_7 + 6\ (CH_2CO)$ requires	66.11	4.08	8.57	26.32
$C_{42}H_{28}N_6C_7 + 7\ (CH_2CO)$ "	65.75	4.11	8.22	29.45

Ultra-Violet Spectrum.

λ max.	Log. ε.	λ min.	Log ε.
233 mu	4.06	330 mu	3.10
366 "	3.32	420 "	3.00
555 "	3.72		

Infra-Red Spectrum.

Bands appear at:-

3268cm^{-1}	(N-H stretching frequency?)
3130cm^{-1}	
1761cm^{-1}	
1704cm^{-1}	
1667cm^{-1}	
1605cm^{-1}	(oxindole ring system).

ATTEMPTED REDUCTIVE ACETYLATION OF VIOLACEIN.

Violacein (300mg.) was gently refluxed with a mixture of glacial acetic acid (5ml.) and acetic anhydride (5ml.). Zinc dust (500mg.) was added in the boiling mixture and

after 20 minutes the colour of the solution changed from violet to a red-brown. The mixture was heated for two hours during which time the colour slowly lightened to yellow. The mixture was then filtered from the zinc and zinc acetate which had crystallised out and a brownish red solution obtained. On standing a little while the colour of this solution reverted to violet possibly due to aerial oxidation of a leuco compound. This solution on heating with zinc dust (100mg.) for 5-10mins changed again to a brownish red colour. It was quickly decanted from the zinc and poured onto a mixture of ice and dilute hydrochloric acid. This caused the immediate precipitation of a pale yellow solid which was filtered, washed and dried in an exicator.

Yield. 250mg. M.P. 220°C (d) violet solid formed.

This solid was soluble in the common organic solvents except petroleum ether but solutions of it acquired dark colours on standing or warming. It resisted all attempts to crystallise it and addition of petroleum ether (40-60°C) to a benzene solution caused the precipitation of a whitish material with a violet tinge.

Found	C	H	N
	63.66	5.08	8.87
	63.57	4.99	8.94

METHYLATION OF VIOLACEIN.

Pure crystalline violacein (200mg.) was intimately mixed with potassium carbonate (1gm.) and the mixture refluxed with acetone (100ml.) after dimethyl sulphate (0.3ml.) had been added. The mixture was refluxed for 12 hours, dimethyl sulphate (0.25ml.) being added after four and eight hours, and during this period the colour of the acetone solution changed from purple to royal blue. The mixture was allowed to cool and then filtered yielding a greyish coloured residue and a royal blue filtrate. The residue was washed continuously with warm water which dissolved the potassium carbonate and left a black micro-crystalline solid (80-85mg.). The filtrate was evaporated under reduced pressure almost to dryness, and water (20ml.) added. It was allowed to stand for ½ hour then filtered to yield a dark blue crystalline solid (90mg.). This solid was then extracted with hot benzene (30ml.) and filtered. The residue consisted of a small amount of the black material (5mg.) which was found to be insoluble in benzene. The benzene solution was reduced to 15ml. under reduced pressure and then passed down a neutral alumina column. The column was eluted with benzene until the major band

of the blue solid had been removed from the column. The benzene solution was evaporated under reduced pressure to 5ml. and set aside overnight when it deposited blue needles with a shiny black reflex (50mg.), on standing a further 25mg. separated.

Melting point of this material 126-127°C.

	C	H	N	OMe
Found for dark blue solid Crystallised from benzene M.P.127°C.	74.6	5.6		
Crystallised from ethyl acetate, M.P. 220°C.	69.9	4.9		
Crystallised from ethyl acetate and dried at 1mm 60°C for ¾ hour.	71.83 71.94	5.14 5.25	10.75 10.42	8.19
$C_{21}H_{15}N_3O_3 + 3CH_2$ requires	72.18	5.26	10.53	7.77

Ultra-Violet Spectrum.

λmax.	Log ε.	λmin.	Log ε.
270 mu	4.31	250 mu	4.26
382 "	3.88	333 "	3.65
600 "	4.29	452 "	3.34

Infra-Red Spectrum.

Bands appear at:-

1672cm^{-1}(carbonyl stretching frequency in oxindole)

1605cm^{-1}(oxindole ring system)

In 5% chloroform solution these bands appear at 1670cm^{-1} and 1603cm^{-1} respectively. A band of weak intensity also appears at 2970cm^{-1}.

Found for black micro-crystalline solid.	C	H	N	OMe
C H N O + 2CH				
requires	69.2	4.3	10.5	7.8

Infra-Red Spectrum.

Bands appear at:-

3185cm^{-1} (NH stretching frequency)
(1681cm^{-1} (carbonyl stretching frequency
1672cm^{-1}) in oxindole system)
1642cm^{-1}
1610cm^{-1} (oxindole ring system).

METHYLATION OF VIOLACEIN WITH METHYL IODIDE/POTASSIUM CARBONATE.

The methylation was carried out as in the manner described for the methylation of violacein with dimethyl sulphate. To replenish losses, methyl iodide was added

in 0.3ml. portions after two, four and eight hours refluxing. From violacein (100mg.) the two methyl ethers were obtained in approximately equal amounts.

ATTEMPT TO INTRODUCE MORE METHYL GROUPS INTO THE HIGH-MELTING ETHER BY CONTINUED METHYLATION.

The high-melting ether from violacein (100mg.) was intimately mixed with potassium carbonate (500mg.) and refluxed in acetone (100ml.) with dimethyl sulphate (0.5ml.) for thirty-six hours. During this period the colour of the solution which was purple did not change. The mixture was filtered and the greyish residue washed free from potassium carbonate to yield the original black micro-crystalline solid (70mg.). The purple acetone filtrate on evaporation to dryness yielded the same material (20-25mg.)

ATTEMPTED ACETYLATION OF THE TRIMETHYL DERIVATIVE OF VIOLACEIN.

The trimethyl derivative of violacein (50mg.) was dissolved in pyridine (3ml.) and acetic anhydride (1ml.) added to the solution. The mixture was shaken and allowed to stand at room temperature for 3 days during which time solid material separated. This solid was then filtered off

washed with water and dried. It was highly crystalline having separated in the form of long needles.

Yield. 25mg. M.P.220°-221°C.

A mixed melting point with the starting material was undepressed. The pyridine on pouring on to ice cold hydrochloric acid deposited a blue solid (25mg.) which after filtering and drying was shown to be the original trimethyl derivative of violacein.

The trimethyl derivative of violacein (50mg.) was intimately mixed with anhydrous sodium acetate (100ml.) and the mixture refluxed for 30 minutes with acetic anhydride (3ml.). On allowing to cool the blue solution deposited very little solid. After two days the solution was poured into water and was allowed to stand overnight whereupon blue crystalline solid (30ml.) was obtained.

M.P. 218°-220°C.

A mixed melting point with the starting material was undepressed.

ACETYLATION OF THE HIGH-MELTING ETHER.

The high-melting ether of violacein (100mg.) was intimately mixed with anhydrous sodium acetate (200mg.) and the mixture refluxed gently with acetic anhydride (5ml.).

A blue solution was formed but a large amount of high melting ether appeared reluctant to dissolve. After refluxing for ½ hour a further 5ml. of acetic anhydride was added and the refluxing continued. When after 1 hour a large amount of dark blue solid material was still visible the mixture was filtered. The blue filtrate was set aside overnight but deposited no solid material. The residue was washed with dilute acetic acid then repeatedly with water. A blue black crystalline solid with a green reflex was obtained.

Yield. 90mg. M.P. 315°C

The acetyl derivative was recrystallised by dissolving in a large volume of acetic anhydride (50ml.) and then reducing this volume to (5ml.) under reduced pressure separating in the form of interlaced needles.

In subsequent preparations it was obtained by refluxing the acetylation mixture for twenty minutes.

Found for acetyl deriv.	C	H	N	OAc
of high melting ether	67.4	4.5	9.5	21.0(alkali)
dried in high vacuum 1mm.	67.7	4.1	7.8	18.4 "
16hrs. dried to constant weight 130-140°C .				19.7(CrO_3/H_2SO_4)
30 mins.	67.1	4.5	8.8*	17.6(alkali)

*Double combustion.

	C	H	N	OAc
$C_{21}H_{13}N_3O_4 + 2CH_2 + 1(CH_2CO)$ requires	67.5	4.0	9.8	10.1
$C_{21}H_{13}N_3O_4 + 1CH_2 + 2(CH_2CO)$ requires	66.5	4.1	8.9	18.3
$C_{21}H_{13}N_3O_4 + 2CH_2 + 2(CH_2CO)$ requires	67.1	4.3	8.7	17.8
$C_{21}H_{15}N_3O_3 + 1CH_2 + 2(CH_2CO)$ requires	68.6	4.6	9.2	18.9
$C_{21}H_{15}N_3O_3 + 2CH_2 + 2(CH_2CO)$	69.1	4.9	9.0	18.3

Infra-Red Spectrum.

Bands appear at:-

3185cm^{-1} (NH stretching frequency)
1748cm^{-1}
1684cm^{-1}
1610cm^{-1} (Oxindole ring system).

PROPIONYLATION OF THE HIGH MELTING ETHER FROM VIOLACEIN.

The high melting ether (100mg.) was intimately mixed with sodium propionate (200mg.) and the mixture refluxed (170°C) with propionic anhydride (4ml.) for 20 minutes. The blue mixture was then filtered and the filtrate set aside to cool. The residue was washed well with water and dried. It consisted of a very dark red crystalline solid (40mg.) which was found to be rather insoluble in organic

solvents. After standing some two-three hours the filtrate deposited a blue crystalline solid (40mg.) which was very soluble in organic solvents and could be recrystallised from benzene and ethyl acetate. When crystallised from benzene it retained solvent of crystallisation and melted at 254°(d) but when free from solvent this compound like the other crystalline derivative did not melt below 320°C.

Found	C	H	N
Soluble methyl propionyl	68.19	4.98	
derivative crystallised	68.21	*5.55	
from benzene	68.7	4.9	8.2
$C_{21}H_{13}N_3O_4 + 2CH_2 + 2(C_3H_4O)$	68.10	4.89	8.22
Found for insoluble methyl-	63.9	4.5	
propionyl derivative	63.6	4.5	

*Hydrogen high due to small amount of sample taken.

Infra-Red Spectrum of the Blue Soluble Methyl Propionyl Derivative.

Bands appear at:-

1748cm^{-1}
1678cm^{-1}
1592cm^{-1}

Infra-Red Spectrum of the Methyl Propionyl Derivative Insoluble in Benzene.

Bands appear at:-

3165cm^{-1} (NH stretching frequency)
1742cm^{-1}
1667cm^{-1}
1608cm^{-1}

HYDROGENATION OF THE TRIMETHYL DERIVATIVE FROM VIOLACEIN.

Violacein trimethyl derivative (100mg.) was dissolved in ethyl acetate (40ml.) and shaken in an atmosphere of hydrogen with Adam's catalyst (20mg.). Hydrogen was slowly absorbed but when after 5½ hours shaking and the colour of the solution was still blue, this absorption ceased. The catalyst was filtered off and the solvent removed under reduced pressure. A green coloured gummy material was obtained which was dissolved in benzene and chromatographed. Two distinct bands were obtained one blue the other yellow-green. The bands were removed on eluting with chloroform but on reducing the volume of each eluent under reduced pressure neither yielded crystalline material. Addition of petrol to the yellow green solution caused the precipitation of a grey coloured solid (20mg.),M.P.160°C.

Violacein trimethyl ether (100mg.) was dissolved in glacial acetic acid (30ml.) and shaken in an atmosphere of hydrogen with Adam's catalyst (20mg.). Hydrogen was

slowly absorbed and the colour of the solution slowly changed from blue to dark green and then to light green. After 5 hours no more hydrogen was absorbed and the colour of the solution remained light green. The catalyst was then filtered off, the acetic acid removed under reduced pressure in the presence of nitrogen and a brown green obtained. This was dissolved in ethyl acetate but resisted attempts to crystallise it. Addition of petroleum ether (40-60°C) caused the precipitation of a white solid (80mg.).

This solid dissolved to some extent both in 2N caustic soda solution and in 2N hydrochloric acid. Attempts to crystallise it by solution in benzene and chromatography on a neutral alumina column were not successful.

REDUCTIVE ALKALINE DEGRADATION OF THE LOW MELTING TRIMETHYL DERIVATIVE OF VIOLACEIN.

The low melting trimethyl derivative of violacein (100mg.) was dissolved in absolute alcohol (15ml.) and to the hot blue solution was added 2N caustic soda solution (15ml.) and zinc dust (200mg.) and the mixture gently refluxed under an atmosphere of nitrogen. The colour changed from blue to red after the addition of the alkali

but as the heating continued and the mixture was continually shaken, it slowly lightened to a brownish yellow. After being refluxed for ¾ hour, the mixture was cooled and the solution decanted from the zinc which was washed with a little water. Concentrated hydrochloric acid was slowly added to the filtrate until pH 6.5-7 when the precipitated zinc salts were filtered off. When the yellow filtrate was acidified a white solid was precipitated which was set aside to ripen.

Yield. 60mg. M.P. 260°C.

The mother liquor on standing deposited a further 10mg. of this solid.

The solid was recrystallised from acetone as small colourless prisms.

M.P. 267°(d) decomposing to a red liquid.

This compound was shown to be identical with the trimethyl derivative of the C_{20} acid.

REDUCTIVE ALKALINE DEGRADATION OF THE HIGH MELTING ETHER OF VIOLACEIN.

The high melting ether of violacein (100mg.) was intimately mixed with zinc dust (200mg.) and the mixture gently heated under reflux in an atmosphere of nitrogen with 2N caustic soda solution (15ml.). The ether on

dissolving gave a red coloured solution but this,as the heating continued and the mixture was continuously shaken, slowly changed to a brownish - yellow. If the mixture were not continuously shaken the colour of the solution was found to be considerably darker. After heating for ¾ hour, the mixture was cooled and the alkaline solution decanted from the zinc which was washed with a little water. The solution was then neutralised with concentrated hydrochloric acid and precipitated zinc salts filtered. Acidification of the yellow filtrate caused the precipitation of a pale yellow solid which was set aside in the refrigerator. After filtration the solid was washed with water and dried.

Yield. 90mg.

The solid was shown to be an acid and on heating with acetic anhydride in pyridine a magenta coloured solution was formed. The acid resisted a number of attempts to crystallise it. It could not be recrystallised from acetone separating in an amorphous form nor were attempts from acetone/petroleum ether (40-60°C) any the more successful.

METHYLATION OF THE ACID OBTAINED FROM THE REDUCTIVE ALKALINE DEGRADATION OF THE HIGH MELTING ETHER FROM VIOLACEIN

The acid obtained from the above degradation (200mg.) was dissolved in 2N. caustic soda solution (6ml.) and the solution maintained in anaatmosphere of nitrogen. Dimethyl sulphate (3ml.) was added in 0.5ml. portions at ten minute intervals with continuous shaking to produce homogeneity. After the last addition, the solution became cloudy and a solid material was precipitated. The mixture was allowed to stand for ½ hour after which time 2N. caustic soda was added drop by drop with shaking until the solution remained alkaline. The solid material was then filtered off and the alkaline solution treated again with dimethyl sulphate in 0.5ml. portions until the solution became acid and more of the solid separated. The mixture was then once more allowed to stand for ½ hour before being made alkaline and the solid filtered off. The combined residues were then washed well with water and dried.

Yield. 160mg.

The solid was recrystallised from absolute alcohol as small colourless needles.

M.P. 181°C.

Mixed melting point with the tetramethyl derivative of the C_{20} acid undepressed. When acidified with hydrochloric acid the filtrate yielded 25mg. of the trimethyl derivative of the C_{20} acid.

DEGRADATION OF ACETYLVIOLACEIN WITH HYDROGEN IODIDE.
(Cf. Jennings, Ph.D. Thesis, Liverpool, 1953.)

Acetylviolacein (200mg.) was added to a mixture of hydriodic acid (10ml.) (which had been standing five days after distillation over red phosphorus, and of specific gravity 1.7) and glacial acetic acid (10ml.) and introduced into an oil bath at 120°C. The temperature of the bath was slowly raised to 130°C and the reaction mixture refluxed for 1½ hours. It was then allowed to stand overnight when the hydrogen iodide salt of the methene which had separated was filtered off. The red needles with a bronze reflex were washed with a little glacial acetic acid.

Yield. 20mg. M.P. 320°C.

Infra-Red Spectrum.

Bands appear at:-

3436cm^{-1}	(OH stretching frequency)
3145cm^{-1}	(NH " ")
1684cm^{-1}	
1600cm^{-1}	

DEGRADATION OF THE HIGH MELTING ETHER OF VIOLACEIN WITH HYDROGEN IODIDE.

The high melting ether of violacein (200mg.) was added to a mixture of hydriodic acid (10ml.) and glacial acetic acid (10ml.) and introduced into an oil bath at 120°C. The temperature was slowly raised at 130°C and the reaction mixture refluxed for 1½ hours. It was then set aside overnight when a dark red crystalline solid separated out.

Yield. 20mg. M.P. 320°C.

This solid dissolved readily in caustic soda solution to give a yellow solution which only darkened to a light brown over the course of 2-3 days.

Infra-Red Spectrum.

Bands appear at:-

3155cm^{-1}
1689cm^{-1}
1667cm^{-1}
1603cm^{-1}

DEGRADATION OF THE LOW MELTING TRIMETHYL DERIVATIVE WITH HYDROGEN IODIDE.

The trimethyl derivative of violacein (100mg.) was added to a mixture of hydriodic acid (which had been

standing five days after distillation over red phosphorus and of specific gravity1.7) (2ml.) and glacial acetic acid (2ml.) and introduced into an oil bath at 120°C. The temperature of the bath was raised to 130°C, and the reaction mixture refluxed for 1½ hours. After standing overnight a yellow-brown amorphous solid (50mg.) separated. When treated with 2N caustic soda solution this solid appeared to undergo some change into a blue solid. This blue solid was insoluble in water and in alkali but gave royal blue solutions in the common organic solvents. The alkaline solution gave a positive test for iodide ion and the yellow-brown solid would thus appear to be a hydrogen-iodide salt of the blue solid.

ACTION OF ALKALI ON VIOLACEIN.

Violacein (300mg.) was carefully powdered and added to 0.1N caustic soda solution (30ml.) which was maintained in an atmosphere of nitrogen. The mixture was gently warmed when the bright green colour changed to red and the reddish solution heated gently with continual shaking for ½ hour. The mixture was then quickly filtered and the red filtrate returned to the flask and maintained in the atmosphere of nitrogen. The undissolved violacein was washed liberally with water and dried (60mg.). Solid CO_2 was then added to the red filtrate and this caused the precipitation of a red solid which was filtered off, washed and dried (100mg.). The filtrate which still possessed a slightly red colour returned quickly to the nitrogen atmosphere and dilute hydrochloric acid added. This caused the precipitation of a yellow solid which was quickly filtered off , washed and dried (20mg.). On standing the filtrate deposited a little more of this yellow material which soon changed to a much darker colour.

More Vigorous Action of Alkali on Violacein.

Violacein (400mg.) was carefully powdered and added to 0.1N caustic soda solution (50ml.) which was maintained

in an atmosphere of nitrogen. The mixture was boiled quite vigorously under reflux for ½ hour during which time only a little of the violacein remained undissolved. The reaction mixture was then cooled and quickly filtered, the red filtrate being returned to the flask and maintained under nitrogen. The undissolved violacein was washed well with water and dried (25-30mg.). Solid CO_2 was then added to the red filtrate and this caused the precipitation of the red solid which was filtered washed and dried (60mg.).

On addition of dilute hydrochloric acid to the red filtrate, the yellow solid was precipitated and this was quickly filtered, washed, and dried (160mg.). More solid separated on leaving the filtrate to stand and this quickly acquired a much darker colour.

ACETYLATION OF THE RED SOLID OBTAINED FROM VIOLACEIN.

The red solid (obtained from violacein by the action of alkali/CO_2)50mg. was intimately mixed with sodium acetate (50mg.) and the mixture refluxed gently with acetic anhydride (3ml.) for an hour. On cooling the mixture set semi-solid and a dark red crystalline solid with a green reflex was filtered off, washed and dried (35-40mg.). The

mother liquor on standing deposited a further 5mg. of this material. M.P. 280°C.

The infra-red spectrum of this compound is identical with that of acetylviolacein.

METHYLATION OF THE RED SOLID OBTAINED FROM VIOLACEIN.

The red solid (obtained from violacein by the action of alkali/CO_2) (100mg.) was mixed intimately with potassium carbonate (500mg.) and the mixture refluxed with acetone (20ml.) and dimethyl sulphate (0.2ml.). A red solution was initially obtained but after refluxing for some two-three hours slowly changed colour to purple. After four hours refluxing a further quantity of dimethyl sulphate (0.2ml.) was added. The reaction mixture was then refluxed for a further 8 hours making a total reaction period of 12 hours. After this period the mixture was filtered and the grey residue washed repeatedly with water to remove all traces of potassium carbonate. This left a blue black microcrystalline solid (65mg.) which was later shown to have an identical infra-red spectrum with the high melting methyl ether obtained from violacein.

On removal of the acetone under reduced pressure a blue solid (25mg.) was obtained which was shown to be

soluble in all the common solvents benzene, ethyl acetate, chloroform, alcohol etc.

ACETYLATION OF THE YELLOW SOLID OBTAINED FROM VIOLACEIN.

The yellow solid (obtained from violacein by the action of alkali/dilute hydrochloric acid) 50mg. was mixed intimately with sodium acetate (50mg.) and the mixture refluxed gently with acetic anhydride (3ml.) for an hour. On cooling the bright red solution set to a solid crystalline mass which was filtered, washed and dried.

Yield. 40mg. M.P. 280°C.

This solid was recrystallised from benzene as small red needles.

Found	C	H	N	OAc
	66.09	4.18	5.4	24.04 19.28
$C_{20}H_{12}N_2O_4 + 3(CH_2CO)$ requires	66.38	3.83	5.95	27.4

Infra-red Spectrum.

Bands appear at:-

3100cm^{-1} (W)
1764cm^{-1}
1712cm^{-1}
1667cm^{-1}
1623cm^{-1}
1595cm^{-1}

CONVERSION OF YELLOW SOLID TO DARKER MATERIAL AND SUBSEQUENT ACETYLATION.

The yellow solid (140mg.) was warmed gently in dilute hydrochloric acid and after a few minutes changed its appearance to a black material. This solid was filtered, dried and mixed intimately with sodium acetate (140mg.). The mixture was gently refluxed with acetic anhydride (4ml.) when a bright red solution was obtained. After heating for ½ hour the solution was set aside to cool and set to a semi crystalline mass. This was filtered washed and dried and a red crystalline solid obtained.

Yield. 140mg. M.P. begins to decompose above 280°C.

The infra-red spectrum of this solid was shown to be identical with that of the acetate obtained from the yellow solid.

METHYLATION OF THE YELLOW SOLID OBTAINED FROM VIOLACEIN.

The yellow solid (obtained from violacein by the action of alkali/hydrochloric acid) 100mg. was intimately mixed with potassium carbonate (500mg.) and the mixture refluxed with acetone (20ml.) and dimethyl sulphate (0.2ml.). A red solution was obtained which did not change colour during the methylation. After fifteen hours refluxing,

the mixture was filtered to remove the potassium carbonate and no insoluble residue remained. The acetone was removed under reduced pressure and the sticky residue triturated with hot water. On filtration an amorphous solid was obtained M.P. 130°C.

A crystalline derivative could be obtained from this material on heating with sodium acetate/acetic anhydride.

ACTION OF ALKALI ON THE TRIMETHYL DERIVATIVE FROM VIOLACEIN.

The trimethyl derivative from violacein (120mg.) was dissolved in acetone (30ml.) and to this solution was added slowly hot N caustic soda solution (30ml.). The colour of the solution changed from blue to red and the mixture was heated under reflux and in an atmosphere of nitrogen for 1 hour. It was then cooled and the violacein trimethyl derivative which had separated during the addition of alkali quickly filtered off (15-20mg.). The red filtrate was then acidified with mineral acid and this caused the precipitation of a yellow solid which very rapidly darkened to blue (80mg.).

ATTEMPTED ACETYLATION OF THE BLUE SOLID OBTAINED FROM THE TRIMETHYL DERIVATIVE OF VIOLACEIN.

The blue solid(obtained from the trimethyl derivative of violacein by the action of alkali/mineral acid)(80mg.)

was mixed intimately with sodium acetate (80mg.) and the mixture refluxed gently with acetic anhydride (3ml.)for ½ hour. The red solution which was obtained was set aside to cool for 1 hour during which time it deposited a crystalline solid. This was filtered off, washed and dried. Yield. 60mg. M.P.297°C.

This solid was recrystallised from benzene in the form of needles with a bright green reflex.

Found	C	H	N	OAc
	70.49	5.17	6.52	0.8
$C_{20}H_{12}N_2O_4 + 3CH_2$	71.5	4.7	7.25	

Infra-Red Spectrum.

Bands appear at:-

3086cm^{-1} (W)
1764cm^{-1} (M)
1667cm^{-1}
1623cm^{-1}
1595cm^{-1}

ACTION OF ALKALI ON THE HIGH MELTING ETHER OF VIOLACEIN.

The high melting ether from violacein (100mg.) was finely powdered and added to 0.5N caustic soda (30ml.) which had been maintained in an atmosphere of nitrogen. The mixture was heated under reflux for ½ hour with continuous shaking but the solid seemed reluctant to

dissolve. The mixture was cooled and the unchanged high melting ether filtered off, washed and dried (60mg.). The red filtrate was returned to the flask and maintained under an atmosphere of nitrogen. Dilute hydrochloric acid was then added and this precipitated a yellow brown solid which was quickly filtered, washed and dried (40mg.).

It was found that the high melting ether dissolved more readily in 10% alkali and gave a higher conversion to the yellow brown solid.

ACETYLATION OF THE BROWN SOLID OBTAINED FROM THE HIGH MELTING ETHER OF VIOLACEIN.

The yellow-brown solid(obtained from the high melting ether of violacein by the action of alkali/hydrochloric acid) (30mg.) was mixed intimately with sodium acetate (30mg.) and heated with acetic anhydride (2ml.) for an hour. A red solution was obtained which on allowing to cool deposited crystalline solid. This solid was filtered, washed well with water and dried.

Yield. 25mg.

The crystals are in the form of needles and have a green reflex, they dissolve in benzene to give a magenta coloured solution.

Infra-Red Spectrum.

Bands appear at:-

3086cm^{-1}	(W)
1751cm^{-1}	
1709cm^{-1}	
1695cm^{-1}	
1672cm^{-1}	
1623cm^{-1}	
1587cm^{-1}	

REFERENCES

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1. Rep. Comm.Amer. Bacteriologists, J. Bact, 1920, 5, 191.
2. Topley and Wilson, Principles of Bacteriology & Immunity, 1945, Third Edition Vol.1. Page 634.
3. C.Bergonzini. Annuario della Societa dei Naturalisti in Modena 1881, Ser.2, 14, 149.
4. Black & Shahan. J.A.M.A. 110, 1270-1271.
5. H.C.Lichstein & V.F. Van de Sand. J. Infectious diseases,1945, 76. 47.
6. Friedheim. Compt. rend. Soc. Biol., 1932, 110, 352.
7. Sartory, Meyer & Waeldele. Compt. rend., 1938, 206, 950.
8. Hartley. Sci. Proc. Roy. Dub. Soc., 1913, 14, 63.
9. Lasseur & Girardet. Bull. Trimistriel Soc. Sci. Nancy, 1926, 3, Series 4, 1-77.
10. Reilly & Pyne. J. Biochem., 1927, 21, 1059.
11. Kögl. Handbuch der Pflanzenanalyze, 1932, Vol.II, Pt.2, 1443.
12. Wrede. Z. Physiol Chem., 223, 113. Arch. Exptl. Path. Pharm., 1937, 186, 532.
13. Tobie. J. Bact., 1938, 35, 11.
    Ramchandini, Annals of Botany,44, 1930, 975-87.
    Dessenowa & Zichow, Cent. Bakt. Abt.1,119,1930, 35-8.
    Preisz, Mathem. es Termeszettudomanyi, Erte sito
    A Magyar Tudomanyos Akadema 43, 1926,188-204.
    Dmitrevskia, Microbiologica 5, 1936, 82-6.

REFERENCES (cont.)

14. Beer, Clarke, Khorana & Robertson. J.C.S., 1949,885.

15. Clarke, Ph.D. Thesis, Liverpool, 1949.

16. Khorana. Ph.D. Thesis, Liverpool, 1949.

17. Beer, Jennings & Robertson. J.C.S., 1954, 2679.

18. Boggiano. Ph.D. Thesis, Liverpool, 1953.

19. Strong, Science, 1944. 100, 287.

20. Julian etul., J.A.C.S. 1953, 75, 5305.

21. Aeschlimann, J.C.S., 1926, 2902.

22. Braude & Jones, J.C.S., 1945, 498.

23. Randall, Fowler, Fuson & Dangl. Infra-red Determination of Organic Structures, Van Nostrand.

24. Cromwell et al., J.A.C.S. 1949, 71, 3337.

25. Djerassi & Fishman, Chemistry and Industry, 1955, 627.

26. Baker, J.C.S., 1946, 461.

27. Jackson & Manske, J.A.C.S., 1930, 52, 5029.

28. Jones & Co-workers, J.A.C.S., 1949,71, 241., 1950,72 956, 1952, 74, 2820.

29. Barnes et al., Infra-red Spectroscopy,Reinhold 1944.

30. Hartwell et al., J.C.S., 1948, 1436.

31. L.J. Bellamy, The Infra-red of Complex Molecules , Methuen 1954.

32. Wrede, Z. physiol.Chem., 1934, 226, 1934.

33. J.C.McGowan,J.C.S. 1949,777.

34. H.Fischer & H.Rose,Ber.,45,1579,(1912).,Ber 47,794,(1914) O.Piloty & S.J.Thannhauser,Ann., 390,191, (1912).