ORGANIC CHEMISTRY: PHYSICAL METHODS AND BIOLOGICAL RELATIONSHIPS*

By SIR ROBERT ROBINSON, PRES.R.S.

THE future historian of science will certainly characterize the first half of the twentieth century as an age of unsurpassed progress of discovery in physics. He will also note the *crescendo* in the elaboration of physical techniques and the decisive part they played in the dramatic developments of the sister sciences. Examples are the commonplaces of our scientific practice, and could be culled from almost any active region of investigation. That almost self-evident fact is well illustrated by the record of the Royal Society's medallists of 1946, and I invite reflexion on the extent to which their distinguished experimental contributions have been rendered possible by a quick appreciation of the potentialities of new physical methods. The thermionic valve, the photo-electric cell, high-vacuum technique, high-pressure technique, production and management of very low and very high temperatures, X-rays, and the use of isotopic and radioactive tracers, are but a few of the tools which modern physics has placed at our disposal.

The vastness of the subject is very significant, and even if, as is necessary, I confine myself to organic chemistry, only a small part of it can be mentioned.

The forty years of my own experience have seen a revolution in the methods of experiment, and unquestionably the great waves of advance are clearly identified with the introduction of new techniques. The improvement of balances and the pioneering work of Pregl brought in microanalysis and, following in its wake, microchemical manipulation. It is safe to say that this has increased the output of a given laboratory man-power by at least 100 per cent because of the saving of time and energy expended previously on pure routine. But even more important is the fact that microchemistry has made possible the successful attack of problems, especially in the field of biochemistry, which could not even be attempted thirty years ago.

Many of the more spectacular researches concerned substances of high biological activity, and a vital part was played by the co-operation of botanists, zoologists, physiologists and bacteriologists. But equally necessary was the help of physicists in the provision of methods of investigation of 1-2 mgm. of material.

Among the more valuable of these new resources are ultra-violet and infra-red spectroscopy and X-ray crystal analysis. The triumphs of the latter are well known, and I will only add that the last details of the constitution of penicillin were revealed by the X-rays in the hands of Crowfoot and Rogers at Oxford and Bunn and Turner-Jones at Northwich. The laborious Fourier analysis which the complete mapping of electronic densities still demands will soon be carried out by machines, and it is not at all improbable that molecular structures will eventually be ascertained with ease, and almost by inspection. That will not close the organic chemical and biochemical laboratories, but, on the contrary, will give impetus to their work in many fascinating directions.

Ultra-violet spectroscopy, once the concern of specialists, is now practised universally; for many purposes, however, the study of infra-red absorption promises even greater usefulness.

Although subject to constitutive influences, the bands in the infra-red are far less so than those in the ultra-violet, and the method provides a kind of elementary analysis of the simpler groups contained in the molecule. It has been used *inter alia* to follow the course of polymerization, for the analysis and characterization of hydrocarbons, such as the isomeric octanes or butanes, and in the everyday control of industrial processes.

We were very impressed by, and grateful for, a recent demonstration of the power of infra-red spectroscopic analysis. A crucial test was devised in order to establish a detail of the constitution of strychnine, and the outcome depended on the unequivocal identification of a degradation product obtained in very small quantity. Our own work indicated that it was carbazole mixed with one of the four C-methylcarbazoles, and probably with 3-methylcarbazole. But we could not be quite certain. Mr. Pausacker made the four methylcarbazoles, of which one was new, and Mr. Richards kindly studied their infra-red spectra. They were characteristic and differed also from that of carbazole. Using only 1.5 mgm. and a novel technique, Richards showed conclusively that the specimen was essentially carbazole containing about 10 per cent of 3-methylcarbazole. The probable course of events recalls the stages through which mountains have been said to pass—an inaccessible peak, an interesting course for experts, an easy day for a lady.

In many directions there have been notable advances in the processes of purification and analysis, but I will merely mention in passing the so-called molecular still, the ultra-centrifuge, polarography and electrophoresis.

I would, however, like to direct attention to a recent series of researches which foreshadow a leap forward in our knowledge of the proteins, again because of the introduction of a new technique. In doing this, I hope to make some amends for having recently bemoaned in another place the relatively small contribution of British men of science to protein research. The equipment for those who venture to follow the pioneers is not elaborate. I gather that the chief requirements are a lead tray, an earthenware drain-pipe and a sheet of paper.

Although the use of animal charcoal for the removal of coloured impurities from solutions has a respectable antiquity, and the separation of dyes in solution on filter paper has long been employed as a method of analysis, modern chromatography was introduced by Tswett forty years ago. He showed that coloured substances are selectively adsorbed from suitable solutions and that distinct bands are formed in a vertical column when the solution of a mixture is poured in at the top and allowed to fall through the adsorbant. In this way Tswett showed that leaf-green chlorophyll consists of two substances, later investigated by Willstätter and Stoll.

The many developments have included various devices for applying the method to colourless substances. A coloured group may be added to the molecule, the fluorescence of the bands may be observed instead of the colour, the adsorbant may be pre-coated with a fluorescent substance (Brockmann), or the column may be streaked with a reagent to produce a visible effect.

* From the presidential address to the Royal Society delivered on November 30.

Chromatography is now a standard laboratory procedure, and in Great Britain Sir Ian Heilbron was the first to perceive its advantages.

Another well-known method of separation of substances depends on their partition between immiscible or partially immiscible solvents, and an apparatus for carrying out a large number of successive partitions has been devised by L. C. Craig at the Rockefeller Institute for Medical Research.

A still more ingenious idea is that of the partition chromatography which A. J. P. Martin and R. L. M. Synge (1941) worked out in the laboratories of the Wool Industries Research Association. It makes use of a Tswett column but is based on the principle of partition rather than on that of adsorption. This distinction is evidently valid in reference to the phases concerned, but it is not so certain that the two processes are not basically similar at the molecular level. As one example, particles of silica gel can be impregnated with a buffer solution on the alkaline side and placed in a column through which the substances to be separated, dissolved in a suitable immiscible solvent, are passed. The effect is obviously that of a large number of successive extractions, and bands analogous to those of a chromatogram are produced; the order of the bands from top to bottom will be one of decreasing acidity of the components of the mixture. *Ceteris paribus*, the most acid constituent, will be found in the top layer. This technique has been found to be well adapted for the separation of the penicillins on the laboratory scale. In 1944, Martin, in collaboration with R. Consden and A. H. Gordon, made a further step forward by the use of water-saturated cellulose as the stationary phase and a mobile phase consisting of a solvent such as phenol or collidine, partially miscible with water. Gordon, Martin and Synge had already shown in 1943 that strips of filter paper could be used to separate amino-acids, and the later work is an extension of this observation for the same purpose. The development may be one-dimensional or, preferably, two-dimensional, in which procedure the first solvent is removed by drying and a second solvent is allowed to ascend the paper at right angles to the direction taken by the first. A drop of protein hydrolysate suffices, and its constituent amino-acids become segregated in definite areas the position of which is dependent on the nature of the amino-acid and the solvents used. The well-known colour reaction with ninhydrin is used to show up the spots. Thus a rapid qualitative analysis of protein *bausteine* is achieved and, moreover, the presence of a new amino-acid will be indicated and a rough idea of its constitution will perhaps be obtained. Furthermore, the simpler peptides are separated and by subsequent hydrolysis and repartition their amino-acids can be recognized.

These researches will, I believe, be recognized as the greatest contribution to the study of the structure of the proteins made since the classical work of Emil Fischer.

How the method can be used is well shown by an outstanding investigation of the molecular structure of gramicidin-S by Consden, Gordon, Martin and Synge (1946). A partial hydrolysate was fractionated on two-dimensional paper chromatograms. The location of dipeptides and tripeptides having been determined, these were taken from a duplicate paper, hydrolysed, both before and after deamination, and the amino-acids identified by means of further chromatograms. The dipeptides so recognized were

synthesized, and their behaviour on partition paper chromatography was found to be identical with that of the respective constituents of the partial hydrolysate. The method of ionophoresis was also used and the findings were consistent. From the chain *ABCDE*, *AB*, *BC*, *CD*, *DE*, *ABC* and *DEA* were obtained and identified.

Hence, not only is the order of the five amino-acids established but also it is rendered very probable that the substance is a cyclic polypeptide. The crystallographic results of Crowfoot and Schmidt are compatible with the hypothesis that the ring contains ten amino-acid groups.

At the Liverpool meeting of the British Association for the Advancement of Science (Section B, 1923) I mooted the idea that many high molecular weight substances of repeating pattern type should be regarded as mammoth rings, basing this speculation mainly on the absence of end-groups required on the open-chain hypothesis. A cyclic decapeptide would include a ring of thirty members.

I will now refer to a subject pursued in my own laboratory in collaboration with biologists, namely, Dr. C. E. Coulthard, of the Research Department of Boots Pure Drug Company, Ltd., and Dr. J. Ungar, of Glaxo Laboratories, Ltd.

The tubercle bacilli are characterized by the possession of a waxy envelope which has often been considered to confer some degree of immunity against the attack of chemotherapeutic agents. Consequently, it has been sought to endow the latter with fat-soluble groups in the hope of penetrating the supposed protective covering. Actually, it may be doubted whether this scheme, which has brought little success, is based on a sound conception, for it may be argued that all that could be achieved would be the establishment of a reservoir of the agent in the lipins. On these lines it would seem necessary to link the fatty part of the molecule to the water-soluble part, which it is hoped will attack the organism, by a readily hydrolysable linkage. Several variations of this theme can be envisaged. Be this as it may, it is obvious that the chemical nature of the lipins of the bacteria deserve close attention, and the first chemist to attack the problem, and with important results, was R. J. Anderson (1929 and later). The fatty acids obtained by hydrolysis of the waxes from the bacterial bodies were fractionated, and one of them, tuberculostearic acid, was found by Spielman, a colleague of Anderson, to be 10-methylstearic acid.

Important constituents of the mixture were acids of the formulae $C_{26}H_{52}O_2$ and $C_{30}H_{60}O_2$; the former, termed *phthioic acid*, has been the more closely studied. Anderson was of the opinion that it was a branched-chain acid similar in constitution to tuberculostearic acid; but the evidence garnered by him and his collaborators and by Wagner-Jauregg was insufficient to establish the details.

E. Stenhagen and S. Stållberg then studied the behaviour of phthioic acid in monomolecular films and also the X-ray reflexions from barium phthioate. They came to the conclusion that the acid is ethyl-decylododecylacetic acid, or something very similar, but the synthesis of this substance by N. Polgar showed that this was an error probably due to the unusual degree of tilt of the molecules.

I will not burden you with the organic chemical details of Polgar's further work; but combined analytic and synthetic attack of the degradation

products made it very probable that phthioic acid is 3:13:19-trimethyltricosanoic acid, a straight chain of twenty-three carbon atoms with three methyl branches. Phthioic acid is feebly optically active, but the optically inactive, synthetic 3:13:19-trimethyltricosanoic acid closely resembles phthioic acid in respect of its physical properties, including the behaviour of monomolecular films on water, and in the melting-points of its derivatives. We thus returned to the original general hypothesis of Anderson.

It has been known for some years that phthioic acid possesses toxic properties (F. Sabin, of the Rockefeller Institute for Medical Research, New York, and others) and that it produces lesions when suitably injected into experimental animals, for

site structural features. A working hypothesis is that the methyl groups block β -oxidation, and some relation to physiologically active unsaturated substances may well be brought to light in the future.

An extremely interesting discovery, quite unrelated to this work in its origin, has been announced by R. P. Cook from the Biochemistry Laboratory, University of Cambridge. He has obtained an acid, or a mixture of acids, $C_{25}H_{50}O_2$, by feeding cholesterol to rats. This is very suggestive of an extraordinary process of unwinding of the tetracyclic nucleus of the sterinoid by breaks at the points where the rings are fused, and also at some peripheral point, and in the side-chain. For example, one possible degradation is illustrated below:

example, the guinea pig. But the observations of Coulthard and Ungar are new in that they have been able to reproduce, by a single intraperitoneal injection of synthetic acids of known constitution, a pathological picture which is almost identical with that of tuberculosis, in respect of the particular manifestations observed. There is no doubt whatever of the reality of the phenomenon, and it is highly significant.

The study of a range of synthetic branched long-chain fatty acids from this point of view is in its infancy, but the following results can be cited. The acids have been synthesized by N. Polgar, partly with the collaboration of S. David and E. Seijo. 3:12:15-Trimethyldocosanoic acid is even more active than phthioic acid, or synthetical 3:13:19-tricosanoic acid, which are equal within the limits of the method. On the other hand, 2:13:17:21-tetramethyldocosanoic acid is inactive. 13:17:21-Tricosanoic acid is inactive, and so is 2:13-dimethylpentacosanoic acid. 13:16-Tricosanoic acid is active, but it was suspected that the specimen contained a 3-methyl-substituted impurity. A purified specimen exhibited greatly diminished activity. 4:13:16-Tricosanoic acid is very active, and though here again the presence of some 3-methyl substituent is not excluded, the activity is such that it can scarcely be due to an impurity. 3:13:19- $\Delta^{13:19}$ -Tricosadienic acid is active but less so than the related saturated substance. It is probable that the specimen contains several geometrical isomerides. The syntheses are very laborious and the biological tests are prolonged, so that progress is necessarily slow.

At present it looks as if a methyl substituent in the 3- or 4-position is necessary. The biological property is evidently highly constitutive, but it is too early to attempt an identification of all the requi-

We are unable to equate the constitution of any substance that could be obtained in this way with that of phthioic acid, but such a direct relation was scarcely to be anticipated. If Cook's acid is really derived from the cholesterol molecule by some transformation, it must be a branched-chain acid, and the determination of its structure is a most urgent problem, the solution of which must surely shed some light on an aspect of the biochemistry of phthioic acid.

The temptation to carry speculation a little further cannot be resisted. In 1926, Prof. L. Ruzicka elucidated the constitution of civetone, the odoriferous principle of the civet cat, and made the dramatic discovery of the existence of large carbocyclic rings in Nature. He also noted the structural relation of civetone with oleic acid. Since the ketone has one less carbon atom than the acid and the latter is widely distributed, the degradation of oleic acid to civetone is more probable than the reverse synthesis. It may be suggested that oleic acid suffers ω -oxidation, a biochemical process to which Verkade has paid attention, and that this is followed by a familiar ketonization.

But analogy then leads us to assume a similar mechanism for muscone from the musk-rat, which was also studied by Ruzicka. We find that its progenitor should be a 3-methylpalmitic acid,

The occurrence of the 3-methyl-substituent is interesting in relation to phthioic acid. Further, Prof. Hans T. Clarke and his collaborators at Columbia University, New York, have shown by the use of C^{13} that the fatty acids are produced in the organism from acetic acid only. In parenthesis, this is a remarkable experimental justification of J. N. Collie's speculations on the role of keto-methylene chains in biosynthesis. An additional molecule of acetic acid could be used to introduce methyl substituents by the mechanism:

3 : 13 : 19-Tricosanoic acid is not a possible product of this scheme of biosynthesis. It would therefore not be surprising to find that phthioic acid, as at present known, is a mixture of a trimethyldocosanoic acid and a trimethyltetracosanoic acid. On this hypothesis the chain should in any event be even-numbered and the methyl groups can only be attached to the odd-numbered carbon atoms. The constitution proposed for tuberculostearic acid conforms to the first condition, but not to the second. It could, however, be 9-methylstearic acid, if oxidation occurs at carbon atoms 9 and 10 and is accompanied by a pinacol-pinacolone migration. Alternative views to that already mentioned involve the intervention of molecules of propionic acid or formaldehyde (or an equivalent) in order to provide the methyl substituents. These, however, fix the methyls on even-numbered carbon atoms and, though tuberculostearic acid then falls into line, they are at variance with our own deductions in regard to the constitution of phthioic acid.

Following the clue afforded by chaulmoogric and hydnocarpic acids in the treatment of leprosy, Roger Adams prepared a range of substituted fatty acids, some of which had considerable action on *B. leprae* (or possibly an analogous organism) *in vitro*. The irritating action of these substances precluded their use in practice.

Our first efforts in the field of tubercle fatty acids had a similar objective, but we have now abandoned the idea of a frontal attack on the organism in favour of an attempt to alleviate the symptoms of the disease. If this can be achieved by an immunity method it is probable that the body resistance will be strengthened. That investigation has not gone beyond the planning stage, but we are glad to know that it will be in the capable hands of Prof. M. Stacey at the University of Birmingham.

There are indications that the pathological role of abnormal lipins may not be confined to tuberculosis. Thus Novak and Grey (1938) found tuberculous tissue, with lesions, associated with granulosa cell tumours, and suggests that these effects were due to lipins produced by the malignant growths. These observations have very recently been confirmed and extended in the United States.

ADAPTATION OF STAPHYLOCOCCUS AUREUS TO GROWTH IN THE PRESENCE OF CERTAIN ANTIBIOTICS

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IT has been noticed that certain antibiotics, when tested against *Staphylococcus aureus* (H strain, N.C.T.C. No. 6571) by the cylinder-plate method¹, give zones of inhibition which have clear edges, but which contain, scattered through them, small numbers of isolated colonies of *Staphylococci* (Fig. 1). Among the first antibiotics found to show this phenomenon was one obtained from an organism of the *subtilis* group (N.C.T.C. No. 7197) isolated from the soil at Oxford (referred to here as *S*) and one which Chain and Callow² discovered in extracts of *Polystictus versicolor* and have named polystictin (referred to here as *P*). Both are water-soluble substances which are not extracted at any pH by common organic solvents and which have only been partially purified. They do not appear to be identical with any of the antibiotics described in the literature.

The formation of isolated colonies in the presence of *S* and *P* involved two modifications of the *Staphylococcus* which were clearly distinct. This is shown in the three plates which are illustrated. Plate 1 (Fig. 1) was seeded with the normal strain of *Staphylococcus*. Plate 2 (Fig. 2) was seeded with a strain derived from the normal strain by picking off a colony from inside the inhibition zone caused by *P* on plate 1. When tested on plate 2, *P* was apparently inactive, whereas *S* produced a zone of inhibition similar to that which it gave on plate 1. Similarly, by starting with a colony from within the inhibition zone caused by *S* on plate 1, a plate could be prepared on which *P* produced a normal zone of inhibition but *S* gave no inhibition. Lastly, by picking

Fig. 1. SEEDED WITH NORMAL *Staphylococcus* STRAIN
Top: Helvolic acid (1 in 5,000). Bottom left: *S* (1 in 2,000).
Bottom right: *P* (1 in 200)