First Symposium of the
British Society for Parasitology

Techniques in Parasitology

EDITED BY
ANGELA E. R. TAYLOR

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FIRST SYMPOSIUM OF THE

BRITISH SOCIETY FOR PARASITOLOGY

TECHNIQUES IN PARASITOLOGY

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PREFACE

The British Society for Parasitology was formed in April 1962 to replace the Parasitology Group (Institute of Biology) which had been in existence for five years. The policy of the Society at present is to hold two scientific meetings each year, a Spring meeting at which 20-30 contributed papers are read (these are not intended for publication but merely to report current work and promote discussion) and a one-day Autumn meeting at which a few specialists are invited to give longer papers on their own subjects, in the form of a symposium.

The first symposium was held on October 19th, 1962, at the Zoological Society, London. The title of the symposium *The Application of New Techniques to Parasitology* was chosen because the Council felt that there was a growing need to bring to the attention of parasitologists new techniques which are available in other disciplines that could equally well be applied to parasitological problems. It was also felt to be desirable that such techniques should be introduced by specialists so that an evaluation of the usefulness of the various techniques could be made at the time of presentation.

The large attendance at the meeting and the lively discussions promoted by each paper was an encouraging indication of the interest of members of the new Society in this symposium. It is hoped that this will be the first of a series of annual symposia in Parasitology.

The Editor would like to thank the contributors to the symposium as well as those people whose help enabled the symposium to be held and this report to be produced. She

would like to pay a special tribute to Professor Sir John Randall F.R.S., for the skilful way he chaired the meeting and handled the discussions.

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THE APPLICATION OF BIOCHEMICAL TECHNIQUES TO PARASITOLOGY

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For some time the biochemist has been influenced in his approach by the concept that in all organisms there is to be found a fundamental similarity of composition and molecular function, a common 'ground plan', growing out of which are innumerable secondary diversities, some reflecting acquisition and others resulting from loss. A study of parasites, or of any other group of animals, without reference to what is known about all other forms of life will inevitably limit the knowledge gained by such a study. In our examinations of parasites results should be regarded, right from the start, not only as relating to the parasite's ways of life but as telling us a little more about life in its more general sense.

It thus follows that the most profitable and satisfying approach to the biochemistry of parasites is a comparative one, in which we seek similarities and differences in details of the 'ground plan' as well as in the nature of the secondary diversities. In a rather more limited context it is possible to outline at least five main types of specialized comparison.

1. Contrasts can be made between parasite species and their most closely related free-living counterparts. Perhaps the development of parasitism can be imagined as involving a long initial period of association of the potential parasite with its future host, during which it was possible for the former to

return to a free-living, saprophytic existence, but during which various changes could occur which would introduce parasitism as a condition for survival. Genetic mutation far more frequently results in the loss than in the gain of an attribute; when such mutation is expressed in failure to complete a biosynthetic sequence it becomes lethal unless the final product of the lost pathway can be derived from the external environment. If this should happen to one organism living in association with another, from which the one could derive the missing metabolite, a type of dependence would emerge and the path to parasitism would be established. Further losses occurring over numerous generations would make the possibility of the parasite's return to the outer world even more remote. But there is no reason to think that the parasite's potentiality for genetic change involving gain is any less than that of free-living organisms. It might be postulated then, that parasites possess certain secondary attributes which have given them a selective advantage in the environment to which they have become committed. On this basis of comparison some insight should be gained into the evolutionary progress from free-living species to parasite and from parasite to more specialized parasite.

2. Within a complex host both closely related and vastly different parasites are to be found in the same environment. Can we find common biochemical attributes between, for instance, trypanosomes and schistosomes in the blood stream or between nematodes and cestodes in the intestine, which have a direct bearing on the suitability of these environments or on 'adaptation' to them?

3. Similarly differences can be sought between adults of related parasite species which dwell in very different host environments, as for instance between trematodes occupying the blood stream and those selectively living in the bile duct.

4. Transition from one stage to another in a parasite's life-cycle may be associated with striking morphological changes and with the occupation of a new situation where the environ-

ment is profoundly different in its physicochemical properties. Associated biochemical changes may be equally striking, as is already indicated in the few instances where comparison has been made between different forms of the same species; the differences may in fact be greater here than between the same or comparable stages of different species.

5. Finally, comparisons between a parasite and its host can reveal differences which are of interest not only in the general framework of comparative biochemistry but which may also have specific bearing on chemotherapy.

MATERIAL FOR STUDY

LEVELS OF INVESTIGATION

When discussing problems of bacterial metabolism Marjory Stephenson, a pioneer in that field, used to describe five 'levels' of changing complexity at which studies could be made. With little modification the scheme is applicable to investigation of the parasitic protozoa and, to a more limited extent, to the helminths; it may be represented (after Woods, 1953) as follows:

(a) Organism growing in a mixed population in its natural environment

(b) Organism growing in pure culture in a complex undefined medium

(c) Organism growing in pure culture in a chemically defined medium

(d) Suspensions of viable but non-proliferating cells ('resting' cells)

(e) Cell-free extracts

(i) 'complete': subcellular particles intact; mixtures of enzyme systems

(ii) 'fractions': subcellular particles or multienzyme systems separated

(iii) 'purified': isolated enzymes.

It should be emphasized that none of these levels is to be regarded as 'higher' or 'lower' than the others; integrated information from all levels of investigation will ultimately have to contribute towards a comprehensive understanding of the biology of parasites.

In Vitro *Culture of Parasites* (*b and c above*)

A great many biological investigations, by analogy with bacteria, can be made with parasites growing at the second level. Cultures of this type can also be regarded as a convenient source of material for biochemical study, particularly of protozoa. From the biochemical point of view, however, it is in passing from this to the next level that the most interesting growth requirements are to be defined, for it is here that nutritional studies are made. The chemical aim is to devise the simplest medium of known chemical composition in which the parasite can consistently reproduce. Dependence upon the endogenous availability of a particular substance is a reflexion of the organism's failure to synthesize that substance so the complexity of an organism's nutritional requirements becomes an inverse index of its biosynthetic capabilities. By comparing the nutritional requirements of parasites with those of free-living organisms, we may hope to find some clues to the nature of parasitism.

(i) *Helminths.* Elsewhere Silverman (p. 45) describes how an encouraging start has been made in the culture of helminths. Quite long survival of the adults of several species has been reported and complete or partial maturation of nematodes and platyhelminths has been observed, but there is as yet little information of nutritional requirements. Only in the case of *Neoaplactana glaseri* has continuous, axenic, successive culture in a defined medium been achieved. This nematode, which has

been studied extensively by Glaser, by Stoll and more recently by Jackson (1962), amongst others, can complete its life cycle in a complex medium containing about 70 defined components. How many of these are essential, merely stimulatory, or unnecessary, is still to be determined. A requirement for as yet undefined factors is indicated by the fact that growth is improved about ten-fold by adding to the defined medium a raw extract of liver from a pregnant rabbit.

(ii) *Protozoa.* Two species of Trypanosomidae can be grown readily in a defined medium. These are *Crithidia fasciculata* (Nathan and Cowperthwaite, 1954), parasitic in mosquitoes and *Strigomonas oncopelti* (Newton, 1956) a parasite of Hemiptera and latex plants; the nutritional requirements of both these parasites are surprisingly simple. Of the haemoflagellates only the monogenetic insect form is culturable *in vitro;* so far growth has only been obtained, and with varying success, in undefined media which tells us little about their nutritional requirements. Their bloodstream forms have so far defied all attempts at culture, as they revert rapidly to the insect form *in vitro.* In spite of this limited success, ability to culture the insect forms of trypanosomes has made possible some rather fundamental biochemical studies.

Progress in the culture of malaria parasites is likely to be even slower than with trypanosomes. In the best conditions described (Trager, 1957), *Plasmodium lophurae* can be maintained, with some nuclear division and segmentation, for up to 4 days. The organism has an apparent requirement for non-dialysable factors present in concentrated extracts of duck erythrocytes.

Rapid advances in this field cannot be hoped for, but there is every hope that a patient, step-wise approach, following the several procedures found so successful in the study of bacterial nutrition, will be successful in making possible the culture of a wide range of parasites. Without letting it discourage us, we should remember that it took 10 years to elucidate the nutri-

tional requirements of the free-living protozoon *Tetrahymena pyriformis* (see Elliott, 1959). One complicating possibility which should be born in mind, particularly when dealing with intracellular parasites, is that the organism is dependent not just on relatively simple and stable products of the host's metabolism but on host enzymes or on products which are so inherently unstable as to demand a close spatial relationship with host enzyme systems.

Cell Suspensions (section d above)

Parasites for biochemical study must, in the absence of methods for *in vitro* culture, be derived from a suitable host animal and should be uncontaminated by host material. With regard to intracellular parasites Bowman *et al.* (1960) have pointed out that conclusions based on a comparison of normal and parasitized erythrocytes may be misleading, since the assumption that the parasites have no influence on host cell metabolism may not be a valid one. Moreover *Plasmodium berghei* has a predilection for reticulocytes, which introduces the complication that one is dealing with a mixed population of host cells.

Separation of protozoa from host blood containing an anticoagulant is based on differential centrifuging. To obtain concentrated suspensions of reticulocytes parasitized with *P. berghei*, Fulton and Spooner (1956) centrifuged blood through a density gradient of egg albumin, a procedure introduced by Ferrebee and Geiman (1946) who had used more costly bovine albumin. Plasmodia can be liberated from erythrocytes by lysing the latter with specific rabbit antiserum in the presence of guinea pig serum as a source of complement and can be further cleaned up by alternate centrifuging and resuspension. When a suitable suspending medium is used the parasites appear not to be damaged by these manipulations (Bowman *et al.*, 1960). Contaminating leucocytes can be removed by percolation of

suspensions through small columns of cellular powder (Fulton and Grant, 1956; Bowman *et al.*, 1960).

Removal of erythrocytes from crude suspensions of trypanosomes derived from centrifuged blood is greatly helped by causing them to agglutinate with rabbit antiserum in the absence of complement. Leucocytes have been removed from such suspensions by percolation through cellulose powder (Grant and Fulton, 1957) or by inducing the formation of a fibrin clot followed by gentle filtration through sintered glass (Fulton and Spooner, 1959).

Fulton and Spooner (1960) have prepared suspensions of *Toxoplasma gondii* from the peritoneal fluid of infected cotton rats. After centrifuging, the residue was resuspended in a suitable medium by shaking briefly with glass beads; this resulted in liberation of the parasites. On standing, many leucocytes agglutinated and were removed; erythrocytes were similarly removed after they had been agglutinated by rabbit antiserum. When too few erythrocytes were present to allow agglutination they were lysed by adding guinea pig serum. The parasites, now free of host cells, were further purified by washing. In all such procedures the suspending medium must be carefully chosen, particularly with regard to ionic strength and balance, to pH, to glucose content and to freedom from trace contamination from metals; the interval between removal from the host and the start of the experiment should be kept to a minimum; and adequate temperature control should be maintained throughout.

Cell-free Extracts (*section c above*)

(i) *Protozoa* have been disrupted by a variety of means; they are a great deal more fragile than most bacteria and all procedures that have succeeded with bacteria are probably applicable to protozoa. Simple lysis in distilled water or rupture of the cell membrane by alternate freezing and thawing will

serve many purposes and require no special apparatus. Crushing of frozen suspensions may be preferable; this can be done, for instance, by adding a suspension of organisms dropwise into liquid nitrogen and grinding the frozen particles manually or by making use of the Hughes (1951) press. In this type of procedure (as also in freeze-thawing) the mechanical effect of ice crystals is probably largely responsible for disruption; in the Hughes press sudden thawing caused by high pressure also plays a part. Mechanical shaking at high frequency in the presence of ballotini beads is a well-tried method with bacteria and has been used successfully with parasitic protozoa. Exposure to ultrasound has also been used and will probably have increasing applicability.

No single technique is more generally useful than any of the others and for any particular purpose the simplest effective procedure will have to be established empirically. Liberation of cell contents often results in some inactivation of enzymes or in disorganization of subcellular organelles. A high degree of cellular disintegration is thus not synonymous with a high yield of enzymic activity and very often one will have to arrive at a compromise.

(ii) *Helminths* can usually be treated in the same way as are pieces of vertebrate tissue. A most useful apparatus (introduced by Potter and Elvehjem, 1936), consists of a cylindrical pestle rotating rapidly within a close-fitting mortar. As the suspended tissue is forced backward and forward in the small annular space between the rotating surfaces it is exposed to high shearing forces. These cause rapid disruption of most cells but leave subcellular particles in a relatively intact state.

The apparatus can be made in a wide range of sizes to suit various purposes. Originally it was made wholly of glass but there is now a trend towards making the pestle from a hard plastic, such as polytetrafluorethylene, which can be machined on an ordinary lathe. A micro-modification of the all-glass apparatus has been described by Hess and Pope (1953).

TECHNIQUES FOR QUANTITATIVE STUDY OF CELLULAR METABOLISM

There is available a huge range of methods for precise measurement of reactions catalysed by single enzymes or by multienzyme systems *in vitro*. Most depend upon measuring the rate either of disappearance of substrate or of appearance of products. In general the second is greatly to be preferred; the appearance of product corresponding to a small percentage of the substrate originally present can usually be measured with much more confidence than can the small change in concentration of substrate itself. The most generally valuable parameter of an enzymically catalysed reaction is its initial velocity, which can only be established in conditions where decrease in substrate concentration is small. These conditions are often difficult to satisfy when the rate of substrate disappearance is to be measured.

Measurements can be made either continuously or discontinuously. When it can be used, a continuous method has several advantages: the system is undisturbed by the act of measurement, successive measurements make for an accurate determination of reaction velocity and the experiment can be stopped when the necessary data have been obtained. Because these are immediately available a second experiment, the design of which is dependent on results obtained from the first, can be set up with little delay. Very often, though, changes cannot be observed continuously and a sampling procedure must be used.

GASOMETRIC TECHNIQUES

Numerous studies have been made on the respiration of parasites by measuring changes in gas volume with the Barcroft (constant pressure) type of apparatus or changes in pressure with the classic Warburg (constant volume) apparatus. These techniques are familiar to most workers and their principles

and practice have been well described, for instance by Dixon (1951) and by Umbrit *et al.* (1957). It should be remembered that the use of gasometric techniques is not confined to measurements of O_2 uptake or CO_2 output; most reactions in which an acid is formed can be followed by measuring displacement of CO_2 from a bicarbonate buffer.

Much valuable information about parasites has been gained by the use of such techniques, which will undoubtedly have an increasingly wide application in this field. Some modifications of the classical technique will be outlined later, in the belief that their adoption will allow studies of a type not yet undertaken.

SPECTROPHOTOMETRIC TECHNIQUES

The development of instruments of high precision capable of isolating narrow band-widths in regions within and on either side of the visible range has gone hand in hand with the development of very sensitive and specific spectrophotometric methods for enzyme assay.

Continuous observation of a reaction can be made in most cases where there exists a difference in light-absorbing properties as between substrate and product. In suitable conditions the rate of change in light absorption at a selected wavelength is a direct measure of reaction velocity. For many purposes experiments can be done at room temperature but for more refined work it may be necessary to maintain the cuvettes at constant temperature by incorporating a thermo-regulating device into the instrument. A simple example is seen in the conversion of fumarate to malate as catalysed by the enzyme fumarase. The substrate (fumarate) absorbs strongly at 300 mμ whereas the product (malate) does not. With a suitable initial concentration of fumarate the decline in absorption at 300 mμ is a direct index of the course of the reaction. Similarly the reduction of the nicotinamide adenine dinucleotides is accompanied by the appearance of an absorption band with a

peak at 340 mμ Reactions catalysed by a large number of dehydrogenases can thus be studied by recording changes in absorption at this wavelength. By coupling them they can also be exploited in a most elegant fashion for the measurement of certain reactions which are not themselves attended by a change in absorption. Several reliable assays take advantage of this type of manouvre and with increasing availability of purified enzymes of 'reagent grade' an extension of it is likely to be seen.

In the same sort of way other oxido-reductions involving natural electron acceptors, particularly the cytochromes, can be followed spectrophotometrically. In some situations artificial acceptors operating at an appropriate potential may be preferred; if the complication of using anaerobic cuvettes is to be avoided an acceptor having a low rate of autoxidation must be used. Methylene blue, which played such a valuable part in the earlier studies on dehydrogenases, is readily reoxidized from its reduced (colourless) state in the presence of atmospheric oxygen. On the other hand the leuco form of certain other dyes, such as 2,6-dichlorophenolindophenol, is reoxidized at a rate which in many conditions may be negligible.

Esters and glycosides of substituted phenols are useful in the study of a number of hydrolytic enzymes including esterases, phosphatases, sulphatases and glycosidases. The principle is basically a simple one and depends on the fact that the free phenol or phenate ion liberated by hydrolysis has a peak absorption at a wavelength where the substrate absorbs relatively little light. Such chromogenic substrates are most widely used in the discontinuous type of measurement, mainly because the pH value necessary to produce a useful differential light absorption between substrate and product is well above that of optimal enzymic activity.

Discontinuous application of many of the spectrophotometric principles indicated above is feasible and may be more convenient, for instance where absolute activity is not high