COMPARATIVE BIOCHEMISTRY OF PARASITES

edited by H. Van den Bossche

Comparative Biochemistry of Parasites

Proceedings of an International Symposium organized by the
Janssen Research Foundation and held at Janssen Pharmaceutica
Beerse, Belgium, September 1-3, 1971

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H. Van den Bossche

Department of Comparative Biochemistry
Janssen Pharmaceutica
Beerse, Belgium

Academic Press
New York and London
1972

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 72-187249

PRINTED IN THE UNITED STATES OF AMERICA

CONTENTS

PARTICIPANTS

*Asterisk denotes chairmen

Bafort, J. M., Instituut voor Tropische Geneeskunde, 2000 Antwerp, Belgium

*Beames, Calvin G., Jr.,** Department of Physiology, Oklahoma State University, Stillwater, Oklahoma 74074

Bone, G. J., Rue Baron de Castro 7, 1040 Brussels, Belgium

Borgers, M., Janssen Pharmaceutica, 2340 Beerse, Belgium

Borst, P., Laboratorium voor Biochemie, Jan Swammerdam Instituut, Amsterdam, The Netherlands

Bout, D. T. J., Service d'Immunologie et de Biologie Parasitaire – Université de Lille, Lille, France

Bowman, I. B. R., Department of Biochemistry, University of Edinburgh Medical School, Edinburgh EH 8 9AG, Scotland

*Bryant, C.,** Department of Zoology, Box 4 G.P.O., Canberra A.C.T. 2600, Australia

*Bueding, Ernest,** The Johns Hopkins University, Baltimore, Maryland 21205

Cheah, K. S., A.R.C. Meat Research Institute, Langford, Bristol BS18 7DY, England

Chowdhury, N., Laboratorium voor Zoöfysiologie, Rijksuniversiteit Gent, 9000 Gent, Belgium

Coles, G. C., MRC Biochemical Parasitology Unit, The Molteno Institute, University of Cambridge, Cambridge CB2 – 3EE, England

Davey, K. G., Institute of Parasitology, MacDonald College 800, Province of Quebec, Canada

De Zwaan, A., Laboratorium voor Scheikunde en Dierfysiologie, Universiteit Utrecht, Utrecht, The Netherlands

Dierick, W. S. H., Laboratorium voor Biochemie, RUCA–2020 Antwerp, Belgium.

Eckert, J., Institut für Parasitologie, Universität Zürich, Zürich, Switzerland

Eeckhout, Yves, Laboratoire de Chimie Physiologique, Université de Louvain, 3000 Leuven, Belgium

Gutteridge, W. E., Biological Laboratory, University of Kent, Canterbury, Kent, England

Haese, W. H., The Johns Hopkins Hospital, Baltimore, Maryland 21205

Hill, George C., MRC Biochemical Parasitology Unit, The Molteno Institute, Cambridge, CB2-3EE, England

Howells, R. E., Liverpool School of Tropical Medicine, Liverpool L3 5QA, England

Jaffe, Julian J., University of Vermont College of Medicine, Given Building, Burlington, Vermont 05401

*Janssen, Paul A. J.,** Janssen Pharmaceutica, 2340 Beerse, Belgium

Janssens, P. G., Instituut voor Tropische Geneeskunde, 2000 Antwerp, Belgium

Kaba, A., Laboratoire de Pharmacodynamie général, Université de Louvain, 3000 Leuven, Belgium

Kohler, P., Institut für Parasitologie, Universität Zürich, Zürich, Switzerland

*Lee, D. L.,** Department of Pure and Applied Zoology, University of Leeds, Leeds LS2 9JT, England

Le Ray, D., Instituut voor Tropische Geneeskunde, 2000 Antwerp, Belgium

Meyer, Franz, Upstate Medical Center, Syracuse, New York 13210

Meyer, Haruko, Upstate Medical Center, Syracuse, New York 13210

Moors, A., Instituut voor Tropische Geneeskunde, 2000 Antwerp, Belgium

Mortelmans, J., Instituut voor Tropische Geneeskunde, 2000 Antwerp, Belgium

*Newton, B.A.,** MRC Biochemical Parasitology Unit, The Molteno Institute, University of Cambridge, Cambridge, CB2-3EE, England

Prins, R. A., Laboratorium voor Veterinaire Biochemie, Universiteit Utrecht, Utrecht, The Netherlands

Reeves, R. E., Louisiana School of Medicine, New Orleans, Louisiana 70112

Riou, G., Institut Gustave, Roussy, Villejuif 94, France

Rombouts, W. A. J. A., Medische Fakulteit, Universiteit Louvain, 3000 Louvain, Belgium

Ruitenberg, E. J., Rijksinstituut voor de Volksgezondheid, Utrecht, The Netherlands

Ryley, J. F., Pharmaceutical Division, I.C.I., Macclesfield, Cheshire SK10 4TG, England

*Saz, Howard J.,** College of Science, University of Notre Dame, Notre Dame, Indiana 46556

Steinert, M., Faculté des Sciences, ULB, 1640 St. Genesius-Rode, Belgium

Tollenaere, J. P., Janssen Pharmaceutica, 2340 Beerse, Belgium

*Trager, W.,** The Rockefeller University, New York, New York 10021

Van den Bossche, H., Department of Comparative Biochemistry, Janssen Pharmaceutica, 2340 Beerse, Belgium

Van de Vijver, G. H., Instituut voor Zoöfysiologie, Universiteit Gent, 9000 Gent, Belgium

Van Meirvenne, N., Instituut voor Tropische Geneeskunde, 2000 Antwerp, Belgium

Van Miert, A., Laboratorium voor Veterinaire Farmacologie, Universiteit Utrecht, Utrecht, The Netherlands

Van Nueten, J. M., Janssen Pharmaceutica, 2340 Beerse, Belgium

Veenendaal, G. H., Laboratorium voor Veterinaire Farmacologie, Universiteit Utrecht, Utrecht, The Netherlands

Vercauteren, R. E., Fakulteit voor de Diergeneeskunde, Universiteit Gent, 9000 Gent, Belgium *von Brand, T. C.*;* 8606 Hempstead Avenue, Bethesda, Maryland 20034

Wattiaux, R., Facultés Universitaires Notre Dame de la Paix, 5000 Namur, Belgium

Weinbach, Eugene C., Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Maryland 20014

PREFACE

In the last two decades a tremendous amount of progress has been made in the field of parasite physiology and biochemistry. Studies on the intermediate metabolisms, on the characterization of nucleic acids, on transport mechanisms, and on the mode of action of chemotherapeutic agents have contributed to a better understanding of the biochemistry and physiology of protozoa and helminths. The main object of the Symposium on the Comparative Biochemistry of Parasites, which was held at Janssen Pharmaceutica from September 1 to 3, 1971, was to bring together experts on various aspects of protozoa and helminth biochemistry and physiology in order to evaluate present knowledge, to stimulate further progress in this field, and to find new approaches for the rational design of new chemotherapeutic agents.

It is always difficult to ascertain whether a symposium is organized at an appropriate date. The most important reason for organizing this symposium in September 1971 was that Dr. Theodor von Brand was able to participate then. Dr. von Brand has worked in the field of parasite physiology and biochemistry for nearly half a century and, as Professor Weinstein [*J. Parasitol.* **56** (1970), 625] pointed out recently, has acted as a leavening agent in this discipline. This book, which is comprised of the papers presented at the symposium, is dedicated to his interest and honor.

It is a pleasure to thank the participants and the chairmen, and particularly Dr. Paul A. J. Janssen for providing the opportunity to organize this symposium and for his continued encouragement. I am pleased to acknowledge the advice of E. Bueding, D. Fairbairn, H. J. Saz, D. Thienpont, and T. von Brand in the preparation of the symposium, and the efficient help of a great number of my colleagues at Janssen Pharmaceutica.

I sincerely hope that this book fulfills its purpose as a tool for future research.

H. Van den Bossche

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Comparative
Biochemistry
of Parasites

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GLIMPSES AT THE EARLY DAYS OF PARASITE BIOCHEMISTRY.

Theodor von Brand
8606 Hempstead Avenue
Bethesda, Maryland 20034, U.S.A.

Parasitology, constitutes only a tiny segment of natural science or medicine. No wonder then that parasitology is mentioned only in a very cursory manner in books dealing with the history of science or medicine. Many newer books on these subjects exist and I looked at a few of them taken at random from the library shelves. This is what I found. The index to the two volumes of Packard's (1963) "History of medicine in the United States" does not contain the words parasites or parasitic diseases. The book gives, however, an account of an early 17th century case of a large uterine hydatid cyst in an unfortunate woman. Lloyd's (1968) "A hundred years of medicine" has a 4 1/2 pages long chapter entitled "Some larger parasites". In Major's (1954) two volumes of "A history of medicine" and in Singer and Underwood's (1962) "A short history of medicine" one finds brief discussions of various tropical diseases, such as malaria, trypanosomiasis, ancylostomiasis, and others. Needless to say the physiology or biochemistry of parasites is not discussed.

If one looks for books dealing specifically with the history of parasitology one is struck by the fact that only very few have been written. Of very great interest are the scholarly works of Hoeppli "Parasites and parasitic infections in early medicine and science" (1959) and "Parasitic diseases in Africa and the Western hemisphere" (1969). Both make for fascinating reading, but for obvious reasons they do not contain and cannot be expected to contain a discussion of the development of parasite biochemistry. As far as I am aware only one other relevant book exists. It is Foster's (1965) "A history of parasitology". Foster traces the development of our knowledge concerning certain parasites and groups of parasites without, however, mentioning any data on parasite biochemistry.

Interesting historical data abound of course in biographies and autobiographies of such men as Sir Patrick Manson (Manson-Bahr and Alcock, 1927), Sir Ronald Ross (Ross, 1923), Geheimrat Bernhard Nocht (Martini, 1957) and others. But these men were not parasite physiologists and therefore the topics parasite physiology or biochemistry have no place in such accounts. In short, we just do not have as yet a history of parasite physiology and biochemistry.

I am not a trained historian and I do not pretend that I can fill this gap adequately, especially not in the confines of a brief lecture. I have, however, been active in the field for nearly half a century, have been interested for a long time in some of the old findings and am therefore to some extent familiar with historical developments. I am also familiar with the reasons why old findings frequently are forgotten. Younger investigators often discount old data as not being relevant to modern approaches and they seem reluctant to read the old literature. I think a survey of parasite physiologists asking them how many have read in the original Weinland's (1901b) classical paper "Uber Kohlenhydratzersetzung ohne Sauerstoffaufnahme bei ***Ascaris,*** einen tierischen Gärungsprozess" would be quite illuminating. Furthermore practically all journals discourage or reject long historical introductions and I certainly do not advocate that the literature citations of each research paper should go back to what amounts to be prehistoric times. I do want, however, to draw your attention to the fact that one can find in the old literature significant data which, if known to subsequent workers, could have accelerated later developments and I would not be too surprised if future historians would find more recent examples of the same type.

A case in point is the cuticle of ***Ascaris.*** Grube (1850) stated that it consisted of chitin and the terms chitin or chitinous were later often used in connection with it and other structures of the nematode body, such as the lining of the stoma and esophagus. And this despite the fact that Lassaigne had proven in 1843 that the cuticle of ***Ascaris,*** in contrast to the chitin of insects, was solubilized by potassium hydroxyde and despite the fact that he stated unequivocally that both do not have the same structure. In fairness to Grube it should be mentioned that Lassaigne's observations on ***Ascaris*** were contained in a paper entitled "Sur le tissue tégumentaire des insectes de differents ordres", that is in a paper where one would not expect to find data on worms.

Another quite instructive example concerns the polysaccharides of ascarids. The great French physiologist Claude Bernard established as early as 1859 that glycogen occurs not only in the liver of vertebrates, but that it can be demonstrated readily by qualitative chemical methods and also histochemically by means of the iodine reaction in such parasites as ***Ascaris, Fasciola, Taenia,*** and larval cestodes. These findings were immediately forgotten as indicated by the fact that the English physiologist Sir Michael Foster published in 1865 a paper in which he states explicitly that, although glycogen had been reported from numerous invertebrates, nobody so far noticed its occurrence in parasitic worms. He himself demonstrated it qualitatively in an undetermined tapeworm and quantitatively in ***Ascaris lumbricoides,*** but his findings also made no lasting impression on his contemporaries.

Gustav von Bunge, a scion of an old aristocratic Baltic family who worked first in Dorpat, Estonia and later took the newly established chair of physiological chemistry at Basel, Switzerland, did not know about these old observations. He had first become interested in the oxygen requirements of intestinal worms because he believed on theoretical grounds that the helminths of warm-blooded hosts would have only minimal needs for oxygen. This view was based on the assumption that fermentative processes were the source of muscular activity and that oxidative processes were related primarily to heat production. He observed experimentally that various ascarid species survived anaerobic periods rather well and that the worms produced anaerobically large amounts of CO_2 and an unidentified volatile acid (Bunge, 1883, 1889). He was however unable to hazard a guess as to the source of these metabolites. Had he been aware of the older work of Bernard or Foster, he undoubtedly would have put two and two together and would thus have been in a position to anticipate Weinland's later findings by more than a decade.

Up to the time of Bunge the chemical studies on helminths were confined to observations on their chemical composition; he was the first to carry out experimental metabolic studies. There are two reasons why Bunge did not pursue this work further. First he found it difficult to secure sufficient fresh worms at Basel and secondly he immersed himself in his later years completely in the temperance movement. A biography of Bunge written years after his death by an admirer of his antialcoholic activities (Graeter, 1952) does not mention even with a single word his work on free-living and parasitic invertebrates.

Before turning to Weinland's achievements, I would like to emphasize that quite a few additional chemical data can be found in the old helminthological literature. Time does not permit to review them in any detail, but a few examples may illustrate the type of data one can unearth. I mention first the calcareous corpuscles of cestodes. In an abstract of a paper presented by Doyère (1840) before the Société philomatique de Paris one finds the statement that those of ***Echinococcus*** consist of calcium carbonate. At the same time Gulliver (1840, 1841) studied the corpuscles of ***Cysticercus*** which he considered as the worms's eggs. He observed that they dissolve quickly upon treatment with hydrochloric ("muriatic") and acetic acid with plentiful evolution of a gas. When the solution was treated with sulfuric or oxalic acid a white precipitate was obtained. Küchenmeister (1851) did not share Gulliver's view concerning the significance of these structures. He remarked that not all corpuscles consist of calcium carbonate, since he found that those of some species dissolve in acid without the evolvement of gas, indicating that they consist of calcium phosphate. Leuckart (1863) mentions in the first edition of his famous book "Die menschlichen Parasiten und die von ihnen herrührenden Krankheiten" that a Dr. Naumann had investigated on his suggestion the inorganic substances of the ***Taenia marginata*** body which he thought could be referred largely to calcareous corpuscles. Dr. Naumann found mainly calcium salts accompanied by small amounts of magnesium, ironoxyde, sodium and potassium which were bound to carbonic, phosphoric, hydrochloric, and sulfuric acid. I must admit that I was totally unaware of these old observations when I first isolated cestode calcareous corpuscles some 90 years after Gulliver's and Doyère's papers appeared.

Quite a few additional old data can also be located for the cycts of ***Echinococcus,*** of which I will mention only two. Heintz showed as early as 1850 that the hydatid fluid contained succinic acid in the form of sodium succinate, a finding never mentioned when modern investigators discuss the production of succinic acid by cestodes. Some of the old investigators were also aware that the hydatid fluid contains sugar. This was shown first for human infections by Bernard and Axenfeld in 1857 and confirmed in 1860 by Lücke who also showed that sugar could be liberated from the membranes by treatment with acid. Incidentally, the Bernard just mentioned is not the famous Claude Bernard, but an unknown Charles Bernard. The note by Bernard and Axenfeld seemingly was not prepared by themselves, but appears to be a summary of a talk written by the Secretary of the Société de Biologie de Paris. It was perhaps the latter who pointed out that the master himself, Claude Bernard, had already found previously sugar in the hydatid fluid of sheep.

With the exception of Bunge's studies the above findings had no significant influence on subsequent developments in the field of helminth physiology. Bunge's studies are the one exception because they stimulated Weinland to take up his justly famous ***Ascaris*** investigations. Ernst Weinland worked at the turn of the century at the Physiological Institute of the University Munich. It is not surprising that he was interested in this aspect of comparative physiology since as a son of the well known helminthologist David Weinland he had been exposed early to parasitology and since he had not only an MD degree, but had also earned a Ph.D degree in zoology. He favored throughout his career the chemical approach to various life processes and relied almost exclusively on quantitative gravimetric methods, while having little faith in the then available colorimetric procedures. His interest centered largely around the metabolism of parasitic worms, but they were not confined to them. He published for instance a series of papers dealing with the metabolism of the fly ***Calliphora*** and one of his last papers dealt with the chemical composition of hedgehogs after various periods of hibernation (Weinland, 1925). His thorough knowledge of the field of comparative chemical physiology and his typical critical approach are well documented in a review he published in 1910 (Weinland, 1910). He had prepared a much more comprehensive treatise on the subject for Winterstein's Handbuch der vergleichenden Physiologie, when the outbreak of the first world war prevented its publication. After the war Weinland tried to bring the manuscript up to date, but because of rather poor health and his preoccupation with preparing his daily physiology lecture to medical students and his involvement with the turbulent politics of these years he never finished it. Finally Winterstein commissioned others and the result was a rather hastily prepared and incomplete account of the vast field of comparative metabolism (Kestner and Plaut, 1924).

However, on this occasion we are interested primarily in Weinland's parasitological work (Weinland, 1901 a, b, 1902 a, b, 1903, 1904, Weinland and Ritter, 1903). He started out by determining, without knowledge of the earlier comparable studies, the glycogen content of various parasitic helminths and was impressed by the enormous quantities stored by them. He then concentrated on ***Ascaris lumbricoides,*** establishing quantitative relationships between glycogen disappearance during starvation and the production of both carbon dioxide and volatile acids. He first thought that valeric acid was excreted almost exclusively; later on he recognized the acids as a mixture of valeric and caproic acids. Since he observed that the metabolic rate was about the same under aerobic and anaerobic conditions, he expressed the rather revolutionary idea that ***Ascaris,*** other intestinal worms, but also free-living mud- and swampdwellers do not require any molecular oxygen. He expressed this view perhaps most clearly in the lecture which he delivered according to German custom before the assembled faculty shortly after assuming the Professorship of Physiology at the University of Erlangen ("Akademische Antrittsrede", Weinland, 1913). He stated then: In these animals a supply of and a requirement for molecular oxygen is unnecessary for the functioning of their vital processes ("Bei diesen Tieren ist eine Zufuhr und ein Bedarf von elementarem Sauerstoff für den Ablauf der Lebensvorgänge nicht erforderlich").

Weinland's approach to the ***Ascaris*** problem was comprehensive. Besides studying the overall carbohydrate metabolism, he also showed that the worms do not utilize lipids for energy production, that they do decompose nitrogenous substances, that they produce antienzymes, and that cell-free preparations show still a fermentative metabolism. This work established him as the foremost authority on parasite metabolism of his day and his work stimulated some other investigators to take up studies in the field. I mention here only a few of them: Schimmelpfennig (1903) studied the chemical composition of ***Parascaris,*** von Kemnitz (1912) published a beautiful paper on the histochemical distribution of glycogen in ***Ascaris*** tissues, Ortner-Schönbach (1913) performed a similar study on trematodes and cestodes, and Krummacher (1918) investigated the heat production of ***Ascaris***. In these same years a few papers were also published that were only indirectly influenced by Weinland's work, or had no connection with it at all. The best known examples are the investigations of the German pharmacologist Flury (1912) on the chemistry and toxicology of ***Ascaris*** and the outstanding study of the French biologist Fauré-Fremiet (1913) on the chemistry of the ***Parascaris*** reproductive cycle. Fauré-Fremiet, incidentally, is the only one of the early group of workers still alive and still active in research, although having left the field of parasite physiology years ago to concentrate on the fine structure of protozoa.

We will now examine briefly the reception accorded Weinland's views by the early workers. Not too surprisingly they were not accepted immediately and fully, but were questioned on various grounds. The first point challenged concerned the production of volatile acids by an animal. Fischer (1924) and Slater (1925, 1928) maintained that the true metabolic endproduct of ***Parascaris*** and ***Ascaris*** was lactic acid, just as it is in the anaerobic metabolism of vertebrate tissues. The former found this to be true when he studied minced worms and in retrospect one can say that his observation was correct, but his interpretation was not. Saz and Lescure (1969) showed recently that the shift in metabolism observed in minced or homogenized worms is due to disturbed segregation of enzymes. Slater (1925, 1928), on the other hand, thought that the volatile acids found regularly in incubates of ascarids were formed by bacterial activity. We know now that this assumption was incorrect, since Epps et al. showed in 1950 that axenized specimens of ***Ascaris*** produce large amounts of volatile acids.

In this respect then Weinland's views were fully vindicated. They fared less well when his thesis was challenged that intestinal worms do not consume oxygen. Various workers working independently at about the same time disproved the point for various helminths. In so far as ***Ascaris*** specifically is concerned, a Belgian worker, Adam, now Head of the Section of recent Invertebrates at the Institut Royal des Sciences Naturelles de Belgique and working then in Jordan's laboratory at Utrecht showed in 1932 that female and male specimens as well as homogenized muscle tissues consume appreciable amounts of oxygen. Even earlier, namely in 1931, had oxygen consumption been demonstrated for the cestode ***Moniezia expansa*** by the Americans Alt and Tischer and in 1932 the German Harnisch showed that the trematode ***Fasciola hepatica*** uses oxygen. These, I believe, are the earliest relevant papers. Since then similar observations were reported for many additional species and today nobody doubts any more that intestinal as well as tissue helminths are capable of utilizing oxygen if they have access to it.

Another controversial point was, and to some extent still is, the question whether intestinal worms lead in situ an anaerobic or an aerobic life. Weinland of course maintained that they do not require oxygen, but this view was challenged repeatedly, in the older days primarily by Slater (1925) and Davey (1938). The former had observed that electrically stimulated ascarids survived better in the presence than the absence of oxygen and the latter established that small nematodes of the sheep intestine could be kept in vitro for longer periods aerobically than anaerobically. As I pointed out a long time ago (von Brand 1938 a) these opposing views are really not mutually exclusive but can be reconciled if one assumes that large intestinal worms because of their organisation and the low oxygen concentrations in their environment lead in nature a predominantly anaerobic life while small parasites can gain in the same habitat significant amounts of oxygen.

It should be emphasized that the assumption of a predominantly anaerobic life of large helminths refers only to the mode of energy production. It is possible and even probable that the small amounts of oxygen which they can acquire under natural conditions may be quite important. Fairbairn (1970) emphasized the point in respect to the collagen formation of ***Ascaris*** which depends on the functioning of an oxygen requiring hydroxylase and the activity of the phenolase responsible for the tanning of the ***Fasciola*** egg shell may according to Moss (1970) account for quite a high percentage of the worm's oxygen consumption. But I dont want to say more about these new findings since it is today my task to review the accomplishments of the research done in days gone by.

Looking back from the vantage point of the seventies on the research in helminth physiology done in the old days, that is about to the start of the second World War, one is struck by several facts. First only relatively few species served as experimental tools. These were the nematodes ***Ascaris*** and ***Parascaris,*** the trematode ***Fasciola,*** and the cestode ***Moniezia.*** Other species were studied only in isolated instances. Mention may be made of the interesting study of the ***Dioctophyme renale*** hemoglobin by Aducco (1889), the study of Bondouy (1910) on the chemical composition of ***Sclerostomum equinum,*** Schopfer's (1932) investigations on the body fluids of various parasites or McCoy's (1930) analysis of the repiration of ***Ancylostoma*** larvae in dependence of temperature and other factors. During these years many more papers dealing with one or the other physiological aspect of the main experimental animals mentioned previously appeared. I list at this point only a few of them. Keilin described in 1925 the occurrence of cytochromes in ***Ascaris*** and Pintner (1922) and Stepanow-Grigoriew and Hoeppli (1926) voiced different views concerning the physiological basis of the nematode's larval migration through the host body.

These last studies already have the second characteristic of the old studies. I refer to the fact that usually entire animals, but occasionally also minced materials were used to investigate some phase of the overall metabolism, but that no studies on the intermediate metabolism were carried out. Examples are the studies on the respiration of ***Ascaris*** by Krueger (1936, 1937) or of ***Triaenophorus*** by Harnisch (1933), the metabolic studies on ***Ascaris*** by Schulte (1917), von Brand (1934) or Oesterlin (1937), the studies on the overall and respiratory metabolism of ***Fasciola*** by Flury and Leeb (1926) or Weinland and von Brand (1926) and of ***Moniezia*** by von Brand (1929, 1933 a) and Cook and Sharman (1930).

The third striking fact about the old research is that very little was done to study in dept the influence of helminths on the metabolism of the host. True enough, relevant data can be found in the old medical literature, for example data on some blood or tissue constituents of human patients infected with ***Trichinella*** (Fuchs, 1922) or hookworms

(Rake, 1894, Donomae, 1927) or data on the metabolism, especially the nitrogenous excretions during these infections (Padoa, 1909, Markowicz and Bock, 1931, Bohland, 1894). However, these and similar investigations did not contribute materially to an analysis of the influence of the parasites on the physiological processes of the host. The only really old experimental studies known to me are the investigations of Flury (1913) and Flury and Groll (1913) on the influence of a ***Trichinella*** infection on the metabolism, especially the nitrogen metabolism of the host.

If we turn now to the biochemistry of parasitic protozoa we find that its development differed in various respects from that just described for helminth biochemistry. First, only very few really old investigations of a clearly chemical nature exist. This of course is not surprising but simply a consequence of the fact that protozoa were difficult to secure in sufficient amounts and sufficient purity in the days when modern micro-methods of analysis had not yet been developed. It is the more remarkable therefore that the justly famous zoologist Bütschli was able to demonstrate in 1885 that the gregarine polysaccharide was water soluble, could be precipitated by alcohol and yielded a reducing sugar upon hydrolysis. The only other truly old studies are dated 1911 and 1913 and are due to Panzer who studied in Vienna primarily the lipids but to some extent also the proteins of the coccidian ***Goussia gadi***. I mention here only that he identified cholesterol and that he established the fact that the fatty acids and glycerides of the parasites differed distinctly from those of the host.

Besides these isolated chemical studies quite a few histochemical data concerning parasitic protozoa can be located in the old literature. Examples are the demonstration of polysaccharides in gregarines by Maupas (1886), in rumen ciliates by Certes (1889) and parasitic amebas by Kuenen and Swellengrebel (1913), or the demonstration of lipid droplets and glycogen in verious sporozoa by Thélohan (1894), Cohn (1896), Brault and Loeper (1904), and others. All these studies however had no discernible influence on the subsequent developments. In contrast to what I said a moment ago about helminth physiology, physiological and biochemical studies of parasitic protozoa received their impetus from early investigations dealing with physiological and biochemical alterations sustained by parasitized hosts. These started early and in general preceded biochemical investigations on the protozoa themselves. To give an extreme example: The malarial pigment deposited in the organs of malarious patients was known for many years before the possible existence of an organism like a ***Plasmodium*** was even dreamed of.

The history of the malaria pigment is long and exemplifies the fact that refinements in experimental technique can make older conclusions obsolete. A full discussion of this topic would require a special lecture; I can mention on this occasion only a few of the old workers in this specialized field. Discoloration of the internal organs of malarious patients has been known for a long time. The earliest references quoted in the literature, which however were not available to me for checking, are the reports by Lancisi (1717), Stoll (1797), and some others. The origin of this dark pigment was widely discussed because two opposing views were proposed during the 19th century: The theory of splenic origin promulgated by Meckel (1847) and Virchow (1849) and the theory of hematogenous origin, usually ascribed to Planer (1854).

Incidentally, it is by no means certain that all the above workers were always dealing with cases of chronic malaria. Indeed Meckel's (1847) autopsy report concerned an insane woman who had been confined for many years in a mental institution and who was not known to have suffered from malaria. This was emphasized by Meckel himself when he described a few years later (Meckel, 1850) the regular appearance of pigment in the spleen and the blood of malarious patients, but Virchow (1849) stated clearly that some of his autopsy cases had suffered from intermittens as he called malaria. Neither Meckel (1847, 1850) nor Virchow (1849) expressed definite views as to the chemical nature of the pigment. The former, however, made solubility tests and observed color changes of the pigment under the influence of acids and alkali. From reading his papers one gets the impression that he allied the pigment to the socalled melanotic pigments. For obvious reasons these old investigators took it for granted that the human body itself produced the pigment. This changed of course very soon after the malaria parasites had been detected and the view was generally accepted that the pigment was derived from the hemoglobin of the host erythrocytes and was formed within the parasites.

For years the view persisted that the malarial pigment was a melanin, that is, an iron-free pigment (e.g. Schridde, 1921), but doubts began to appear as evidenced by the fact that new names were coined for it. Ross (1910) called it plasmodin, Askanazy (1921) haemo-melanin, and eventually the now current name, hemozoin, was generally accepted. The reasons for finally abandoning the old view were on the one hand differences in solubilities and reactions to the bleaching action of oxidizing between the malarial pigment and genuine melanin (Brown, 1911, and others) and on the other hand chemical and spectroscopic data which seemed to indicate close resemblance or even identity of malaria pigment and hematin (Carbone, 1891, Ascoli, 1910, Brown, 1911, and