MICROBIOLOGICAL REVIEWS, Dec. 1988, p. 412–432
0146-0749/88/040412-21$02.00/0

Vol. 52, No. 4

Biochemistry of the *Leishmania* Species

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INTRODUCTION

Since 1980, there has been a burst of published reports describing the biochemistry of various human parasites. Interest in the biochemistry and molecular biology of the human parasites has increased greatly, in part because of the growing awareness of the morbidity and mortality they cause worldwide, especially in developing countries, and because of increased support for parasite research in the developing countries through programs sponsored by governments and various foundations and philanthropic groups concerned about global health problems.

This review focuses mainly on the enzymes and metabolic machinery of the *Leishmania* spp., the trypanosomatid genus responsible for the various forms of leishmaniasis in humans: visceral leishmaniasis (kala azar), mucocutaneous leishmaniasis (Espundia), and cutaneous leishmaniasis (Old World, oriental sore; New World, South and Central America) (23). It is appropriate to open this review with a quotation from an article that appeared in 1987 (68): "It has been estimated that 12 million new cases of leishmaniasis occur each year, yet remarkably little is known about the intermediary metabolism" of the causative organism. Our purpose is to provide a comprehensive view of the metabolic capabilities of the *Leishmania* spp. Because of our own particular interest in the cell surface phosphatases of these organisms, emphasis is placed on cell surface and secreted enzymes. We also summarize what is known about carbohydrate, fat, and nucleotide metabolism in the *Leishmania* spp. Discussion of the structure and metabolism of the nucleic acids is beyond the scope of this review.

New knowledge about the enzymes, proteins, and other macromolecules (e.g., leishmanial excreted factor) synthesized by parasites in general is important for at least three reasons. First, such information may provide insight about the function of these macromolecules or inform us of which ones play some key role in the life cycle or contribute to the infectivity of the parasite. Second, these proteins, especially if they are essential to the organism living extracellularly or if they play some important pathophysiologic role in the host-parasite relationship, could provide targets for innovative therapeutic strategies. Third, assays for these macromolecules, be they enzymologic or immunologic, might be useful in the diagnosis of leishmaniasis and in the identification of various strains within a particular clinical form of the disease, thereby helping guide the selection of the most appropriate therapy.

We have divided the biochemistry of the *Leishmania* spp. into three sections: plasma membrane-bound enzymes, intracellular metabolic pathways, and excreted extracellular proteins.

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TABLE 1. Classification of leishmaniasis

Disease complexes	Major parasites	Synonyms	Geographic distribution
Visceral	*L. donovani*	Kala azar	South America, Africa, Mediterranean basin, Asia
Cutaneous	*L. mexicana* *L. tropica* *L. major*	Chiclero ulcer Oriental sore	Central and South America, southern Europe, Africa, Asia, middle Asia, southern USSR
Mucocutaneous	*L. braziliensis*	Espundia	South America

CLINICAL MANIFESTATIONS OF LEISHMANIASIS

For many years, the taxonomy of the genus *Leishmania* was based primarily on clinical factors, geographic distribution, and differences in development in the vector and virulence in experimental hosts. Since these criteria provide an insufficient basis of classification, the development of recent techniques involving monoclonal antibody typing (87), isoenzyme analysis (74), deoxyribonucleic acid (DNA) buoyant density analysis (19), and restriction endonuclease analysis of nuclear and mitochondrial DNAs (4) has resulted in a more complex subdivision of the genus (84, 137). Since the taxonomy of the *Leishmania* spp. based upon newer, more molecular approaches is relatively complex, we present a more simplified and traditional classification based on clinical criteria with prototypal species discussed in each class. Excellent treatises on leishmaniasis have appeared recently, and they cover much of the clinical, epidemiologic, and taxonomic aspects (22) as well as the biochemistry and cell biology of the parasite (36).

On clinical grounds *Leishmania* spp. are divided into groups which cause (i) visceral, (ii) cutaneous, and (iii) mucocutaneous leishmaniasis (Table 1).

Leishmania donovani (sensu lato), the etiologic agent of visceral leishmaniasis, replicates in mononuclear phagocytic cells of the spleen, liver, lymph glands, and bone marrow and produces a chronic disease which usually results in death in untreated cases. Cutaneous leishmaniasis is caused by *L. tropica*, *L. aethiopica*, *L. major*, and the several subspecies of *L. mexicana*. The cutaneous leishmania replicate in dermal histiocytes and produce self-limiting skin lesions. *L. braziliensis* also causes cutaneous disease, but one subspecies (*L. braziliensis* subsp. *braziliensis*) has a tropism to macrophages of the oro-naso-pharyngeal area, where it causes disfiguring and potentially lethal mucocutaneous leishmaniasis.

Collectively, the *Leishmania* spp. are responsible for one of the major communicable diseases of the world, and it is for this reason that the World Health Organization has included leishmaniasis among the six major diseases targeted for intensive research and control efforts. Based on overall medical significance, among the protozoal diseases of humans (22), leishmaniasis is regarded second only to malaria.

MORPHOLOGY AND LIFE CYCLE OF THE ORGANISM

The *Leishmania* spp. are unicellular protozoa that exist in two distinct morphologic forms. In the alimentary tract of their insect vectors, the parasite exists extracellularly as the flagellated, motile promastigote (70). In the phagolysosomal system of host mononuclear phagocytes, the parasite occurs intracellularly in the nonmotile amastigote form (20). The environment of the phagosome becomes acidified after parasites or bacteria are ingested by polymorphonuclear leukocytes. It seems the amastigote has adapted to survive and multiply in an acid environment. Mukkada and co-workers (93) have shown that certain catabolic and anabolic reactions of the amastigote form of *L. donovani* proceed optimally near pH 5.0; the pH of the phagolysomal contents is between 4.0 and 5.0. Specifically, they showed that incorporation of radiolabeled thymidine and uridine into nucleic acids, oxidation of glucose and proline, and respiration in the presence of succinate were all maximal when the amastigotes were incubated at pH 4.5 to 5.5. In contrast, promastigotes performed these metabolic processes maximally when the pH of the incubation medium was near 7.0.

Except for *L. enriettii*, which is not a human pathogen and infects guinea pigs, all species of *Leishmania* are essentially indistinguishable morphologically (5). Promastigotes have a spindle-shaped body with a single anterior flagellum; they are 10 to 15 μm in length and measure 1.5 to 3.5 μm at their widest part. The monoflagellated amastigote is spherically shaped, with a diameter of approximately 2 to 3 μm. The average amount of DNA per cell is 91×10^6 and 242×10^6 base pairs for the promastigotes of *L. donovani* and *L. braziliensis*, respectively. For *L. mexicana*, the quantity of protein per cell is 5.3 pg for promastigote and 1.3 pg for amastigote (22).

The life cycle of the *Leishmania* spp. is depicted in Fig. 1 and reviewed in detail elsewhere (22). Bloodsucking sand flies of the genera *Phlebotomus* and *Lutzomyia* are the vectors for the disease. The amastigote, when ingested by the sand fly during a blood meal taken from an infected vertebrate host, migrates to the midgut of the sand fly, where it transforms into the promastigote in a process that takes about 3 days. After a period of days to weeks, as a result of replication by means of binary fission and subsequent migration to the foregut of the insect, the promastigotes partially obstruct the digestive tract of the insect. When the infected sand fly takes a second blood meal, it regurgitates infectious promastigotes from its pharynx into the bloodstream of the vertebrate. Once inside the bloodstream of the vertebrate, such as humans or the various other animals that serve as reservoirs for the disease (e.g., canines, marsupials, eden-

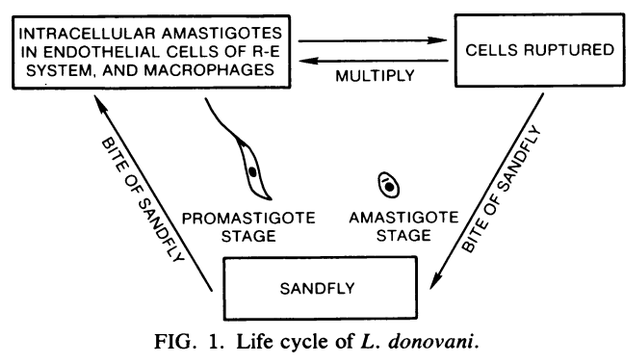

FIG. 1. Life cycle of *L. donovani*.

TABLE 2. Summary of some of the properties of membrane-bound *L. donovani* acid phosphatases

Phosphatase	Relative abundance (%)	Isoelectric point	pH optimum	Mol wt	Energy of activation (kcal/mol)[a]	Inhibitors (I_{50}, mM)[b]		
						Sodium-L-(+)-tartrate	Sodium fluoride	Ammonium molybdate
ACP-P_1	80	4.1 ± 0.25	5.5	120,000	7.46 ± 0.88	>10	<5.0	0.016
ACP-P_2	10	5.4 ± 0.14	5.2	108,000	11.10 ± 1.21	0.221	<5.0	0.009
ACP-P_3	10	7.1 ± 0.05	5.0	133,000	7.30 ± 0.43	0.217	<5.0	0.064

[a] 1 cal = 4.184 J.
[b] I_{50}, 50% inhibitory concentration.

tates, and rodents), promastigotes are phagocytosed by the mononuclear phagocytic cells of the host, whereupon they transform into amastigotes and begin replicating within modified phagolysosomes designated parasitopherous vacuoles; eventually, the host cells lyse, releasing free parasites. The newly released amastigotes then infect other cells or are taken up by sand flies, thereby completing the life cycle of the parasite.

It was demonstrated recently that the saliva of the sand fly plays a role in the transmission of leishmaniasis. Titus and Ribeiro (125) showed that injecting mice with *L. major* parasites mixed with homogenized salivary glands from *Lutzomyia longipalpis* resulted in cutaneous lesions of *L. major* that were much more extensive than those produced by controls, that is, an inoculum of parasites that had not been exposed to the salivary gland homogenate. The nature of the active principle of the sand fly salivary gland is obscure, as is the mechanism by which it exacerbates cutaneous leishmaniasis.

The three clinical forms of leishmaniasis result from the different affinities of the various species of *Leishmania* for macrophages located in different parts of the body. Temperature may be one of the major factors involved in this tropism. The *Leishmania* species that produce cutaneous and mucocutaneous diseases grow better at the slightly cooler temperatures (35°C) of the skin. In contrast, *L. donovani*, the cause of visceral leishmaniasis, prefers the slightly higher temperature of 39°C for growth (11).

SURFACE ENZYMES

Hydrolases

Since Dwyer and Gottlieb (36) wrote their informative and comprehensive review of the biochemistry of *Leishmania* surface membranes, a number of useful reports have been published on the enzymes and proteins that are localized to the outer surface of the various species of *Leishmania*.

Phosphomonoesterases. (i) Phosphatases. *(a) Membrane-bound acid phosphatases: purification and properties.* Promastigotes of one particular clone of *L. donovani* contain at least three distinct surface membrane-bound acid phosphatases (46, 74). Using histochemical-electron microscopic and subcellular fractionation techniques, Gottlieb and Dwyer (50, 51) demonstrated convincingly that the majority of the cell-associated acid phosphatase activity in *L. donovani* promastigotes is located on the external surface of the parasite.

The membrane-bound acid phosphatase activity can be solubilized and resolved into one major and two minor phosphatase isoenzymes (111). The first step in the purification scheme requires the extraction and solubilization of the acid phosphatase activity from parasite membranes. Extraction with sodium cholate solubilizes about 80% of the total particulate acid phosphatase activity. The three acid phosphatases can be separated from each other by chromatography on QAE-Sephadex. The major phosphatase component is resistant to inhibition by L-(+)-sodium tartrate (111). It had been shown earlier by Dwyer and Gottlieb (36) that most of the acid phosphatase activity on the promastigote surface is tartrate resistant. The predominant acid phosphatase, designated ACP-P_1, was purified to homogeneity by a series of gel filtration and lectin (concanavalin A) chromatography steps, followed by hydrophobic affinity chromatography on a phenyl-Sepharose column (111). Properties of acid phosphatases are summarized in Table 2.

ACP-P_1 runs as a single band and exhibits a molecular weight of 60,000 on sodium-dodecyl sulfate-polyacrylamide gels. The native molecular weight of ACP-P_1 is 120,000, indicating that native ACP-P_1 is a 120,000-dalton dimer composed of identical 60,000-dalton subunits. Its isoelectric point is 4.1, and it has a pH optimum between 5.0 and 5.5 when assayed with 4-methylumbelliferylphosphate as the substrate. Some of the preferred natural phosphomonoester substrates for ACP-P_1 are phosphotyrosine, fructose 1,6-diphosphate, and adenosine monophosphate (AMP). The level of tartrate-resistant acid phosphatase activity parallels cell number during the growth cycle, while tartrate-sensitive acid phosphatase peaks during the middle of the log phase of growth (Fig. 2).

The physiologic role of these acid phosphatases is not well understood. Gottlieb and Dwyer (49) have proposed that the acid phosphatases, by hydrolyzing phosphomonoesters, may provide the parasite with a source of inorganic phosphate for growth. It has also been suggested, because of their surface orientation, that the leishmanial phosphatases may be involved in pathophysiologically significant surface interactions between the host cell and the parasite (22, 105). In fact, there is some recent evidence in support of this last hypothesis and this will be discussed in the following section.

Few studies have been carried out on the lysosomal hydrolases of *Leishmania* amastigotes. Antoine et al. (3) have shown that *L. mexicana* subsp. *amazonensis* amastigotes contain large amounts of acid phosphatase activity, some of which is contributed by an ectoenzyme associated with the plasma membrane or the flagellar pocket. It would be useful to isolate and characterize the acid phosphatase of the amastigote and to compare it with promastigote phosphatases.

In a comprehensive comparative study of various phosphomonoesterases in various *Leishmania* species, Hassan and Coombs (61) found high levels of acid phosphatase activity in extracts of *L. donovani*, *L. mexicana* subsp. *mexicana*, and *L. mexicana* subsp. *amazonesis*. They also demonstrated that *L. mexicana* subsp. *mexicana* amastigotes and promastigotes contain acid phosphatase on their

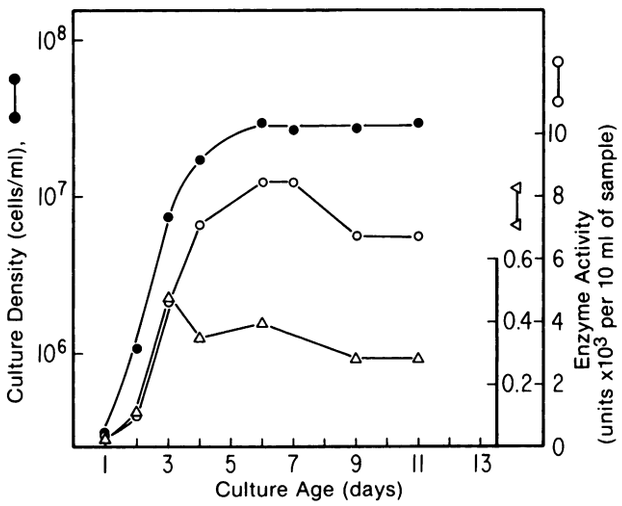

FIG. 2. Acid phosphatase activity during growth of *L. donovani* promastigotes. A culture flask containing 200 ml of medium 199 and 20% fetal calf serum was inoculated with 3.2×10^5 cells. Samples were removed at the indicated times, and cell density was determined by hemacytometry. The particulate fractions were assayed for acid phosphatase activity in the absence and presence of 12 mM L-(+)-sodium tartrate from which tartrate-resistant (○) and tartrate-sensitive (△) acid phosphatase activity was determined.

external surfaces. However, inhibitor and substrate specificity studies revealed qualitative differences in the particulate acid phosphatase activities of promastigotes and amastigotes. Furthermore, by using the electron microscope in a semiquantitative histochemical technique, it was observed that surface staining of acid phosphatase was less intense for amastigotes than for promastigotes.

Effect on host cell metabolism. We proposed that the leishmanial phosphatase might mediate surface interactions between host cells and parasites and account, in part at least, for the ability of the parasite to escape killing by host cells (31). More specifically, our original hypothesis stated that the cell surface phosphatase of the parasite, by dephosphorylating constituents of the host cell during phagocytosis, would interfere with the microbicidal response of the host cell. This hypothesis was generated in part because others had demonstrated that phosphoproteins, potential substrates for phosphatases on the surface of phagocytic cells, participate in the stimulus-response coupling exhibited by these cells (2, 119).

One of the critical microbicidal actions of phagocytic cells is the generation of toxic oxidative metabolites, including superoxide anion (O_2^-) and H_2O_2. In vitro (95, 104, 117) and in vivo (21, 97) experiments have both shown that *L. donovani* cells are killed by oxidative radicals. Therefore, the possibility that a leishmanial acid phosphatase acting on substrates in host phagocytic cells could alter the production of toxic oxidative metabolites by the latter was one of the first possibilities we explored.

In the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase reaction, phagocytosis perturbs the plasma membrane of macrophages and granulocytes and results in a rapid increase in the rate of oxygen consumption that is termed the respiratory burst. Most of that oxygen consumed by these phagocytic cells is committed directly to the production of reactive oxygen metabolites such as O_2^- and H_2O_2, which in turn are precursors to other cidal substances (e.g., hypochlorite and hydroxyl radical).

Superoxide anion alone is not very leishmaniacidal as it is not especially reactive. In addition, many strains of *Leishmania* spp. and other trypanosomatids possess a superoxide dismutase which converts O_2^- to H_2O_2 (75). However, the H_2O_2 that is formed from O_2^- by spontaneous dismutation or by the action of phagocyte superoxide dismutase and which can diffuse into the extracellular spaces does kill *Leishmania* spp. Promastigotes are generally more sensitive than amastigotes to H_2O_2 and O_2^- (55, 95, 103). The reaction catalyzed by superoxide dismutase is as follows:

$$O_2^{\bullet -} + HO_2^{\bullet} + H^+ \rightarrow H_2O_2 + O_2.$$

The enzyme responsible for the respiratory burst is called NADPH oxidase (for review, see reference 96). It is actually not a single enzyme protein but a multiprotein electron transport-enzyme complex embedded in the plasma membrane of the phagocyte. One at a time, electrons from NADPH generated by the $NADP^+$-linked dehydrogenases of the hexose monophosphate shunt (pentose phosphate pathway) are thought to be channeled sequentially through a flavoprotein enzyme, cytochrome b_{559}, and ubiquinone to O_2, thereby yielding O_2^-. The cytochrome component of the system has a dual location in granulocytes: some is localized to the plasma membrane, whereas the remainder occurs in the membranes of intracellular granules. It is generally believed that stimulation of phagocytes with particulate matter or soluble agents (e.g., *n*-formyl-methionyl-leucyl-phenylalanine [FMLP]) results in the migration to and fusion of these cytochrome *b*-containing granules with the plasma membrane, thereby reconstituting a functional NADPH oxidase system. Granulocytes of patients with chronic granulomatous disease have a decreased ability to produce reactive oxygen intermediates and consequently a decreased ability to kill microorganisms, including various *Leishmania* strains (97). This observation underscores the importance of the NADPH oxidase reaction to microbicidal killing and the cellular defense against infection.

Enzyme studies have suggested that the increased superoxide-generating capacity of activated macrophages is the result of an increase in the affinity of the NADPH oxidase for NADPH; the K_m of the oxidase of stimulated macrophages is about 10-fold lower than that of resting macrophages (54, 127). The mechanism underlying this increase in the affinity of the oxidase for NADPH is unknown; it could be that the concentration of some inhibitor of the oxidase is decreased upon activation of macrophages or that the enzyme is activated by covalent modification (e.g., phosphorylation) or by allosteric modifiers (127).

Based on what is known about the NADPH oxidase reaction in phagocytic cells, there are at least four ways a parasite might interfere with O_2^- production and thereby disarm the host cell: (i) reducing the rate of production or availability of NADPH, by inhibiting the dehydrogenases of the hexose monophosphate shunt or by stimulating the NADPH-utilizing enzymes that would compete for and consume NADPH; (ii) blocking the migration of the cytochrome b_{559}-containing granules to, or their proper fusion with, the plasma membrane of macrophages and granulocytes, possibly through interference with fluxes in intracellular calcium ion concentrations, thus preventing calcium-dependent reconstitution of NADPH oxidase; (iii) preventing the marked decrease in the K_m of the NADPH oxidase for NADPH that normally occurs when macrophages are stimulated (94); and (iv) destroying or inactivating NADPH oxidase or the cytochrome component of the

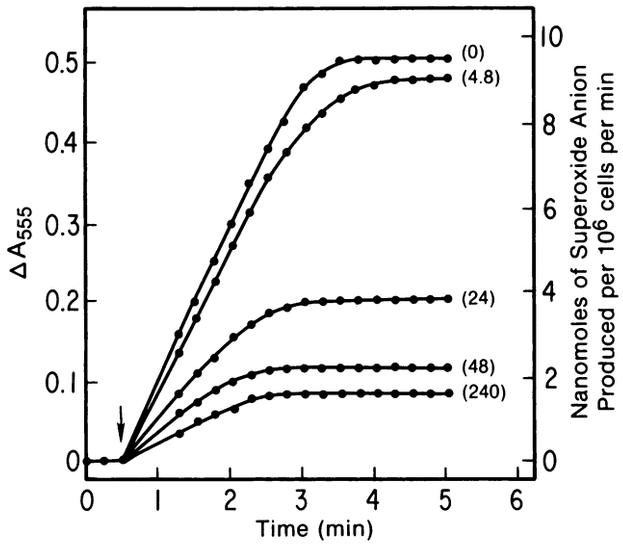

FIG. 3. Effect of acid phosphatase treatment on superoxide anion production. A suspension of 2.5×10^6 neutrophils in Krebs-Ringer phosphate buffer (pH 7.4) containing 5 mM glucose and 0.05 mM cytochrome *c* was incubated for 15 min at 37°C with 0 to 240 U of acid phosphatase as indicated by the numbers in parentheses. Neutrophils were stimulated with 10^{-7} M FMLP at the time shown by the arrow, and superoxide anion production was calculated from the change in absorbance at 550 nm (ΔA_{550}).

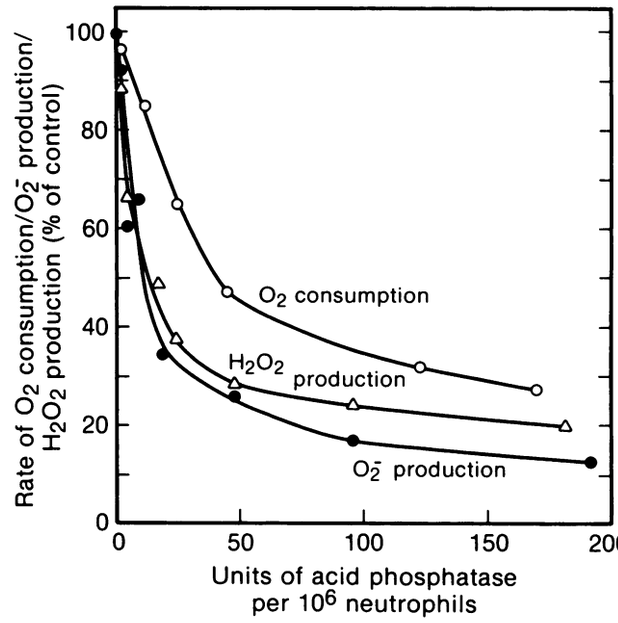

FIG. 4. Effect of *L. donovani* acid phosphatase pretreatment on human neutrophil oxidative metabolism. Prior to stimulation with 10^{-7} M FMLP, a suspension of 2.5×10^6 neutrophils per ml in Krebs-Ringer phosphate buffer (pH 7.4) containing 5 mM glucose was incubated for 15 min at 37°C with the indicated amounts of acid phosphatase units. The initial rates of O_2 consumption (○), O_2^- production (●), and H_2O_2 production (△) were determined.

reaction. That one can solubilize and isolate the various components of the NADPH oxidase system and then reconstitute them should facilitate studies which address these possibilities (106).

Regarding inhibition of the oxygen burst, Fig. 3 shows the effect of a pure preparation of leishmanial acid phosphatase (ACP-P_1) on O_2^- production by human neutrophils; preincubation of human neutrophils for 15 min at 37°C with purified ACP-P_1 markedly reduced both the rate and extent of superoxide anion production by neutrophils stimulated with the bacterial chemotactic peptide FMLP, a well-known soluble stimulator of phagocytes (113). The amount of ACP-P_1 needed to inhibit neutrophil superoxide anion production is physiologically relevant; we estimate that under these experimental conditions the phosphatase activity of one parasite is capable of reducing by one-half the rate of O_2^- production of three neutrophils.

Two other leishmanial acid phosphatases that we have purified from *L. donovani* membranes, designated ACP-P_2 and ACP-P_3, have no effect on the ability of neutrophils to produce O_2^- when tested at levels 50-fold above those at which ACP-P_1 is inhibitory. Since the production of superoxide anions depends upon the consumption of oxygen and results in the subsequent generation of other toxic oxidative radicals, it should not be surprising to find that preincubation of neutrophils with acid phosphatase inhibits both O_2^- and H_2O_2 production and blocks the oxygen burst; in fact, the dose-response curves for all three parameters are similar (Fig. 4) (112).

Hexose monophosphate shunt activity is stimulated by FMLP, thereby providing the NADPH reducing equivalents which reduce molecular oxygen in the NADPH oxidase reaction. Estimation of hexose monophosphate shunt activity is therefore a sensitive correlate of the respiratory burst in phagocytes. Table 3 shows that an amount of ACP-P_1 similar to that required to inhibit neutrophil O_2^- production also inhibits hexose monophosphate shunt activity to the same extent.

Our hypothesis is that the leishmanial phosphatase acting on host phagocytic cells interferes with the production of toxic oxidative radicals, thereby enhancing the survival of the parasite.

If ACP-P_1 does interfere with the respiratory burst of host phagocytic cells, one might expect that ACP-P_1 treatment might blunt the response of phagocytes to other stimuli. Table 4 shows that ACP-P_1 pretreatment does block concanavalin A-stimulated oxygen metabolism just as it did with FMLP. However, the leishmanial phosphatase did not block O_2^- consumption when the tumor promoter phorbol myristate acetate was used as the stimulatory agent.

It was also determined from direct binding experiments that ACP-P_1 treatment has no effect on receptor-mediated

TABLE 3. Effect of *L. donovani* acid phosphatase treatment on neutrophil hexose monophosphate shunt activity of human neutrophils[a]

Condition	Stimulus	dpm
5×10^6 neutrophils/ml	None	4,050 ± 170
5×10^6 neutrophils/ml plus 1,280 U of heat-killed ACP-P_1	10^{-7} M FMLP	21,700 ± 640
5×10^6 neutrophils/ml plus 1,280 U of ACP-P_1	10^{-7} M FMLP	5,500 ± 550

[a] Human neutrophils were pretreated with *L. donovani* acid phosphatase (ACP-P_1) 15 min prior to stimulation. Cells were then incubated at 37°C in Krebs-Ringer phosphate medium with 0.20 μCi of 1-[^{14}C]glucose for 30 min. The incubation was terminated by addition of H_2SO_4, and the $^{14}CO_2$ generated was trapped in hydroxide of hyamine and counted by liquid scintillation. All values are means ± standard deviations of triplicate determinations.

TABLE 4. Effect of various treatments on O_2^- production by human neutrophils[a]

Condition	Phosphatase activity (U)	Activator	O_2^- production (nmol/10^6 cells per min)[b]
Neutrophils plus acid phosphatase	0	10^{-7} M FMLP	4.9
	10		2.9
	48		1.7
	240		0.8
Neutrophils	0	10^{-7} M FMLP preincubated with 110 U of acid phosphatase for 15 min, 37°C	4.3
	110[c]	10^{-7} M FMLP	4.4
Neutrophils plus acid phosphatase	0	500 μg of concanavalin A per ml	1.14
	128		0.84
	320		0.57
	640		0.26
Neutrophils plus acid phosphatase[c]	0	1 μg of phorbol myristate acetate per ml	6.1
	160		5.7
	320		6.1
	480		6.3

[a] All samples were preincubated for 15 min at 37°C under conditions described in the legend to Fig. 4 in the presence or absence of *L. donovani* acid phosphatase.
[b] Measured by continuous assay.
[c] Acid phosphatase was added separately immediately before stimulation with FMLP.

binding of FMLP by leukocytes, thereby excluding the possibility that inhibition of the respiratory burst by ACP-P_1 is a result of its interaction with the receptor for FMLP (113).

The lack of an effect of ACP-P_1 pretreatment on the phorbol myristate acetate-stimulated oxygen burst is especially interesting. Phorbol myristate acetate, by acting as a diglyceride analog, activates protein kinase C in the plasma membrane of phagocytic cells, leading ultimately to the activation of these cells (98). By activating cells in this manner, phorbol myristate acetate appears to bypass some early event in the activation process which may be the target of the leishmanial acid phosphatase.

Additional evidence in support of the hypothesis that the *Leishmania* spp. evade intracellular killing by impairing oxygen-dependent defense mechanisms of the host was obtained recently by Buchmuller-Rouiller and Mauel (18). They demonstrated that mouse peritoneal macrophages infected with *L. enriettii* or *L. major* exhibited a markedly reduced oxygen burst when stimulated in vitro with macrophage-activating factor or lipopolysaccharide. The presence of these intracellular parasites in the phagocytes inhibited NADPH oxidase activity when the latter was estimated by chemiluminescence, reduction of cytochrome *c* or Nitro Blue Tetrazolium, or hexose monophosphate shunt activity. To demonstrate that the effect was parasite specific, they showed that inert intracellular particles (e.g., latex beads) did not block the respiratory burst. Although the macrophages they used in their study were infected with promastigotes, by the time the cells were tested for respiratory burst activity, the parasites had transformed into amastigotes. The authors speculated that some enzyme on the parasite surface membrane, such as acid phosphatase, might play a role in the survival of the parasites within host cells. As noted elsewhere in this review, amastigotes also contain acid phosphatase activity on their surface.

Hassan and Coombs (61) have questioned whether the tartrate-resistant surface acid phosphatase is crucial for survival in macrophages simply by virtue of the fact that it inhibits oxidative metabolism in neutrophils (112). They point out that, if this is the case, one would expect it to be a *Leishmania*-specific feature and a key enzyme in the stages of the parasite that inhibits macrophages, namely, the metacyclics and amastigotes. However, when they compared the specific activities of acid phosphatase in crude homogenates of promastigote and amastigote forms of *L. mexicana* subsp. *mexicana*, the two values were nearly the same (88 versus 66 U/mg of protein, respectively). They also stressed the need for additional data regarding the correlation between infectivity and cell surface acid phosphatase activity. There is at least one published report which shows higher acid phosphatase activity in a more virulent strain of *L. donovani* (67). However, in a collaborative study with David Sacks, we have observed that the specific activity of tartrate-resistant acid phosphatase is about the same in highly infective metacycles and low-infectivity promastigotes (R. H. Glew and D. Sacks, unpublished observations).

It is likely that inhibition of the macrophage's oxygen burst by cell surface acid phosphatase may not be the only means by which the *Leishmania* spp. escape killing. As pointed out by Meshnick and Eaton (89), the remarkable capacity of *Leishmania* spp. to survive and replicate inside host cells despite exposure to activated oxygen may be due in part to the superoxide dismutase they contain in their cytoplasm. These investigators argue that enzymatic degradation of O_2^- would explain the ability of the parasite to resist destruction by macrophages. On a specific activity basis (units of activity per milligram of protein), *L. donovani* amastigotes contain about threefold-higher levels of superoxide dismutase than promastigotes (96). The H_2O_2 generated by parasite superoxide dismutase in turn could be decomposed to H_2O and O_2 by catalase present in the parasite. Amastigotes and promastigotes of *L. donovani* do, in fact, contain catalase; however, the catalase specific activity in extracts of amastigotes is two to three times higher in extracts of amastigotes.

It has been suggested that parasite glutathione peroxidase may also play a role in parasite survival by eliminating H_2O_2; interestingly, amastigotes of *L. donovani* contain 10 times the level of glutathione peroxidase as promastigotes (96).

There is evidence which calls into the question the issue of

whether the surface acid phosphatase of *Leishmania* spp. is effective in blocking the oxygen burst that occurs when the parasites are actually being phagocytized. First, Haidaris and Bonventre (56) showed that superoxide anion production (as determined by Nitro Blue Tetrazolium reduction) by activated mouse macrophages was only slightly less than that exhibited by zymosan-stimulated macrophages. Second, Meshnick and Eaton (89) demonstrated that *L. tropica* promastigotes stimulated mouse macrophages to produce twice as much combined H_2O_2 and superoxide anion as that elicited by phorbol myristate acetate. The latter investigators argued that the superoxide dismutase present in the promastigote cytoplasm inactivates the O_2^- generated during the oxygen burst.

Apart from the possible pathophysiologic role of the leishmanial phosphatase, the ability of the enzyme to modulate the respiratory burst of phagocytes is interesting in terms of the mechanism of action of the phosphatase. As stated earlier, our hypothesis is that the phosphatase mediates its effect by acting on phosphorylated constituents of the phagocyte membrane. One of the requirements for defining the mechanism of action of the acid phosphatase is identification of physiologic substrates on phagocytes. In view of the recent insights that have been gained (1, 110, 126) concerning the stimulus-response coupling of phagocytes, we and others (13, 31) have pointed out that phosphoproteins and phosphoinositides represent potential substrates for leishmanial phosphatases.

In this regard, we have shown that the tartrate-resistant leishmanial phosphatase is capable in vitro of dephosphorylating ^{32}P-radiolabeled phosphorylase kinase, pyruvate kinase, and histones. The activities (V_{max}) on the three proteins, albeit very low, varied greatly; pyruvate kinase supported the highest activity. However, even the best phosphoprotein (pyruvate kinase) was <1% as effective a substrate as the nonphysiologic substrate, 4-methylumbelliferylphosphate. Unless it can be shown that phagocytes contain a specific phosphoprotein that is a substrate for the leishmanial phosphatase, these preliminary results indicate that phosphoproteins are not especially good substrates for the enzyme.

Another class of phosphorylated phagocytic cell constituents that have been considered as leishmanial phosphatase substrates are the phosphoinositides. It is believed that early in the process of macrophage activation inositoltriphosphate (IP_3) is released by the action of phospholipase C acting on phosphoinositide-4,5-bisphosphate. FMLP receptor-stimulated hydrolysis of phosphoinositide-4,5-bisphosphate to generate IP_3 and diglyceride is an important event in phagocyte activation (130), and IP_3 is believed to be a second messenger which triggers the release of stored calcium in cells (12, 13). We have observed (31) that phosphoinositide-4,5-bisphosphate and IP_3 are both excellent substrates for the leishmanial acid phosphatase. Surprisingly, however, in contrast to nonphysiologic 4-methylumbelliferylphosphate which is hydrolyzed most rapidly at pH 5.5, phosphoinositide-4,5-bisphosphate is hydrolyzed optimally by the *L. donovani* phosphatase at pH 6.8 to 7.0. The leishmanial phosphatase, ACP-P_1, also utilizes IP_3 and inositoldiphosphate as substrates (31).

The mechanism of action of the leishmanial acid phosphatase seems to involve alterations in second-messenger (e.g., calcium ions, inositol triphosphate, diacylglyceride) levels in phosphatase-treated phagocytes. Early on in the phagocyte activation process, there is a release of membrane-bound stores of calcium. This release of calcium precedes the generation of reduced oxygen metabolites and is believed to play a role in several processes, such as activation of protein kinase (34, 98; C. K. Huang, J. Hill, W. M. Mackin, B. J. Bormann, and E. L. Becker, Fed. Proc. **42**:1080, 1983), phagosome formation (20), and translocation of a cytochrome to the plasma membrane to reconstitute the NADPH oxidase (99). A sensitive fluorometric assay which permits one to monitor this release of calcium uses chlorotetracycline, which is a lipophilic probe for calcium. When calcium ions bind to the antibiotic, there is diminished fluorescence. Figure 5 shows that neutrophils preloaded with chlorotetracycline and then treated with the leishmanial phosphatase exhibit a diminished fluorescence response to FMLP stimulation when compared with control cells treated with boiled, inactivated acid phosphatase. These data indicate that the leishmanial acid phosphatase blocks calcium mobilization from intracellular membrane-bound stores in a dose-dependent manner that is similar to its effect on the respiratory burst. It appears the leishmanial acid phosphatase blocks the production of reduced oxygen metabolites in neutrophils by interfering with calcium-dependent steps in the activation process. This hypothesis is intriguing in light of the observation reported above that the leishmanial phosphatase hydrolyzes IP_3, a second messenger that causes the release of calcium from intracellular storage sites.

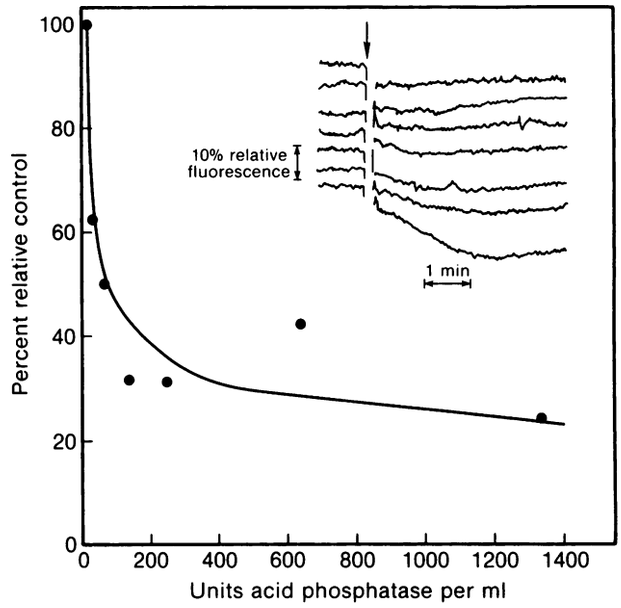

FIG. 5. Effect of acid phosphatase on chlorotetracycline response of neutrophils. A suspension of 5×10^6 neutrophils per ml stimulated with 10^{-7} M FMLP, which were previously loaded with chlorotetracycline, were monitored fluorometrically. (Inset) Change in fluorescence with time after a preincubation of 15 min with the units of acid phosphatase indicated in the derived plot. The lower trace is the control, while upper traces were treated with increasing numbers of units of ACP-P_1. Values (percent relative control) in the derived plot were obtained by subtraction of the minimum level of fluorescence after stimulation (arrow) from resting levels of fluorescence.

A potentially useful tool for studying the role of various glycoproteins in the virulence of the *Leishmania* spp. was revealed when Kink and Chang (72) showed that tunicamycin-resistant mutants of *L. mexicana* subsp. *amazonensis* were much more virulent than their wild type in mice. The

mutants, selected by gradual acclimation of cells to increasing concentrations of tunicamycin, contained amplified DNA and 15-fold more *N*-acetylglucosaminyl transferase than the parental strain. This suggests that leishmanial virulence may be related to expression of genes encoding enzymes involved in the biosynthesis of glycoproteins composed of N-linked oligosaccharide chains (73). It would be interesting to compare the glycoproteins present in the tunicamycin-resistant mutants and the parental wild type with specific emphasis on glycoproteinaceous enzymes that are secreted and localized to the outer surface of the parasite (e.g., tartrate-resistant acid phosphatase and 3′-nucleotidase).

(b) Secreted extracellular acid phosphatase. More than 90% of the acid phosphatase activity produced by the same clone of *L. donovani* from which the tartrate-resistant phosphatase was isolated accumulates in the extracellular growth medium (52). The extracellular enzyme arises not as a consequence of membrane damage or cell lysis but instead as a result of secretion by active, growing cells. Large amounts of the acid phosphatase are secreted regardless of whether the organism is grown in a serum-supplemented or a chemically defined medium.

In 1986, Lovelace et al. (78) described a three-step isolation scheme that yields a pure preparation of extracellular leishmanial acid phosphatase of high specific activity (>200 μmol of 4-methylumbelliferylphosphate cleaved/min per mg of protein). The enzyme binds to lentil lectin, suggesting that it is a glycoprotein, and direct carbohydrate analysis indicated that it contains 0.37 mg of hexose per mg of protein. Studies with tunicamycin have suggested that N-linked oligosaccharides are required for expression of catalytic activity (81). The molecular weight of the native enzyme, estimated by sucrose density gradient ultracentrifugation, is 134,000; however, when analyzed by gel filtration chromatography on Sephacryl S-500, it appears much larger (>700,000), presumably because of its glycosylation, shape, large Stoke's radius, and tendency to aggregate.

The secreted leishmanial acid phosphatase is a relatively acidic protein (pI 4.5), and its optimum activity on a variety of low-molecular-weight phosphomonoester substrates is in the vicinity of pH 5.0. The extracellular phosphatase hydrolyzes sugar phosphates (e.g., glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate), adenosine triphosphate (ATP), 5′-AMP, and 3′-AMP. Although phosphotyrosine and, to a lesser extent, phosphoserine and phosphothreonine are good substrates for the enzyme, the extracellular phosphatase does not dephosphorylate the phosphoprotein phosvitin C. The most outstanding difference between the secreted acid phosphatase and the one localized to the outer surface of the parasite lies in their sensitivity to inhibition by L-(+)-sodium tartrate, the cell surface enzyme being approximately 40 times more resistant to the effects of this inhibitor than the extracellular enzyme (52).

Monospecific antibodies to the soluble, extracellular acid phosphatase of *L. donovani* have been prepared; they do not recognize the surface membrane-bound acid phosphatase or any of the five major human acid phosphatases (6).

Lovelace and Gottlieb (79) examined 40 isolates of *Leishmania* spp., representing all of the major species infectious to humans, for the ability to secrete an extracellular acid phosphatase. All species, except *L. major* and *L. tarantolae*, secrete acid phosphatase into the culture medium. The amount of acid phosphatase into the growth medium is strain dependent; whereas *L. donovani*, *L. tropica*, *L. brasiliensis*, and several *L. mexicana* isolates produce large amounts of the enzyme, certain *L. mexicana* and *L. major* isolates produce one-fifth (or less) as much activity. The extracellular acid phosphatases produced by the various strains also differed in terms of their electrophoretic mobility and sensitivity to inhibition by L-(+)-sodium tartrate. Noteworthy, but of unknown significance, is the fact that the secreted acid phosphatase of *L. donovani* occurs in several phosphorylated forms (81).

(ii) Nucleotidases. Attached to the outer surface of the plasma membrane of *L. donovani* along with the acid phosphatase are two distinct nucleotidases, 5′-nucleotidase and 3′-nucleotidase. Nucleotidases are phosphomonoesterases which remove the phosphate group from phosphorylated sugars (ribose or deoxyribose) linked by an N-glycosidic bond to a purine or a pyrimidine. In the case of 3′-nucleotides, phosphate is attached to the hydroxyl group on C-3 of the pentose; in the 5′-nucleotide series, the phosphate moiety is attached to the hydroxyl group on C-5 of the sugar. The 5′- and 3′-nucleotidases are phosphomonoesterases that catalyze the reactions indicated below, both of which generate orthophosphate (P_i) and a nucleoside (base-pentose):

$$\text{base-pentose-3′-phosphate} + H_2O \xrightarrow{\text{3′-nucleotidase}} \text{base-pentose} + P_i$$

$$\text{base-pentose-5′-phosphate} + H_2O \xrightarrow{\text{5′-nucleotidase}} \text{base-pentose} + P_i$$

On a specific activity basis, plasma membranes from *L. donovani* promastigotes contain 40 times more 3′-nucleotidase than 5′-nucleotidase (35). However, crude extracts of *L. mexicana* subsp. *mexicana* promastigotes or amastigotes contain only two- to threefold more 3′-nucleotidase than 5′-nucleotidase (61). In terms of function, it is thought that these nucleotidases play a nutritional role. *Leishmania* spp., as well as other trypanosomatid protozoa (e.g., *Trypanosoma* and *Crithidia* spp.), cannot synthesize purines de novo and are therefore dependent upon an exogenous supply of preformed purines which are utilized by means of a purine "salvage" pathway. Nucleotides are not taken up by these organisms, but nucleosides are. Therefore, the leishmanial 5′-nucleotidase, which can hydrolyze both ribonucleotides and deoxyribonucleotides, and the 3′-nucleotidase, which is specific for ribonucleotides, appear to provide the parasite with the purine nucleosides required for growth. Hassan and Coombs (61) have shown that the specific activities of 3′-nucleotidase and 5′-nucleotidase are nearly the same in promastigotes and amastigotes.

Additional support for the idea that the 3′-nucleotidase plays a nutritional role in the trypanosomatid was provided by Gottlieb's observation (48) that the level of *Crithidia fasciculata* 3′-nucleotidase is regulated by the purine concentration of the growth medium; specifically, *C. fasciculata* grown in a purine-depleted medium contained 1,000-fold more 3′-nucleotidase activity than organisms grown in a purine-replete medium. Furthermore, loss of 3′-nucleotidase activity was observed following transfer of the organism from the purine-deficient medium to one rich in adenosine; the loss of activity was due to degradation or inactivation of the enzyme and not simply to cessation of additional 3′-nucleotidase synthesis (35).

It is presumed that the nucleotide substrates for the nucleotidases arise in the gut of the insect vector and lysosomes of host macrophages from the nuclease-mediated hydrolysis of DNA and ribonucleic acid (35).

The specificity of the 3′-nucleotidase is such that the enzyme could also play a role in the acquisition of coenzyme A by the parasite, since the enzyme is capable of catalyzing

the hydrolysis of the phosphate group that is linked to the 3′-position of the ribose moiety of coenzyme A (35).

The leishmanial 3′-nucleotidase has the following properties: pH optimum, 8.0 to 9.0; bivalent cation requirement, $Co^{+2} > Ca^{2+} > Mn^{2+} > Ca^{2+}$; substrate specificity, 3′-AMP > uridine 3′-monophosphate > guanosine 3′-monophosphate (3′-GMP) > cytidine 3′-monophosphate; not inhibited by fluoride ions. The alkaline pH optimum of the enzyme raises the question of whether it could function in the acid environment of the phagolysosome.

Gottlieb and Zlotnick (53) demonstrated that the 3′-nucleotidase of *L. donovani* promastigotes also possesses nuclease activity and speculated that the enzyme might provide the parasite with a source of purines.

The properties of the 5′-nucleotidase are as follows: pH optimum, 6.5 to 7.0; bivalent cation requirement, none; substrate specificity, 5′-GMP > 5′-AMP = uridine 5′-monophosphate > cytidine 5′-monophosphate; inhibited by fluoride ions.

Proteases. There are few reports of studies of the proteases of *Leishmania* species. Extracts of *L. mexicana* subsp. *mexicana* promastigotes and amastigotes possess proteases that are capable of hydrolyzing protein substrates that include azocasein, hide powder azure, and α-casein (108). The two leishmanial stages can be distinguished on two grounds: first, the specific activities of the amastigote proteases are much higher than those of promastigotes; second, the pH optimum on azocasein is at pH 5.5 for the amastigotes and near neutrality for the promastigotes. Synthetic protease substrates such as *N*-benzoyl-Arg-4-nitroanilide were also cleaved by crude extracts of both forms of the parasite; for *N*-benzoyl-Arg-4-nitroanilide, the pH optimum for each was 8.0 and the specific activities of the two were similar.

Chromatography of the soluble fraction of the amastigote extract on a Sephadex G-75 column fractionated two azocaseinases (designated peaks A and B), whereas chromatography of the corresponding fraction from the promastigotes yielded a single peak of azocaseinase (peak C). Peaks A and C had the same apparent molecular weight (50,000), whereas peak B was smaller (10,000). To the extent they were compared, peaks A and C appeared quite similar: their pH optima were the same (pH 5.5) and both were inhibited by dithiothreitol and chelators (ethylenediaminetetraacetic acid and 1,10-phenanthroline), indicating that they are metalloproteinases. The specificities of the partially purified proteases were compared by using a variety of synthetic peptide substrates. The low-molecular-weight proteinase of amastigotes (peak B) was highly specific, preferring *N*-benzoyl-Pro-Phe-Arg-nitroanilide as substrate. In contrast, proteinases A and C had similar but relatively low levels of activity against a number of nitroanilide derivatives of arginine.

Pupkis and Coombs (108) interpreted the stage-specific nature of the *L. mexicana* subsp. *mexicana* proteinases as indicating that the low-molecular-weight enzymes play some essential role in the survival of the parasite inside host macrophages. There is some evidence for this hypothesis; inhibitors of the amastigote proteinases have antileishmanial activity (108).

Etges and co-workers (40) recently demonstrated protease activity associated with a 62- to 65-kilodalton glycoprotein from *L. major* and which had been shown previously by others (25, 115) to be the major amphiphilic surface protein of many different *Leishmania* species; this proteolytic enzyme has been termed p63, gp63, and "promastigote surface protease." The enzyme is probably not a serine esterase since its activity is unaffected by serine-active site reagents such as diisopropylfluorophosphate or phenylmethylsulfonyl fluoride. On a molar basis, in terms of activity against azocasein substrate, the promastigote surface protease is 1.5 times more active than trypsin and it has a broad pH optimum which extends from pH 7 to 10. It does not attack low-molecular-weight peptides such as *N*α-benzoyl-L-arginine *p*-nitroanilide, *N*α-benzoyl-L-lysine thiobenzyl ester, or succinyl-L-Ala-Ala-Pro-Phe-thiobenzyl ester. The glycoprotein is anchored to the promastigote surface through a phospholipase C-sensitive, hydrophobic anchor similar to that present in the variant surface glycoprotein of *Trypanosoma brucei* (15). It has been speculated (41) that one function of the protease is to protect the promastigote from microbicidal enzymes in the gut of the insect vector or while the parasite is establishing itself in the host.

In 1987, Russell (114) identified a 63,000-dalton glycoprotein, designated gp63, on the surface of *L. mexicana* promastigotes as the predominant receptor for the C3b component of complement; it is generally accepted that gp63 is identical to p63. Although the nature of the interaction between gp63 and C3b has not yet been elucidated, its significance was alluded to by Russell. He pointed out that, from the promastigote's perspective, the complement receptor provides the ideal kind of molecule to modulate parasite binding and uptake because it does not provoke the oxygen burst and the generation of toxic oxygen metabolites known to be associated with phagocytosis mediated by other receptors. Characterization of the nature of the interaction between gp63 and the complement receptor on phagocytes should yield important insights into the critical biochemical steps involved in the host-parasite relationship.

Chaudhuri and Chang (24) confirmed the findings of Etges et al. (40), using *L. mexicana* promastigotes as their source of gp63 protease. However, when Chaudhuri and Chang used native proteins (e.g., albumin) rather than low-molecular-weight peptides as substrates, the pH optimum of the purified cell surface protease from *L. mexicana* exhibited a very sharp optimum near pH 4.0. Furthermore, gp63 appears to be an endopeptidase. Interestingly, the mass of gp63 and its protease activity are two to three times higher on the surface of virulent cells as compared with avirulent cells. It has been postulated (40) that, upon entry of promastigotes into a macrophage, the acid environment of the phagolysosome activates the surface gp63 protease of the parasite, thereby enabling the organisms to inactivate microbicidal proteins and enzymes located therein. However, because gp63 also exhibits protease activity in the pH range 7.0 to 7.5, it may also function to hydrolyze and inactivate immunoglobulins and complement C3 (40).

Protein Kinases

Leishmania spp. contain relatively high levels of protein kinase activity. Protein kinases are a class of enzymes which catalyze the transfer of the γ-phosphate group from nucleoside triphosphates (usually ATP) to the hydroxyl group of serine, threonine, or tyrosine residues in proteins.

Protein kinases have been described in protozoa (83) and bacteria (109), and we have partially purified and characterized two protein kinases that are present in the intracellular bacterium that causes *Legionella* pneumonia, namely, *Legionella micdadei* (118). Several excellent reviews of protein kinases recently appeared (17, 135). *Plasmodium berghi* and *Babesia bovis* contain protein kinase activity, and infection by these organisms results in phosphorylation of erythrocyte membrane proteins (136, 138). *Trypanosoma*

brucei contains at least two different protein kinases (131). In addition, the variant surface glycoproteins of *T. brucei* are rapidly phosphorylated (33). Preliminary studies indicate that phosphorylation-dephosphorylation reactions may control the release of these variable surface glycoproteins from trypanosomes (33).

It can be shown by using radioactive phosphorous and metabolically labeled cultures of promastigotes that the membrane proteins of *L. donovani* undergo rapid phosphorylation-dephosphorylation in vivo (33). Furthermore, motile and live *L. donovani* promastigotes, when incubated at pH 7.0 in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, catalyze the transfer of ^{32}P from [γ-^{32}P]ATP to exogenous histone acceptors present in the extracellular medium. Comparing these results with those obtained with broken cells, we estimated that more than half of the total protein kinase of *L. donovani* promastigotes is localized to the outer surface of the parasite. When the histone acceptor was excluded from the incubation medium, we found that the cell surface protein kinase phosphorylated at least a dozen parasite proteins, presumably those localized to the outer surface of its plasma membrane. Extracellular ATP has been demonstrated in blood (see reference 47 for review), and it has been argued that the concentration of pericellular ATP could easily reach the high micromolar range. Thus, it is not unreasonable to suppose that a *Leishmania* ectokinase might phosphorylate surface proteins of the host cell.

One can solubilize 80% or more of the particulate leishmanial kinase by freeze-thawing washed promastigotes and purify the enzyme extensively by chromatographing the extract on a series of columns containing QAE-Sephadex, Sephadex G-150, and hydroxyapatite (30). The *L. donovani* protein kinase has a molecular weight of about 75,000 and phosphorylates exclusively serine residue when histones are used as the substrate. The enzyme prefers the H2b and H4 subfractions of histone; pyruvate kinase, phosphorylase kinase, and rabbit muscle myosin are not phosphorylated. The pH optimum of the *L. donovani* protein kinase is 7.0, and the K_ms for ATP and mixed calf thymus histones are 0.80 and 0.15 mM, respectively. The leishmanial protein kinase is not stimulated by calmodulin, cyclic AMP, or cyclic GMP.

With regard to the growth curve, protein kinase activity per cell is relatively low and remains so during the exponential phase of growth; however, as the promastigotes enter the stationary phase, kinase activity per cell increases about sixfold. Late in the stationary phase there is a precipitous decline in protein kinase activity.

The demonstration of ectokinase activity in *L. donovani* promastigotes, together with previous reports documenting the presence of intense acid phosphatase activity on the outer surface of the organism, indicates that the parasite possesses the capability of regulating the properties and function of not only its own cell surface macromolecules, but also those of host cells through phosphorylation and dephosphorylation reactions.

It was mentioned earlier how the tartrate-resistant leishmanial acid phosphatase has the ability to block superoxide anion production by phagocytic host cells; perhaps the ectokinase of *L. donovani*, too, plays some role in disarming host cells. We have demonstrated (N. Mukhopadhyay and R. Glew, unpublished observation) that calf brain tubulin is an excellent substrate for the protein kinase from *L. donovani* promastigotes. Bordier et al. (16) demonstrated that the microtubules of the pellicular membrane of *L. tropica* are composed of α- and β-tubulin subunits that are nearly identical to those of mammalian brain tubulin. Since phosphorylation of tubulin monomers inhibits their ability to assemble into microtubules (132), it is conceivable that the parasite, through the activity of its ectokinase, modifies the cytoskeletal apparatus of the host.

Keithly and Bienen (69) have shown that log- and stationary-phase primary culture promastigotes behave as a continuously infective population for hamsters and the infectivity depends on morphological type. Sacks and Perkins (116) reported that *Leishmania* promastigotes taken from the stationary phase of growth are much more infectious than exponential-phase promastigotes. Perhaps the sixfold increase in protein kinase activity which occurs as the organism enters the stationary phase (30) contributes, at least in part, to the increased virulence of stationary-phase promastigotes. It is interesting that highly infective metacyclic forms of *L. major*, isolated from stationary culture by using the peanut agglutinin, contained eightfold more protein kinase activity than noninfective log-phase cells (Glew and Sacks, unpublished data).

There are additional questions about the kinase that are worthy of pursuit. Does the parasite retain the histone-dependent kinase when it undergoes transformation, inside the host cells, from the promastigote into the amastigote form? Do other *Leishmania* species possess this kinase? Do host cells contain proteins that can function as phosphate acceptors for the ectokinase? Does incubation of the solubilized, purified, histone-specific kinase with phagocytes have any effect on the metabolism of the latter?

METABOLISM

Lipids and Lipid Metabolism

The appraisal expressed in 1979 by Beach et al. (7) that knowledge of the lipids of the *Leishmania* spp. is fragmentary is still valid today. What is known about the lipids of *Leishmania* spp. has been derived largely from two kinds of experimental approaches: direct quantitative and qualitative chemical analysis of the lipids in the organism and various of its subcellular fractions, and metabolic studies involving the incorporation of various radioactive precursors (e.g., [^{14}C]acetate, $^{32}P_i$, [^{3}H]glycerol) into lipid constituents of the organisms.

The crude lipid fraction obtained by exhaustively extracting promastigotes with chloroform-methanol (2:1, vol/vol) accounts for 2 to 15% of the dry weight of a number of species of *Leishmania*, including *L. donovani*, *L. braziliensis*, *L. mexicana*, *L. tropica*, *L. enrietti*, *L. hertigi*, *L. adleri*, and *L. tarentolae* (7); neutral lipids and polar lipids accounted for 14 to 55% and 45 to 86% of the total lipid, respectively. For most strains, the total lipid content and the neutral/polar lipid ratio was the same for cultures grown in minimum essential medium plus 10% fetal calf serum versus defined medium RE III plus 0.06% defatted peptone (Difco Laboratories).

The neutral lipid fraction of *L. tarentolae* (grown in brain heart infusion), when analyzed by silica gel thin-layer chromatography, exhibited the following composition (by weight): sterols (ergosterol), 43.3%; triacylglycerols (triglycerides), 43.0%; sterol esters, 9.1%; alkoxydiacyl glycerols, 1.7%; diacylglycerols, 1.5%; monoacylglycerols, 1.0%; and a trace of free fatty acids. Wassef et al. (134) reported that neutral lipids accounted for about 25% of the total lipids of *L. donovani* promastigotes; 60% of the neutral lipid fraction

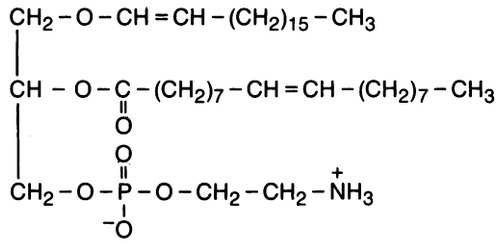

FIG. 6. Structure of ethanolamine plasmalogen (alk-1-enylacyl form).

was accounted for by sterols and 30% was accounted for by diglycerides (diaclyglycerols). Alkoxy lipids (Fig. 6) were first detected in *Leishmania* spp. by Gercken et al. (44) in 1976.

When Beach and co-workers (7) subjected the polar lipid fraction to two-dimensional thin-layer chromatography and triethylaminoethyl cellulose column chromatography, they observed diacylglycerophospholipids, plasmalogens (two-thirds alk-1-enylacyl glycerols and one-third alkylacyl forms), and traces of lysophosphatides. The four major diacylglycerolphosholipids present included phosphatidylethanolamine (PE) (20% by weight), phosphatidylcholine (49%), phosphatidylinositol (8%), and diphosphatidylglycerol (3%). The plasmalogens were almost exclusively of the PE variety, and they comprised 10 to 12% of the mass of the crude phospholipid fraction (Fig. 6).

Hermann and Gercken (63) analyzed the phospholipids of *L. donovani* promastigotes grown in a lipid-free medium and found that the phospholipid composition was the same for log-phase and stationary-phase cells; relative to phosphatidylcholine (49.5% by weight), plasmenyl ethanolamine (alk-1-enylacyl plasmologen) and plasmanylethanolamine (alkylacyl-plasmalogen) comprised 18.7 and 1.4%, respectively, of total phospholipids. Using $^{32}P_i$ to study phospholipid metabolism, these authors concluded that *L. donovani* does not synthesize choline or inositol plasmalogens (63).

From their compositional analyses and metabolic labeling studies, Hermann and Gercken (63) were impressed by the fact that the choline phospholipids of *L. donovani* promastigotes exist solely in the diacyl form, indicating a high degree of specificity of the enzymes involved in the biosynthesis of this phospholipid.

On a dry-weight basis, the surface membrane of *L. donovani* contains about four times as much lipid as whole cells (134). Phospholipids are the major lipid class of the surface membrane of *L. donovani* promastigotes, accounting for about 70% of the crude lipid fraction; neutral lipids (24.7%) and glycolipids (4.7%) account for the rest of the lipid of the surface membrane. The phospholipid composition of the surface membrane is as follows: phosphatidylcholine (14.9%), PE (37.7%), phosphatidylinositol (17.9%), and phosphatidylserine (9.9%), with traces of cardiolipin and phosphatidylglycerol. An unidentified phosphophingolipid was reported to constitute 19% of the total phospholipid of the surface membrane.

Beach et al. (7) could find no major qualitative or quantitative differences in the glycerolipids among eight *Leishmania* species or in a given species grown in different laboratory media. To our knowledge, the lipids of *Leishmania* amastigotes have not yet been reported.

The temperature at which the promastigotes are cultured influences their lipid content and fatty acid composition. Greenblatt and Wetzel (55) and Beach et al. (8) observed a

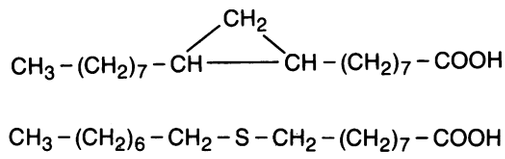

FIG. 7. Structures of dihydrosterculic acid (top) and 10-thiastearic acid (bottom).

temperature-dependent increase in the total lipid content of *L. enrietii* and *L. donovani*. *L. donovani* promastigotes grown in Tobie biphasic medium at 37°C contained nearly twice as much total lipid as cells grown at 25°C (16 versus 9%). Furthermore, the neutral/polar lipid ratio was 0.59 at 37°C and 1.63 at 25°C, indicating a larger amount of phospholipid on a dry-weight basis in cells grown at the higher temperature.

The crude lipid fraction derived from *L. donovani* grown at 25°C had the following fatty acid composition: saturated fatty acids were palmitic (16:0), 3.9%, and stearic (18:0), 7.8%, acids; unsaturated fatty acids were oleic 18:1(Δ9), 24.0%; linoleic 18:2(Δ9,12), 20.0% and linolenic 18:3(Δ9,12,15), 15.5%, acids. At 37°C, the crude lipid fraction contained higher percentages of palmitic (2.4-fold) and stearic (1.8-fold) acids and lower percentages (15.5 versus 6.4%) of linolenic acid. However, the oleic acid content of the crude lipid fraction was slightly higher at 37 than at 25°C (30.8 versus 24.0%, respectively). There was not much difference in the linoleic acid content of cells grown at the two temperatures. Thus, while there was little difference in the average chain length of the fatty acids at 25 and 37°C (73.2 and 77.6% C_{18} chain length, respectively), the overall degree of unsaturation of the fatty acids was higher at the lower temperature. It was postulated (55) that the fatty acid desaturase pathway that converts oleic acid to linolenic acid is impaired at the higher temperature. In addition, the C_{19} cyclopropane fatty acid, dihydrosterculic acid (9,10-methyleneoctadecanoic acid; Fig. 7), which is present in small concentrations (0.6%) in cells grown at 25°C, is not found in promastigotes grown at 37°C, indicating an impairment in cyclopropane fatty acid biosynthesis at the higher temperature. The cyclopropane fatty acid is confined exclusively to PE.

Marked quantitative differences exist in fatty acid composition among various leishmanias, but no pattern to the differences between species was apparent (7). In *L. donovani*, the major phospholipids, PE and phosphatidylinositol, contained mostly C_{18} fatty acids. Phosphatidylcholine was rich in C_{18}, C_{20}, and C_{22} polysaturated fatty acids and was the phospholipid that contributed the most linolenic acid.

With regard to the cyclopropane fatty acids, Pascal et al. (102) reported recently that a sulfur-containing analog of dihydrosterculic acid called 10-thiastearic acid (Fig. 7) was a potent inhibitor of dihydrostercolate biosynthesis in the trypanosomatic protozoan *C. fasciculata*. Furthermore, micromolar concentrations of 10-thiastearic acid inhibited growth of the protozoan in laboratory media. It was suggested that, since mammals do not synthesize or require cyclopropane fatty acids, specific inhibitors of dihydrostercolic acid biosynthesis should not be harmful to them and consequently should be exploited in the design of antiprotozoal drugs.

That dihydrosterculic acid is exclusively confined to PE should stimulate investigators interested in the lipid metabolism of the *Leishmania* spp. to determine the molecular

basis for this observation. Considering the pathway of PE biosynthesis that operates in most eucaryotes, the incorporation of the cyclopropane fatty selectively into PE could be accounted for by highly specific transferases which catalyze key reactions in the pathway; for example, the cytidyl transferase which catalyzes the transfer of the phosphorylethanolamine moiety from cytidine diphosphate-ethanolamine to a diacylglycerol moiety which contains dihydrosterculic acid might have a high degree of specificity for a cyclopropane fatty acid-containing diglyceride acceptor. Alternatively, if the cyclopropane fatty acid is introduced into PE by an acyltransferase that utilizes lyso-PE as the acceptor, then it is conceivable that the *Leishmania* spp. might possess a dihydrostercoyl-coenzyme A:lyso-PE acyltransferase that is highly specific for lyso-PE and dihydrostercoyl-coenzyme A. It would be worthwhile investigating the enzymes involved in PE metabolism in the *Leishmania* spp.

Since *Leishmania* spp. can be grown on lipid-free synthetic and semidefined media, the organism must be capable of synthesizing its own fatty acids and complex lipids. Several groups of investigators (7, 134) have confirmed the assumption that *Leishmania* spp. synthesize the fatty acids of their phospholipids. Jacobs et al. (65) followed the time course of [^{14}C]acetate incorporation into lipids in *L. donovani*; incorporation of the isotope into lipids was higher during the logarithmic phase of growth than in the stationary phase, indicating the increased need for aliphatic moieties in propagating cells. In short-term incubations, of the order of minutes, it was found that neutral glycerides (mono-, di-, and triacylglycerols) were labeled first; by 20 s, 77.4% of the label was in neutral lipids and 21.6% was in the phospholipid fraction. Over the next 5 min, as radioactivity decreased in the neutral lipid fraction, it increased in the phospholipid fraction, and after 5 min and for up to 16 days, lipid-associated radioactivity was distributed equally between the neutral lipid and phospholipid fractions. It seems that fatty acyl moieties in lipids are not used mainly for energy production and that the fatty acids contained in triacylglycerols (triglycerides) serve primarily as sources of acyl and alkoxy moieties of phospholipids, wax esters and sterol esters.

It would be useful to pursue the question of why long-chain fatty acids are apparently not oxidized very rapidly by *Leishmania* promastigotes that have been grown in lipid-free media. Do they contain less carnitine, carnitine acyltransferase activity, or enzymes of the β-oxidation pathway than amastigotes? We have demonstrated that *L. donovani* promastigotes do contain L-(+)-carnitine (H. Paul and R. H. Glew, unpublished observation). More will be said about fatty acid oxidation in the next section.

L. donovani promastigotes incorporate [^{14}C]acetate into lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic and C_{20} and C_{22} fatty acids, indicating that they contain not only the apparatus for the de novo synthesis of fatty acids, but also the desaturases and elongating systems required for the conversion of stearic acid into unsaturated and very long-chain fatty acids.

Taking the approach of metabolic labeling with [^{14}C] myoinositol, Kaneshiro and co-workers (66) focused on the inositol lipids of *L. donovani* promastigotes and made an interesting discovery; they found that the organism contains an inositol sphingophospholipid (InSL). The inositol lipid composition was as follows: phosphatidylinositol (45.1%), InSL (37.9%), phosphatidylinositolphosphate (13.0%), phosphatidylinositoldiphosphate (2.2%), and lysophosphatidylinositolphosphate (2.6%). The fatty acyl groups of *Leishmania* inositol glycerolipids are different from those commonly found in mammalian cells (i.e., 18:0 at C-1; 20:4 at C-2). The *Leishmania* inositol lipids contain only traces of arachidonic acid (20:4); most of the acyl groups are accounted for by stearic and oleic acids. The N-acyl group of leishmanial InSL was contributed by stearic acid. The InSL discovered by Kaneshiro et al. (66) may be the same as the "unidentified" lipid described earlier by Wassef et al. (134). The biosynthesis and function of this interesting InSL warrant further investigation. In addition, it would be interesting to study the fate of radioactively labeled promastigote lipids once the parasites have been phagocytized.

Energy Metabolism

Glucose metabolism. Despite the excellent and comprehensive review of carbohydrate metabolism in the *Leishmania* spp. that appeared in 1980 (85), many gaps remain in our knowledge of the products and pathways of glucose metabolism. In that review, Marr concluded that, (i) whereas carbohydrates were metabolized primarily by aerobic fermentation in the promastigotes, they oxidize sugars incompletely to a mixture of organic acids (e.g., succinate, acetate, and pyruvate) and CO_2; (ii) they do not store polysaccharides; (iii) glucose is an energy substrate of secondary importance; and (iv) aerobic fermentation is greater in stationary-phase promastigotes than in logarithmic-phase cells.

Having seen elsewhere in this review that promastigotes (grown in lipid-free media) do not oxidize fatty acids appreciably, and finding that glucose is a "substrate of secondary importance" and metabolized only by stationary-phase cells, the reader might wonder what the primary fuel source is in propagatory *Leishmania* spp. The answer seems to be amino acids (85). It appears that rapidly dividing promastigotes take up amino acids, deaminate them by means of transaminases to the corresponding α-keto acid carbon skeletons, and then oxidize the latter in the Krebs cycle. The metabolism of amino acids by *Leishmania* spp. will be discussed below.

Nevertheless, the fact remains that glucose is taken up and utilized by the *Leishmania* spp. However, different species oxidize glucose at different rates. *L. donovani*, for example, consumes glucose more raidly than *L. braziliensis* (123). Summarizing decades of investigation involving tracer studies and enzymologic studies with extracts of various *Leishmania* spp., Marr (85) concluded that (i) glucose is most likely converted to triose phosphates by a combination of the pathway of glycolysis and the pentose phosphate pathway (10); (ii) triose phosphates are metabolized to pyruvate via the intermediary of phosphoenolpyruvate; (iii) pyruvate is converted to oxaloacetate, most likely by means of the pyruvate carboxylase reaction; and (iv) oxaloacetate is converted sequentially to malate, fumarate, and succinate. It is not clear whether mitochondrial or cytoplasmic enzymes are involved in the conversion of oxaloacetate to succinate.

Darling and co-workers (29) recently demonstrated that *L. braziliensis* produces D-lactate anaerobically; however, the pathway responsible for D-lactate production is obscure. These workers hypothesized that survival of *Leishmania* spp. under anaerobic conditions may be mediated by increased production of D-lactate which generates NAD^+ from NADH. The physical-chemical and kinetic properties of some of the enzymes involved in glucose metabolism in the *Leishmania* spp. were reviewed by Marr (85).

The pentose phosphate pathway or hexosomonophosphate shunt is present in *Leishmania* promastigotes. The

ratio of $^{14}CO_2$ produced from [1-^{14}C]glucose to that from [6-^{14}C]glucose is taken as a crude measure of the ability of a cell or tissue to oxidize glucose by means of the pentose phosphate pathway. Several teams of investigators have shown that various *Leishmania* species oxidize [1-^{14}C]glucose to $^{14}CO_2$ two to six times faster than they do [6-^{14}C]glucose, indicating that they consume an appreciable fraction of the glucose they use via the pentose phosphate pathway (10, 68). With increasing culture age of *L. donovani*, there is a decrease in the ratio of $^{14}CO_2$ produced from [1-^{14}C]glucose to that from [6-^{14}C]glucose, indicating decreased commitment of glucose to the pentose phosphate pathway relative to the glycolytic pathway as the organism enters the stationary phase.

The glycolytic enzymes in the trypanosomatids, including promastigotes and amastigotes of *Leishmania* species, are contained in a unique organelle called the glycosome (69), which was discovered by Opperdoes and Borst (100). Mottram and Coombs (91) found that the early enzymes of the glycolytic pathway, those that catalyze the reactions between and including hexokinase and glyceraldehyde-3-phosphate dehydrogenase, are confined largely to the glycosome. In contrast, most of the phosphoglycerate kinase and pyruvate kinase activities were found to be cytosolic in both stages of the parasite. The first enzyme in the pentose phosphate pathway, namely, glucose-6-phosphate dehydrogenase, is largely cytosolic.

Etges and Mukkada have devoted a number of years to the study of carbohydrate and energy metabolism in the trypanosomatids (42). That *Leishmania* promastigotes are capable of performing both glycolysis and gluconeogenesis raises questions about metabolic regulation. Etges and Mukkada (42) purified pyruvate kinase from *L. major* promastigotes and, through a series of kinetic studies, concluded that the enzyme is an important regulatory site in glycolysis; specifically, the enzyme is activated by its substrate, phosphenolpyruvate, in a positively cooperative fashion and heterotropically by fructose 1,6-bisphosphate. The activity of the purified *L. major* pyruvate kinase is inhibited by ATP, guanosine triphosphate, inosine triphosphate, and, to a lesser degree, citrate.

Mottram and Coombs (91) made the interesting observation that in *L. mexicana* subsp. *mexicana*, during the transformation from the promastigote to the amastigote form, the levels of PEP carboxykinase and malate dehydrogenase activities increase greatly while that of pyruvate kinase decreases markedly. The activities of malate dehydrogenase and phosphoenolpyruvate carboxykinase in *L. mexicana* subsp. *mexicana* amastigotes are particulate, presumably localized to the glycosome (90). The physiological implications of these changes are unclear. Mottram and Coombs (91) purified and characterized the two enzymes and suggested that, while the precise role of malate dehydrogenase in amastigotes is presently unclear, the extremely high malate dehydrogenase activity in this form of the parasite make it an attractive target for chemotherapeutic attack.

All of the tricarboxylic acid cycle enzymes can be found in all of the *Leishmania* species that have been examined in this regard. However, although a complete tricarboxylic acid cycle apparatus exists, the levels of certain key enzymes of the pathway (e.g., citrate synthase and α-ketoglutarate dehydrogenase) are so low that it appears that the role of the tricarboxylic acid cycle is to trap reducing equivalents through reduction of oxaloacetate to succinate, excretion of the latter, and conversion of isocitrate to glutamic acid, which would detoxify or organify ammonia and make glutamate available for further transamination reactions.

Much remains to be learned about the regulation and function of carbohydrate and organic acid metabolism in *Leishmania* spp. and about how energy and redox metabolism are integrated in the organism.

Fatty acid oxidation. In a comprehensive study replete with interesting observations, Hart and Coombs (59) compared the utilization of various energy substrates, including nonesterified fatty acids, in amastigotes and promastigotes of *L. mexicana*. As noted elsewhere in this review, promastigotes do not oxidize free fatty acids very rapidly. In contrast, however, amastigotes have a well-developed capacity for oxidizing long-chain fatty acids to CO_2 and water; for example, an equimolar mixture of stearate, palmitate, and oleate (0.07 mM each) was oxidized at rates of 3.1 and 0.2 nmol/h per 10^8 cells by amastigotes and promastigotes, respectively. Compared with amastigotes, promastigotes contain less of the activities of β-ketothiolase and 3-β-hydroxylacyl-coenzyme A dehydrogenase, enzymes of the fatty acid β-oxidation pathway (26). Interestingly, when offered linoleate, the difference between the rate of fatty acid oxidation by amastigotes and promastigotes was not nearly as great (5.0 versus 2.0 nmol/h per 10^8 cells, respectively). Exogenous triglycerides were also oxidized by *L. mexicana*; again, amastigotes were more active than promastigotes.

Hart and Coombs (59) suggested that the high capacity of amastigotes for fatty acid oxidation may reflect an adaptation to the kind of substrate they encounter in their intracellular habitat. Blood monocytes and macrophages take up and degrade the triacylglycerols contained in lipoproteins, thereby providing a potential supply of lipids for the amastigote.

Blum and co-workers (14, 28) compared fatty acid oxidation in promastigotes and ellipsoidal, amastigotelike forms of *L. braziliensis* subsp. *panamensis* which were produced by heating 26°C-grown promastigotes at 34°C for 12 h. Like amastigotes derived from infected macrophages, the heat-induced ellipsoidal forms oxidized medium (C_{12}) and long-chain (C_{18}) fatty acids labeled in their carboxyl groups with ^{14}C more rapidly (1.5- to 2.0-fold) than promastigotes did; however, the differences were not as great as they were for promastigotes and macrophage-derived amastigotes, in which this difference is on the order of 10-fold (59).

In that same study (14), Blum observed that promastigotes release $^{14}CO_2$ much more rapidly from [1-^{14}C]lauric acid than from [12-^{14}C]lauric acid, indicating that the fatty acid is not completely oxidized to CO_2 and that the omega (methyl) end of the fatty acid may be used for some other, but unknown, purpose (e.g., production of ketone bodies or chain elongation for the synthesis of other fatty acids).

The inhibition of fatty acid oxidation in *L. mexicana* by compounds that are known to inhibit cytochromes or β-oxidation enzymes (59) suggests that classic mitochondrial β-oxidation is the pathway responsible for the degradation of long-chain fatty acids in mitochondria. Nevertheless, studies in recent years have documented that a variety of eucaryotic cells oxidize very long-chain fatty acids by a peroxisomal pathway distinct from the mitochondrial one; it would be interesting to determine whether *Leishmania* forms possess a peroxisomal fatty acid-oxidizing apparatus.

While on this subject of fatty acid oxidation, it is worth mentioning that investigators exploring the effects of cyclopropane fatty acid analogs such as 10-thiosterculic acid on leishmanias should examine the effects of these fatty acids on fatty acid oxidation.

Amino acid catabolism. We have seen that fatty acids, but probably not hexoses (e.g., glucose), are important fuels for the *Leishmania* spp. Another significant source of energy, perhaps the most important, are the carbon skeletons of the amino acids, especially in promastigotes. Rates of amino acid utilization in general are higher in promastigotes than in amastigotes. In their study with *L. mexicana*, Hart and Coombs (59) observed that proline was a poor substrate for both amastigotes and promastigotes; in fact, promastigotes appear to synthesize and excrete proline. However, the following amino acids were rapidly extracted from the growth medium and degraded: asparagine, glutamine, glutamate, leucine, lysine, methionine, and threonine.

It appears that a L-arginine is assimilated by *L. donovani* promastigotes by means of the α-oxoglutarate pathway that involves the intermediary of γ-guanidinobutyramide and the enzyme L-arginine decarboxyoxidase (9). By this pathway, L-arginine is converted to succinate.

Zilberstein and Dwyer (140) investigated the mechanism for the uptake of amino acids in *Leishmania* promastigotes and the nature of the driving force responsible for their transport. Specifically, they demonstrated that the transport of L-proline and D-glucose by mid-log-phase *L. donovani* promastigotes is carrier mediated and that the energy is provided by a proton electrochemical gradient. Presumably, the hydrolysis of ATP on the inside of the parasite's plasma membrane results in the secretion of a proton into the extracellular fluid and symport translocation of a molecule of amino acid or glucose into the cell.

Purine and Pyrimidine Metabolism

Purines. Since Hitchings (64) and Marr and Berens (86) have recently reviewed the subject of purine metabolism in *Leishmania* spp., only the highlights of what is known about the topic will be summarized here. The *Leishmania* spp., like other hemoflagellates, appear to be incapable of synthesizing the purine nucleus de novo and therefore require exogenous purines for growth.

Mammalian cells possess two purine salvage pathways. The first is provided by enzymes of the phosphoribosyltransferase family which catalyze the following type of reaction, where PP_i represents pyrophosphate:

$$\text{base} + \text{PP-ribose-P} \rightarrow \text{base-ribose-P} + PP_i$$

For example, if the base is adenine, then the product of the phosphoribosyltransferase reaction will be the nucleotide AMP. In this pathway, the ribose and phosphate moieties are transferred to the purine in one step. It seems that, like mammalian cells, the *Leishmania* spp. possess two distinct phosphoribosyltransferases, one specific for adenine and another specific for guanine.

The second salvage pathway is composed of two enzymes, where ADP is adenosine diphosphate:

$$\text{base} + \text{ribose-1-P} \rightarrow \text{base-ribose} + P_i$$

$$\text{base-ribose} + \text{ATP} \rightarrow \text{base-ribose-P} + \text{ADP}$$

The first reaction, which attaches ribose to the purine, is catalyzed by a phosphorylase (operating in an anabolic direction), and the second reaction, which attaches a phosphate group to the nucleoside, is catalyzed by a nucleoside kinase.

Adenosine kinase is one such nucleoside kinase, and it was recently isolated in pure form from *L. donovani* by Datta et al. (32); it catalyzes the following reaction:

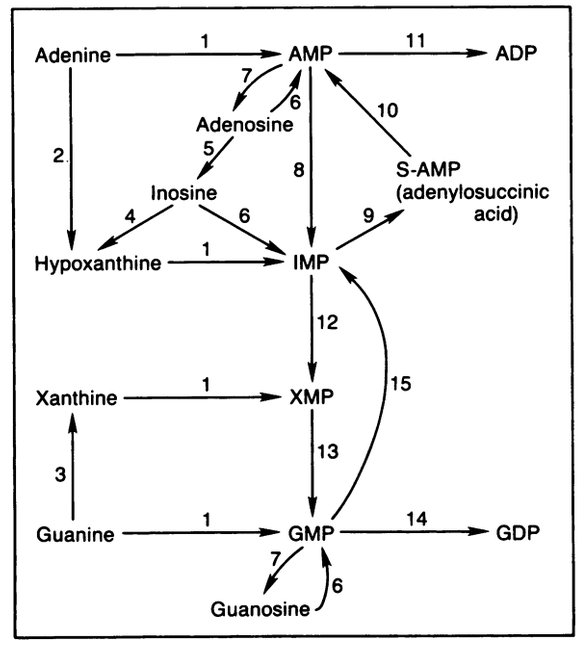

FIG. 8. Purine salvage pathways. Enzymes: 1, phosphoribosyltransferase; 2, adenine amidohydrolase (deaminase); 3, guanine deaminase (aminohydrolase); 4, inosine nucleotidase (phosphorylase; catabolic); 5, adenosine deaminase; 6, nucleoside kinase; 7, nucleotidase; 8, AMP deaminase; 9, adenylosuccinate synthetase; 10, adenylosuccinate lyase; 11, AMP kinase; 12, IMP dehydrogenase; 13, GMP synthetase; 14, GMP kinase; 15, GMP reductase.

$$\text{adenosine} + \text{ATP} \xrightarrow{Mg^{2+}} \text{5'-AMP} + \text{ADP}$$

The *L. donovani* adenosine kinase has physical and kinetic properties which distinguish it from adenosine kinases of other eucaryotic cells. Since nucleotides do not enter into cells readily, it is thought that adenine nucleoside (adenine-ribose) permeates the plasma membrane first and then is converted to its nucleotide (AMP).

The absence of phosphorylase activity in some *Leishmania* species means that these organisms can salvage purines only be means of the phosphoribosyltransferase reaction. Thus, the *Leishmania* spp. preferentially salvage purine bases instead of nucleosides.

The purine salvage pathways the *Leishmania* spp. use are different from those used by mammalian cells. Promastigotes and amastigotes of *L. mexicana* subsp. *mexicana* utilize the salvage pathway summarized in Fig. 8 (60). Key enzymes in this process are adenine deaminase and guanine deaminase, which convert adenine and guanine to hypoxanthine and xanthine, respectively. However, stage-specific differences exist for some *Leishmania* species, with promastigotes containing adenine aminohydrolase and amastigotes containing adenosine deaminase. The phosphoribosyltransferases of the parasite then convert hypoxanthine and xanthine to inosine monophosphate (IMP) and xanthine monophosphate, respectively. These nucleotides can be converted to AMP and GMP by the following enzymes: adenylosuccinate synthetase, adenylosuccinate lyase, IMP dehydrogenase, and GMP synthetase. *L. donovani* and other *Leishmania* species also salvage purines by this pathway. However, *L. mexicana* subsp. *mexicana* and *L. donovani* differ in other respects in terms of purine metabolism. The pathway of adenosine metabolism present in the amastigote form of *L.*

donovani does not occur in *L. mexicana* subsp. *mexicana* (76). In *L. mexicana* subsp. *mexicana* it appears that both stages of the organism convert exogenous adenosine either to AMP by the action of adenosine kinase or to hypoxanthine via adenosine deaminase and inosine nucleotidase. During transformation of promastigote to amastigote, adenosine kinase activity increases nearly 50-fold (59). It has been suggested that adenosine kinase plays a key role in the process of transformation (32).

Another difference in the two species is that, whereas extracts of *L. donovani* lack nucleoside kinase activities, kinase activities for all four purine nucleosides are present in promastigotes and amastigotes of *L. mexicana* subsp. *mexicana* (60).

Phosphorylase activity in the anabolic direction (i.e., in the presence of ribose-1-phosphate, conversion of adenine to adenosine) was not demonstrable in *L. mexicana* subsp. *mexicana*, indicating that bases can be salvaged only by phosphoribosyltransferases. Except for adenosine, nucleosides could be cleaved by nucleotidase or phosphorylase, thereby permitting the resulting bases to be converted to their respective nucleotides by phosphoribosyltransferases. The phosphoribosyltransferases for adenine and guanine appear to be separate enzymes.

In *L. mexicana* subsp. *mexicana* there is compartmentation of some of the key purine-metabolizing enzymes; it appears the enzymes involved in the catabolism of purines and nucleosides occur mainly in the cytosol, whereas the anabolic pathways that provide nucleotides for DNA and ribonucleic acid syntheses are associated with glycosomes (62).

As pointed out by Hassan and Coombs (60), Wang (133), and Marr (85), differences in the properties of leishmanial and host cell purine-metabolizing enzymes may be exploited in chemotherapy. The main difference in purine metabolism between the *Leishmania* spp. and humans is that the hypoxanthine-guanine phosphoribosyltransferases of *Leishmania* spp., but not of humans, utilize allopurinol as a substrate and convert the drug to the corresponding ribonucleotide. The amastigote is the main target for this type of leishmanial drug. The most extensively studied of these drugs are the pyrazolopyrimidines.

In this regard, Looker and co-workers (77) have shown that allopurinol (4-hydroxypyrazolo-[3,4-*d*]pyrimidine) and other pyrazolopyrimidines are effective antileishmanial agents in vitro. Allopurinol, an analog of the purine inosine, is converted to the IMP analog 4-hydroxypyrazolo-[3,4-*d*]pyrimidine ribonucleoside monophosphate (HPPR-MP) by hypoxanthine-guanine phosphoribosyltransferase (Fig. 8, enzyme 1). Adenylosuccinate synthase and adenylosuccinate lyase then convert HPPR-MP to APP-ribonucleoside monophosphate, which in turn is phosphorylated to its triphosphate form and finally incorporated into ribonucleic acid.

The differences in purine metabolism between host and parasite account for the selective toxicity of allopurinol between host and parasite. In humans, inosine is phosphorylized to hypoxanthine and ribose-1-phosphate; the hypoxanthine is then converted to inosinic acid by the action of hypoxanthine:guanine phosphoribosyltransferase. In contrast, when allopurinol is metabolized to allopurinol riboside, it gives the appearance of being relatively stable metabolically because its formation is favored in the presence of purine nucleoside phosphorylase. In humans, very little allopurinol ribonucleotide is produced. In the *Leishmania* spp., the extensive allopurinol ribonucleotide that is produced is converted readily into 4-aminopyrazolo-[3,4-*d*]pyrimidine ribonucleoside mono-, di-, and triphosphates. It appears that the adenolosuccinate synthetases and lyases of the *Leishmania* spp. are less substrate specific than those of the mammalian host which barely carry out these reactions (76).

It is thought that allopurinol exerts its antileishmanial action by upsetting nucleic acid biosynthesis in at least two ways. First, the IMP analog HPPR-MR inhibits GMP reductase; this blocks synthesis of ATP from guanine. HPPR-MP also inhibits IMP dehydrogenase. Thus, allopurinol interferes with the interconversion of purines and creates a condition analogous to purine starvation. Second, by unknown mechanisms, pyrazolopyrimidines inhibit uracil and ribonucleic acid metabolism.

Pyrimidines. Whereas the kinetoplastida protozoa cannot synthesize purines de novo, they do possess the enzymatic apparatus required to synthesize pyrimidines; all six enzymes of the pyrimidine biosynthetic pathway have been demonstrated in *L. mexicana* promastigotes (57). It was shown by Mukherjee and co-workers (92) that, unlike what one finds in most other eucaryotes, the first three enzymes of the de novo pyrimidine pathway are not organized into a multienzyme complex. One of these enzymes, namely, aspartate carbamoyltransferase (aspartate transcarbamylase), is very sensitive to inhibition by *N*-(phosphonoacetyl)-L-aspartic acid, a transition-state analog.

The *Leishmania* spp. appear to possess a unique thymidylate synthase, an enzyme which catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) to produce (deoxythymidine monophosphate (dTMP), which is essential for DNA synthesis:

$$dUMP + CH_2H_4 \text{ folate} \rightarrow dTMP + H_2 \text{ folate}$$

In this reaction, the cofactor is the source of both the one-carbon unit and the reducing equivalents. What is unique about the leishmanial thymidylate synthase is that it is a bifunctional protein: it occurs as a dimer of identical subunits (30 to 35 kilodaltons), each of which contains thymidylate synthase activity and dihydrofolate reductase (43). Bifunctional dihydrofolate reductase-thymidylate synthase is the target of methotrexate. Ellenberger and Beverley (37) have contributed much to our knowledge of methotrexate resistance in the *Leishamania* spp.; however, a discussion of this phenomenon is beyond the scope of the present review. An interesting property that distinguishes the *L. major* thymidylate synthase from the enzyme from mammalian sources is its ability to form relatively stable dUMP-thymidylate synthase complexes. This property indicates that there may be significant differences between the thymidylate synthases of the parasite and its host and that the enzyme may be a target for antileishmanial drugs.

Another potentially useful target for new antileishmanial drugs is dihydroorotate dehydrogenase, the enzyme which catalyzes the conversion of L-5,6-dihydroorotate to orotate in both amastigotes and promastigotes (45). The conversion of L-5,6-dihydroorotate to orotate is an integral part of the de novo pyrimidine pathway. The properties of L-5,6-dihydroorotate dehydrogenase in *L. mexicana* subsp. *mexicana* are very different from those of the host mammal.

EXCRETED FACTOR

Leishmania spp. growing in culture produce a polysaccharide or glycoconjugate called excreted factor which occurs on the cell surface and in the culture medium (120). It is the

major antigenic glycoconjugate released from the surface of the leishmanial parasite. Excreted factor is immunologically active and forms very tight complexes with albumin which can be dissociated by treatment with trichloroacetic acid into two fragments whose molecular weights are in the 15,000 to 30,000 range. The polysaccharide character of the material is indicated by its ability to react with certain lectins and the fact that it shows positive reaction with the periodic acid-Schiff stain. The trichloroacetic acid-released polysaccharide factor reacts with peanut lectin, indicating the presence of nonreducing galactose residues. The soluble, secreted form of excreted factor appears to be immunologically identical to a substance that is physically associated with promastigotes (39). The cell-associated form of excreted factor, when chromatographed on a Sephadex G-100 column, elutes very close to a pepsin standard (molecular weight, 32,000). Like the secreted form of excreted factor, the excreted factor isolated from promastigotes appears to be a highly negatively charged polysaccharide.

Definitive proof that leishmanial excreted factor contains terminal nonreducing galactose residues was provided by Slutzky and Greenblatt (122) when they demonstrated that galactosyl groups may be specifically labeled by treatment with galactose oxidase and sodium boro[^{3}H]hydride. Furthermore, they showed that galactose was the immunodominant sugar of excreted factor.

Until recently, the detailed chemical structure of excreted factor was obscure. Pritchard and co-workers (107) analyzed excreted factor purified from the culture medium in which *L. donovani* or *L. tropica* promastigotes had been grown and reported that it contained galactose and xylose in a 6:1 molar ratio; phosphate and *N*-acetylgalactosamine were absent. They proposed a galactanlike structure for the leishmanial polysaccharide.

Turco and co-workers (129), taking primarily a metabolic labeling approach using [^{3}H]galactose and [^{3}H]mannose, investigated the structure of an *L. donovani* promastigote-associated substance whose properties resemble those of excreted factor. They first delipidated the radiolabeled cells by organic solvent extraction and then solubilized the glycoconjugate by treating the lipid-free residue with 0.04 M NH_4OH at 100°C. The highly acidic, anionic product they obtained was resistant to digestion by a variety of proteases, hyaluronidase, chondroitinase ABC, and endoglycosidase and ran on sodium dodecyl sulfate-polyacrylamide gels as though its molecular weight were in the 15,000 to 30,000 range. Characterization of the products generated by partial acid hydrolysis of the radiolabeled leishmanial glycoconjugate revealed a dissaccharide repeat unit consisting of phosphorylated galactosyl-β-mannose, suggesting a polymer of galactosyl-β-mannose units linked together by phosphate bridges (Fig. 9). Turco and co-workers (129) refer to this acidic glycoconjugate as lipophosphoglycan (LPG).

In a subsequent study in which they used a galactose oxidase-NaB(^{3}H)$_4$ labeling technique whch tritiates exposed, nonreducing galactose residues, King et al. (71) demonstrated that the LPG of *L. donovani* promastigotes is localized to the parasite's cell surface. They showed too that LPG could be released from the cell surface into the culture medium. The observation that the presence of albumin in the medium enhanced the rate of release of LPG from cells prompted King et al. (71) to postulate that the lipid moiety of LPG interacts with some hydrophobic binding site on albumin. Furthermore, although LPG was present on cells throughout the growth curve, it was preferentially expressed late in the logarithmic and stationary phases.

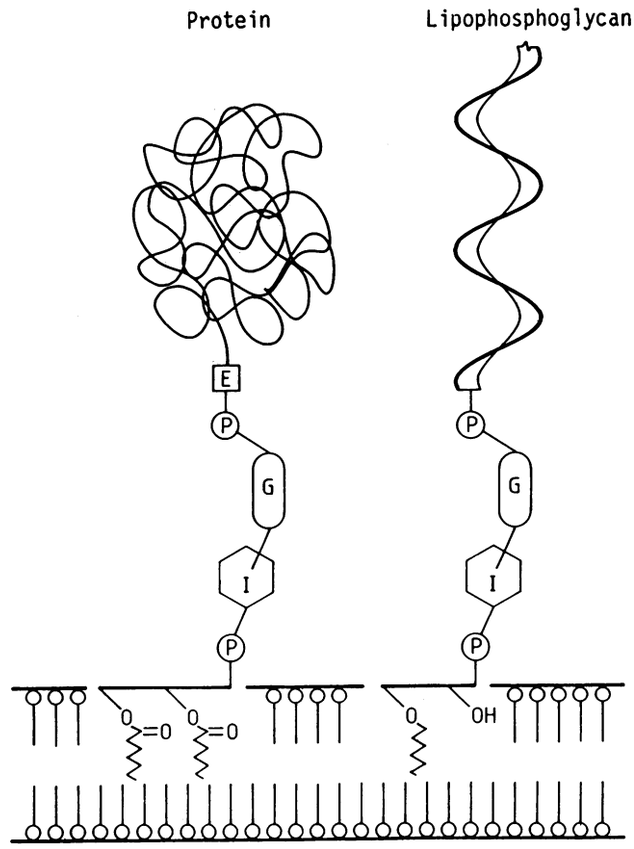

FIG. 9. Schematic representation of proteins and LPG anchored in membranes. I, Inositol; G, glycan core; P, phosphate; E, ethanolamine. In this schematic representation, all 16 phosphorylated disaccharide units are illustrated as a ribbonlike, consecutive sequence. However, it is possible that one or more of the units are attached to a second site in the heptasaccharide core (G) (101).

Orlandi and Turco (101) subjected the lipid moiety of *L. donovani* LPG to comprehensive structural analysis, using a combination of controlled chemical degradations and nuclear magnetic resonance spectroscopy. The lipid moiety of LPG was released by treatment with (0.25 M nitrous acid, pH 4 (25°C, 16 h). They concluded that the lipid domain of LPG has the structure of a lyso-alkyl-phosphatidylinositol (Fig. 9).

In an equally informative companion paper, Turco and co-workers (128) tackled the structure of the carbohydrate domain of *L. donovani* LPG. The carbohydrate domain of LPG was released by mild acid hydrolysis (0.02 N HCl, 5 min, 100°C) and then purified by chromatography on a DE-52 cellulose ion-exchange column and a silica gel thin-layer plate. The structure of the carbohydrate fragment was determined to be $PO_4 \rightarrow 6Gal(\beta 1 \rightarrow 4)Man\alpha 1$. It was calculated that 16 of these phosphorylated disaccharide units occur in LPG and they appear to be linked together by α-glycosidic bonds. Thus, the disaccharide repeat units seem to occur as a linear structure in LPG.

Based on the results of these two structural studies, Orlandi and Turco (101) have proposed the model shown in Fig. 9. It is noteworthy that the lipid domain of LPG is similar to the carbohydrate-containing phosphatidylinositol anchor that has been found in a number of eucaryotic membrane proteins (27, 82, 121), the major difference being

that for most proteins the phosphatidylinositol moiety is diacylated, whereas in LPG one finds lyso-alkyl-phosphatidylinositol.

A number of functions have been suggested for excreted factor. First, it has been shown that excreted factor inhibits the activity of the lysosomal β-galactosidase of macrophages (38). The inhibition is probably due to the terminal nonreducing galactosyl moieties present in excreted factor. The mechanism of the inhibition has not been characterized, but two explanations are likely. The excreted factor may be either a substrate for mammalian lysosomal β-galactosidase or a competitive inhibitor of the enzyme. It has been proposed that, by inhibiting lysosomal hydrolases of the host phagocyte, the parasite becomes resistant to digestion within phagolysosomes. As King et al. (71) have pointed out, LPG and other cell surface components of the *Leishmania* spp. may play a role in the survival of the parasite during encounters with hydrolytic substances in the alimentary tract of its sandfly vector.

A second possibility is that excreted factor located on the surface of promastigotes plays a key role in the binding and uptake of parasites by host cells. The importance of galactosyl residues on the promastigote surface was shown by Zehavi et al. (139) when they demonstrated that galactose oxidase treatment, which selectively decreases the number of nonreducing galactosyl units on promastigotes surfaces, caused a marked reduction in the attachment of parasites to macrophages. It remains to be seen whether the promastigote surface contains sufficient excreted factor to fulfill this proposed role and whether the material that was oxidized by Zehavi and colleagues (139) was, in fact, excreted factor. In this regard, El-On et al. (39) reported that when they subjected *L. donovani* promastigotes to subcellular fractionation they found excreted factor-related material in the soluble, particulate-free fraction. Handman and Goding (58) have characterized the membrane and extracellular forms of the glycoconjugate and showed that, at least for *L. major*, it is the parasite receptor for macrophages and thus directly involved in the initiation of infection.

There is a third way in which excreted factor might promote the attachment and ingestion of leishmanial parasites to macrophages. Pritchard and co-workers (107) found that excreted factors isolated from the culture fluid in which *L. tropica* and *L. donovani* were grown will precipitate C-reactive protein. As these workers pointed out, while the ability of leishmanial excreted factor to bind C-reactive protein may be of significance in host defense against these organisms, another intriguing possibility is that this interaction actually benefits the parasite since phagocytes generally take up particles coated with C-reactive protein.

Finally, McNeely and Turco (88) have shown that the purified LPG from *L. donovani* is capable of inhibiting the activity of protein kinase C derived from rat brain; the glycoconjugate was a competitive inhibitor with respect to the lipid activator, diolein, and a noncompetitive type inhibitor with respect to the other lipid activator of protein kinase C, namely, phosphatidylserine. In view of the role of protein kinase C in regulating the respiratory burst in macrophages, these investigators speculated that the LPGs might block the production of superoxide anions.

FUTURE WORK

Apart from the satisfaction one normally derives from assembling the fund of knowledge in a particular area, one of the rewards of preparing a review like this is that it gives an appreciation of specific studies that are feasible and which should provide a good deal of new insight into the biochemical capabilities of the *Leishmania* spp.

In the areas of phosphatases and kinases, there are a number of questions begging answers. First, what are the natural substrates of the leishmanial phosphatase, not just the cell surface tartrate-resistant one but also the secreted phosphatase? Are they phosphoproteins or are they phospholipids or inositides that bear phosphomonoester groups? And, of course, there is the issue of the structure of these enzymes. But the most pressing question in this area pertains to the mechanism by which the leishmanial acid phosphatase blocks the NADPH oxidase reaction. Second, there is the protein kinase: does it serve only functions relevant to the free-living parasite or does it play some role in the host-parasite relationship? More specifically, what are the natural acceptors of the protein kinase activity? Since it is unlikely that histones are the physiologically relevant substrate for the enzyme, are there proteins in phagocytes or on their surface which are phosphorylated by the kinase? Heretical as it may seem, could it be that lipids are substrates for the leishmanial protein kinase? Interestingly, we have observed that when phosphatidylinositol is incubated with extensively purified *Legionella micdadei* protein kinase and [γ-^{32}P]ATP, phosphatidylinositolphosphate is formed (A. K. Saha and J. N. Dowling, unpublished data). With regard to the question of protein substrates for the protein kinase, it would be interesting to compare various species of *Leishmania*, as well as infectious and noninfectious strains of the same species, for the number and kinds of proteins which they phosphorylate.

In the area of lipid metabolism, many interesting and readily approachable questions await pursuit. With regard to the metabolism of triglycerides and fatty acids, we need to learn more about the structure and regulation of the enzymes and proteins involved in triglyceride utilization, fatty acid transport into intracellular organelles (e.g., mitochondria) of various species and stage-specific forms of the *Leishmania* spp., and β-oxidation. For example, what sort of lipases in the parasite release fatty acids from triglycerides? Is more than one lipase involved in the degradation of triglycerides by amastigotes? Does carnitine play an essential role in intracellular fatty acid transport in the parasite? Do the *Leishmania* spp. synthesize their own carnitine or do they obtain it from the vector or host? Do peroxisomes participate in fatty acid oxidation in the *Leishmania* spp.? In terms of the fatty acid oxidation pathway(s), how many dehydrogenases are required for the complete oxidation of a long-chain fatty acid and what are their substrate specificities? Zoeller and Raetz (141) have reported a strategy for isolating Chinese hamster ovary cell mutants that are defective in the peroxisomal enzyme dihydroxyacetonephosphate acyltransferase, which catalyzes a key step in the biosynthesis of plasmalogens. If such a strategy could be successfully applied to the *Leishmania* spp., one would have a useful tool for studying the metabolism, function, and significance of ether lipids in those parasites.

With regard to the excreted factor, the recent studies of Turco and co-workers (128) have illuminated the structure of the LPG of the *Leishmania* spp. and should stimulate a variety of studies aimed at elucidating the structure, metabolism, function, and immunology of this interesting substance. For example, how is the polysaccharide domain of LPG synthesized? Is it produced via a dolichol-linked dissacharide repeat unit? Also, how does LPG interact with host cells? Are there specific LPG receptors on phagocytes? How

is LPG metabolized by host cells? What effects does LPG have on the metabolism of host cells and does it contribute to the survival of the parasite inside the phagocytes?

These questions and many more await the efforts of imaginative cell biologists, biochemists, immunologists, and molecular biologists interested in unraveling the secrets of one of the world's most troublesome but interesting parasites.

ACKNOWLEDGMENTS

We are grateful to Salvatore J. Turco, Michael Gottlieb, David L. Sacks, James K. Lovelace, and Richard D. Pearson for helpful comments on this review. S. J. Turco also kindly provided us with Fig. 9. We acknowledge Teresa Poloka for secretarial assistance.

Original research in our laboratory was supported by Public Health Service research grant AI-18945 from the National Institutes of Health.

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