percentage of recovery for IgM thus isolated. We suggest instead a more time-consuming two-gradient procedure which becomes practical when the total amount of serum available is severely limited. This method consists of direct isolation of IgM from serum using a simple sucrose gradient which ranges up to 45% (w/v) sucrose. The IgM is then removed from the bottom of the gradient tube. The rest of the gradient (protein, sucrose, salts) is dialyzed and concentrated. The IgG in the concentrate then can be purified using the complete SID method.

The SID method also holds promise for the isolation and direct visualization of one enzyme from a mixture of proteins. This application requires that the pH gradient is set up in such a way that no protein reaches its isoelectric pH while other proteins in the mixture are still passing through that segment of the gradient.

Summary. A high-purity micromethod called Salt—Isoelectric—Density Method (SID) for simultaneous isolation of multiple IgG (or IgG and IgM) samples is described. Kinetics of the immune response to mixed flagellate antigens isolated by the SID method is described.

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Received Sept. 9, 1968. P.S.E.B.M., 1969, Vol. 130.

The Influence of Three Analogs of Isoleucine on *in Vitro* Growth and Protein Synthesis of Erythrocytic Forms of *Plasmodium Knowlesi** (33612)

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Recently we reported that L-isoleucine is essential for *in vitro* growth of erythrocytic forms of *Plasmodium knowlesi* (1). In the present communication it was shown that L-isoleucine was more avidly incorporated into plasmodial protein than seventeen other radiolabeled amino acids. On the basis of these observations, two isoleucine antagonists, L-*O*-methylthreonine and alloisoleucine, known to inhibit *in vitro* growth of certain organisms (2, 3), were examined for their effect on DNA and protein synthesis in erythrocytic forms of *P. knowlesi in vitro*. *N*-Acetylisoleucine, thought on a structural basis to act as an antagonist of isoleucine, was similarly tested. It was found that L-*O*-methylthreonine (L-OMT) strongly inhibited *in vitro* growth of *P. knowlesi*. Certain aspects of the mode of action of L-OMT were investigated and discussed.

Materials and Methods. The methods of preparing plasmodial cultures, dialyzing serum, and determining DNA have been described (1).

Incorporation of isotopically labeled amino acids into protein. Plasmodia in the trophozoite stage were incubated for 15–16 hr in Eagle's basal medium containing 0.2 mmole of each L-amino acid, including L-alanine, L-aspartic acid, L-glutamic acid, glycine, L-proline, and L-serine and supplemented with 10% dialyzed human AB serum. One half or 1

* Contribution No. 459 from the Army Research Program on malaria.

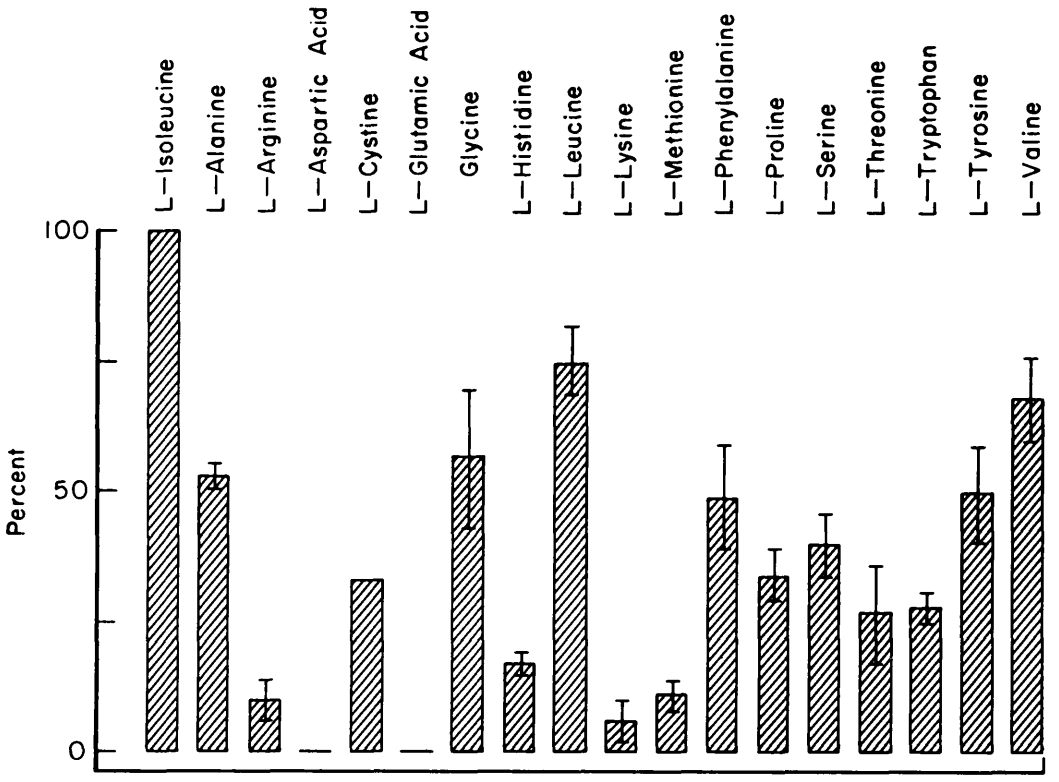

FIG. 1. Quantitative comparison of the incorporation of isotopically labeled amino acids into protein of *P. knowlesi*. Experimental conditions as described in "Methods." The incorporation of each amino acid into protein is depicted as a percentage of the amount of L-isoleucine-U^{14}C incorporated into protein. Each value represents an average of 10 cultures with SD (two cultures per experiment repeated five times, except for cystine and tryptophan, which were examined in one and two experiments, respectively).

μCi of each of the following U-^{14}C L-amino acids were added to separate cultures in duplicate at time 0: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine.[1] Three amino acids were labeled differently: DL-tryptophan-2-^{14}C, L-methionine-^{35}S, and L-cystine-^{35}S. The specific activity of each amino acid after mixing the labeled with the unlabeled amino acid in the medium was 4.54–4.76 mCi/mmole. In the calculations of the results of Fig. 1, we assumed that only the L-isomer of DL-tryptophan-2-^{14}C was incorporated into protein of *P. knowlesi*. Incorporation of the labeled amino acids into the white blood cells and reticulocytes present in the parasitized cultures was measured in control cultures containing an equal volume of normal blood cells. The number of white blood cells and reticulocytes of parasitized and nonparasitized monkeys were similar (4).

Following incubation, the cells were dissolved in 0.05% sodium lauryl sulfate in water. Trichloroacetic acid (TCA) was added to bring the final concentration to 10% and the cell fragments were sedimented into a pellet at 46,000*g* for 20 min. The cell pellet was washed once with 10% TCA, once with 10% TCA at 90° for 15 min, twice with ethanol and twice with ethanol:ether 3:1. The residual pellet called protein, was dissolved in 0.1 *N* NaOH, thinly plated in duplicate and

[1] All labeled substrates used in these experiments were purchased from New England Nuclear Corp., Boston, Mass., Nuclear Chicago Corp., Des Plaines, Ill., or Schwartz BioResearch, Orangeburg, N. Y.

counted for at least 1000 counts with a low background gas flow counter (model 4342 Nuclear Chicago Corp., Des Plaines, Ill.). The incorporation values of radionuclide into plasmodial protein shown in Fig. 1 were obtained by subtracting the incorporation values of nonparasitized from the parasitized cultures.

Results. During *in vitro* growth, erythrocytic forms of *P. knowlesi* incorporated the following L-amino acids from the medium into protein (Fig. 1): alanine, arginine, cystine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. No significant amounts of aspartic acid and glutamic acid were incorporated into protein. Quantitative comparison of the utilization of various amino acids for protein synthesis were made and showed that L-isoleucine-U-^{14}C was more avidly incorporated into the protein of *P. knowlesi* than the other amino acids.

In the following experiments the influence of L-OMT ,alloisoleucine and *N*-acetylisoleucine on the incorporation of orotic-6-^{14}C acid into DNA, L-isoleucine-U-^{14}C and L-methionine-$^{14}CH_3$ into the protein of *P. knowlesi* were determined and compared.

L-OMT in a concentration 20 (1.4 m*M*) and 60 (4.2 m*M*) times greater than L-isoleucine (0.07 mM) inhibited orotic-6-^{14}C acid incorporation into plasmodial DNA to 70 and 15% of the control values, respectively, (Fig. 2). L-Alloisoleucine, in a concentration 80 (5.5 m*M*) times greater than L-isoleucine (0.07 m*M*) decreased the formation of DNA to 70%, while *N*-acetyl-L-isoleucine in a concentration 60 (4.2 m*M*) times greater than L-isoleucine (0.07 m*M*) had no effect on the incorporation of orotic-6-^{14}C acid into DNA. D-Alloisoleucine and *N*-acetyl-D-isoleucine, used in the above concentrations had no effect on orotic-6-^{14}C acid incorporation.

Concentrations of L-OMT 56 times (2.8 m*M*) greater than L-isoleucine (0.05 m*M*) inhibited the incorporation of L-isoleucine-U-^{14}C and L-methionine-$^{14}CH_3$ into protein; i.e., 25 and 23%, respectively (Fig. 3). Similar concentrations of L-alloisoleucine inhibited L-isoleucine-U-^{14}C incorporation to 15%, without significantly affecting L-methionine-$^{14}CH_3$ incorporation into protein. A concentration of *N*-acetyl-L-isoleucine 42 times (2.1 m*M*) greater than L-isoleucine (0.05 m*M*) reduced L-isoleucine-U-^{14}C incorporation to 81%, while greater concentrations of the analog had no effect on L-methionine-$^{14}CH_3$ incorporation. Similar concentrations of the D-isomers of the latter two analogs had no effect on the incorporation of either L-isoleucine-U-^{14}C or L-methionine-$^{14}CH_3$ into protein.

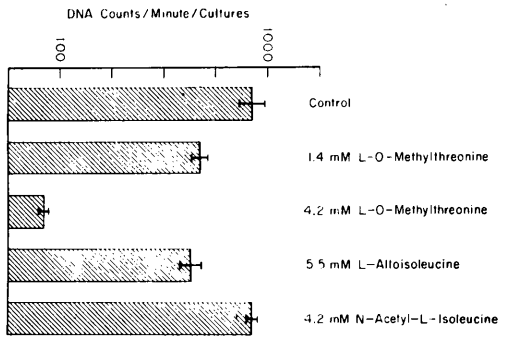

FIG. 2. Effect of L-isoleucine antagonists on orotic 6-^{14}C acid incorporation into DNA of *P. knowlesi*. Plasmodia were grown for 16 hr in Eagle's medium containing 0.07 mmole of each amino acid, 10% dialyzed human AB serum and 1 μCi/culture of orotic-6-^{14}C acid (sp act. 30 mCi/mmole). The analogs were added at time 0 in the concentrations indicated. The DNA was determined as described previously (1). Each value represents an average of three cultures with SD.

L-Isoleucine, in a concentration of 0.65 m*M* markedly diminished the inhibitory effect of 2.8 m*M* L-OMT on the incorporation of L-methionine-$^{14}CH_3$ into protein, but only slightly affected the inhibition of orotic-6-^{14}C acid incorporation into DNA (Fig. 4). The addition of similar quantities of other amino acids (0.65 mmole) did not affect L-OMT inhibition of protein synthesis. These amino acids (L-isomers) were: cystine, arginine, glutamine, histidine, leucine, lysine, phenylalanine, threonine, tryptophan, and valine tested separately and alanine, glycine, proline, and serine which were tested simultaneously.

Discussion. Previous investigations have

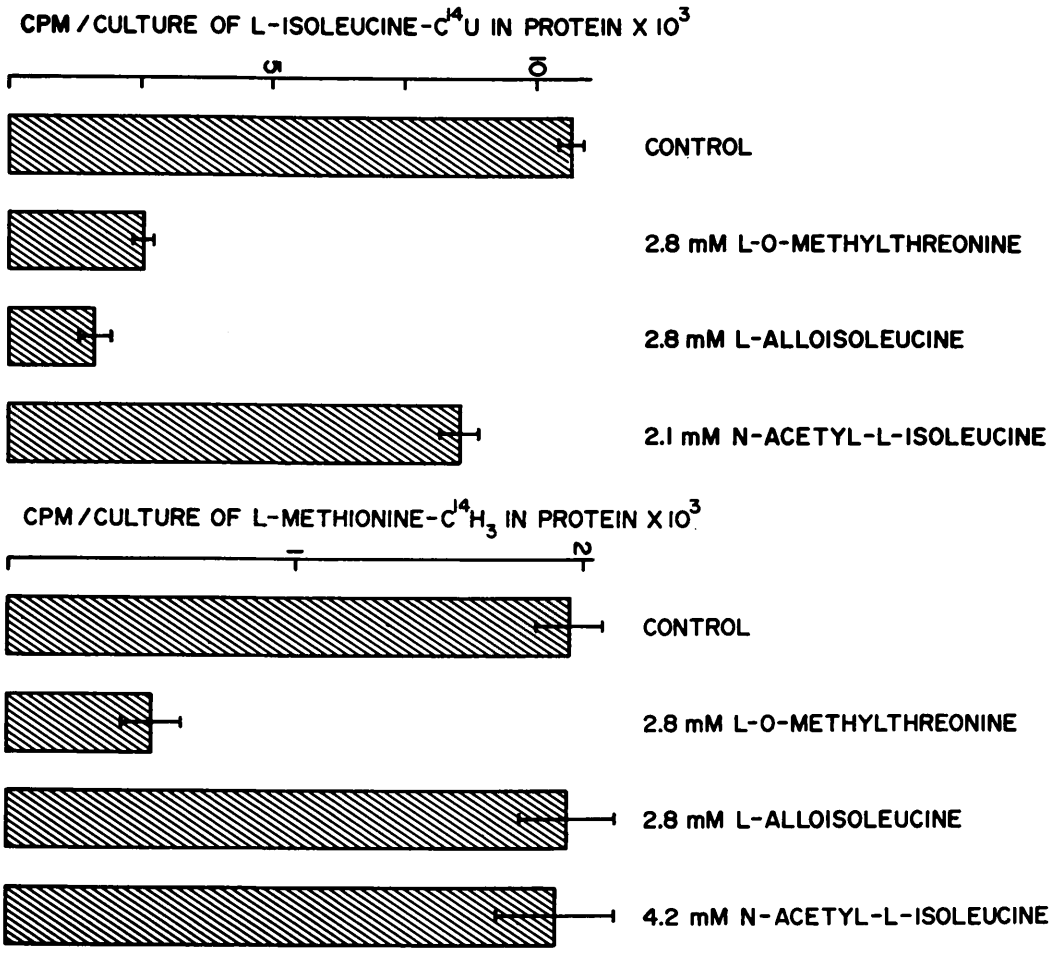

FIG. 3. Effect of L-isoleucine analogs on L-isoleucine-U^{14}C (upper) L-methionine-$^{14}CH_3$ (lower) incorporation into protein of *P. knowlesi*. Plasmodia were incubated for 16 hr in Hanks salt solution containing 100 mg/100 ml of glucose, 0.05 mmole of both L-isoleucine and L-methionine and 10% dialyzed human serum. To one half of the cultures 0.5 μCi of L-isoleucine-^{14}C (sp act. 261.35 mCi/mmole and to the other half 1 μCi of L-methionine-$^{14}CH_3$ (sp act. 14.9 mCi/mmole) were added at time 0. The isoleucine analogs were added at time 0 in the concentrations cited; none was added to the control cultures. Protein was determined as described in methods. Each value represents an average of 3 cultures with SD.

shown that erythrocytic forms of malarial parasites phagocytize red blood cell fragments (5–7) and digest hemoglobin (8, 9), suggesting that erythrocytic forms of malaria plasmodia use hemoglobin as a source of amino acids for protein synthesis. It was also shown that parasitized erythrocytes concentrate amino acids above the levels present in normal blood cells (10, 11). The present results, that *P. knowlesi* incorporated into protein 16 of the 18 amino acids added to the media, indicates that plasmodia use the growth medium as a second source of amino acids for protein synthesis. In addition our results showed that *P. knowlesi* does not use significant amounts of extracellular L-aspartic acid and L-glutamic acid; the plasmodium may obtain both amino acids either from digested hemoglobin or may derive them biosynthetically from carbohydrates. The latter possibility which would constitute a third possible source of amino acids for plasmodial

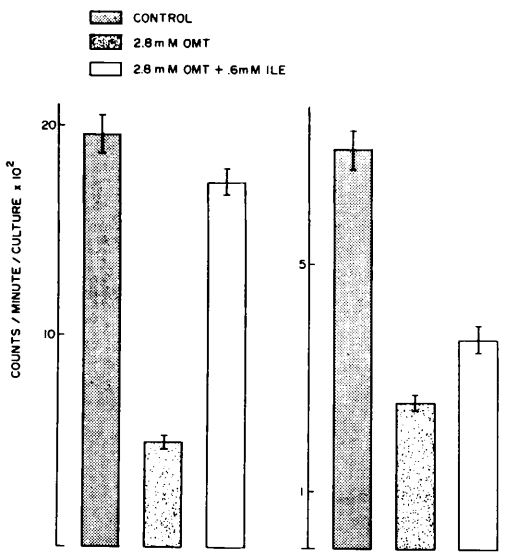

FIG. 4. Effect of L-isoleucine (ILE) on L-OMT inhibition of orotic-6-^{14}C acid incorporation into DNA (left) and L-methionine-$^{14}CH_3$ incorporation into protein (right) of *P. knowlesi*. Experimental technique was similar to Fig. 3. To one half of the cultures 1 μCi of orotic-6-^{14}C acid (sp act. 30 mCi/mmole) and to the other half 1 μCi of L-methionine-$^{14}CH_3$ (sp act. 14.9 mCi/mmole) were added per culture. The DNA and protein were measured as described in methods and previously (1). Each value represents an average of 4 cultures with SD.

protein synthesis, has not yet been investigated.

The observation that L-isoleucine is essential for *in vitro* growth of *P. knowlesi* and that this amino acid is more avidly incorporated into plasmodial protein from growth medium than the 17 other amino acids examined, suggested that isoleucine antagonists might be potent antimalarial drugs.

Previous investigators have shown that DL-OMT or L-OMT inhibits the growth of plants (12) and bacteria (2, 3), and that it decreases protein synthesis in mammalian cells (13, 14). The present results (Fig. 2) showed that L-OMT inhibits *in vitro* growth of erythrocytic forms of *P. knowlesi* and that this effect can only be reversed by L-isoleucine. Smulson *et al.* (3) showed that L-OMT inhibition of growth of *E. coli* could be reversed by L-valine, L-leucine, L-isoleucine, L-threonine, and L-vallylleucine, while Neale and Tristram (2) reported reversal of DL-OMT induced growth inhibition of *E. coli* C_4 by methionine or threonine and not by isoleucine. Both L-isoleucine and L-leucine reversed L-OMT induced growth inhibition of *Euglena gracilis* (12).

The mechanism of growth inhibition by the isoleucine antagonists has not been precisely determined. That the number of amino acids reversing the growth inhibiting effect of L-OMT is variable, could be explained on the basis that L-OMT shares with a variable number of amino acids a common site of entry into the cell and that consequently, L-OMT competes at this site with these amino acids for entry (3). Why L-isoleucine incompletely reverses L-OMT inhibition of orotic-6-^{14}C acid incorporation into DNA while reversal of L-methionine-$^{14}CH_3$ incorporation into protein is complete is not clear (Fig. 4).

The observation that L-alloisoleucine and *N*-acetyl-L-alloisoleucine inhibit L-methionine-$^{14}CH_3$ incorporation less than L-isoleucine-U-^{14}C incorporation into protein (Fig. 2) suggests that these two analogs compete with L-isoleucine for incorporation into plasmodial protein. The finding that L-OMT inhibits L-isoleucine-U-^{14}C and L-methionine-$^{14}CH_3$ incorporation into protein to a similar degree, suggests that L-OMT does not compete with L-isoleucine for incorporation into protein. Also in mammalian cells L-OMT appears not to be incorporated into protein, because it has been shown that although L-OMT is activated by isoleucyl-RNA synthetase from mammalian cells, the enzyme is incapable of effective transfer of the analog to isoleucine transfer RNA (15). In bacteria, however, it was shown that L-OMT specifically competes with isoleucine for incorporation into protein (3), while DL-OMT was not incorporated into *E. coli* protein (2).

Summary. On the basis that L-isoleucine is essential for *in vitro* growth of erythrocytic forms of *P. knowlesi* and that the same amino acid is more avidly incorporated into plasmodial protein than 17 other amino acids; three antagonists of L-isoleucine: L-*O*-methylthreonine (L-OMT), alloisoleucine and *N*-

acetylisoleucine were examined for their *in vitro* effect on DNA and protein synthesis of erythrocytic forms of *P. knowlesi*. L-OMT inhibited markedly and L-alloisoleucine slightly the incorporation of orotic-6-^{14}C acid into DNA. The inhibitory effect of L-OMT was reversed by L-isoleucine. L-Alloisoleucine and *N*-acetyl-L-isoleucine did, while L-OMT probably did not compete with isoleucine for incorporation into plasmodial protein. The L-isoleucine antagonists are proposed as a possible new class of antimalarial drugs.

We wish to thank Dr. M. Rabinovitz, National Cancer Institute, National Institutes of Health, for providing the L-*O*-methylthreonine; and Dr. D. Jacobus, Walter Reed Army Institute of Research for providing N-acetylisoleucine used in this study.

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Received Sept. 4, 1968. P.S.E.B.M., 1969, Vol. 130.

Detection of Virus-Like Particles in Germinal Centers of Normal Guinea Pigs* (33613)

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In a previous electron microscopy (EM) study (1) we reported that C-type virus particles were localized throughout the preleukemic stage in the germinal centers of the spleen and lymph nodes of C58 mice. Similar studies of AKR mice (2), a strain which also has a high natural incidence of leukemia, revealed that C-type virus particles also appeared to be focally accumulated in the germinal centers of the spleen, mesenteric lymph nodes, and Peyer's patches of mice 1–6 months of age. Recently the occurrence of virus-like particles has been reported (3) in splenic germinal centers of BC3F mice, a strain with a low natural incidence of leukemia. There is substantial evidence (4) to support the concept that specialized reticular cells in germinal centers possessing elaborate dendritic processes (5–7) play an important role in the trapping of antigens. As an extension of our preliminary findings (1) we

* Supported by grants from Elsa U. Pardee Foundation and the Special Virus Leukemia Program of the National Cancer Institute, Contract PH43-65-639.

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