was also found that the original ECHO-13 prototype strain was a mixture which contained predominantly ECHO-1 virus in late passages(10).

Although no grouping of the ECHO viruses has been designated by the Enterovirus Committee, ECHO-12 virus, because of its ability to produce plaques in both patas and rhesus MKCC, falls into the proposed group B of Hsiung and Melnick which contains types 7, 8 and 12(8). The ability of ECHO-12 virus to hemagglutinate human type O erythrocytes at 4, 25 and 37°C but not chick erythrocytes is also typical of type 7 ECHO virus but not type 8; the latter virus apparently does not hemagglutinate(9).

ECHO-12 virus was not found capable of producing observable illness in suckling mice, adult guinea pigs, rabbits, monkeys or embryonated eggs.

The only association of this virus with human disease to date is limited to serological rises in 2 patients with the aseptic meningitis syndrome(1,4).

Summary. 1) ECHO-12 prototype virus, 2-85-4 Travis, was isolated from rectal swab of a normal American child in the Philippines, collected in 1953. A rise in NA was demonstrated in serial serum specimens. This virus was antigenically distinct from all other currently recognized enteroviruses which are cytopathogenic for rhesus MKCC, as determined by reciprocal NA tests with all these viruses and their respective monkey or rabbit antisera. 2) Specificity and biologic properties of ECHO-12 virus in certain laboratory animals, chick embryos, and cell cultures are described. The ability of ECHO-12 virus to form plaques in both rhesus and patas MKCC and to hemagglutinate human type O erythrocytes at 4, 25 and 37°C further distinguishes this virus from the first 19 of the 24 recognized ECHO types. Prototype ECHO-12 infected rhesus MKCC fluids can be used in CF, NA and HAI tests. 3) This virus has now been repeatedly isolated from individuals in the Philippines, from United States and Mexico. Antibodies are present in Japanese gamma globulin. One isolation of virus was made from a throat swab.

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Action of Certain Tetrapyrrole Derivatives in Experimental *Trypanosoma congolense* Infections. (24765)

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Since the work of Pfeiffer(1) on *Hemophilus influenzae*, and of Novy and MacNeal (2) on *Trypanosoma brucei*, the nature of factors occurring in blood necessary for growth of certain microorganisms has been progressively elucidated Studies on the influenza organism by Granick and Gilder(3) and on non-pathogenic members of the Trypanosomatidae by Lwoff(4,5) have revealed certain differences between porphyrin re-

quirements of those bacteria and protozoa which cannot synthesize this type of compound. The literature in this field has been reviewed(6-7). All investigations on the function of porphyrins as growth factors have been conducted *in vitro*. Although some of above authors have mentioned the possibility that competitive inhibition of these substances might serve as an approach to chemotherapy we have not found any reference to attempts to influence the course of experimental infection in animals by antagonism of these metabolites. This article reports on effects produced by certain tetrapyrroles and their relatives in experimental infections with *Trypanosoma congolense* in mice, and our attempts to demonstrate their activity in other parasitic infections.

Materials and methods. Compounds. The tetrapyrrole derivatives tested, their sources, and lot numbers (if known) are listed in Table I. Phthalocyanin and a number of simpler pyrrole derivatives synthesized in our chemical laboratories, both in Basel and Summit, were also tested. None of the latter group was active. The general nature of these compounds will be discussed below. *Test Organisms.* The "Wellcome FN" strain of *Trypanosoma congolense* Broden, 1904, has been used recently because it produces, in mice, a subacute infection, less fulminating than that produced by parasites of the *brucei-evansi* group, but nevertheless fatal. Since deaths do not take place as rapidly as in infections with the latter group, more prolonged medication is possible and more subtle effects on course of disease, such as those produced by anti-organ sera(8), or reticulo-endothelial blocking agents(9), which do not alter the course of infection with *T. equiperdum* or *T. gambiense,* can be observed. This strain has been used, therefore, for preliminary screening of certain types of compounds because of its sensitivity, not as representative of trypanosomes in general. Compounds active against above strain were also tested in mice against other parasites: *Trypanosoma gambiense* ("Wellcome TS" strain), *Trypanosoma equiperdum* ("Pearce-Brown" strain), *Trypanosoma cruzi* ("Brazil" strain), *Plasmodium berghei* ("KBG^{173}" strain), pinworms (*Syphacia obvelata* and *Aspiculuris tetraptera*), and *Nematospiroides dubius. Trypanosoma congolense* ("Lumu" strain) was tested in rats, *Leishmania donovani* ("Khartoum" strain) in hamsters, and *in vitro* tests were made with *Entamoeba histolytica* ("F22T" strain). The compounds were dissolved or suspended in water whenever possible. Certain of those not readily soluble were put into solution with a small amount of potassium hydroxide and diluted to desired strength with horse serum, to maintain compounds in solution, and having no effect on parasitic infections as proved by controlled experiments. In preliminary screening against *T. congolense* the test compounds were injected intravenously into DBA mice, at maximum tolerated doses, a single dose each of 4 consecutive days. On day after first medication, the animals were infected by intraperitoneal inoculation with 500,000 parasites/mouse. Usually 5 animals were used for each compound. In all tests all controls were dead by 9th day after infection. In about half the tests all controls were dead by 6th day. A record was kept of mortality up to 60 days. Animals surviving at that time were considered cured. They harboured no parasites and their bloods were not infective on subinoculation. After preliminary screening revealed activity in certain compounds, dose levels and schedules were varied to determine their influence on results. In experiments, in which 2 compounds were used, the medication period was limited to 3 days because when 2 injections of these highly colored compounds were given each day (total of 6 injections) it became progressively difficult to locate and inject the tail veins. In *in vivo* tests with other parasites, listed above, the compounds were given at dose levels equivalent to or greater than those which produced effects in *T. congolense* infections with "Wellcome FN" strain.

Results. Chlorophyllin and related compounds. Experimental infections with *T. congolense* in mice could be cured by intravenous injections of sodium-potassium chlorophyllin. The results of tests involving various dose levels and schedules, are given in Table I. Some cure were effected even when the compound was given as late as 2 days after infec-

TABLE I. List of Tetrapyrrole Derivatives Used, Their Sources and Lot Numbers.

Compound	Source*	Lot No.†
Bilirubin	NBC	8339
Biliverdin	Mann	4099
Chlorophyllin, sodium potassium	NBC	9473
Chlorophyllin, magnesium water soluble (W-4)	Keystone	K-1140
Chlorophyllin, sodium potassium copper	"	
Chlorophyllin, sodium copper (100%)	Allen	D-360
Chlorophyll, crude, water soluble	National	
Chlorophyll, oil soluble	Delta	
Deuteroporphyrin	"	
Hematoporphyrin	Mann	1483
	NBC	8359
	"	3151
Hemin	"	8278
	"	2820
Mesoporphyrin	Delta	
Phaeophorbide a	K. & K.	11850 S
" b	"	11851 S
Phylloporphyrin	"	11852 S
Protoporphyrin	H. & M.	117.12.29
		117.19.40
Pyrroporphyrin	K. & K.	11854 S
Rhodoporphyrin	"	11853 S
Urobilin	Delta	
Urobilingen	"	
Uroporphyrin, dimethyl ester	K. & K.	11720 S

* Commercial sources as follows: Allen Chlorophyll Co., Ltd., London; Delta Chemical Works, N. Y.; H. & M. Chemical Co., Santa Monica, Cal.; K. & K. Labs., N. Y.; Keystone Chemurgic Corp., Bethlehem, Pa.; Mann Research Labs., N. Y.; National Chlorophyll & Chemical Co., Lamar, Col.; Nutritional Biochemicals Corp., Cleveland, O.

† Blanks in this column indicate that lot numbers were not designated for products so marked.

tion. Chlorophyllins in which magnesium had been replaced by copper, were also active against *T. congolense* at maximum tolerated doses but chlorophyll itself, in which the phytol group was still intact, was ineffective. The phaeophorbides, a and b, were inactive. When tested against *Trypanosoma cruzi, T. equiperdum, T. gambiense,* and *L. donovani,* sodium-potassium chlorophyllin did not show activity. It was likewise ineffective against mouse malaria and helminths studied and did not affect amebae *in vitro,* except at low dilutions (1:100).

Porphyrins and bile pigments. Among porphyrins tested only one, hematoporphyrin, had significant activity. In Table III the results of some tests are shown. It is apparent that hematoporphyrin acted similar to chlorophyllin, producing some cures with as little as 400 mg/kg. Comparable or greater amounts of deuteroporphyrin, mesoporphyrin, phylloporphyrin, protoporphyrin, rhodoporphyrin, and uroporphyrin were ineffective. Although no special precautions for avoiding sunlight were taken, no signs of light toxicity were noted. Certain differences in toxicity and antitrypanosomal effect were noted between different lots of hematoporphyrin.* Hematoporphyrin was also tested against *T. cruzi* infection in mice; no activity could be detected. Of bile pigments, biliverdin and bilirubin affected *T. congolense* infections very slightly but urobilin and urobilinogen were completely ineffective at equivalent doses.

Other compounds. A very slight delay during *T. congolense* infections was observed when phthalocyanin was used, but it is considered possible that this effect is related to stimulation of the reticuloendothelial system which sometimes results when macromolecular substances are injected intravenously(9). In addition to this substance which simulates the porphin nucleus, 4 porphyrin-like condensation products of pyrrole with other organic radicals were completely inactive. Further, to explore the possibility that there might be activity inherent in the pyrrole nucleus, 33 other compounds were tested, 7 of which were pyrroles (2 of them polymers), 5 pyrrolines and pyrrolenines, 16 pyrrolidines (2 with 2 pyrrolidine groups) and 5 pyrrolidones. None of these had significant activity.

Reversal experiments. In a number of experiments to show that the activity of chlorophyllin and hematoporphyrin lies in their ability to interfere with porphyrin metabolism by substituting for hemin or protoporphyrin in synthesis of hemoprotein enzymes, hemin could completely reverse the effects of chlorophyllin at certain dose levels.

* Some authors(10) use the term "hematoporphyrin" in quotation marks and point out that all "hematoporphyrins" they tested consisted of a large number of very closely related porphyrins. This heterogeneity may account for the differences we observed between lots.

TABLE II. Survival of DBA Mice Infected with *Trypanosoma congolense* and Given Sodium Potassium Chlorophyllin Intravenously.

Total dose (mg/kg)	Dose levels (mg/kg) and No. of administrations	Day on which medication began	% survivors: On day last control died	% survivors: At 30 days
1000	250 × 4	1 day before infection	100	100
"	"	1 after	"	"
800	200 × 4	1 before	"	80
"	"	1 "	"	20
750	250 × 3	1 after	"	100
600	150 × 4	1 before	"	40
"	"	1 "	"	20
"	200 × 3	1 after	"	60
"	"	2 "	"	40
"	"	3 "	"	0
500	250 × 2	1 "	"	40
"	125 × 4	1 before	60	20
450	150 × 3	1 after	80	0
400	100 × 4	1 before	100	0
"	"	1 "	60	20
300	100 × 3	1 after	80	40
250	250 × 1	1 "	100	0
200	200 × 1	1 before	60	0
"	"	Same day as	100	40
"	50 × 4	1 day before	40	20
150	50 × 3	1 after	80	40

In all tests reported here and in Table III there were 5 mice in a group and all controls were dead by 9th day after infection. In about half of the tests all controls were dead by 6th day.

The ratio of hemin to chlorophyllin which demonstrated reversal, was 2 to 1. The number of possible ratios which could be tested in mice was limited by the maximum tolerated dose of hemin on one hand and by minimum effective dose of chlorophyllin on the other. The latter dose, which could be depended upon to produce a demonstrable effect in all tests, was 75/mg/kg/day for 3 days, and this effect could be completely reversed by 150 mg/kg/day of hemin. A test in which these dosages were used is shown in Fig. 1.

Hemin was also able to counteract the antitrypanosomal effect of hematoporphyrin, but not completely, as it did with chlorophyllin. Protoporphyrin, however, was unable to reverse the effects of either hematoporphyrin or chlorophyllin. All these effects and reversals first studied in mice infected with "Wellcome FN" strain and medicated intravenously, were

TABLE III. Survival of DBA Mice Infected with *Trypanosoma congolense* and Given Hematoporphyrin Intravenously.

Total dose (mg/kg)	Dose levels (mg/kg) and No. of administrations	Day on which medication began	% survivors: On day last control died	% survivors: At 30 days
800	200 × 4	1 day before infection	100	100
"	"	*Idem*	"	80
600	150 × 4	"	"	"
"	200 × 3	"	"	0
"	"	Same day as infection	"	100
"	"	1 day after "	"	60
450	150 × 3	1 "	"	"
400	100 × 4	1 before	"	"
300	100 × 3	1 after	"	0
"	75 × 4	1 "	"	0
200	50 × 4	1 before	60	0

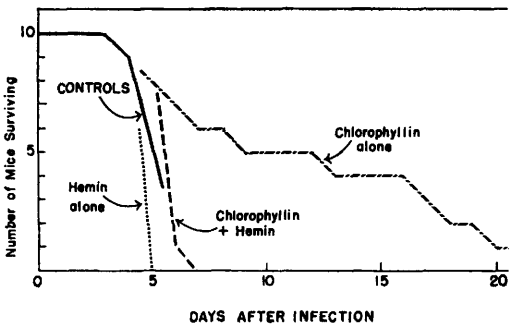

FIG. 1. Reversal of effects of 75 mg/kg of chlorophyllin by concurrent administration of 150 mg/kg of hemin in *T. congolense* infection in mice. Compounds were both given on 4 consecutive days, beginning on day before infection.

subsequently confirmed in rats, infected with "Lumu" strain and medicated intraperitoneally.

Discussion. Granick and Gilder(6) pointed out that chlorophyll, which occurs in chloroplasts which also contain heme enzymes, is prevented from competing with heme for specific heme apoproteins by having its propionic acid groups either esterified with phytol or cyclized, with the resulting free carboxyl group methylated. In chlorophyllin these conditions no longer exist; there is one ionizable propionic acid group and the carboxyl group is also free. It seems logical, therefore, to assume its ability to compete with heme for positions in the metal containing enzymes or vitamins.

Lwoff and Lwoff(11) concluded from respiratory studies that hematin was needed by *H. influenzae* for synthesis of cytochrome, cytochrome oxidase, catalase and peroxidases. Granick and Gilder(3) found that growth of the influenza organism was supported, however, not only by heme but also by protoporphyrin, into which the organism was able to insert iron. They also found that growth of this organism was supported by iron hematoporphyrin, iron deuteroporphyrin and iron mesoporphyrin, but that none of these porphyrins without the iron would support growth. Upon this work is based the conclusion that the vinyl groups of protoporphyrin are important to insertion of iron into the porphin nucleus by the organism.

In Trypanosomatidae, however, the picture is somewhat different. *Strigomonas fasciculata* requires for growth either iron protoporphyrin or protoporphyrin alone, into which we can assume it is able to insert iron. But this organism cannot substitute deuteroporphyrin, mesoporphyrin or hematoporphyrin or their corresponding iron containing compounds for the protoporphyrin requirement (4). *T. congolense* appears to have an even more specific requirement. Ability of hemin to reverse the action of chlorophyllin and hematoporphyrin in infections with this organism and the inability of protoporphyrin to reverse the effect suggest that this parasite has a requirement that can only be satisfied by an iron porphyrin.

It is a matter for some speculation as to why *T. congolense* is the only one of the pathogenic trypanosomes tested which can be affected by this competitive inhibition of an essential factor. According to Agosin and von Brand(12) *T. congolense* occupies an intermediate position, in respect to its physiological characteristics, between *T. cruzi* on the one hand and the *brucei-evansi* group on the other hand. Members of the *brucei-evansi* group are sensitive to arsenicals and to iodoacetate and this is taken as evidence of the importance of sulfhydryl enzymes of the glycolytic chain. *T. cruzi* on the other hand is insensitive to sulfhydryl inhibitors, and *T. congolense* is partially sensitive to these agents. Members of the *brucei-evansi* group are insensitive to cyanide, however, while *T. cruzi* is cyanide sensitive. Again, *T. congolense* occupies an intermediate position, being partially sensitive to cyanide. Sensitivity to cyanide is taken as evidence of the presence of an enzyme system catalyzed by heavy metals and it is presumed this is a cytochrome system, although there is evidence that the oxidase of *T. cruzi* differs somewhat from the conventional cytochrome C oxidase(13).

Rate of oxygen consumption of *T. congolense* is nearly as high as that of organisms of the *brucei-evansi* group; also high is the respiratory quotient which with the parasites of the *brucei-evansi* group is very low. It appears that in maintenance of its high oxygen consumption and more complete glucose degradation *T. congolense* is dependent on both

dehydrogenase and oxidase systems and it seems possible that interference with the latter by means of porphyrin antagonism is more critical for *T. congolense* because of the demands of its metabolism.

It might be conjectured that the effects on an oxidase system should be readily detectable, if not more profound, in infections with *T. cruzi*, the blood form of which is more sensitive to cyanide than *T. congolense*. Our experiments did not show this to be the case, however, and it will be recalled that not only is *T. cruzi*, in the blood stream, a consumer of less oxygen and much less glucose than *T. congolense* but also, in its intracellular phase, concerning the physiology of which little is known, it is protected from attempts to change its environment.

Summary. A number of tetrapyrrole derivatives of both plant and animal origin were tested for ability to influence the course of *Trypanosoma congolense* infections in mice. Of these, chlorophyllins, containing either magnesium or copper, and hematoporphyrin were effective in delaying deaths from trypanosomiasis and with prolonged administration cures could be effected. The non-metallic phaeophorbide relatives of chlorophyllin were ineffective as were deuteroporphyrin, mesoporphyrin, phylloporphyrin, pyrroporphyrin, rhodoporphyrin and uroporphyrin, as well as certain bile pigments. The antitrypanosomal effect of chlorophyllin and hematoporphyrin could be reversed, either completely or partially, by hemin, but not by protoporphyrin. The hypothesis that the mechanism of action of these tetrapyrrole derivatives in suppressing trypanosome infection involves competitive inhibition of a growth factor essential for the parasite (apparently hemin, rather than protoporphyrin) seems to be supported by our experiments. Other species of hemoflagellates and certain other parasites were uninfluenced by compounds active in *T. congolense* infections. It is conjectured that sensitivity of this particular parasite may be attributable to a certain combination of physiological attributes which is not possessed by its relatives.

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Induction and Maintenance of Mammary Growth and Lactation in Rats with Acetylcholine or Epinephrine.*† (24766)

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Mammary growth and lactation can proceed independently of connections to the nervous system, as suggested by experiments involving mammary transplantation or ligation of nerves to the mammary glands(1). The nervous system can influence mammary function, however, as indicated by observations

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