ameboid astrocytes are evident. Perivascular inflammatory reaction is not a constant finding in this disease, but when it occurs it is probably a secondary reaction in response to disintegration of myelin(8).

Summary. Fluorescent antibody studies show that the virus of canine distemper is localized in astrocytic and possibly other glial nuclei. The astrocytes exhibit degenerative changes consistent with viral invasion. Astrocytic involvement appears to be followed by demyelination although this causative relationship cannot be explained.

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Monolayer Cultures of Trypsinized Monkey Kidney Cells in Synthetic Medium. Application to Poliovirus Synthesis.* (22294)

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The outgrowth of monkey kidney cells from suspensions of trypsinized tissue has always been found to require substantial amounts of serum in the medium. The necessity for adding serum has precluded many kinds of studies and has restricted the kind of cultures available for study. A simple synthetic medium is described which, in the absence of any added protein, supports outgrowth of monkey kidney cell suspensions. After outgrowth in this medium, the cells support synthesis of poliovirus and may be used for physiological studies under defined conditions in the absence of serum.

Methods. Cell suspensions. Trypsinization of kidneys taken from rhesus monkeys was done by a modification(1) of the Youngner procedure(2). The cells were harvested from the trypsin fluid by centrifugation at 200 rpm for 30 minutes, resuspended in 500 x their volume of Basic Salt #2 without bicarbonate (as described in Table I), and recentrifuged. The washed cells, resuspended in 100-200 ml of synthetic medium, were filtered through 2 layers of cheese cloth. A count of those cells showing both nuclei and attached cytoplasm was made by staining with 0.1% crystal violet-0.1 M citric acid, and the suspensions were diluted to 350,000 cells per ml. Amounts of 0.5 ml were seeded into 16 x 150 mm test tubes, 7 ml into 2 oz and 12 ml into 4 oz prescription bottles, and 80-100 ml into Roux flasks. These amounts are also in the optimal range for cells suspended in the medium which contains serum (1). In experiments where outgrowth in the synthetic medium was to be compared to outgrowth in a serum-containing medium, the suspension in PBS was divided into 2 portions and the second half after recentrifuging was diluted into the serum-containing medium. The serum-containing, "complete" medium (called "M") used for comparison was the 0.5% lactalbumin hydrolysate-2% calf serum medium in Hanks' salt solution which has been used extensively in this laboratory(3).

Virus titrations. These were made by the

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TABLE I. Synthetic Medium (SM-1) for Outgrowth of Monkey Kidney Cell Suspensions.

Basic salt sol. (B.S. #2)*		Trace elements†	
	g/l		M × 10^{-6}/l
NaCl	8.0	$ZnSO_4$	3
KCl	.4	$Fe(NO_3)_3$	2
$MgSO_4$ 7 H_2O	.1	$CoCl_2$	.2
$MgCl$ 6 H_2O	.1	$MnCl_2$	.5
Na_2HPO_4	.08	$CuSO_4$	.4
NaH_2PO_4	.02		
$CaCl_2$	.42		
$NaHCO_3$	.78		
Glucose	2.0		

Organic supplements‡		Supplementary buffer§
	mg/l	
l-cysteine • HCl	30	Tris (hydroxymethyl
l-isoleucine	60	aminomethane)
d-ribose	30	8 × 10^{-3} M, pH 7.6

* Made in 10 × concentrated, combined stock without $CaCl_2$ and $NaHCO_3$. Sterilized by filtration. $CaCl_2$ is made at 100 × concentrated; $NaHCO_3$ is made at 100 × concentrated. Glucose in 20% stock solution, sterilized by filtration.

† Held as separate stocks, 0.05% concentrations. Sterilized by filtration.

‡ Stocks 100 × concentrated. Sterilized by filtration. May be held as combined stock 40 × concentrated in H_2O.

§ Conveniently stored as 2 M stock adjusted to pH 7.6 with HCl. May be sterilized by autoclaving at 15 lb for 15 min.

Dulbecco-Vogt plaque method on cultures grown out in the indicated medium in 4 oz prescription bottles(3). The culture fluid was decanted, and the inoculum of virus was added from the same pipette to a series of 12-20 bottles. After incubation for 1 hr at 37°C, bottles of each series were randomly selected and overlaid with calf serum agar overlay, "C.S.", or with amino acid supplement agar overlay "AAS", made as follows: *C.S.*: Agar (2.7 g/60 ml), 33%; calf serum, 2.5%; neutral red (1/1000 of 80% dye), 1.8%; Earle's salt solution 10 x concentrated, 10%; H_2O, 50%; $NaHCO_3$ (58 g/50 ml solution), 2.05%. *AAS*: (solutions as in C.S. overlay) Agar, 33%; basic salt #2 (Table I) 2 x concentrated, 50%; H_2O, 8%; neutral red, 1.8%; amino acid supplement 40 x concentrated, 2.5%; Na_2HPO_4 (5% solution), 0.28%; $NaHCO_3$, 3.8%. Solutions added in order given.

Experimental. The synthetic medium, SM-1, which has been used routinely for outgrowth of monkey kidney cell suspensions, is described in Table I. It consists of a salt solution containing a higher concentration of calcium than is widely used; trace elements; and the organic constituents, cysteine, isoleucine, and d-ribose. The buffering capacity of the medium is increased by the addition of 8 x 10^{-3} M Tris (hydroxymethyl aminomethane). It may be supplemented with penicillin and streptomycin if desirable. The medium is stable for at least one week at 4°C, and is not used once a precipitate forms on standing. The medium cannot be frozen.

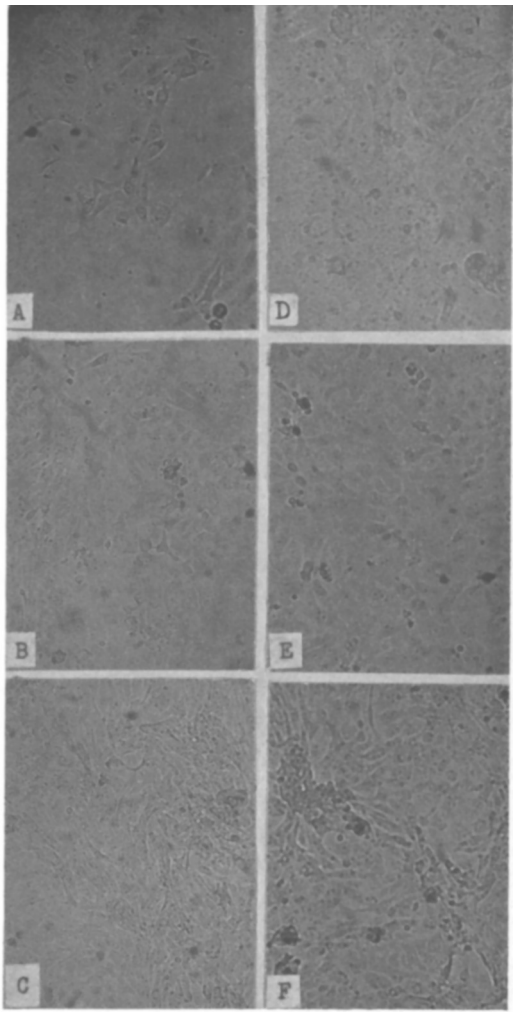

FIG. 1. Outgrowth of monkey kidney cells in SM-1 and comparison with parallel culture in lactalbumin hydrolysate-calf serum "M" medium. A-C = Outgrowth after 2½, 5, and 10 days in "M." D-F = Outgrowth after 2½, 5, and 10 days in SM-1.

The outgrowth of identical suspensions of monkey kidney cells seeded in SM-1 and in the complete medium, M, is shown in the accompanying photographs (Fig. 1). Cells seeded in SM-1 attach more slowly to the glass. The outgrowth in the first 48 hours may be inferior compared to that obtained in the serum-containing medium. By the third day, however, as indicated in the photographs, the outgrowth in SM-1 compares favorably in both amount and quality with the outgrowth in the complete medium. The two sets of cultures are comparable up to the 10th-12th day after seeding. The complete medium must be changed between the 5th and 7th days because of the decline in pH. It is not necessary to change the SM-1 medium before the 8th-10th day.

The choice of medium for replenishment is determined by the studies to be undertaken. Replenishment of cells grown in SM-1 with the M medium results in a further proliferation of the cells after a lag period of 2 days, and survival for a month or more. On the other hand, for studies uncomplicated by the addition of serum, replenishment with an amino acid mixture such as that given in Table II, in the same basic salt solution, gives a useful additional lifetime of 1-3 weeks.

The medium SM-1 was developed on the assumption that freshly isolated kidney tissue, possessing considerable endogenous reserves and biochemical capacities, would require very few organic supplements for outgrowth and survival if placed into a properly adjusted physico-chemical environment. It may be useful to summarize the steps in development. (i) Suspensions of washed monkey kidney cells in Hanks' salt solution were supplemented with single organic nitrogen sources over a range of concentrations and pH. It was found that cysteine was the only supplement in more than 50 tried which gave a significant response; cells suspended in Hanks' solution alone, or in Hanks' solution supplemented with most other nitrogen sources, disintegrated within 24 hours. (ii) The Hanks'-cysteine medium was supplemented with single nitrogen and carbon sources. Several unrelated nitrogen sources increased the extent and duration of outgrowth over that obtained with cysteine alone. On the other hand, d-ribose and several other carbon compounds improved the quality rather than the extent of outgrowth. (iii) The Hanks' cysteine medium, supplemented —rather arbitrarily—with isoleucine and d-ribose, was chosen to investigate the effects of salts and trace elements on outgrowth. The concentration of salts, particularly calcium, was found to be a critical factor for outgrowth in the absence of serum. Both ferric and cupric ions had pronounced beneficial effects. It was also found that when the pH of the medium fell below 7.2-7.3 during the first 4 days, outgrowth was inhibited; cells failed to attach to the glass and the cells already proliferating along the glass became thickened and granulated. Attachment to the glass was also retarded when the medium was more highly fortified with amino acids, phosphates, traces of certain carboxylic acids, or after prolonged metabolism. The morphological changes and lack of attachment have been found to be completely reversible. Cells held in suspensions at 37°C for several weeks, and then adjusted to pH 7.6-7.8 with bicarbonate or supplemented with calcium or zinc, were found to attach and grow into clear monolayers.

TABLE II. Amino Acid Supplement.

	mg/l
l-cysteine • HCl	30
l-isoleucine	40
l-histidine • HCl	10
l-arginine • HCl	7
l-lysine • HCl	8
l-methionine	6
l-threonine	14

Conveniently made from sterile stocks to give 40 × concentrated mixture in H_2O and diluted into salt solution with trace elements and glucose as given in Table I, with 0.9 g $NaHCO_3$ per liter. May be buffered with 8×10^{-3} M Tris at pH 7.6.

The cellular monolayers grown out in SM-1 are so firmly attached to the glass that it has not been possible to remove them by the use of trypsin or versene, or by scraping, without substantial losses. Accurate cell counts have not been obtained. As an estimation of the total number of cells recovered after outgrowth, we have relied on (i) morphological

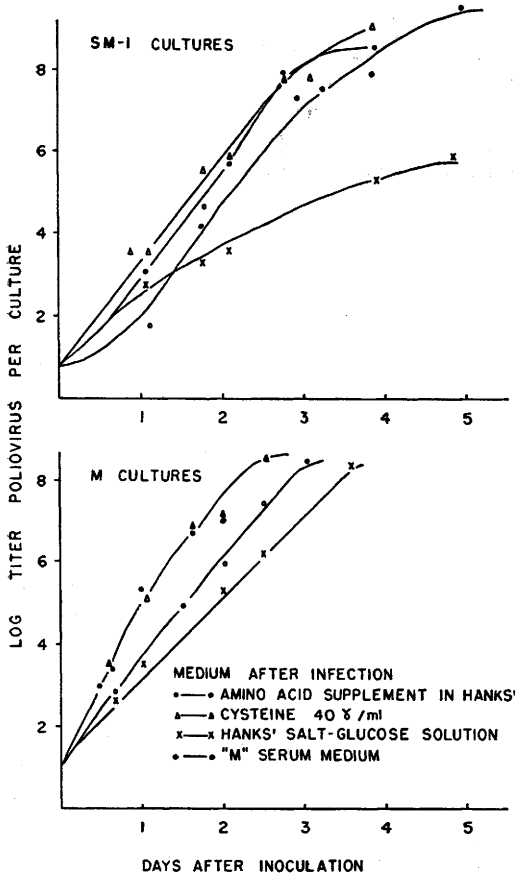

FIG. 2. Production of poliovirus in cultures grown out in SM-1 (top) and in M (bottom); and effect of medium after infection on rate of propagation. A series of 9-day-old cultures grown out in SM-1 and in M in 2 oz prescription bottles, from an inoculum of 1.7×10^6 cells/bottle, were infected with 10 plaque forming units of Type 1 poliovirus suspended in the amino acid supplement. Virus adsorbed 30 min. at 37°C. Cultures washed 3 times with PBS and in series of 6 bottles each replenished with 5 ml of medium indicated, supplemented with 8×10^{-3} M Tris pH 7.6. Arrow indicates time at which pH was readjusted to 7.4-7.6 with bicarbonate. Virus titered by plaque method, sampling from a different bottle at each point. Last point is pooled sample.

appearance of the culture and (ii) RNA and DNA recovered after a modified Schmidt-Tannhauser fractionation. The RNA and DNA recoveries indicate that after 7-10 days in SM-1, from 0.8-1.5 times the number of cells are recovered as were seeded. This compares with a recovery of 1.0-2.0 obtained in the complete medium changed at the 5th-7th day. The results obtained here for the complete medium agree with those obtained by Youngner(2) on the basis of cell count.

Virus synthesis. As a criterion of the physiological state of the cells after outgrowth in SM-1, the synthesis of poliovirus was studied. The propagation of poliovirus, Type 1, from an initial inoculum of an average of 10 plaque-forming units per culture, was followed in cultures grown out in SM-1 and in cultures of the same suspension grown out in the complete M medium. The propagation of virus throughout the two cultures, and details of the protocol, are shown in Fig. 2 and its legend. It is seen that the total yield of virus obtained from cells grown in the simple medium and inoculated and transferred to the amino acid supplement is the same as that from the control culture grown and infected in the presence of serum. The propagation of virus in the amino acid supplement is somewhat faster than in the M medium, and is more striking when the cells have been previously grown in the serum-containing medium. In this case, there may be as much as 1000 times more virus present in the cultures in the amino acid supplement at the end of 48 hours than in cultures with the complete medium. However, after further incubation of 2 or 3 days, the yield of virus from the M medium approaches that obtained in the amino acid supplement.

Since the amino acid supplement has a higher concentration of cysteine than the M medium, or other media usually used for virus cultivation, the effect of cysteine on virus propagation was studied. The data presented in Fig. 2 show that cysteine can completely replace the amino acid supplement and, even in the case of cells previously grown out in the simple medium, supports a more rapid propagation of virus than the M medium. Preliminary studies(4) indicate that the more rapid propagation of virus is due to a shorter virus growth cycle.

The amino acid supplement may be used advantageously as the nutrient in the agar overlay when assaying virus by the Dulbecco-Vogt plaque technic. The rate of appearance of plaques during assay of poliovirus Types 1, 2, and 3, on M cells overlaid with the

TABLE III. Effect of Amino Acid Supplement in Agar Overlay on Plaque Formation by Polioviruses on Monkey Kidney "M" Cells.

Exp. #*	Virus stock	Overlay medium	Plaques per bottle																				Titer (plaque forming units per ml of stock)
			Day 2							Day 3							Day 5						
1	Type 1, Brunhilde	C.S.†	1	3	2	7	3	2	5	6	3	0	18	9	10	11	7	8	6	5	19	13	14	12	13	12	12	10	10	7	62
		AAS†	6	6	7	9	15	12	10	5	5	13	10	9	13	14	21	14	13	10	11	18	12	11	17	16	21	14	13	11	11	18	70
2	Type 2, M.E.F.	C.S.	0	0	0	0	0	0					2	1	1	0	0	3			4	7	2	9	10	8							88
		AAS	7	11	3	5	5	4					8	15	4	11	16	5			10	15	20	14	20	17							220
3	*Idem*	C.S.	0	0	2	0	2	0	1	1	1	0	12	17	16	8	11	16	9	13	5	8	16	23	19	10	17	13	16	9	17		195
		AAS	11	14	13	20	17	14	19	8	12		28	24	17	30	29	30	27	20	23		29	28	20	34	31	32	31	22	29		390
4	"	C.S.	1	1	1	0	0	0	0	0	0	0	2	2	2	1	3	0	0	0	0	0	2	2	4	4	2	0	0	0	0	0	49
		AAS	2	3	2	2	3	3	1	3	2	1	2	6	2	5	5	3	5	3	3	1	23	10	6	10	9	7	9	4	4	6	310
5	Type 3, Saukett (Stock A)	C.S.	4	1	2	4	3	6	2	4	0	0	21	12	11	13	9	14	11	11	12	7	24	17	14	17	17	20	21	19	14	13	88
		AAS	11	16	16	16	13	15	3	11	8	4	23	26	23	22	21	20	13	19	20	13	28	29	26	27	23	27	16	22	21	20	119
6	*Idem*	C.S.	0	0	0	0	0	0	0	0	0	0	10	6	3	4	6	6	4	5	7	10	14	13	9	5	9	11	6	8	12	11	49
		AAS	12	2	3	12	4	0	6	5	5	4	22	13	6	8	14	10	7	9	13	4	23	18	11	13	20	16	13	12	19	8	78
7	Type 3, Saukett (Stock B)	C.S.											0	0	1	1	0	0					2	1	3	3	1						10
		AAS											2	1	5	6	1	6					6	9	11	10	5	7					30

* Exp. 1: .2 ml inoculum/bottle. Exp. 2, 3, 4: Repeat titrations with stock diluted 1.33×, 3×, and 7×, using .1, .2, and .2 ml inoculum/bottle, respectively. Exp. 5, 6: Repeat titrations with Stock A Saukett, .2 ml inoculum/bottle. Exp. 7: .2 ml inoculum/bottle, Stock B Saukett.

† C.S. = Calf serum in agar overlay. AAS = Amino acid supplement in agar overlay.

amino acid supplement in agar compared to the rate of appearance in the calf serum-agar overlay (5) is given by the data in Table III. Each assay was done on outgrowth cultures from kidney cells from different monkeys. The variation in final titer obtained for Types 2 and 3 indicates the variable susceptibility of the different cultures to these types. With all 3 poliovirus types, plaques appeared more rapidly in the amino acid supplement-agar overlay than in the calf serum-agar overlay. This acceleration was particularly striking with the slower growing Types 2 and 3. The final titer on Types 2 and 3 was also higher in every assay in the amino acid supplement than in the calf serum.

The susceptibility of SM-1 cells to poliovirus has been studied by the plaque technic. The data already presented in Table III indicate the rate and number of plaques expected with the different viruses on M cells with the amino acid supplement overlay. A comparative assay on SM-1 cells and M cells with this overlay would indicate any difference in the intrinsic cellular susceptibility to the virus. The SM-1 cells are sensitive to neutral red, and it is necessary to add the neutral red several days after the agar to develop the plaques. Types 1, 2, and 3 were assayed on a series of 10 bottles each of SM-1 cells and of M cells, grown in parallel from the same suspension of trypsinized kidneys. After decanting the growth fluid, the bottles were mixed so that virus inoculum was added from the same pipette in a random order to the series of 20 bottles. Neutral red was added 3 days after the agar overlay, and the plaques were read 4 hrs later. The data in Table IV show that the number of plaques developed on SM-1 cells was the same as the number developed on the M cells. This finding indicates that the cultures grown out in the simple medium have the same intrinsic susceptibility to poliovirus as the usual cultures grown out in serum. It also suggests that the difference in plaque count between M cultures in the calf serum-agar overlay and in the amino acid supplement-agar overlay is a reflection of the metabolism of the cells prevailing in these two media.

TABLE IV. Comparative Susceptibility of SM-1 and M Cultures to Poliovirus.

Virus type	Culture type	Overlay medium	No. of plaques/bottle after 3 days										Avg plaques per bottle
1	SM-1	AAS	12	13	10	11	18	14	11	16	13	27	14.5
Brunhilde	M	AAS	11	10	14	13	14	21	13	11	10	17	13.4
2	SM-1	AAS	9	9	5	11	8	13	8	14	6	7	9.0
M.E.F.	M	AAS	11	10	9	8	8	11	9	13	10	5	9.4
3	SM-1	AAS	10	6	8	4	4	1	8	7	5		5.3
Saukett	M	AAS	9	10	3	9	2	6	2	6	2	5	5.4

Discussion. The present studies indicate that suspensions of monkey kidney cells may be grown out in a simple synthetic medium in the absence of any added protein and compare favorably, in appearance and capacity to support poliovirus synthesis, with suspensions grown in the serum-containing M medium.

Under the conditions which have been studied, cysteine was found to be a specific requirement for successful outgrowth. The function of cysteine is not clear. It has not been possible so far to replace it with inorganic reducing agents, or with other organic sulfur compounds. In view of the effect of slight changes in the ionic balance of the medium during outgrowth, it is possible that these efforts were unsuccessful because the agents interfered with the necessary ionic conditions.

Outgrowth in the absence of serum was found to be sensitive to the concentration of the different salts, particularly calcium. A concentration of calcium as high as 4 times that occurring in normal serum has been incorporated into the medium. Although calcium may regulate many metabolic activities, it is considered that its beneficial effects during outgrowth may be due in part to protection of the cells during the physico-chemical adjustment to *in vitro* conditions. It may easily be observed that monkey kidney cells agglutinate at pH 4.5, suggesting a high concentration of carboxylic acid residues at their surface. If this is the case, at an alkaline pH, calcium may serve to stabilize the cells by shielding of the charged groups. It is also possible that calcium serves as a bridge through which the carboxylic acid groups are bound to the negatively charged glass. If this is a prime mechanism for the anchoring and spreading of cells along the glass surface, the pH, calcium concentration, and anionic components of the medium would be regulating factors for successful outgrowth.

Serum has heretofore been an absolute requirement for the outgrowth of monkey kidney cells. The fact that cells may be grown out and maintained for 2 to 3 weeks in the absence of serum suggests that its role is not predominantly nutritive during this period. It is possible that serum is largely protective, serving as an effective buffer to maintain the particular environment necessary for outgrowth. Biophysical studies have indicated that the elasticity of the mammalian cell membrane is probably due to tightly bound serum protein(6). It should be possible to simulate to some extent the stability afforded by bound protein by the adjustment of the physico-chemical environment. Consideration of some of the physical factors involved in stability and reversible attachment may enable one to design the medium of choice for the particular studies in tissue culture to be undertaken.

One immediate application of these findings would seem to be the production of vaccine from high titer virus obtained from monkey kidney cells grown in a medium free of added animal proteins. The binding of serum protein by animal cells means that after outgrowth in serum, even after washing, there is considerable contamination with serum protein. Although there has been little evidence of protein sensitization after inoculation of vaccines grown in monkey kidney cultures, the problem has raised a considerable controversy, particularly as to the effect of repeated injections over the years.

Summary. A synthetic medium containing only cysteine, iso-leucine, d-ribose in a salt-glucose medium, buffered with Tris (hydroxymethyl aminomethane) supports the outgrowth of monkey kidney cells. The outgrowth is comparable in both rate and amount with the lactalbumin hydrolysate-calf serum (M) medium and supports the synthesis of poliovirus. Propagation of poliovirus throughout a culture in either liquid or agar medium occurs more rapidly in the presence of a simple amino acid supplement than in the serum-containing medium. Cysteine can completely replace the amino acid supplement for propagation of Type 1 in liquid cultures.

The technical assistance of Miss Selva Reissig for the virus assays is gratefully acknowledged.

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Effects of 2-Ethylamino-1,3,4 Thiadiazole HCl on Uric Acid Production in Man.* (22295)

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(Introduced by Joseph H. Burchenal.)

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A compound having the formula, 2-ethylamino-1, 3, 4 thiadiazole HCl, was found by Oleson, *et al.*(1) to have antitumor activity in various laboratory animals. Further testing by Clarke(2) confirmed the activity against sarcoma 180 and Burchenal and Dagg (3) demonstrated a definite prolongation of the survival time of mice with transplanted leukemias 82 and 8174. Because of its antitumor effect in animals, Farber(4) initiated therapeutic trials in humans. He has supplied us with preliminary clinical dosage data.

Materials and methods. Clinical evaluation by our group was started in patients with far-advanced cancer. This report deals with the observations made in the 8 adult patients treated to date. The thiadiazole was given once daily by mouth, in doses of 1-4 mg/kilo/day. In most cases therapy was continued until the development of oral toxicity. Frequent physical examinations, appropriate roentgenograms, and hematological and biochemical determinations were performed before, during and after treatment. In 5 of the 8 patients herein reported daily serum and urine uric acid determinations were performed. The method employed for the determination of uric acid was that of Archibald as described in detail by Forsham, *et al.*(5). For the urine determinations this was modified as follows: Duplicate specimens were set up. The first specimen was treated in the usual manner. To the second specimen, after dilution 0.01 ml Worthington liquid uricase† was added to 5 ml diluted urine in a Coleman, Jr. cuvette and the specimen was incubated at 37°C for 90 minutes. After incubation the procedure was completed as with the first specimen. The color remaining in the second specimen represented non-uric acid chromogens and was subtracted from the total. All

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† Worthington Biochemical Co., Freehold, N. J.