# THE MODE OF ACTION OF A NOVEL 18-MEMBERED MACROLIDE, VIRUSTOMYCIN A (AM-2604 A), ON *TRICHOMONAS FOETUS*

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The mode of action of virustomycin A, a novel 18-membered macrolide, on *Trichomonas foetus* was investigated. The antibiotic inhibited the biosynthesis of RNA, DNA and protein in the organism. The inhibition of RNA biosynthesis was the most severe. Virustomycin A repressed the incorporation of [³H]uridine into both acid-soluble and insoluble fractions, whereas actinomycin D inhibited the incorporation of [³H]uridine into acid-insoluble fraction alone. Furthermore, it was found that virustomycin A interfered with nucleotide formation from uridine and adenosine but not with their transport to the cells. On the other hand, the antibiotic did not inhibit the activities of uridine kinase and uracil phosphoribosyltransferase in a cell-free extract from the organism. These data suggest that the antibiotic interferes with the formation of phosphate donor(s) (possibly ATP-forming system) of the organism.

Virustomycin A (AM-2604 A) is a new antibiotic isolated from the culture broth of *Streptomyces* sp. AM-2604 by the authors[1]. It is active against trichomonads and various RNA and DNA viruses, and weakly active against some fungi. Our recent work on the structure elucidation revealed that the antibiotic is a novel 18-membered macrolide constructed from flavensomycinoic acid and the aglycone of concanamycin A as shown in Fig. 1[2]. The insecticidal and fungicidal antibiotics flavensomycin and prasinons A and B are known to possess the flavensomycinoic acid moiety. INOUE and GOTTLIEB[3] reported that flavensomycin interfered with electron transfer in *Penicillium oxalicum*. KINASHI et al.[4] discovered concanamycins A, B and C as inhibitors of the proliferation of mouse splenic lymphocytes stimulated by concanavalin A. However, they have not reported the mode of action against trichomonads.
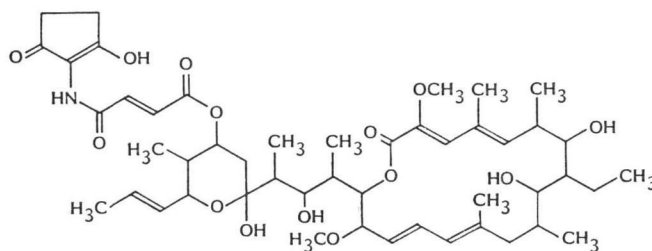
The present paper describes the mode of action of virustomycin A on *Trichomonas foetus*.

## Materials and Methods

### Organism and Media

A strain of *Trichomonas foetus* provided from Merck Sharp & Dohme Res. Labs., U. S. A. was

Fig. 1. Structure of virustomycin A.

maintained at 37°C in DIAMOND's medium[5] supplemented with 10% heat-inactivated calf serum, 100 U/ml of benzylpenicillin and 1.0 mg/ml of streptomycin sulfate, using 48-hour transfers.    A 24-hour culture grown in DIAMOND's medium (without agar) supplemented with 10% heat-inactivated calf serum was used in the experiments.    The number of cells was counted with a hematocytometer.

### Antitrichomonal Activity

Minimum inhibitory concentration (MIC) and minimum "cidal" concentration (MCC) against the organism were determined in DIAMOND's medium (without agar) according to the method described by ASAMI et al.[6]    The MIC was read as the least concentration of drug showing growth below twofold number of inoculated cells (inoculum size, $1 \times 10^4$ cells/ml).    After the MIC experiment, an aliquot of the drug-treated culture was transferred into a fresh medium without drug, and incubated at 37°C for 42 hours.    The MCC was read as the least concentration of drug showing no growth in the fresh medium.

### Incorporation of Radioactive Precursors into Acid-soluble and Insoluble Fractions in *T. foetus*

A radioactive precursor was added to a growing culture ($3 \sim 3.4 \times 10^6$ cells/ml) of *T. foetus* in DIAMOND's medium (without agar) and the culture was incubated at 37°C for given period.    The cells were harvested by centrifugation, and washed three times in ice-cold saline at 0°C.    Then, the cells were resuspended in ice-cold saline and 1 volume of ice-cold 0.6 N perchloric acid (PCA) was added to the suspension.    The resulted precipitate (acid-insoluble fraction) was collected on a Toyo membrane filter (type TM-2) and then washed with ice-cold 0.3 N PCA.    The filter was dried and the radioactivity was counted by a liquid scintillation spectrometer using a toluene scintillation system (5 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-[2-methylstyryl]benzene in 1 liter of toluene).    An aliquot of the filtrate (acidsoluble fraction) was mixed with ACS II Aqueous Counting Scintillant (Amersham Co.) and the radioactivity was measured with a liquid scintillation spectrometer.

### Analysis of Nucleotides in an Acid-soluble Fraction

UMP, UDP, UTP, uridine and uracil contained in an acid-soluble fraction were analyzed by paper chromatography using Toyo No. 51A filter paper and *iso*-butyric acid - $CH_3COOH$ - 1 N $NH_4OH$ (10: 1: 5)[7] as a developing solvent.    After the development, UV-absorbing spots on the paper were cut off to analyze for radioactivity.    Rf values are: UTP 0.14, UDP-glucose 0.16, UDP 0.22, UMP 0.32, uridine 0.54, uracil 0.66.

### Preparation of a Cell-free Extract[8]

Cells were harvested by centrifugation in the late logarithmic phase of growth and washed three times with 1/15 M phosphate buffered saline (pH 7.6).    Then, the cells were resuspended in 50 mM Tris-HCl (pH 7.6) containing 10 mM 2-mercaptoethanol, broken by sonication (30 seconds, five times) under cooling conditions, and centrifuged at 4°C and $105,000 \times g$ for 1 hour.    The supernatant fluid was used as an enzyme preparation for the following assays.

### Enzyme Assays[8,9]

Uridine Kinases:    A reaction mixture (200 μl) containing 62.5 mM Tris-HCl (pH 7.6), 2.5 mM phosphoglyceric acid, 12.5 mM $MgCl_2$, 1 mM ATP, 10 mM 2-mercaptoethanol, 72 nM [³H]uridine and 100 μl of enzyme solution was incubated for 30 minutes at 37°C, and the reaction was terminated by adding 0.25 ml of ice-cold 4.0 M PCA.    The labeled UMP in the reaction mixture was analyzed by paper chromatography as described above.

Uracil Phosphoribosyltransferase:    A reaction mixture (200 μl) containing 100 mM Tris-HCl (pH 7.8), 7 mM $MgCl_2$, 1 mM 5-phosphoribosyl-1-pyrophosphate, 1 μg of bovine serum albumin, 4 μM [¹⁴C]uracil and 30 μl of enzyme solution was incubated for 30 minutes at 30°C.    A suspension (3 ml) of icecold polyethyleneimine cellulose in 1 mM $CH_3COONH_4$ was added to the reaction mixture.    The cellulose was collected on a Whatman GF/C glass microfiber filter, and washed three times with ice-cold 1 mM $CH_3COONH_4$.    The filter was dried and the radioactivity was counted.

### Chemicals

Virustomycin A was prepared as reported previously[1].    Metronidazole and actinomycin D were obtained from Merck Sharp & Dohme Res. Labs., U. S. A., and Wako Pure Chemical Ind., Japan, respectively.

These agents were dissolved in dimethyl sulfoxide (DMSO) and then water was added to the solutions. At the final concentration of 0.1 % (v/v), DMSO did not affect the growth of the trichomonad cells and the incorporation of radioactive precursors into acid-soluble and acid-insoluble fractions of the trichomonad cells. [5-³H]Uridine (28 Ci/mmol), [6-³H]thymidine (26 Ci/mmol), L-[U-¹⁴C]leucine (342 mCi/mmol), [6-³H]uracil (21 Ci/mmol), [2-¹⁴C]uracil (55.2 mCi/mmol), [2-³H]adenosine (22 Ci/mmol) and [2-³H]adenine (19 Ci/mmol) were purchased from Amersham International Ltd., U.K. 5-Phosphoribosyl-1-pyrophosphate was purchased from Calbiochem-Behring Co., California. Polyethylene-imine cellulose was purchased from Serva Feinbiochemica GmbH. & Co., West Germany. Other chemicals were obtained commercially.

## Results and Discussion

### Antitrichomonal Activity of Virustomycin A against *T. foetus*

The activity of virustomycin A against *T. foetus* was examined by the method of ASAMI *et al.*[6] Table 1 shows that the MIC and MCC were almost equivalent to those of metronidazole, which is used as an antitrichomonal agent in the medical field. The MIC (1.6 $\mu$g/ml) of virustomycin A is different from that (25 $\mu$g/ml) of the previous report[1]. The difference seems to be due to the use of a different method including a different medium for the MIC determination. Fig. 2 shows the effect of virustomycin A on the growing culture of *T. foetus*. When virustomycin A was added to the culture in the initial stage, the growth was significantly repressed at the concentration of 0.3 $\mu$g/ml, and the cells were lysed at 3.0 $\mu$g/ml. When it was added to a logarithmic phase culture, the cells were also lysed at the concentrations of both 0.3 and 3.0 $\mu$g/ml. These results indicate that a logarithmic phase culture is more susceptible to the antibiotic than is an initial stage culture just after being inoculated with cells from a stationary phase.

Fig. 2. Effect of virustomycin A on a growing culture of *T. foetus* when it was added at initial stage (A) and in middle logarithmic phase (B).

The arrow indicates the time of virustomycin A addition. ○, control (without virustomycin A); ●, 0.3 $\mu$g/ml; ■, 3.0 $\mu$g/ml.



Table 1. Antitrichomonal activities of virustomycin A and metronidazole against *T. foetus*.

| Compound | MIC ($\mu$g/ml) | MCC ($\mu$g/ml) |
|---|---|---|
| Virustomycin A | 1.6 | 3.1 |
| Metronidazole | 1.0 | 2.0 |

Medium: DIAMOND's medium without agar.
Incubation: at 37°C for 42 hours.
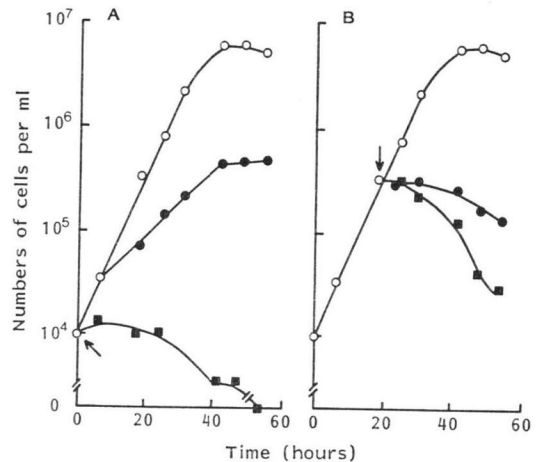
### Effect of Virustomycin A on Biosynthesis of Macromolecules

The effects of the antibiotic on nucleic acid and protein syntheses were examined by the incorporation of [³H]uridine, [³H]thymidine and [¹⁴C]leucine into the acid-insoluble fraction of *T. foetus*. As shown in Fig. 3, although virustomycin A interfered with the incorporation of [³H]uridine, [³H]thymidine and [¹⁴C]leucine, the inhibition of the incorporation of [³H]uridine was the most severe. The inhibition reached about 50% after 60 minutes of incubation in the presence of 0.03 $\mu$g/ml of virustomycin A. About 50% inhibition of the incorporation of [³H]thymidine or [¹⁴C]leucine was observed after 120 or

Fig. 3. Effect of virustomycin A on the incorporation of radioactive precursors into acid-insoluble macro-
molecular fraction in *T. foetus*.

○, Control (without virustomycin A); ●, 0.03 μg/ml; ■, 0.3 μg/ml.
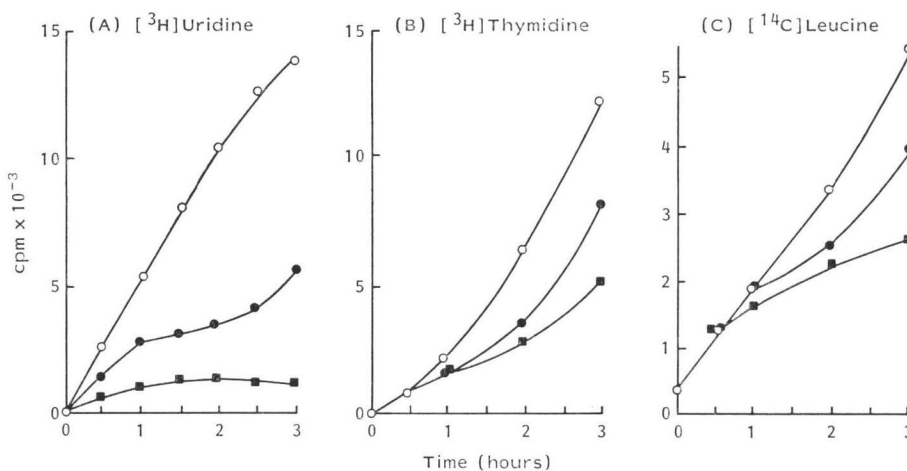


180 minutes, respectively, at the concentration of 0.3 μg/ml, but the incorporation of them was not in-
hibited until 30 minutes.

The above results seem to indicate that virustomycin A inhibits primarily RNA synthesis in *T. foetus*.
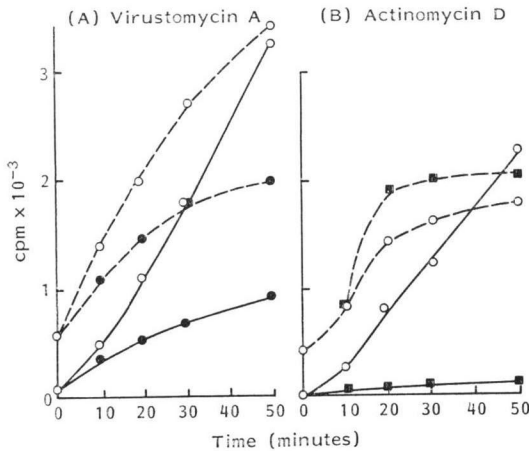
## Effect of Virustomycin A on RNA Synthesis

Next, the effect of the antibiotic on the incorporation of [³H]uridine into acid-soluble and acid-
insoluble fractions was examined compared with that of actinomycin D, which is well known to bind to
DNA and to inhibit DNA-directed RNA synthesis[10]. Actinomycin D is known to inhibit the growth of
mammalian cells at relatively low concentrations. However, the *Trichomonas* cells were less susceptible
to the antibiotic. At the concentration of 10 μg/ml, it hardly affected the incorporation of [³H]uridine
into both acid-soluble and acid-insoluble fractions (data not shown), but, at 100 μg/ml, it inhibited the
incorporation into the acid-insoluble fraction while it rather enhanced somewhat that into acid-soluble
fraction as shown in Fig. 4B. On the other hand, virustomycin A repressed the incorporation of [³H]-
uridine into both acid-soluble and acid-insoluble fractions (Fig. 4A). From these data, it was speculated
that the site of inhibition by virustomycin A is differentiated from that of actinomycin D and lies on steps
of formation of nucleotides from uridine. In fact, the speculation was confirmed by the results of pulse-
chase experiment shown in Fig. 5.

Cells were prelabeled with [³H]uracil at 20°C for 60 minutes. The cells were then collected by cen-
trifugation, washed and further incubated in a fresh medium at 37°C with and without virustomycin A.
[³H]Uracil instead of [³H]uridine was used because the incorporation rate of the former was higher than
that of the latter. At 20°C, the incorporation of [³H]uracil into the acid-soluble fraction occurred at an
appreciable rate while the rate of incorporation into acid-insoluble fraction was very low. These results
indicate that, at this temperature, transport of [³H]uracil and nucleotide formation from [³H]uracil and
further phosphorylation of nucleotide continues while RNA synthesis is arrested. Fig. 5 shows that, at
37°C, the labeled nucleotides were transferred from the pool into nucleic acids and that virustomycin
A had no effect on the transfer.

Fig. 4. Effects of virustomycin A (A) and actino-
mycin D (B) on the incorporation of [³H]uridine
into acid-soluble and acid-insoluble fraction in *T. foetus.*

———, Acid-insoluble fraction; ----, acid-soluble
fraction; ○, control (without drug); ●, 0.1 μg/ml;
■, 100 μg/ml.



Fig. 5. Effect of virustomycin A on the incorpora-
tion of [³H]uracil into acid-soluble and acid-insolu-
ble fractions in *T. foetus.*

The cell suspension supplemented with 0.2 μM
[³H]uracil (21 Ci/mmol) was incubated at 20°C.
After 60 minutes of incubation, the cells were col-
lected by centrifugation, washed three times with
ice-cold saline at 0°C, and were suspended to the
original cell density in warm (37°C) DIAMOND's
medium (without agar) containing 1.0 μg/ml of
virustomycin A. The suspensions were further
incubated at 37°C and monitored for radioactivity
in the acid-insoluble and acid-soluble fractions.

———, Acid-insoluble fraction; ------, acid-soluble
fraction; ○, control (without virustomycin A);
●, 1.0 μg/ml of virustomycin A.



## Effect of Virustomycin A on Transport of Base and Nucleoside

The rate of the incorporation of [³H]uracil
and [³H]adenosine into acid-soluble fraction at
20°C was lower (about one-fifth) than that at
37°C, as shown in Figs. 6 and 7, and was not
inhibited by the antibiotic until at least 30 min-
utes. This indicated that the transport of these labeled compounds was not interfered with by the anti-
biotic. The rate of the incorporation of the labeled compounds into acid-soluble fraction at 37°C was
higher and was repressed in the presence of the antibiotic. However, the incorporation of [³H]uracil
and [³H]adenine at 37°C was not inhibited by the antibiotic until 15 minutes, this also suggests that
the site of action is not on the transport of the bases.

### Effect of Virustomycin A on Nucleotides Formation from [³H]Uracil or [³H]Uridine

Cells were incubated in the presence of [³H]uracil or [³H]uridine, and the acid-soluble fraction pre-
pared from the cells was analyzed for labeled nucleotides by paper chromatography. As shown in Table
2, the formation of UMP, UDP and UTP from uracil or uridine was inhibited by virustomycin A; The
inhibition of nucleotide formation from uridine was stronger than that from uracil. The inhibition of
the incorporation of adenosine into acid-soluble fraction was also stronger than that of adenine.

In general, formation of nucleoside triphosphates (substrates for RNA polymerase) from uracil and
uridine requires uridine kinase, uracil phosphoribosyltransferase and nucleotide-phosphorylating sys-
tems. As shown in Table 3, the activities of uridine kinase and uracil phosphoribosyltransferase in a
cell-free extract from the organism were not affected by the antibiotic. Also, since the rates of inhibi-
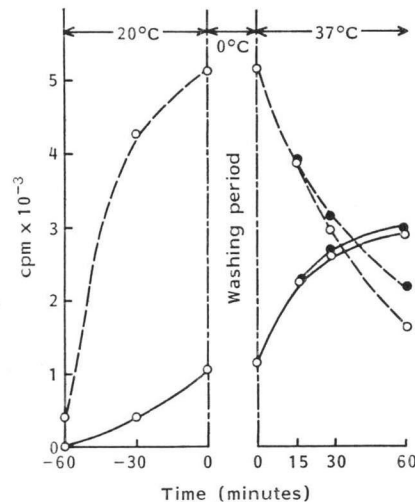tion of nucleotides (UMP, UDP and UTP) formation shown in Table 3 were almost equivalent each

Table 2.  Effect of virustomycin A on nucleotide formation from uridine or uracil with a growing culture of
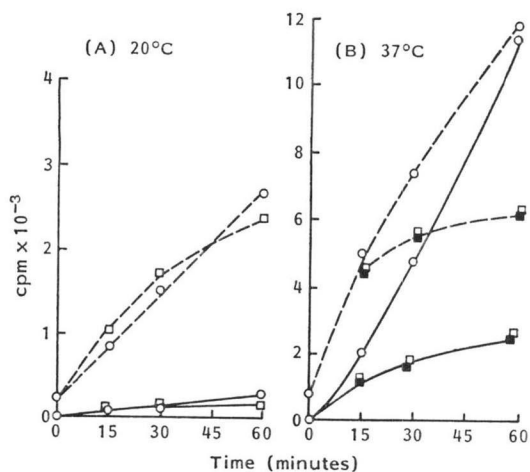T. foetus.

The nucleotides in the acid-soluble fraction were analyzed by the methods described in the text.

| Precursor | Virustomycin A added ($\mu$g/ml) | Nucleotides formed (% of control) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Uracil | Uridine | UMP | UDP | UTP* | Total nucleotide |
| [³H]Uridine | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| | | (704 cpm) | (1,175) | (3,365) | (5,789) | (6,589) | (15,743) |
| | 0.1 | 103 | 90 | 30 | 48 | 35 | 38 |
| | 1.0 | 57 | 64 | 23 | 19 | 27 | 23 |
| [³H]Uracil | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| | | (3,050) | (13) | (2,625) | (5,168) | (8,505) | (16,298) |
| | 0.1 | 83 | 72 | 63 | 53 | 60 | 58 |
| | 1.0 | 91 | 79 | 48 | 42 | 53 | 48 |

* Contains UDP-sugars.

Fig. 6.  Effect of virustomycin A on the incorpora-
tion of [³H]uracil into acid-soluble and acid-in-
soluble fractions at 20°C (A) and 37°C (B) in T.
foetus.

——, Acid-insoluble fraction; ------, acid-soluble
fraction; ○, control (without virustomycin A); ■,
0.1 $\mu$g/ml; □, 1.0 $\mu$g/ml.



Table 3.  Effect of virustomycin A on UMP forma-
tion from uridine or uracil with a cell-free extract
from T. foetus.
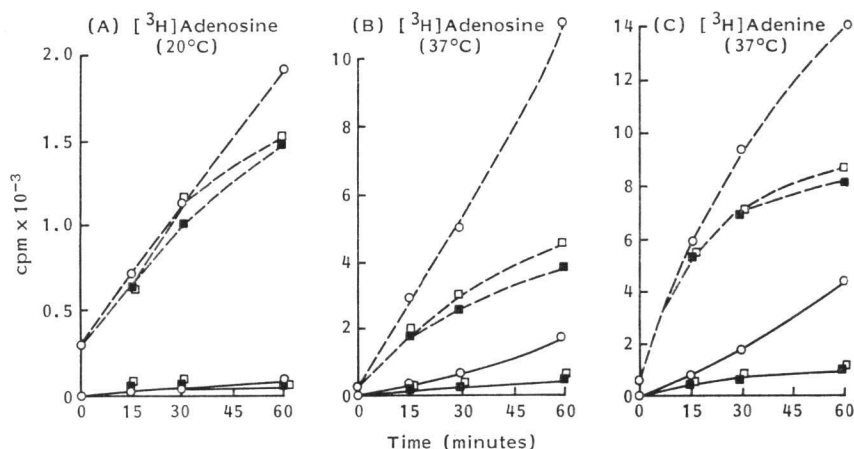
The methods are described in the text.

| Precursor | Virustomycin A added ($\mu$g/ml) | UMP formed (% of control) |
|---|---|---|
| [³H]Uridine | 0 | 100 |
| | | (14,475 cpm) |
| | 1 | 88 |
| | 10 | 80 |
| [¹⁴C]Uracil | 0 | 100 |
| | | (1,919 cpm) |
| | 1 | 97 |
| | 10 | 99 |

other, the antibiotic does not seem to inhibit
nucleotide phosphorylating system.

These results suggest that virustomycin A
does not interfere with enzymes involved in nu-
cleotide formation, but instead with the forma-
tion of a phosphate donor(s), required for
nucleotide formation.  Since the most likely
phosphate donor is ATP, it is possible that virustomycin A interferes with an ATP-forming system.
The low inhibition rate of the incorporation of bases ([³H]uracil and [³H]adenine) into the acid-soluble
fraction (Table 2, Figs .6 and 7) may reflect the presence of 5-phosphoribosyl-1-pyrophosphate pooled in
the cells.   After the latter was consumed, the inhibition rate became higher.   With the incorporation of
nucleosides ([³H]uridine and [³H]adenosine), the rate of inhibition by the antibiotic was relatively low
at a initial stage of incubation compared with that at a later stage.   This may reflect the presence of
ATP pooled in the cells.

To confirm the above speculation, the site of action of virustomycin A on an ATP-forming sys-
tem(s) in T. foetus must be determined.   Experiments with a cell-free extract from the organism are now
in progress.

Fig. 7. Effect of virustomycin A on the incorporation of [³H]adenosine and [³H]adenine into acid-soluble and acid-insoluble fractions in *T. foetus*.

——, Acid-insoluble fraction; ------, acid-soluble fraction; ○, control (without virustomycin A); ■, 0.1 µg/ml; □, 1.0 µg/ml.

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